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The role of TLRs and T cells in modulating glial activation; Implications for Alzheimer's disease

Tara Browne

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

Supervisor: Prof. Marina Lynch
Trinity College Institute of Neuroscience
2013
“You have to begin to lose your memory, if only in bits and pieces, to realise that memory is what makes our lives. Life without memory is no life at all, just as an intelligence without the possibility of expression is not really an intelligence. Our memory is our coherence, our reason, our feeling, even our action. Without it, we are nothing.”

Luis Bunuel 1900-1993
I. Declaration

This thesis is submitted by the undersigned for the degree of Doctor of Philosophy at the University of Dublin. I declare that this thesis is entirely my own work with the following exceptions; certain results were produced in collaboration with Dr. Anthony Lyons, Dr. Keith McQuillan and Roisin McManus. This work has not been submitted in whole or part to this or any other university for any other degree. The author gives permission to the library to lend or copy this work upon request.

Tara Browne
II. Abstract

Microglia are the key immune mediators of the CNS but, while astrocytes are primarily involved in maintaining homeostasis, they also exhibit immune functions. Both microglial and astrocytic activation is associated with autoimmune and neurodegenerative diseases such as multiple sclerosis (MS), Parkinson’s disease and Alzheimer’s disease (AD). Activation by a stimulus, such as a toll like receptor (TLR) agonist, or amyloid-beta (Aβ) results in the rapid production and secretion of pro-inflammatory cytokines and chemokines by these cells. However, under normal conditions, microglia are maintained in a quiescent state by interaction with other cells. Specifically, activation of the microglial receptor CD200R by its ligand, which is expressed on many cells including neurons, is important in this role, and disruption of the CD200-CD200R interaction results in increased microglial activation, neuroinflammation and autoimmune inflammation.

In this study, glial activation was assessed in vitro and in vivo in wildtype and CD200 knockout (CD200"') mice following treatment with a TLR2 agonist, Pam3Csk4. It was found that Pam3Csk4 induced a significant increase in the expression of markers of glial activation and the production of pro-inflammatory cytokines and chemokines in vitro and in vivo. Furthermore, the loss of CD200 significantly enhanced cytokine and chemokine production in response to Pam3Csk4 in vitro and in vivo. In addition, it was found that TLR2 expression was increased on microglia cultured from CD200", compared with wildtype, mice suggesting a possible mechanism for the exaggerated response seen in CD200" mice.

There is evidence that Aβ induces some of its actions by interacting with TLR2 and the data from this study revealed that Aβ mimicked many of the effects of Pam3Csk4. Significantly, incubation of cells in the presence of an anti-TLR2 antibody significantly attenuated Aβ-induced cytokine and chemokine production by glia. These data provide evidence that activation of TLR2, either by the commonly-used agonist, Pam3Csk4, or by Aβ triggered released of inflammatory mediators from glia and, importantly, provided support for the hypothesis that Aβ-induced changes are TLR2-mediated.

Infiltration of T cells into the central nervous system (CNS) is believed to be involved in the pathogenesis of MS and a number of studies have reported that T cells infiltrate the brains of patients with AD. This is significant because T cells have been shown to interact with microglia and modulate their function. In this study, Th1, Th2 and Th17 cell lines were assessed for their ability to modulate glial activation in vitro. It was found that Th1 cells and Th17 cells increased glial activation as measured by CD40 and CD86 expression and IL-6 and TNFα production. Th2 cells had no effect on glial activation.

Having established that Th1 cells and Th17 cells impacted on glial activation in vitro, this was assessed in vivo. A transgenic mouse model of AD, which over-expresses amyloid precursor protein (APP) and presenilin-1 (PS1) (APP/PS1 mice) was used to examine the effect of Th1 and Th17 cells in modulating microglial activation and amyloid deposition. The data shows that T cells were present in greater numbers in the brains of APP/PS1 mice compared with wildtype mice and these cells secreted interferon (IFN)-γ and interleukin (IL)-17. Adoptive transfer of Aβ-specific Th1 or Th17 cells into APP/PS1 mice increased microglial activation, as measured by CD11b immunoreactivity, while Th1 cells increased the number of Aβ-containing plaques in hippocampus and cortex of APP/PS1.

In order to further examine the impact of Th1 cells on microglial activation and Aβ plaque burden, mice were injected with an anti-IFN-γ antibody prior to, and on several days after, injection of Aβ-specific Th1 cells. The data show that treatment of mice with anti-IFN-γ antibody attenuated the Th1 cell-induced increase in microglial activation and Aβ deposition.

The results reveal that microglial activation is modulated by several factors including activation of CD200R and TLR2, as well as interaction with T cells. The evidence suggests that glial activation induced by Th1 cells in APP/PS1 mice is a result of release of IFN-γ. It remains to be established whether targeting T cell infiltration or IFN-γ release or activation of IFN-γ receptors may be beneficial in limiting disease progression in APP/PS1 mice and ultimately be of benefit in the treatment of AD.
III. Acknowledgements

Well where to begin, it’s been an amazing chapter of my life that couldn’t have happened without the support of an endless amount of people and to them I am truly grateful.

Firstly and most importantly, I’d like to sincerely thank my supervisor Professor Marina Lynch for her continual support, advice and incredible patience on matters both inside the lab and outside. Thank you so much for everything you have done for me over the last three years!! I’d also like to thank my funding body HRB who have kindly funded this project.

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CD3^CD8^ cells infiltrate the brains of wildtype and APP/PS1 mice.

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Expression of MHC class II on microglia was assessed following co-culture with Th1 cells and incubation with IFN-γ and Aβ_{1-42}.

Expression of CD40 and CD86 on microglia was assessed following co-culture with Th17 cells and incubation with IL-17 and Aβ_{1-42}.

Expression of MHC class II on microglia was assessed following co-culture with Th17 cells and incubation with IL-17 and Aβ_{1-42}.

CD11b expression is increased in cortical sections prepared from APP/PS1, compared to wildtype, mice.

CD11b expression is increased in hippocampal sections prepared from APP/PS1, compared with wildtype, mice.

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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
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<tr>
<td>Aβ</td>
<td>Amyloid-beta peptide</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<td>B cell receptor</td>
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<tr>
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<td>Blood brain barrier</td>
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<td>BSA</td>
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<td>Central nervous system</td>
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<td>CD11b</td>
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<td>CIA</td>
<td>Collagen induces arthritis</td>
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<td>CR3</td>
<td>Complement receptor 3</td>
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<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<td>Complete freund’s adjuvant</td>
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<td>DAB</td>
<td>Diaminobenzidine</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
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<td>Familial Alzheimer’s disease</td>
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<td>FACS</td>
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<td>IL-1R accessory protein</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1R associated kinases</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IP-10</td>
<td>Interferon gamma-induced protein 10</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IL-1R</td>
<td>Interleukin-1 receptor</td>
</tr>
<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneally</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenously</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>+/-</td>
<td>Knock out</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine rich repeats</td>
</tr>
<tr>
<td>L</td>
<td>Ligand</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MIP-1</td>
<td>Macrophage inflammatory protein-1</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------------------</td>
</tr>
<tr>
<td>MHC class II</td>
<td>Major histocompatibility complex class II</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>μ</td>
<td>Micro</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>mM</td>
<td>Molar</td>
</tr>
<tr>
<td>OX6</td>
<td>Monoclonal antibody clone for MHC class II</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation factor 88</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
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<tr>
<td>NHS</td>
<td>Normal horse serum</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cooling temperature</td>
</tr>
<tr>
<td>Pam₃Csk₄</td>
<td>Pam₃CysSerLys₄</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pathogen recognition receptors</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate buffered saline containing tween</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PS</td>
<td>Presenilin</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated and normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Strep-HRP</td>
<td>Streptavidin-horseradish peroxidase linked</td>
</tr>
<tr>
<td>H$_2$SO$_4$</td>
<td>Sulphuric acid</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory Cell</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>ThT</td>
<td>Thioflavin T</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNF receptor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TE</td>
<td><em>Toxoplasma</em> encephalitis</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenic</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>BACE</td>
<td>β-APP Cleaving Enzyme</td>
</tr>
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Chapter 1

Introduction
Chapter 1

1.1. The immune system

The immune system, an organism's defence mechanisms against invading or self pathogens, is fundamental to survival. It consists of a network of proteins, cells, tissue and organs that respond to a given insult in a manner which preserves homeostasis and prevents tissue damage. The immune system has evolved to distinguish between host cells and pathogens and comprises a non-specific innate immune system and a specific adaptive immune system. The innate immune system is the first line of defence and consists of physical barriers and cells that react if the physical barriers are breached. Although the innate immune system responds rapidly following pathogen detection it is unable to generate immunological memory as it is non-specific. Immunological memory is generated by the adaptive immune system which requires antigen presentation for its activation. While defined as separate systems, they are functionally intertwined and the action of one system has a direct effect on the other (Becher et al., 2000).

1.1.1. The innate immune system

The functions of the innate system include directly killing a pathogen with its own cells or initiating an appropriate adaptive immune response that will clear the pathogen (Medzhitov and Janeway, 1997). The cells involved are derived from the myeloid lineage such as granulocytes (neutrophils, basophils, eosinophils and mast cells) and monocytes (dendritic cells and monocytes). These cells act as antigen presenting cells (APCs) and phagocytes, and secrete cytokines to communicate with other cells of the immune system (Becher et al., 2000). Once a pathogen has breached the physical barriers, pathogen recognition receptors (PRR) found on the cells of the innate immune system are required to identify pathogens by their specific pathogen-associated molecular patterns (PAMPs) (Medzhitov & Janeway, 1997). Recognition of PAMPs by immune cells results in the activation of signalling pathways leading to antigen presentation, costimulatory molecule upregulation and the release of cytokines and chemokines.
The most-studied PRRs are the Toll-like receptors (TLRs) (Laflamme et al., 2001; Takeda & Akira, 2004).

1.1.1.1. TLRs

TLRs were first identified in Drosophila as receptors involved in dorso-ventral patterning in embryos and, since then, evidence for a role in immunity was provided by Hoffmann and colleagues in 1996 who found that Toll-mutant flies were highly susceptible to fungal infection (Takeda & Akira, 2004). TLRs play a pivotal role in identifying invading pathogens and producing the appropriate immune response (Pandey & Agrawal, 2006). TLRs are expressed on a wide array of immune cell types including B cells, T cells, macrophages, monocytes, neutrophils, basophils, dendritic cells and natural killer cells (Hopkins and Sriskandan, 2005). Furthermore, TLRs are expressed by cells of the central nervous system (CNS) including microglia (Applequist et al., 2002) and astrocytes (Bowman et al., 2003). TLRs are expressed both on the cell surface (TLRs 1, 2, 4, 5 and 6) and intracellularly (TLRs 3, 7, 8 and 9) (Pandey & Agrawal, 2006). Although the activation of TLRs is crucial to mount an appropriate immune response, their activation or dysregulation is also implicated in the pathogenesis of autoimmune, chronic inflammatory and infectious diseases including cancer, asthma, psoriasis, inflammatory bowel disease, Alzheimer’s disease (AD) and multiple sclerosis (MS) (Chen et al., 2007; O’Neill et al., 2009). The activation of TLRs and their implication in the pathogenesis of diseases, be it beneficial or detrimental, has lead to widespread research investigating the precise role of individual TLRs in diverse diseases as they may be targets for therapeutic intervention (Chen et al., 2007).

1.1.1.2. TLR structural characteristics and signalling pathways

TLRs are a family of type 1 integral membrane glycoproteins which consist of an extracellular domain made up of leucine-rich repeats (LRR) in the pathogen-binding ectodomains (ECD), and a cytoplasmic domain referred to as Toll/IL-1R (TIR) domain as it shows striking similarity to the cytoplasmic domain of the interleukin (IL)-1 receptor family (Takeda & Akira, 2004; Pandey &
Agrawal, 2006). To date, thirteen mammalian TLRs (TLR1-13) have been identified, each with their own set of PAMPs and signalling pathways. TLR signalling has been widely reviewed and it is known that TLRs may signal via the myeloid differentiation factor 88 (MyD88)-dependent pathway (all TLRs) or via the MyD88-independent pathway (selective for TLR3 and TLR4) (Takeda & Akira, 2004; Pandey & Agrawal, 2006; Chen et al., 2007). The TIR domain of the TLRs acts as a scaffold for protein-protein interactions allowing for the activation of signalling cascades including mitogen-activated protein kinases (MAPKs), phosphoinositide 3-kinase (P13K) and nuclear factor-κB (NF-κB) (Chen et al., 2007). These signalling pathways employ different adaptor proteins that are recruited in different combinations to different TLRs allowing for individual responses to diverse pathogens (O'Neill et al., 2009).

The MyD88-dependent pathway employs several molecules to initiate downstream signalling including members of the IL-1R associated kinase (IRAK) family and tumour necrosis factor (TNF) receptor associated factor (TRAF-6) (Pandey & Agrawal, 2006). Furthermore, the linker molecules transforming growth factor β-activated kinases (TAK-1) and TAK-binding protein (TAB)-1 and -2 are recruited, which activate downstream IκB kinase kinases (IKK) (Pandey & Agrawal, 2006). IKK is composed of IKKa, IKKβ and NEMO which induce the phosphorylation of the IκB family proteins and nuclear translocation of NF-κB enabling the upregulation of genes involved in pro-inflammatory cytokine and chemokine production (Pandey & Agrawal, 2006; Chen et al., 2007). TAK-1 can also activate the MAPK pathway such as c-Jun N-terminal kinases (JNK) thereby inducing activating protein-1 (AP-1), resulting in similar upregulation of genes encoding inflammatory cytokines (Takeda & Akira, 2004; Chen et al., 2007).

The MyD88-independent pathway uses the TIR-domain containing adaptor-inducing interferon-β (TRIF)-related adaptor molecule (TRAM) which binds to TRIF which, in turn, mediates signalling in several ways including a TRAF-6-dependent or -independent manner. The TRAF-6-independent pathway is mediated through the kinase receptor-interacting protein (RIP)-1. TRIF also binds with TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK-1) and inducible IκB kinase (IKK-ε), which in turn, phosphorylates interferon regulatory factor (IRF)-3 and IRF-7. Phosphorylation of IRF-3 and IRF-7 allows for their nuclear translocation resulting in the
upregulation of type 1 interferon (IFN) genes (Pandey & Agrawal, 2006). Figure 1.1 illustrates the essential components of TLR activation inducing the MyD88-dependent and MyD88-independent signalling cascades.
TLRs mediate cytokine and chemokine production through MyD88-dependent and MyD88-independent pathway. These pathways result in downstream recruitment of signalling molecules with eventual translocation of NF-κB, AP-1 and IRF-3/7 to the nucleus and the upregulation of genes encoding cytokines and chemokines.

Adapted from Pandey & Agrawal (2006)

Figure 1.1. TLR signalling via MyD88-dependent and MyD88-independent signalling cascades.
1.1.1.3. TLR ligands

TLRs respond to numerous ligands (Pandey & Agrawal, 2006). It has been suggested that TLR2 and TLR4 primarily recognise bacteria through differential cell wall components (Chen et al., 2007), while TLR3, TLR7, TLR8 and TLR9 are endosomal and sense nucleic acid-based ligands, through their LRR domains (O'Neill, 2008). TLR3, TLR5 and TLR9 are involved in the recognition of double-stranded ribonucleic acid (RNA), bacterial flagellin and bacterial CpG-containing oligodeoxynucleotide (CpG) deoxyribonucleic acid (DNA), respectively (Takeda et al., 2002). TLR4 recognises a component of the cell wall of gram-negative bacteria, lipopolysaccharide (LPS), and is critical for an LPS-induced immune response as shown in studies in which TLR4 was absent (Takeuchi et al., 1999). TLR2 recognises the widest array of PAMPs including surface proteins on gram-negative and gram-positive bacteria, lipoteichoic acids, peptidoglycans (PGN), mycoplasma, mycobacteria and zymosan (Kielian, 2006). However, TLR2 has been shown to work as a heterodimer, with TLR1 and TLR6, to discriminate between lipopeptides (Ozinsky et al., 2000). MALP-2, a synthetic mycoplasmal lipopeptide, which contains a diacylated cysteine residue is recognised by TLR2/TLR6 heterodimer while, Pam₃CysSerLys₄ (Pam₃Csk₄) which contains a triacylated cysteine residue is recognised by TLR2/TLR1 heterodimer (Takeda et al., 2002). Triacylated lipopeptides interact with the TLR2/TLR1 heterodimer through the insertion of two ester-bound lipid chains into a pocket in TLR2 and the insertion of an amide-bound lipid chain into the hydrophobic channel in TLR1 (O'Neill, 2008).

1.1.2. The adaptive immune system

The adaptive immune system is more complex than the innate immune system in that it involves antigen-specific responses and the development of immunological memory for specific pathogens. Activation of the adaptive immune system depends on cells of the lymphoid lineage namely B and T progenitor cells. B cells develop in the foetal liver and later mature in the bone marrow and are mainly involved in humoral responses whereas T cells mature in the thymus and are responsible for cell-mediated immune responses.
B cells require signalling from the B cell receptor (BCR) and an additional signal from T helper (Th) cells to trigger their activation (Geisberger et al., 2006). Once activated, they differentiate into either plasma cells or memory B cells. Plasma cells are terminally-differentiated B cells that secrete five different antibodies, including immunoglobulin (Ig) M, IgD, IgG, IgA, and IgE, which provide immediate and long-term protection against pathogens (Hoyer et al., 2005). Once these antibodies are secreted they bind to their specific antigen, in a process called opsonisation, marking pathogens for phagocytosis.

T cells are involved in cell-mediated immune responses and are subdivided into cluster of differentiation (CD)8⁺ T cells or CD4⁺ T cells. Naïve CD8⁺ T cells can be divided into cytotoxic effector CD8⁺ cells or memory CD8⁺ T cells. CD8⁺ T cells recognise endogenous antigens, mainly viral particles, through major histocompatibility complex (MHC) class I allowing for the secretion of perforin and granulysin which lyse infected cells. Similarly CD4⁺ T cells can be divided into effector or memory cells. Activation of naïve CD4⁺ cells into appropriate effector cells requires two signals. The first signal is delivered through interaction of MHC class II on cells of the innate immune system with their specific T cell receptors (TCRs) (Sharpe & Freeman, 2002). This process is accompanied by a secondary signal from co-stimulatory molecules CD80 and CD86 on innate immune cells, to CD28 on T cells. Once both signals are present, naïve CD4⁺ T cells will rapidly divide into a subpopulation of T cells with effector functions (Sharpe & Freeman, 2002). In the absence of the second signal from co-stimulatory molecules, on APCs, T cells fail to respond and are rendered anergic or, if the secondary signal is a negative signal, T cell tolerance appears to be induced (Sharpe & Freeman, 2002). The phenotype of the activated CD4⁺ T cells generated depends not only on the antigen presented but on the cytokine environment present at the time of clonal expansion (Mosmann et al., 1986). These CD4⁺ T cells subpopulations include Th1, Th2, Th17 and regulatory T (Treg) cells.

~ 8 ~
1.1.2.1. CD4⁺ T cell subpopulation induction and cytokine secretion

The distinct CD4⁺ T cell subtypes, termed Th1 and Th2 cells, were first discovered by Mosmann and colleagues (1986) and since then several other subtypes have been discovered, including Treg and Th17 cells (Mills, 2008).

Th1 cells are derived from naïve CD4⁺ T in the presence of interleukin (IL)-12, transforming growth factor-β (TGF-β) and interferon-γ (IFN-γ). IL-12 is thought to be the main factor in directing Th1 cell development and is produced by macrophages in response to microbial products (O'Garra, 1998). Th1 cells secrete an array of cytokines which correlate well with their function including IFN-γ, IL-2 and lymphotoxin (LT). Th1 cell cytokines are involved in macrophage activation as well as cytotoxic and inflammatory functions leading to the eradication of pathogens (Mosmann & Sad, 1996), however their dysregulation is also involved in immunopathologies and autoimmune diseases (O'Garra, 1998). Th2 cells divide in the presence of IL-4 which is secreted by mast cells and basophils (Constant & Bottomly, 1997). Th2 cells secrete cytokines including IL-4, IL-6, IL-10 and IL-5 which, in turn, can activate eosinophils and mast cells and induce B cells to produce IgE. Th2 cells are thought to control parasitic infections and their dysregulation has been implicated in atopy and allergic inflammation (O'Garra, 1998; Harrington et al., 2005). Th17 cells divide in the presence of IL-1 and IL-23 and secrete IL-17, IL-17A, IL-21, IL-22, TNFα and IL-6. Th17 cells play an important role, through their secreted cytokines, in anti-microbial immunity at epithelial and mucosal barriers. Th17 cells are also involved in inflammation and autoimmunity (Mills, 2008).

Th1, Th2 and Th17 cells are able to regulate the induction of each other through the secretion of their cytokines in that Th1 cell-secreted IFN-γ inhibits Th2 cell development and humoral responses while, Th2 cell-secreted IL-4 and IL-10 inhibits Th1 development and macrophage activation (O'Garra, 1998). Similarly Th17 cells are regulated by Th1 cells (Mills, 2008). Figure 1.2 illustrates CD4⁺ clonal expansion.
Figure 1.2. Naïve CD4\(^+\) T cell clonal expansion following antigen presentation and co-stimulation.

Following antigen presentation and the interaction of MHC class II with the TCR and co-stimulation from the CD28 and CD80/CD86 interaction naïve CD4\(^+\) T cell will differentiate into different T cell subsets depending on the cytokine environment present at the time of naïve T cell activation. CD4\(^+\) T cells activated in the presence of IL-12 will divide into Th1 effector cells, whereas IL-4 will shift the immune response towards Th2 effector cells. While, IL-1, IL-23 and IL-6 allow for Th17 effector cell development.

Adapted from Mills (2008) and Benveniste et al., (2001)
1.1.2.2. CD4⁺ T cells and disease

Despite the role of T cells in host defence their dysregulation or unchecked activation has been associated with a range of immune pathologies such as MS and type II collagen-induced arthritis (Harrington et al., 2005). Knowledge of the role of specific T cell subgroups in MS has come from studies in the animal model experimental autoimmune encephalomyelitis (EAE) (Finsen & Owens, 2011). Originally studies in the EAE model suggested that Th1 cells mediated disease progression however more recently this view has been redefined (Becher et al., 2006). This is based on the evidence from Billiau and colleagues (1987) demonstrating that blocking IFN-γ enhanced EAE and that mice deficient in IFN-γ or its receptor developed EAE (Chu et al., 2000). It is now thought that both Th1 and Th17 cells can induce EAE but the pathology induced by Th1 cells differs to that of Th17 cells. For example Th1 cells have been found to facilitate macrophage recruitment into the CNS while, Th17 cells facilitate neutrophil recruitment (Kroenke et al., 2008). Furthermore it has been shown that Th17 and CD4⁺ cells secreting both IFN-γ and IL-17 infiltrate the CNS of mice undergoing EAE and exacerbate clinical symptoms accompanied by the activation of microglia and the secretion of pro-inflammatory cytokines (Murphy et al., 2010). A role for Th2 cells in EAE is more controversial. Lafaille and colleagues (1997) suggest that Th2 cells can induce EAE in immuno-compromised RAG-1 knockout mice, whereas others suggest an anti-inflammatory role for Th2 cells and that Th2 cells can reverse Th1 cell-induced inflammatory changes (Gimsa et al., 2001).

1.2. The CNS

The brain has evolved to be one of the most sophisticated and complex organs that nature has derived. Neurons roughly make up about 10% of the total cell number whereas glia make up about 90% of the total cell number and provide nutrients, support and defense for neurons but also interact with neurons in a complex and intricate fashion, such that deficits in glial function profoundly impact on neuronal function (Streit, 2005). Malfunction or disruption of this mutually-beneficial interaction is implicated in every major neurodegenerative disease (Moore & Thanos, 1996).
1.2.1. CNS immunity and inflammation

The CNS has developed both physiologically and anatomically to protect itself from damaging immune mediated inflammation (Aloisi, 2001). The proposal that the brain was “immunologically privileged” isolated from the periphery by the blood brain barrier (BBB) was supported with evidence from several investigators demonstrating that tissue transplanted into the brain survived for a longer period than tissue transplanted into the peripheral system (Medawar, 1948). The presence of the BBB, lack of lymphatic drainage and the lack of MHC antigens added to the proposed “immunoprivileged” notion however, this concept has now been revised. It is now accepted that the CNS can only employ a limited array of immune-defence components and that peripheral immune cells can cross the BBB and aid the resident immune cells in specific immune responses (Ransohoff et al., 2003). These resident immune cells include astrocytes (Dong & Benveniste, 2001) and microglia. Microglia are considered the chief resident immunocompetent cells within the brain (Aloisi, 2001) and, as such assist in the recruitment of peripheral immune cells; however both microglia and astrocytes can upregulate the expression of adhesion molecules, as well as cytokines and chemokines which orchestrate the invasion of peripheral immune cells into the CNS through chemotaxis (Aloisi, 2001). Thus, regardless of the presence of the BBB, the CNS can facilitate its own immune surveillance (Hickey, 2001).

1.2.2. The BBB

The BBB consists of brain microvascular endothelial cells, a basement membrane, pericytes and glial cell elements, specifically astrocytic endfeet (Prat et al., 2001). The specialized endothelial cells are linked together by tight junctions which are comprised of adhesion molecules including cadherins, occludin and claudins (Wilson et al., 2010). These endothelial cells need constant input from the other neuroglia cells to maintain their BBB related properties (Prat et al., 2001). The BBB is designed to selectively minimize the trafficking of cells and macromolecules into the CNS, however peripheral cells possess the ability to cross the BBB in search of specific antigens (Hickey, 2001). It is thought that leukocytes can migrate into the CNS through several routes including the choroid.
plexus, subarachnoid space and the parenchymal perivascular space (Wilson et al., 2010). Migration into the CNS is dependent on interacting pairs of selectins and their ligands, integrins and adhesion molecules, and chemokines and chemokine receptors (Ransohoff et al., 2003). Figure 1.3 illustrates this process of migration.

Several studies suggest that CNS inflammation can disrupt the BBB integrity. Interestingly studies have found that both the expression of adhesion molecules and chemokines are increased in normal ageing and in response to cytokines which are released during CNS inflammation (Xu et al., 2010). Furthermore, activation of endothelial cells and astrocytes during CNS injury can reduce tight junction integrity and increase BBB permeability aiding in the recruitment of peripheral cells (Wilson et al., 2010). For instance, animal models of stroke exhibit decreased occludin expression following cerebral ischemia (Hua et al., 2008). Previous studies from this laboratory have observed decreased expression of the tight junction molecules, occludin and claudin, and increased BBB permeability, measured by gadolinium extravasation, in APP/PS1 mice and CD200 knockout (CD200<sup>−/−</sup>) mice, two mouse models which exhibit exacerbated inflammation (Kelly et al., unpublished data). Importantly dysfunction of the BBB integrity has been described in several pathologies including AD, MS and vascular dementia (Campbell et al., 2010), as well as in normal ageing (Farrall & Wardlaw, 2009).
Leukocyte extravasation into areas of inflammation has been separated into several steps. The first step is referred to as tethering and requires contact between the leukocyte and the endothelial cell followed by rolling. Both of these steps require interaction of selectins with their ligands. The next step, activation, requires the interaction of chemokines and their receptors which activate integrins. Integrin interaction with cell adhesion molecules results in the flattening of the leukocyte. The final stage is diapedesis which requires cytoskeletal reorganization that requires signalling from chemokines through their receptors.

Adapted from Ransohoff et al., (2003)
1.2.3. Astrocytes

Astrocytes belong to the family of macroglia and are the most abundant glial cells within the CNS. Astrocytes are essential in development, maintaining homeostasis, survival of neurons and efficient running of the CNS (Bowman et al., 2003). However, there is now considerable evidence suggesting a role for astrocytes in immune functions and inflammation (Dong & Benveniste, 2001). These cells express several TLRs which are important in recognizing bacterial products and mounting an effective immune response (Bowman et al., 2003; Carpentier et al., 2005). Furthermore, studies have found that astrocytes can produce chemokines (Babcock et al., 2003; Dimitrijevic et al., 2006) as well as cytokines which are integral to modulate immune responses. Astrocytes respond to both innate immune signals (e.g. TLR agonists) and adaptive immune signals (e.g. IFN-γ); studies from Carpentier and colleagues (2005) observed increased cytokine and chemokines secretion, as well as increased adhesion molecule, antigen presentation machinery and co-stimulatory molecule expression following stimulation with polyinosinic-polycytidylic acid (Poly I:C), LPS, IFN-α, and TNFα. Additionally, both the secretion of TNFα and IL-6 by astrocytes can lead to increased BBB permeability allowing for T cell entry (Carpentier et al., 2005), and astrocytes have been shown to secrete IL-12 which is an important cytokine in T cell proliferation (Gurley et al., 2008). In turn, T cells by releasing their cytokines can increase astrocyte activation (Giuliani et al., 2003).

Evidence suggests that exacerbated astrocytic activation may have a role in neurodegenerative disease; reactive astrocytes are observed in the AD brain and are found in close association with amyloid (Aβ) deposits (Xia et al., 2000), while it has been suggested that astrocytes respond more profoundly in the aged brain to inflammatory insults due to an age-related increase in basal astrocytic activation (Deng et al., 2006).

1.2.4. Microglia

Microglial cells are the principal resident immune cells of the brain that were first described by Franz Nissl in the 1880s. As microglia are the resident brain macrophages they exhibit many of the features of other tissue macrophages
including phagocytosis, antigen presentation, production of chemokines and cytokines, complement components and reactive oxygen species (Benveniste et al., 2001). They respond rapidly to endogenous insults (e.g. signals from stressed or dying cells as well as immune signals like cytokines and T cells) and exogenous insults such as those of pathogens (Aloisi, 2001). Under normal conditions they demonstrate a downregulated immunophenotype tailored to the specific microenvironment of the CNS. Interaction with neurons through ligand-receptor interactions is believed to aid in the maintenance of a downregulated phenotype. These ligand-receptor pairs include; CD200-CD200R, fractalkine-fractalkine receptor and signal regulatory protein-a (SIRPα)-CD47 (Hanisch & Kettenmann, 2007). However, microglia respond rapidly to a range of signalling molecules suggesting that their apparent quiescence represents a state of vigilance to disruptions in their extracellular surroundings (Kreutzberg, 1996). Traditionally, it was thought that microglia possessed only two states of activation a non-phagocytic phenotype and a phagocytic phenotype however, it is now acknowledged that this is not the case and multiple activation states exist (Lynch, 2009). Indeed microglia are very plastic and their activation state depends on the inflicted insult/pathology (Kreutzberg, 1996) and, more importantly, different phenotypes means functional diversity (Hanisch & Kettenmann, 2007). The transformation of microglia into an activated phenotype is characterized by increased expression of cell surface markers including those involved in antigen presentation and phagocytosis, and increased secretion of several inflammatory mediators namely cytokines and chemokines (Gehrmann et al., 1995; Lynch, 2009).

Microglial activation is associated with an array of disease pathologies including neurodegenerative diseases such as MS, AD and Parkinson’s disease (PD). Furthermore, studies suggest that microglial activation increases with ageing and this can be associated with an exacerbated response to an inflammatory insult (Deng et al., 2006). Despite extensive research, the role of microglia in neurodegenerative diseases still remains unclear and their activation has been associated with both neuroprotective and neurotoxic effects (Hanisch & Kettenmann, 2007).
1.2.5. Markers of glial activation

Under resting conditions, glia express low basal levels of markers including CD11b (microglia only), CD40, CD86, CD80, MHC class II and TLRs however, CNS pathologies and systemic infections upregulates expression of these markers (Frank et al., 2007). Further it has been established that microglia display different expression of these markers depending in the state of activation (Lynch, 2009).

1.2.5.1. CD11b

The integrin, CD11b, forms a heterodimer with CD18 to make up the complement receptor-3 (CR3), also known as MAC-1 (Lynch, 2009). The extent of CD11b expression is reported to correlate with the degree of microglial activation (Roy et al., 2008). The CR3 receptor uses a lectin binding site to bind to an array of microbial molecules and behaves as a powerful mediator of the immune response particularly as it activates protein kinase cascades that are crucial for leukocyte activation (Aloisi, 2001). Recent studies have reported that CD11b is upregulated on microglia in response to an array of pro-inflammatory cytokines including TNFα, IFNα, IL-1β and IL-6 and by fibronectin and vitronectin, which are extracellular matrix substrates that are released during BBB breakdown (Milner & Campbell, 2003). Consistently, LPS increases CD11b expression on microglial cell lines and also in vivo in the striatum of mice injected with LPS (Roy et al., 2008). In addition, microglia have been observed expressing CD11b in close proximity to Aβ-plaques in a mouse model of AD (Bornemann et al., 2001).

1.2.5.2. MHC class II and co-stimulatory molecules CD80 and CD86

MHC class II expression is a hallmark of APCs and is expressed at low levels on resting microglia however, its upregulation is an early consequence of microglial activation that may even precede morphological changes (McGeer et al., 1993). Although MHC class II expression is generally found on professional APCs, such as microglia, it is also expressed on astrocytes although to a much
lesser extent (Lynch, 2009). MHC class II interaction with the TCR, expressed on
CD4+ T cells, in the presence of co-stimulatory molecules initiates the activation
of signalling cascades as well as the upregulation of CD40 and CD40L on their
respective cells (Dong & Benveniste, 2001). Several factors increase the
expression of MHC class II; IFN-γ, the Th1 cell secreted cytokine, is perhaps the
most potent inducer of MHC class II expression on microglia (Xu & Ling, 1995),
and astrocytes (Dong & Benveniste, 2001). Studies from Milner and Campbell
(2003) demonstrated that microglia expressed MHC class II following IFN-γ
stimulation. Others have observed basal expression of MHC class II on ex vivo
human microglia and its upregulation following IFN-γ and LPS stimulation
(Becher & Antel, 1996). MHC class II expression is enhanced in mixed glia
treated with both IFN-γ and Aβ (McQuillan et al., 2010). In addition, MHC class
II expression is upregulated on microglia during ageing (Henry et al., 2009) and
following inescapable shock (Frank et al., 2007).

The expression of MHC class II has been linked with neurodegenerative
diseases. Lopes and colleagues (2008) reported MHC class II-positive microglia
in close proximity to senile plaques in post-mortem tissue from AD patients.
Although aberrant expression of MHC class II is implicated in the pathogenesis of
autoimmune diseases its expression is also essential for normal immune function,
as seen in Bare Lymphocyte Syndrome, where these individuals have severe
immunodeficiency due to the lack of MHC class II (Benveniste et al., 2001).

CD80 (B7-1) and CD86 (B7-2) are transmembrane glycoproteins which
are expressed on APCs, including microglia and astrocytes, and signal via CD28
and cytotoxic T lymphocytes antigen 4 (CTLA-4) expressed on T cells (Lynch,
2009). CD80 and CD86 engagement along with MHC class II and TCR
engagement is essential for T cell proliferation. Several stimuli have been
observed to upregulate CD80 and CD86 expression. CD80 expression is
upregulated in human microglial cultures following IFN-γ and LPS stimulation
(Becher & Antel, 1996) and in microglial cell lines following PGN stimulation
(Kielian et al., 2002). CD86 and CD80 expression are enhanced in murine mixed
glia following Aβ treatment (McQuillan et al., 2010).
1.2.5.3. CD40

CD40 is a member of the TNF-receptor family found on APCs and interacts with its ligand (CD40L), also known as CD154, on activated T cells (Aloisi, 2001; Benveniste et al., 2001) and astrocytes (Townsend et al., 2005). Interaction between CD40-CD40L results in the upregulation of co-stimulatory molecules (CD80 and CD86) and increased secretion of cytokines and chemokines (Aloisi, 2001; Benveniste et al., 2001). CD40 is expressed at low levels on microglia and its expression is greatly enhanced in response to several stimuli including IFN-γ (Benveniste et al., 2001), LPS (Lyons et al., 2009a), and Aβ (Tan et al., 1999).

The expression of CD40 has been linked with neurodegenerative diseases including MS and AD. It has been found that engagement of CD40 on microglia results in the production of IL-12, a cytokine involved with Th1 cell development (Aloisi et al., 2000b; Becher et al., 2000a). Increased CD40 expression has been observed in MS lesions and it has been reported that blocking the CD40-CD40L interaction significantly reduces disease severity in EAE, a mouse model of MS (Gerritse et al., 1996). Similarly decreased microgliosis and astrocytosis has been observed in an APP mouse model of AD with deficiencies in CD40L (Tan et al., 2002). Townsend and colleagues (2005) demonstrated that CD40 shifted microglia away from a phagocytic phenotype to an APC phenotype when stimulated by Aβ. These studies found that, following CD40 ligation and the presence of Th1 cytokines, the expression of MHC class II was upregulated on microglia and these microglia were found to be co-localized with Aβ accumulations (Townsend et al., 2005).

1.2.5.4. TLRs

TLRs are expressed on both microglia (Applequist et al., 2002) and astrocytes (Bowman et al., 2003). Jack and colleagues (2005) observed the expression of TLR1-9 in human glial cells, and microglia had higher TLR expression levels than that of astrocytes. Additionally, studies have shown the expression of TLR co-receptor CD14 in microglial cell lines (Chen et al., 2006) and in human microglial cultures (Becher & Antel, 1996). Comparable to other
cell surface markers TLR expression is upregulated upon glial activation. TLR2 gene transcription is upregulated in several brain regions, including the circumventricular organs, following stimulation with gram-negative and gram-positive cell wall components (Laflamme et al., 2001). Zhou and colleagues (2008) observed enhanced TLR2 expression accompanied by enhanced MHC class II and co-stimulatory molecule expression, in both astrocytes and microglia, following lymphocytic choriomeningitis virus infection (LCMV). There is an extensive literature describing the impact of TLR activation on modulating the production of cytokines and chemokines by glial cells. Stimulation of human glial cultures with TLR agonists, LPS, Pam3Csk4 and a synthetic dsRNA (PIC), results in the secretion of pro-inflammatory cytokines IL-6 and TNFα as well as T cell polarizing cytokines IL-12 and IL-10 (Jack et al., 2005). Stimulation of murine microglial cultures with LPS and Pam3Csk4 results in the secretion of IL-6 and TNFα (Shah et al., 2009), as well as the release of nitric oxide (NO) (Ebert et al., 2005). Other studies have observed the secretion of chemokines in microglial cultures following TLR stimulation with fibronectin and increased phagocytic activity following co-stimulation of microglia with fibronectin and other TLR agonists (Ribes et al., 2010). Murine astrocyte cultures stimulated with TLR agonists secrete cytokines including IL-1β, TNFα, IL-6 and chemokines including macrophage inflammatory protein (MIP)-1β, MIP-2 and monocyte chemotactic protein (MCP)-1 (Esen et al., 2004).

Recent evidence suggests that chronic TLR expression, like other glial cell markers, may have a role in neurodegenerative diseases. TLR expression is increased in MS lesions and microglia isolated from MS patients express several TLRs (Bsibsi et al., 2002). Furthermore studies in EAE have found that TLR1, TLR2, TLR4, TLR6-9 expression is increased in the spinal cord of these animals accompanied by an increase in MyD88 (Prinz et al., 2006). Expression of TLR2 and TLR4 is increased in the brains of AD patients (Walter et al., 2007), and TLR2 and TLR7 expression (Letiembre et al., 2009), as well as CD14 overexpression (Fassbender et al., 2004) is observed in a mouse model of AD. Additionally studies from Letiembre and colleagues (2007) show an age-related increase in the expression of TLR1-7 and CD14 in mice.
1.2.6. Cytokines

Cytokines are a family of pleiotropic proteins that are involved in cell-to-cell communication and cellular activation. They have been described as immunoregulatory and neuromodulators and are subdivided into anti- and pro-inflammatory categories (Szelenyi, 2001). Cytokines are key regulators of innate and adaptive immune responses (Aloisi, 2001), yet chronic cytokine elevation leads to neuroinflammation and is a pathological feature of many neurodegenerative diseases. Cytokines and their receptors are expressed at low levels throughout the brain (Szelenyi, 2001) but are promptly upregulated in response to various environmental stimuli as a consequence of astrocytic (Dong & Benveniste, 2001; Carpentier et al., 2005) and microglial (Aloisi, 2001) activation, and infiltration of monocytes and activated lymphocytes (Ghirnikar et al., 1998).

1.2.6.1. TNFα

TNFα is a multipotent cytokine which is involved in the pathogenesis of inflammatory and autoimmune diseases (Probert et al., 2000). It is described as one of the master pro-inflammatory cytokines and is secreted by microglia and peripheral macrophages during CNS inflammation. TNFα binds to two receptors the TNFRI and TNFRII (Aloisi, 2001). The role of TNFα in CNS inflammation is compounded by its ability to induce cell adhesion molecules allowing for the recruitment of peripheral immune cells; Milner and Campbell (2003) observed that TNFα increased the expression of the integrins α4β1 and CD11b on microglia. TNFα is secreted in vitro in response to an array of stimuli including LPS (Szczepanik et al., 2001) and Pam3Csk4 (Jack et al., 2005; Shah et al., 2009). TNFα has been shown to collaborate with IFN-γ, a Th1 cell cytokine, in the induction of inducible NO synthase (iNOS) and NF-κB activation in astrocytes (Falsig et al., 2004; Hsiao et al., 2007) and microglia (Mir et al., 2008). Furthermore its secretion has been observed following Aβ treatment in mixed glial cultures (McQuillan et al., 2010) and in human macrophages and murine microglial cell lines (Szczepanik et al., 2001). Butovsky and colleagues (2005)
observed upregulated TNFα in rodent organotypic hippocampal slice cultures in response to both LPS and Aβ.

There is a vast amount of literature implicating a role for TNFα in disease pathology. TNFα is secreted by microglia and astrocytes in the AD brain (Lue et al., 2001a). TNFα expression is elevated in the cerebrospinal fluid (CSF) of MS patients (Sharief & Hentges, 1991), and is present in MS lesions as well as in EAE (Probert et al., 2000). While, some studies have suggested that TNFα may have a protective role in disease; Liu and colleagues (1998) observed that TNFΔ/Δ mice developed a more severe disease progression following myelin oligodendrocyte glycoprotein (MOG)-induced EAE.

1.2.6.2. IL-6

IL-6 is a pluripotent cytokine, with diverse biological function, that in recent years has received attention for its involvement in regulating inflammation and immunological responses. IL-6 has been found to be both beneficial and destructive, within the CNS, as it protects neurons from ischemic damage yet promotes astrocyte proliferation (Dong & Benveniste, 2001). IL-6 is expressed at low levels within the CNS however in inflammation, infection and disease, IL-6 levels become rapidly elevated (Van Wagoner & Benveniste, 1999). IL-6 binds to its plasma membrane receptor IL-6R resulting in the activation of signalling cascades JAK and MAPK (Heinrich et al., 2003). IL-6 is produced by glial cells and neurons and its synthesis can be increased by other cytokines, namely IL-1β and TNFα (Gadient & Otten, 1997) and IL-17 (Ma et al., 2010). Aβ increases the expression of IL-6 in murine mixed glia cultures (McQuillan et al., 2010) and in murine microglia cell lines (Szczepanik et al., 2001). Additionally Aβ, linked with advanced glycation endproducts (AGE), in combination with IFN-γ and LPS profoundly induces the secretion of IL-6 from murine microglia and to a far greater extent than any of the stimuli alone (Gasic-Milenkovic et al., 2003).

Like most cytokines, IL-6 dysregulation and overexpression has been implicated in several pathophysiological events (Gadient & Otten, 1997). IL-6 expression is elevated in the CSF of both patients with AD and PD (Blum-Degen et al., 1995). IL-6 has also been shown to have a role in EAE; IL-6-deficient mice are resistant to EAE (Eugster et al., 1998). Because of the dual effects of IL-6 it is
unclear if IL-6 is causative in these neurodegenerative diseases or is merely a reflection of the ongoing chronic inflammation as a result of progressive neuronal damage (Gadient & Otten, 1997).

1.2.6.3. IL-1β

IL-1β was first described in the 1980s by several laboratories working on inflammation. It has been described as a key mediator of immune responses and has been found to affect most cell types within the body resulting in inflammation (O'Neill, 2008). IL-1β belongs to the family of IL-1 which includes IL-1α. It binds to its receptor IL-1 type I receptor (IL-1R1) expressed on an array of cells, resulting in the release of other pro-inflammatory cytokines including IL-6 and TNFα (Mills & Dunne, 2009). IL-1β is formed as pro-IL-β and then cleaved by caspase-1 to produce the activated form of IL-1β (Mills & Dunne, 2009). IL-1β is produced by neuronal and glial cells and is released after their stimulation due to injury, insult or stress (Murray & Lynch, 1998). IL-1β can trigger neuronal cell death and, interestingly, the presence of glial cells is required for IL-1β-induced neuronal cell death (Thornton et al., 2006). Specifically Thornton and colleagues (2006) observed that astrocytes were the primary mediators of IL-1β-induced neuronal death through the release of free radicals and this effect was blocked by IL-1RA the endogenous antagonist for the IL-1R1. IL-1β concentrations are increased in both aged and stressed animals as well as animals treated with IL-1β itself (Murray & Lynch, 1998). In addition, an age-related increase in IL-1β expression and concentration is associated with an age-related increase in caspase-1 activity (Lynch et al., 2007). Furthermore, IL-1β expression is increased in the response to LPS and is significantly exacerbated in aged animals in response to LPS (Henry et al., 2009).

There is evidence implicating IL-1β in neurodegenerative diseases; microglia expressing IL-1 are found in association with plaques in brain tissue and this expression correlates with neuronal death (Mrak, 2012). IL-1β is increased in the hippocampus of wildtype mice (Zaheer et al., 2008), and in murine microglial cultures, following Aβ stimulation (Szczepanik et al., 2001). Studies in IL-1R1-deficient mice suggest a role for IL-1 in mediating neurodegenerative autoimmune diseases; it has been shown that IL-1β in combination with IL-23 promotes Th17
cell expansion, a T cell subtype involved in MS, and the secretion of IL-17 (Sutton et al., 2006).

1.2.7. Chemokines

The infiltration of leukocytes from the periphery into the CNS is crucial for host defense responses as well as the initiation and maintenance of CNS autoimmunity (Aloisi, 2001). Chemokines are 8-14 kDa plurifunctional secreted proteins that are instrumental in this process of leukocyte trafficking (Cartier et al., 2005). Currently there are more than 40 known human chemokines and 19 known G-protein coupled chemokine receptors. Chemokines are divided into 4 subfamilies (CXC, CC, C and CX3C) based on the sequence of their conserved cysteine residue but are also classified into inflammatory or homeostatic according to their function (Aloisi, 2001; Cartier et al., 2005). Inflammatory chemokines are produced in response to cytokines and recruit monocytes, granulocytes and T cells to sites of inflammation. Homeostatic chemokines are expressed in discrete environments and recruit cells of the adaptive immune system (Cartier et al., 2005). During CNS inflammation, several cell types are known to produce chemokines including endothelial cells, astrocytes and microglia (Aloisi, 2001). Chemokines play an important role in the development of CNS pathologies; chemokines and chemokine receptor expression upregulated in neurodegenerative diseases allowing for increased leukocyte infiltration and enhanced inflammation (Cartier et al., 2005). Our knowledge of chemokine production by microglia and astrocytes has been greatly facilitated by studies in MS and EAE (Owens et al., 2005). These studies have established that different chemokines may have diverse roles in disease mechanisms (Tran et al., 2000a). However it must be noted that chemokines also have neuroprotective effects, such as fractalkine which inhibits microglial activation (Biber et al., 2007).

1.2.7.1. IP-10

IP-10, also known as CXCL10, is secreted during inflammation by cells such as monocytes, lymphocytes, neutrophils and endothelial cells (Xia et al., 2000). IP-10 binds to its G coupled receptor, CXCR3, expressed on activated T
cells. Several stimuli increase its expression; IP-10 mRNA expression is upregulated in response to IFN-γ (Xia et al., 2000). Furthermore, Aβ treatment significantly increases the expression of IP-10 hippocampal concentrations in rodents (Clarke et al., 2007). IP-10 plays a significant role in the recruitment of T cells to sites of inflammation and IP-10 expression correlates with T cell infiltration into diseased tissue (Dufour et al., 2002). It has been shown that IP-10−/− mice, infected with hepatitis virus, have decreased cell infiltration into the CNS associated with decreased IFN-γ expression and levels of demyelination (Dufour et al., 2002).

Studies have suggested a significant role for IP-10 in neurodegenerative diseases (Cartier et al., 2005). Xia and colleagues (2000) observed an increase in IP-10-positive astrocytes in post-mortem tissue from AD patients and a frequent association of these IP-10-positive astrocytes with Aβ deposits. In MS, IP-10 is significantly elevated in the CSF of MS patients (Sorensen et al., 1999), as well as in the CNS of animal undergoing MOG-induced EAE (Tran et al., 2000a).

1.2.7.2. MCP-1

MCP-1, also known as CCL2, recruits monocytes, memory T cells and dendritic cells to the site of injury. It binds to its receptor, CCR2, which is expressed on active monocytes, endothelial cells and leukocytes. MCP-1 was one of the first chemokines to be identified and associated with inflammation (Mahad et al., 2006). Studies have implicated MCP-1 not only in leukocyte recruitment but also in BBB disruption; MCP-1 binding to its receptor expressed on endothelial cells, alters endothelial tight junction structure, resulting in increased BBB permeability following ischemic inflammation (Dimitrijevic et al., 2006). Microglia and astrocytes are prominent sources of MCP-1; glial cells produce MCP-1 following axonal injury and this is associated with the infiltration of leukocytes into the CNS (Babcock et al., 2003). Several different stimuli have been shown to increase MCP-1 expression. MCP-1 expression is increased in progenitor-derived astrocytes and neurons in response to the pro-inflammatory cytokines IFN-γ and TNFα (Lawrence et al., 2006). In addition MCP-1 has been shown to be increased in rodent hippocampal tissue following intracerebroventricular injection of Aβ (Clarke et al., 2007). Furthermore, it has
been shown that Aβ can upregulate the expression of MCP-1 receptor on endothelial cells and trigger T cell infiltration (Li et al., 2009).

Evidence suggests that MCP-1 has a role in neurodegenerative diseases. Acute and active MS lesions display MCP-1 and MCP-2 immunoreactivity in post-mortem tissue from MS patients (McManus et al., 1998), and MCP-1 is observed in the CNS of MOG-induced EAE (Tran et al., 2000a). MCP-1 has been reported in mature senile plaques (Ishizuka et al., 1997), and in CSF samples from AD patients (Galimberti et al., 2003).

1.2.7.3. RANTES and MIP-1α

RANTES, also known as CCL5, is involved in the recruitment of granulocytes, monocytes and effector T cells to sites of inflammation (Cartier et al., 2005). Its receptor, CCR5, also acts as a receptor for MIP-1α, and is expressed by activated/memory T cells and upregulated in inflammatory conditions; T cells expression of CCR5 is increased in rheumatoid synovial fluid (Qin et al., 1998). Both astrocytes and microglia produce RANTES following LCMV infection (Zhou et al., 2008). The expression of RANTES is increased following entorhinodentate axotomy (Babcock et al., 2003), as well as traumatic brain injury (Ghirnikar et al., 1998).

Like MCP-1 and IP-10, both RANTES and MIP-1α are associated with neurodegenerative diseases. RANTES is increased in the CSF of MS patients (Tran et al., 2000a), and its receptor has been detected on lymphocytic cells, macrophages and microglia in actively demyelinating MS lesions (Sorensen et al., 1999). CCR5 expression is increased on reactive microglia in AD and this is associated with an increase in MIP-1α expression on reactive astrocytes which are found in association with Aβ deposits (Cartier et al., 2005). Furthermore microglia isolated from AD brain tissue treated with Aβ increase the production of MIP-1α (Lue et al., 2001a).

1.2.8. Modulators of microglial activation: Maintaining microglial quiescence

Many different factors, including secreted factors and cell surface receptors, play a role in modulating the inflammatory and activated states of
microglia. There is extensive literature reviewing the role that neurons play in delivering "on" and "off" signals to microglia. For example CD200, fractalkine, CD47 and high mobility group box 1 (HMGB1) expressed on healthy neurons antagonise the pro-inflammatory activity of microglia, while damaged or dying neurons are thought to produce chemokines that activate microglia (Biber et al., 2007).

1.2.8.1. Fractalkine

Fractalkine, also known as CX3CL1, is a chemokine which is expressed on neurons and its receptor, CX3CR, which is expressed mainly on microglia (Lynch, 2009). Fractalkine can exist as a membrane-bound and soluble form. The membrane bound form displays adhesion properties while, the soluble form is involved in chemotaxis (Gemma et al., 2010). It has been shown that both the soluble and the membrane form of fractalkine can attenuate LPS-induced microglial activation (Lyons et al., 2009a). These authors also demonstrated an age-related decrease in fractalkine expression correlated with an upregulation in CD40 and MHC class II expression and the production of IL-1β. Gemma and colleagues (2010) proposed that decreased fractalkine with ageing results in increased microglial activation and cognitive dysfunction. These authors found that administering soluble fractalkine to 22 month-old rats resulted in decreased MHC class II expression and increased neurogenesis in these animals. Interestingly a decrease in plasma levels of fractalkine has been observed in patients with AD (Kim et al., 2008), and APP transgenic mice display decreased expression of neuronal fractalkine (Duan et al., 2008).

1.2.9. CD200

1.2.9.1. CD200 and CD200R

CD200, previously known as OX2, is a 41-47 kDa type-1 cell surface glycoprotein with two immunoglobulin domains and looks like the TCR β sheet chain or an Ig light chain with a membrane integration segment (Clark et al., 1985). CD200 is unable to signal due to its short cytoplasmic tail and the absence
of any known signalling motifs (Mukhopadhyay et al., 2010). The structure of CD200 is closely related to the T cell co-stimulatory molecules CD80 and CD86 (Jenmalm et al., 2006). CD200 is widely expressed within the CNS including on neurons, endothelial cells, activated T cells (Broderick et al., 2002; Jenmalm et al., 2006) and astrocytes (Costello et al., 2011). CD200 interacts with CD200R expressed on cells of the myeloid lineage such as microglia and macrophages (Meuth et al., 2008). The structure of CD200R is similar to that of CD200 but it contains a larger cytoplasmic tail for signalling (Wright et al., 2000). Currently five CD200Rs have been reported in mice (CD200R1-5) (Gorczynski et al., 2004). The CD200-CD200R interaction is believed to protect the CNS from excessive inflammation by exerting an inhibitory signal suppressing IFN-γ-induced proliferation, activation and secretion of NO, IL-6 and IL-13 by cells (Feuer, 2007; Meuth et al., 2008). Furthermore, it has been demonstrated that disruption of this interaction between CD200-CD200R results in increased microglial activation and neuroinflammation (Masocha, 2009).

1.2.9.2. CD200 signalling

CD200R signalling differs from that of most immune inhibitory receptors, in that it lacks the cytoplasmic acid sequence termed immunoreceptor tyrosine-based inhibitory motif (ITIM), and contains an NPxY sequence in its cytoplasmic domain instead (Zhang et al., 2004). The CD200 signalling pathway has not yet been fully elucidated, although it is proposed that the NPxY motifs interact with adaptor molecules through their phosphotyrosine-binding (PTB) domains. The NPxY motif contains three tyrosine residues as potential phosphorylation sites following CD200-CD200R engagement (Snelgrove et al., 2008). Once phosphorylated adaptor molecules downstream of tyrosine kinase (Dok) 1 and Dok 2, are recruited and in turn are phosphorylated and associate with Ras GTPase-activating protein (RasGAP) and scr homology 2 domain containing phosphatases (SHIP) (Snelgrove et al., 2008). Dok2 primarily recruits RasGAP and non-catalytic region of tyrosine kinase adaptor protein 1 (Nck) while, Dok1 preferentially recruits Crk-like protein (CrkL) (Mihrshahi & Brown, 2010). The downstream signalling events result in the inhibition of the Ras/MAPK pathway including extracellular signal-regulated kinase (ERK), p38 MAPK and JNK.
(Zhang et al., 2004). Ultimately this inhibition of signalling cascades results in the decrease in the production of inflammatory cytokines. Figure 1.4 illustrates the structure of CD200 and its receptor and the signalling cascade initiated following CD200-CD200R interaction.
Figure 1.4. The structure of CD200 and CD200R and the signalling pathway initiated following CD200-CD200R interaction.

CD200 has a short cytoplasmic tail while the CD200R has a longer cytoplasmic tail which contains 3 tyrosine residues which when phosphorylated recruit Dok 1 and Dok 2, in turn these activate SHIP and RasGAP resulting in the inhibition of MAP kinases and inhibition of cytokine production.

Adapted from Miller et al., (2011)
The hypothesis that CD200 binding CD200R induces immune suppression was originally proposed by Hoek and colleagues (2000). Most of the studies which have evaluated the role of CD200 have come from the deletion of the CD200 gene in mice. These CD200$^{-/-}$ mice have a normal lifespan and are grossly normal in appearance but are more susceptible to autoimmune diseases (Hoek et al., 2000). Some of the earliest studies demonstrated that microglia obtained from CD200$^{-/-}$ mice have increased activity compared to microglia obtained from healthy wildtype animals. Hoek and colleagues (2000) used several different models, including facial nerve transection model, EAE and collagen-induced arthritis (CIA), to examine the effect of CD200 on disease progression; the data showed that CD200$^{-/-}$ mice had enhanced microglial responses and activation levels compared with wildtype animals. It was hypothesized that the increase in myeloid activation in CD200$^{-/-}$ animals was due to the lack of inhibitory signalling through CD200R (Hoek et al., 2000).

It has been established that in vivo disruption of the CD200-CD200R interaction aggravated the clinical course and outcome of EAE in rats (Meuth et al., 2008). These authors reported that neutralizing the CD200-CD200R interaction significantly amplify IFN-γ-induced IL-6 response by macrophages. Furthermore it was observed that disruption of the CD200-CD200R interaction was accompanied by a significant increase in infiltrating immune cells into the spinal cord of MOG-induced EAE animals (Meuth et al., 2008). While studies in Wld$^\#$ mice, which exhibit unique protection against neurodegenerative conditions like EAE, have been shown to overexpress CD200 possibly due to impaired protein ubiquitination and degradation (Chitnis et al., 2007). Chitnis and colleagues (2007) observed that increased CD200 expression was able to protect neurons and axon, both in vitro and in vivo, from microglia-induced damage.

The importance of the CD200-CD200R interaction in maintaining microglial quiescence has also been confirmed by the use of CD200R antibodies and by CD200 fusion protein (CD200Fc). It was established that a rat anti-mouse CD200R monoclonal antibody (DX109), which activated CD200 signalling, suppressed experimental autoimmune uveoretinitis (EAU) progression while, CD200$^{-/-}$ mice displayed early onset EAU (Copland et al., 2007). These results
were in consensus with an earlier report from Broderick and colleagues (2002) who reported that CD200<sup>−/−</sup> mice had accelerated onset of EAU associated with increased microglial activation, and increased number of infiltrating cells resulting in photoreceptor death. Similarly in CIA it was found that CD200Fc not only suppressed CIA but also caused decreased levels of TNFα and IFN-γ both of which drive myeloid cell activation and CIA disease progression (Gorczynski <i>et al.</i>, 2002a). More recent evidence, using an influenza model, highlighted the use of CD200Fc and an agonist antibody to CD200R as a method of resolving inflammation in cases of extensive inflammation and myeloid activation (Snelgrove <i>et al.</i>, 2008). These authors reported that a nonlethal dose of the influenza virus delivered to CD200<sup>−/−</sup> mice resulted in death. Delivery of CD200Fc or OX110, an agonist monoclonal antibody to CD200R, to mice following influenza infection did not compromise viral clearance but decreased the expression of IFN-γ and TNFα in the airways and lung tissue of these animals (Snelgrove <i>et al.</i>, 2008).

From the evidence published to date it is clear that the CD200-CD200R interaction indeed plays a key role in immunoregulation. The presence of CD200 in immune-privileged sites and sites accessible to antigen challenge indicates that the CD200-CD200R interaction is involved in anti-inflammatory responses at these specific sites (Minas & Liversidge, 2006).

1.2.9.4. The role of CD200 in neurodegeneration

Several lines of evidence now suggest that the loss of CD200 may contribute to some of the microglial-induced inflammatory changes seen in neurodegenerative diseases like MS and AD. Most neurodegenerative diseases are characterized by an uncontrolled neuroinflammatory environment which has been attributed largely to microglial activation. The majority of these neurodegenerative disorders, but not all, are age-related diseases and evidence suggests that CD200 expression is decreased with age; CD200 expression is decreased in the hippocampus of aged rats accompanied by increased expression of MHC class II and IFN-γ (Frank <i>et al.</i>, 2006).

A role for CD200 has also been described in AD. Studies have found decreased expression of CD200 and CD200R in post-mortem brain tissue from
AD patients (Walker et al., 2009). Specifically, these authors describe a deficit in neuronal CD200 expression in brain areas affected by AD pathology. Though it is possible that this is due to a decrease in viable neurons as a result of neurodegeneration (Masocha, 2009). Furthermore, microglial activation in the hippocampus of aged and Aβ-treated rats correlates with a decrease in neuronal CD200 expression (Lyons et al., 2007). These studies reported that CD200-CD200R interaction decreased expression of MHC class II and the accompanied production of pro-inflammatory cytokines by Aβ-treated glia (Lyons et al., 2007). These authors suggested that the expression of CD200 may be modulated by the anti-inflammatory cytokine IL-4; it was found that IL-4 markedly increases CD200 expression in vitro and that IL-4^{-/-} mice have exacerbated microglial activation associated with a decrease in CD200 expression (Lyons et al., 2007).

Studies in post-mortem brain tissue from MS patients have demonstrated that both the expression of CD200 and its receptor could be found in peri-plaque regions of human MS (Meuth et al., 2008). More importantly, it has been found that the expression of both CD200 and CD47, another macrophage modulatory protein, are downregulated in human MS lesions (Koning et al., 2007). Together, these findings have suggested that enhancing the CD200-CD200R interaction may have therapeutic potential in neurodegenerative diseases such as AD and MS.

1.3. AD

AD is a devastating age-dependent neurodegenerative disease that is the most frequent cause of dementia in the ageing population. The majority of AD cases are sporadic with risk factors including head injury, stroke and even diet although about 5% of all AD cases are familial (FAD). FAD cases have a rare autosomal dominant mutation in genes that effect the expression levels of amyloid precursor protein (APP), presenilin (PS) 1 and 2 genes (Wyss-Coray, 2006). AD is characterised by progressive cognitive decline and memory loss associated with dramatic neuronal death and loss of synapses particularly in brain regions critical for learning and memory including the hippocampus, cerebral cortex and basal forebrain (Nagele et al., 2004). The neuropathological hallmarks include neurofibrillary tau tangles, neuritic plaques, reactive gliosis and inflammation (Nagele et al., 2004). Neurofibrillary tau tangles are inclusions within neurons.
which consist of hyperphosphorylated tau, a protein associated with microtubules, which assembles into paired helical filaments. Neuritic plaques are composed of a dense core of Aβ fibrils, dystrophic neurons, microglia and astrocytes (Benveniste et al., 2001; Nagele et al., 2004).

The identification of these features of AD lead to the development of several hypotheses suggesting the mechanisms by which neurodegeneration occurs in AD including the “Aβ hypothesis”. The “Aβ hypothesis” suggests that an imbalance between the production and clearance of Aβ in the brain gives rise to the accumulation and the formation of Aβ plaques, exacerbated glial activation, prolonged inflammation and neuronal loss which ultimately causes cognitive decline (Karran et al., 2011). The basis of this hypothesis has greatly influenced the research of current AD therapies especially investigating mechanisms which may aid the removal of Aβ or inhibit its overproduction (Karran et al., 2011).

1.3.1. Aβ; processing, production and overproduction

The fact that Aβ accumulates in the brain before disease onset, and that Down syndrome patients that have tri-chromosome 21, suffer from AD suggests a role for Aβ as a mediator of AD (Farfara et al., 2008). Aβ peptides are natural occurring products of metabolism made up of 36-43 amino acids (Querfurth & LaFerla, 2010). Aβ is a 4kD peptide which maps to chromosome 21 and is highly expressed on neuronal cells (Farfara et al., 2008). It is generated from the type I integral membrane protein, APP. APP is cleaved by α- and β-secretase (also known as BACE1) to generate two C-terminal fragments αAPP and βAPP which become inserted into the membrane (Sastre et al., 2008). These fragments are cleaved by the γ-secretase complex (consisting of; PS1, nicastrin, anterior pharynx defective 1 (APH-1) and presenilin enhancer 2 (PEN-2)) resulting in the release of two peptides, P3 and Aβ (Walsh & Selkoe, 2007; Sastre et al., 2008). When βAPP is cleaved by γ-secretase at different points, three types of Aβ namely Aβ1-38, Aβ1-40 and Aβ1-42 are formed (Walsh & Selkoe, 2007). It is the overproduction of all three Aβ peptides, or a bias towards production of Aβ1-42, that is implicated in AD (Walsh & Selkoe, 2007).

Aβ self-aggregates into several coexisting physical forms including oligomers which can form into intermediate assemblies and fibrils, ultimately
forming insoluble fibrils when arranged as β-pleated sheets (Querfurth & LaFerla, 2010). It has been suggested that the assembly state of the Aβ peptide is central in determining the microglial response induced; Aβ1-42 oligomers and fibrils are actively taken up by microglia and induce neuronal toxicity while Aβ1-40 oligomers are not taken up by microglia nor do they induce neuronal toxicity yet they induce cytokine expression by microglia (Parvathy et al., 2009).

Overproduction of Aβ is attributed to altered activation of the enzymes that process APP and several factors have been shown to be involved in altering these enzymes. BACE1 activity increases with age in both mouse models of AD and non-transgenic mouse models (Fukumoto et al., 2004); knocking out BACE1 in APP transgenic mice reduces Aβ production (Ohno et al., 2004). Additionally, factors associated with increased microglial activation alter BACE1; TNFα upregulates BACE1 expression in cultured astrocytes and this effect can be blocked by anti-TNFα antibodies (Yamamoto et al., 2007), while oxidative stress alters PS1 and BACE1 activity (Tamagno et al., 2008).

1.3.2. Role of the innate and adaptive immune system in AD

Neuroinflammatory changes are observed in both familial and sporadic cases of AD (McGeer & McGeer, 2003). Persuasive evidence that the inflammatory process plays an important role in AD comes from studies in which patient populations, treated with non-steroidal anti-inflammatory drugs (NSAIDs), have decreased incidence of AD (Bamberger & Landreth, 2001). Furthermore, administration of NSAIDs to transgenic animal models of AD significantly decreases the amount of reactive microglia in association with Aβ-plaques (Bamberger & Landreth, 2001).

1.3.2.1. Role of Microglia in AD

Although microglia provide the first line of defense in acute situations the function of microglia in the case of chronic situations like AD, is not clear (Streit, 2005). Microglial activation appears to occur in a very early stage of disease even prior to cognitive decline (McGeer & McGeer, 2003). It has been suggested that increased microglial activation, may be accounted for by an age-related decrease
in the neuronal-glial interactions, which maintain microglia in a quiescent state (Biber et al., 2007). One such interaction that may contribute to this is the CD200-CD200R interaction which is altered with age (Lyons et al., 2007) and in AD (Walker et al., 2009).

While activated microglia are found in close proximity to Aβ-plaques in mouse models of AD (Bornemann et al., 2001) and in the brains of AD patients (McGeer et al., 1993), one of the central paradoxes surrounding the role of microglia in AD is their failure to phagocytose Aβ deposits (Bamberger & Landreth, 2001). It has been proposed by Streit (2004) that, in the AD brain, microglia are “dysfunctional” rather than activated and this results in decreased plaque removal and increased production of inflammatory mediators. Thus it has been suggested that activating microglia into a phagocytic phenotype may have beneficial effects in plaque clearance; administration of anti-Aβ antibodies results in decreased plaque load in Tg2576 mice (Wilcock et al., 2004). Similarly following systemic LPS injection, increased microglial phagocytic activation and an associated clearance of diffuse Aβ, but not that of fibrillar Aβ, has been observed in Tg2576 mice (Herber et al., 2007). In contrast, decreased microglial and astrocytic activity is associated with decreased plaque number, Aβ concentration and phosphorylated tau protein in APP transgenic iNOS−/− mice (Nathan et al., 2005).

There is extensive literature describing the effects of Aβ peptides in modulating glial activation both in vivo and in vitro; Aβ induces the production of cytokines, chemokines and the upregulation of APC molecules in vitro (Szczepeanik et al., 2001; Jana et al., 2008; McQuillan et al., 2010) and in vivo (Clarke et al., 2007). The Aβ-induced effect on glial activation is even greater in the presence of augmenting factors such as LPS and IFN-γ (Gasic-Milenkovic et al., 2003). Despite these studies, the mechanisms by which Aβ-induces microglial activation are poorly understood. It has been suggested that Aβ mediates its interaction with microglia through a receptor complex consisting of αvβ3 integrin, CD47, CD36 and the scavenger receptor A (Bamberger et al., 2003). However, another mechanism which is receiving recent consideration is the interaction of TLRs with Aβ. There is a growing body of evidence suggesting that these innate immune receptors, which have significant effects on tailoring glial activation
(Jack et al., 2005), may play a critical role in the pathogenesis of AD (Liu et al., 2012).

1.3.2.2. A role for TLRs in AD

Expression of TLRs and the TLR co-receptor CD14 (Fassbender et al., 2004) is increased in the brains of AD patients (Walter et al., 2007) and in animal models of AD (Letiembre et al., 2009). Furthermore, increased expression of CD14 in areas of the brain which are pathophysiologically relevant to AD has been observed (Liu et al., 2005). The increased expression of TLRs in AD places them as prospective players in neurodegenerative mechanisms and disease progression (Okun et al., 2009). However, the role of TLRs in AD is unclear with reports arguing both beneficial and destructive effects through their ability to tailor microglial responses. Several recent reports have observed that TLR2, TLR4 (Udan et al., 2008), and CD14 deficiency significantly reduces Aβ-induced microglial activation (Reed-Geaghan et al., 2009). Consistently, Aβ1-42 mediates pro-inflammatory cytokine production by microglia and increased expression of integrin markers through its interaction with TLR2 and this effect is blocked in TLR2−/− mice (Jana et al., 2008). Liu and colleagues (2012) established that microglia can interact with Aβ through TLR2/TLR1 heterodimer to induce pro-inflammatory cytokine production and that TLR2 deficiency shifts microglia away from an inflammatory state to a phagocytic phenotype. In addition, it has been observed that Aβ-induced microglial activation via TLR2, TLR4 and CD14 initiates activation of MAP kinases and the induction of phagocytosis, reactive oxygen species (ROS) and NF-κB (Reed-Geaghan et al., 2009). CD14 deficiency or neutralization of CD14 with antibody significantly decreases Aβ-induced microglial production of cytokines and nitric oxide (Fassbender et al., 2004). In addition, TLR2-deficient APP/PS1 mice exhibit decreased plaque burden at 6 months of age although they have comparable plaque burden to wildtype animals by 9 months of age (Richard et al., 2008).

In vitro studies suggest that activation of TLR2, TLR4 and TLR9 with respective ligands PGN, LPS and CpG-ODN, increases the removal of Aβ from cultures (Tahara et al., 2006). These authors also observed increased plaque burden in TLR4 mutant APP/PS1 mice at 14-16 months of age. The current
literature suggests that a better understanding of the interaction of innate immune TLRs with Aβ in AD may be useful in establishing effective therapeutic strategies.

1.3.2.3. A role for T cells in AD

While peripheral T cells were not traditionally associated with the progression of AD, this view has changed following several observations. T cells are present in the AD brain in a greater number than healthy individuals (Rogers et al., 1988; Togo et al., 2002; Monsonego et al., 2003), particularly in areas of the brain that are devastated during AD such as the hippocampus and limbic structures (Togo et al., 2002). Furthermore, these studies have established that these T cells are activated and in the process of becoming effector T cells (Togo et al., 2002). However, the role of T cells in disease pathology is still unclear with contradictory reports in the literature suggesting both beneficial and detrimental effects by T cell subtypes (Th1, Th2 and Th17). The possibility that T cell activation is influenced by their interaction with glia, and vice versa, needs to be explored.

A maladaptive role for T cells is mainly attributed to Th1 cells and their signature cytokine, IFN-γ, as well as Th17 cells. Th1 cells interact with microglia and upregulate the expression of antigen presentation machinery, co-stimulatory molecules and adhesion molecules resulting in Th1 cell activation (Aloisi et al., 2000a). Similarly, studies from McQuillan and colleagues (2010) observed that both Aβ-specific Th1 and Th17 cells increased the expression of MHC class II and co-stimulatory molecules and an associated increase in the release of pro-inflammatory cytokine production. Supernatants from Th1 cells upregulate a similar pattern of antigen presentation molecules on microglia and the secretion of cytokines and chemokines and these effects can be blocked by anti-IFN-γ treatment (Seguin et al., 2003). In addition treatment of microglial cultures with CD40L and IFN-γ increased chemokine secretion (D'Aversa et al., 2002), as well as inhibiting microglial uptake of Aβ1-42 (Townsend et al., 2005); interestingly the effect of IFN-γ and IL-17 on glial activation is greater in the presence of Aβ (McQuillan et al., 2010) Furthermore, IFN-γ deficient APP mice have significantly reduced Aβ deposition in the cortex and hippocampus compared with
controls and this correlated with decreased astrogliosis and microgliosis (Yamamoto et al., 2007).

T cells may have a beneficial effect through enhanced Aβ clearance and decreased production of glial inflammatory mediators. *In vitro* studies have suggested that Th2 cells and Th2 cell-secreted cytokines fail to induce antigen presentation machinery on microglia (Aloisi et al., 2000a; Seguin et al., 2003) but can enhance microglial uptake of Aβ1-42 (Townsend et al., 2005). IL-4 has been shown to selectively inhibit the secretion of IL-1β and IL-6 from microglia stimulated with Aβ or LPS (Szczepanik et al., 2001). In addition Th2 cells can attenuate Th1 and Th17-induced glial activation (McQuillan et al., 2010). Moreover, APP/PS1 mice which received Aβ-stimulated T cells exhibit decreased numbers of plaque-associated microglia and decreased cognitive decline, although interestingly this effect was associated with Th2 cells, and not Th1 cells, suggesting that different T cell subsets mediate different functions in AD (Ethell et al., 2006). Cao and colleagues (2009) observed decreased pro-inflammatory cytokine production and plaque-associated microglia in APP/PS1 mice treated with Aβ-specific Th2 cells.

1.3.3. Transgenic mouse models of AD

Transgenic mouse models that have behavioral and pathological aspects of AD have enabled the generation and testing of therapies for AD (Gelinas et al., 2004). The identification of genes that encode APP, PS1 and PS2 has revolutionized the use of animal models of AD and lead to the generation of several transgenic mouse models. However, these models only exhibit some of the pathological features including Aβ deposition and progressive memory loss, but they lack neurofibrillary tau tangles and neuronal loss (Ashe, 2001). Although it is possible to generate mouse models with both amyloid plaques and neurofibrillary tangles these animals are not suitable for investigating learning and memory as they become paralyzed (Ashe, 2001). The transgenic models currently used are based on mutations in APP, PS1 and PS2 resulting in a greater production and aggregation of Aβ peptides. These genes include the “Swedish” mutation which occurs at the β-secretase site and increases the production of Aβ1-40 and Aβ1-42 through preferential cleavage of APP by β-secretase rather than α-secretase.
Mutations in PS1 and PS2, which play a critical role in \( \gamma \)-secretase activity, also results in the overproduction of \( \alpha\beta_{1-42} \) (Higgins & Jacobsen, 2003).

The first transgenic model generated displaying neuritic \( \alpha\beta \)-plaques was the PDAPP mouse which overexpressed APP containing the V717F mutation. These animals have accelerated production of \( \alpha\beta_{1-40} \) and \( \alpha\beta_{1-42} \) resulting in plaque deposition, astrogliosis and microgliosis (Games et al., 1995). Several other mouse models with mutations in APP exist including the Tg2576 model which was first examined by Hsaio and colleagues (1996), and is one of the most widely-studied mouse model of AD. The Tg2576 expresses the APP695 isoform with the Swedish mutation under the control of the prion protein promoter (Higgins & Jacobsen, 2003). These mice display increased \( \alpha\beta \) production coupled with cognitive impairment in spatial learning tasks (Hsiao et al., 1996). The APP23 developed at Novartis expresses the APP751 isoform with the Swedish mutation under the control of a Thy1 promoter (Higgins & Jacobsen, 2003). Sturchler-Pierrat and colleagues (1997), reported that these APP23 mice display similar features to both the PDAPP and Tg2576 mouse model, including neuritic plaque deposition, astrogliosis and microgliosis particularly in areas devastated in AD including the hippocampus and neocortex.

Following on from these single transgenic models was the development of double transgenic models. Duff and colleagues (1996) reported that mice overexpressing a mutant PS1 gene had accelerated production of \( \alpha\beta_{1-42} \). It was found that crossing Tg2576 mice with mice that overexpressed mutant PS1 (PSAPP) had accelerated plaque development and substantial gliosis by 6 months of age, far earlier than the single transgenic mice (Holcomb et al., 1998). A more recent double transgenic mouse model the APPswe/PS1dE9, and the model of choice in this study, is perhaps the model of preference for AD. The APPswe/PS1dE9 model is generated by co-injection of plasmid constructs encoding the APP695 isoform and the Swedish mutation with exon-9 deleted into pronuclei. These genes are driven by an independent prion protein promoter (Jankowsky et al., 2001). These mice display enhanced \( \alpha\beta_{1-42} \) production and exacerbated plaque burden in the cortex and hippocampus by 6 months of age (Garcia-Alloza et al., 2006). Garcia-Alloza and colleagues (2006) observed increased ratio of insoluble \( \alpha\beta_{1-42} / \alpha\beta_{1-40} \) as well as diffuse \( \alpha\beta \) deposition around

\[ \sim 40 \sim \]
compact Aβ-plaques contributing to the increased size of plaques in mice by 8 months of age. Behavioral testing of these mice indicates significant deficits in spatial memory as assessed by the water maze (Cao et al., 2007).

1.4. Study aims

The aims of this study were:

• To investigate the modulation of glial activation by CD200-CD200R interaction following TLR stimulation in vitro and in vivo.

• To investigate if Aβ-induced glial activation could be modulated by TLR2.

• To investigate the interaction between T cells and glia in vitro and establish if specific T cell subtypes could modulate glial activation.

• To investigate the role of T cells in modulating microglial activation in a mouse model of AD.
Chapter 2

Methods
Chapter 2

2.1. Preparation, treatment and harvesting of primary glia

2.1.1. Preparation of primary mixed glia

Primary glial cells were prepared from 1-day-old wildtype (C57BL/6) and CD200\(^{−/−}\) mice (Bioresource Unit, Trinity College, Dublin 2, Ireland). Neonates were sacrificed by decapitation and the brains were removed into a sterile petri-dish (Fisher Scientific, UK). The tissue was bi-directionally chopped using a sterile scalpel (Swann-Morton, UK) and transferred to 15 ml falcon tubes (Fisher Scientific, UK) containing warm Dulbecco's Modified Eagle Medium (DMEM; 2 ml; Gibco, UK) containing penicillin (100 \(\mu\)g/ml; Gibco, UK) and fetal bovine serum (FBS; 10 % w/v; Gibco, UK). The tissue was incubated for 5 minutes in 5 % CO\(_2\) at 37 °C in a Nuaire Flow CO\(_2\) incubator (Jencons, UK). Using sterile Pasteur pipettes the tissue was filtered through 50 \(\mu\)l sterile nylon mesh filters (BD Bioscience, USA) into 50 ml falcon tubes (Fisher Scientific, UK). The tubes were centrifuged at 1200 x g for 5 minutes at 20 °C, the supernatant was discarded, and the remaining pellet reconstituted in warm DMEM (6 ml). The resuspended glia were plated in 6-well plates (Fisher Scientific, UK) and incubated for 12-14 days in 5 % CO\(_2\) at 37 °C. DMEM was replaced with fresh media every 2-3 days until the glia were ready for treatment.

2.1.2. Preparation of primary astrocytes and microglia

Primary glial cultures were prepared from 1-day-old wildtype mice as per section 2.1.1 with the exception that the mixed glia were maintained in T25 flasks (Fisher Scientific, UK). After 12 days glia were separated by shaking at 110 rpm for 2 hours at room temperature (RT). Supernatants were removed to 50 ml falcon tubes and centrifuged at 1200 x g for 5 minutes at 20 °C. The supernatant was discarded and the remaining pellet was reconstituted in warm DMEM (1 ml) and viable microglial cells were counted using trypan blue (Sigma-Aldrich, UK). Microglia were plated in 24-well plates (1 x 10\(^5\) cells/well: Fisher Scientific, UK) and incubated for 24 hours in 5 % CO\(_2\) at 37 °C before co-culture with specific T
cells. Astrocytes were harvested by trypsinization (0.25% trypsin, 0.02% ethylenediaminetetraacetic acid EDTA; Invitrogen, UK) for 5 minutes. DMEM was then added to the flasks and supernatants were removed to 50 ml falcon tubes and centrifuged at 1200 x g for 5 minutes at 20 °C. Viable astrocytes were counted and plated in 6-well plates (3 x 10^5 cells/well) and incubated for 24 hours in 5 % CO₂ at 37 °C prior to co-culture with specific T cells.

2.1.3. Treatment of mixed glia

After 14 days in culture cells prepared from wildtype and CD200^−/− mice were treated. DMEM was removed from each well and replaced with fresh pre-warmed DMEM (1 ml) before treatment. Each plate was divided into control or treatment groups; controls received DMEM only. Cells were treated with Pam3Csk4 (100 ng/ml; Invivogen, France) for 24 hours prior to harvest.

Cells prepared from wildtype mice for the anti-TLR2 study were prepared for treatment as above. Cells were pre-treated with anti-TLR2 (2.5 µg/ml; Hycult Biotech, Netherlands) for 2 hours prior to treatment with Aβ1-40/1-42 cocktail (10 µM; Invitrogen, US) for 24 hours. After 24 hours the cells were harvested.

2.1.4. Harvesting of mixed glia

Plates were removed from the incubator one at a time and placed on ice for the duration of the harvest. Supernatants were removed through aspiration and stored in 1.5 ml tubes (Sarstedt, Germany) at -80 °C for cytokine analysis. Each well was washed with ice-cold phosphate-buffered saline (PBS; Sigma-Aldrich, UK) and harvested for gene or protein analysis. RA1 buffer (Macherey-Nagel, Germany) containing β-mercaptoethanol (1:100; Sigma-Aldrich, UK) was added to each well (353.5 µl/well), cells were scraped and the buffer removed and stored in sterile RNAse free tubes at -80 °C until required for gene analysis. For protein analysis, cells were harvested for fluorescent activated cell sorter (FACS) analysis. Cells were trypsinised (150 µl/well) for 5 minutes, FACS buffer (800 µl/well; PBS/FBS 2 %), was added, and cells were immediately transferred to FACS tubes (BD Biosciences, UK) for cell marker expression analysis.
2.2. Cell counts

Cell counts were performed by diluting cells (1:10) in Ethidium Bromide Acridine Orange (EBAO) or trypan blue. The cell suspension (10 μl) was loaded into a disposable haemocytometer (Hycor Biomedical, UK). Viable cells which stained green (EBAO method) or did not stain (trypan blue method) were counted under a fluorescent microscope (EBAO method) or light microscope (trypan blue method).

2.3. Amyloid-beta (Aβ)

2.3.1. Preparation of Aβ_{1-42} and Aβ_{1-40}

Aβ_{1-42/40} (Invitrogen, US) was dissolved in high-performance liquid chromatography-grade water and sterile PBS to generate a 1 mg/ml stock solution. Aβ_{1-42} was allowed to aggregate for 48 hours at 37 °C and Aβ_{1-40} was allowed to aggregate for 48 hours at RT. Aβ was used immediately or stored at -20 °C until required.

2.3.2. Testing of Aβ aggregation by thioflavin T assay

The presence of fibrillar Aβ was assessed using a thioflavin T assay (ThT, Sigma-Aldrich, UK). ThT stock (2 mM) was diluted (1:20) to give a working concentration (100 μM). Aliquots of PBS, freshly-reconstituted Aβ and aggregated Aβ were thawed on ice and samples (5 μl) were added in triplicate to a black ELISA plate (Labsystems, Finland). Glycine (185 μl; 50 mM; Sigma-Aldrich, UK) and ThT solution (10 μl) was added to each well and the plates were read immediately at 435-485 nm using a 96-well plate reader (Biotek, Mason Tecnology, Ireland). Aggregation of Aβ was measured by an increase in fluorescence intensity.
Figure 2.1. Measurement of Aβ aggregation by thioflavin T assay.

Aβ was aggregated as described in section 2.3.1 and aggregation was measured as described in section 2.3.2, and the aggregation was expressed as units of fluorescence intensity.
2.4. Animals

Specific pathogen free C57BL/6 mice were obtained from Harlan UK Ltd., Bicester, UK and CD200\textsuperscript{−/−} mice were gifted from Jonathan D Sedgwick. APP/PS1 mice were obtained from the Jackson Laboratory, Maine, US, and subsequently bred in a specific pathogen free units. All animals were housed and maintained under the required guidelines of the Irish Department of Health including veterinary supervision, light and temperature control (12 hour light-dark cycle and 22–23 °C); food and water were available \textit{ad libitum}. All experiments on animals were performed under a license granted by the Minister for Health and Children (Ireland) under the Cruelty to Animals Act 1876.

2.5. Treatment of animals for Pam\textsubscript{3}Csk\textsubscript{4} study

Male and female wildtype and CD200\textsuperscript{−/−} mice (2-3 months old) were randomly assigned to control or treatment groups (n=8). Mice were injected intraperitoneally (i.p) with either saline or Pam\textsubscript{3}Csk\textsubscript{4} (100 μg/100 μl). Animals were sacrificed 5 hours post treatment and tissue was harvested for analysis.

2.6. Genotyping of APP/PS1 mice

2.6.1. Isolation of genomic DNA

Tail snips were taken from APP/PS1 and littermate control mice and stored at -20 °C until required. All solutions and reagents used to isolate genomic DNA were from a DNeasy\textsuperscript{®} blood and tissue kit (Qiagen, US). The buffers AW1, AW2 and AL were prepared by the addition of ethanol (96-100 %) and brought to RT prior to use. Tail snips (0.5 cm) were placed in 1.5 ml microcentrifuge tubes and brought to RT. Buffer ATL (180 μl) and Proteinase K (20 μl) were added and the tubes were incubated overnight at 56 °C. Tubes were vortexed for approximately 15 seconds and buffer AL (200 μl) was added. Tubes were vortexed and ethanol (200 μl, 96-100 %) was added to the tubes. The mixture was transferred to DNeasy Mini spin columns in 2 ml collection tubes. Tubes were centrifuged at 8,000 rpm for 1 minute at RT. The flow-through was discarded and the column
placed in a new 2 ml collection tube. Buffer AW1 (500 µl) was added and tubes were centrifuged at 8,000 rpm for 1 minute at RT. The column was placed in a new 2 ml collection tube and buffer AW2 (500 µl) was added and tubes were centrifuged at 14,000 rpm for 3 minutes at RT to dry the membrane. The column was placed in a fresh 1.5 ml microcentrifuge tube and buffer AE (200 µl) was added and incubated for 1 minute at RT. Tubes were centrifuged at 8,000 rpm for 1 minute at RT to elute the DNA and stored at 4 °C overnight to ensure that the DNA was completely solubilised. DNA concentrations were quantified using a NanoDrop Spectrophotometer (ND-1000 v3.5, NanoDrop Technologies Inc., US).

2.6.2. Polymerase chain reaction (PCR) for APPswe and PS1dE9 genes

The presence of APPswe and PS1dE9 mutations were assessed using PCR. Mastermix was prepared by addition of DNase-free water, GoTaq® qPCR mastermix (Promega, US) and sense and antisense primers (100 µM, MWG Biotech, Germany) to a sterile tube. The mastermix for the PS1dE9 gene also contained sense and anti-sense primers for the PrP genes as an internal control. DNA and mastermix were added to fresh tubes, which were then placed in a thermocycler (MJ Research Peltier Thermal Cycler-200, Biosciences, Ireland). The amplification process consisted of an initial denaturing step at 94 °C for 30 seconds, an annealing step at 67 °C for 1 minute, and an extension step at 72 °C for 1 minute. After 35 cycles of amplification, a final extension step at 72 °C for 10 minutes was applied to ensure complete extension of PCR products. Equal volumes (10 µl) of each sample and a 100 base pair ladder (Promega, US) were mixed with loading buffer (2 µl; Promega, US), and loaded onto a 1 % (w/v) agarose gel containing gel red (1;10,000, Biotium, US). Samples were separated by application of 90 volts for 120 minutes. PCR products were visualised under an ultraviolet light and photographed using an ultraviolet transluminator (Labworks, Ultra Violet, Bioimaging Systems, US). Table 2.1 summarises the primers used for DNA amplification.
Table 2.1. Primers used for DNA amplification.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequence</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APPswe forward:</td>
<td>5'-AGGACTGACCACCTCGACCAG-3'</td>
<td>377bp product</td>
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<tr>
<td>APPswe reverse:</td>
<td>5'-CGGGGGTCTAGTTCTGCAT-3'</td>
<td></td>
</tr>
<tr>
<td>PSEN forward:</td>
<td>5'-GCCATGAGGGCACTAATCAT-3'</td>
<td>608bp product</td>
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<td>Prion forward:</td>
<td>5'-CTAGGCCACAGAATTGAAAGATCT-3'</td>
<td>324bp product</td>
</tr>
<tr>
<td>Prion reverse:</td>
<td>5'-GTAGGTGGAAAATTCTAGCATCATCC-3'</td>
<td></td>
</tr>
</tbody>
</table>
2.7. T cell study protocols

2.7.1. Generation of T cell lines from APP/PS1 and littermate control mice

2.7.1.1. Preparation of spleen cell culture

APP/PS1 and littermate control mice (12-months-old) were sacrificed by cervical dislocation and spleens were harvested to 50 ml falcon tubes containing x-vivo media (2 ml; Lonza, Belgium) containing penicillin (100 μg/ml), L-glutamine (100 mM; Gibco, UK) and β-mercaptoethanol (2 μl). Spleens were homogenized and passed through 40 μm sterile nylon mesh filters to obtain a single cell suspension. Cells were centrifuged at 1,200 rpm for 5 minutes and resuspended in x-vivo media (2 ml). The cell suspension was divided into two 50 ml falcon tubes allowing for the generation of non-specific T cells and Aβ-specific T cells from each spleen. For the generation of specific T cells, x-vivo media (9 ml) was added and cells were counted as per section 2.2. Cells were centrifuged at 1,200 rpm for 5 minutes and supernatants removed. CD4+ T cells were isolated using a magnetic activated cell sorter (MACS) column (Miltenyi Biotec, UK) and a CD4+ T cell isolation kit (Miltenyi Biotec, UK). Cells were resuspended in MACS buffer (40 μl/per 10^7 cells; PBS containing 0.5 % bovine serum albumin (BSA) and 2 mM EDTA; Sigma-Aldrich, UK) and incubated in antibody cocktail (10 μl/per 10^7 cells; Miltenyi Biotec, UK) for 10 minutes at 4 °C. Cells were flooded with MACS buffer (30 μl/per 10^7 cells) and incubated with anti-biotin microbeads (20 μl/per 10^7 cells; Miltenyi Biotec, UK) for 15 minutes at 4 °C. Cells were flooded in MACS buffer (20 x volume of antibody added), centrifuged at 1,200 rpm for 5 minutes and resuspended in MACS buffer (500 μl). The MACS column was flooded with MACS buffer (3 ml) and cells were transferred to the MACS column for magnetic sorting. T cells were centrifuged at 1,200 rpm for 5 minutes and resuspended in x-vivo media (2 ml). Cells were counted and plated in 24-well plates (200 μl/well, 2 x 10^6 cells/ml).

For the generation of T cells that would later be stimulated into Aβ-specific T cell lines, red blood cells were lysed by the addition of ammonium chloride solution (1 ml; 0.87 %) for 2-3 minutes at RT. Cells were flooded with x-vivo media (30 ml) and centrifuged at 1,200 rpm for 5 minutes. Supernatants were
removed and the cell pellet was resuspended in x-vivo media (10 ml). Cells were counted and plated in 24-well plates (250 µl/well, 4 x 10^6 cells/ml).

2.7.1.2. Generation of non-specific Th1, Th2 and Th17 cell lines

T cell lines were cultured for 5 days at 37 °C in a humidified 5 % CO2 environment. All T cell lines were developed in the presence of anti cluster of differentiation 3/28 (αCD3/CD28; 4 µg/ml, BD Biosciences, US) at the beginning of the culture. Th1 cell lines were developed in the presence of IL-12 (500 ng/ml, R&D Systems), Th2 cell lines in the presence of IL-4 (10 ng/ml, R&D Systems) and anti-IFN-γ (5 µg/ml, BD Biosciences US) and Th17 cell lines in the presence of IL-1β (25 ng/ml, R&D Systems), IL-23 (50 ng/ml), anti-IFN-γ (5 µg/ml) and TGFβ (5 ng/ml, R&D Systems). On the 3rd day of the culture media was changed on the T cell lines and cytokines were added in fresh x-vivo media without αCD3/CD28. T cell lines were harvested on the 5th day for co-culture with glia.

2.7.1.3. Generation of Aβ-specific Th1, Th2 and Th17 cell lines

Aβ-specific T cell lines were cultured as per section 2.7.1.2 with the exception that Aβ1-42 (15 µg/ml) was included in the culture media instead of αCD3/CD28 at the beginning of the culture. On the 3rd day of the culture, media was changed on the T cell lines and cytokines were added without Aβ1-42. T cell lines were harvested on the 5th day for co-culture with glia.

2.7.2. Co-culture and treatment of specific T cell lines and glia

2.7.2.1. Non-specific T cell co-culture with glia

Supernatants were removed from T cell lines and stored at -20 °C for later cytokine analysis. T cells were transferred from the 24-well plates to 50 ml falcon tubes by pipetting up and down. Tubes were centrifuged at 1,200 rpm for 5 minutes and resuspended in DMEM (500 µl). Cells were counted and resuspended in DMEM allowing for 1.5 x 10^5 cells/ml. T cells were cultured at a 1:2 ratio with microglia (300 µl/well) and astrocytes (1000 µl/well) for 24 hours at 37 °C in a

~ 51 ~
humidified 5 % CO₂ environment. Supernatants were removed for cytokine analysis by enzyme linked immunosorbent assay (ELISA) and cells were harvested for flow cytometry.

2.7.2.2. Aβ-specific T cells co-culture with glia and incubation with Aβ₁₋₄₂

Supernatants were removed from Aβ-specific T cell lines and stored at -20 °C for later cytokine analysis. T cells were removed from the 24-well plates, as per section 2.7.2.1, and resuspended in x-vivo media (2 ml), counted, and CD4⁺ T cells were isolated using a MACS sort column and a CD4⁺ T cell isolation kit as per section 2.7.1.1. Following MACS sort Aβ-specific T cell lines were recounted and resuspended in DMEM allowing for 1.5 x 10⁵ cells/ml. Aβ-specific T cell lines were cultured at a 1:2 ratio with microglia (300 μl/well) and astrocytes (1000 μl/well) in the presence of Aβ₁₋₄₂ (15 μg/ml) for 24 hours at 37 °C in a humidified 5 % CO₂ environment. Supernatants were removed for cytokine analysis by ELISA and cells were harvested for flow cytometry. Figure 2.2 summarises the generation of T cell lines from APP/PS1 and littermate control mice and co-culture with microglia and astrocytes.
Figure 2.2. Timeline of generation of T cell lines from APP/PS1 and littermate control mice and co-culture with microglia or astrocytes.
2.7.3. Generation of Th1 and Th17 cell line from C57BL/6 mice and co-culture with mixed glia

2.7.3.1. Generation of Th1 and Th17 cell lines

C57BL/6 mice (2 month-old) were sacrificed by cervical dislocation and spleens were harvested for generation of T cell lines as per section 2.7.1.1. T cell lines were cultured for 5 days at 37 °C in a humidified 5% CO₂ environment and generated in the presence of polarising cytokines as per section 2.7.1.2.

2.7.3.2. Co-culture and treatment of Th1 and Th17 cell lines with mixed glia

Supernatants were removed from T cell lines and cells were transferred to 50 ml falcon tubes and centrifuged at 1,200 rpm for 5 minutes. T cell lines were resuspended in DMEM (500 μl), counted and resuspended in the appropriate volume of DMEM allowing for 0.5 x 10⁵ cells/ml. T cells were cultured at a ratio of 1:2 with mixed glia (1x 10⁵ cells/ml). Mixed glia were also treated in the presence or absence of IFN-γ (20 ng/ml; R&D Systems, US) or IL-17 (20 ng/ml; R&D Systems, US). After 1 hour, co-cultures were stimulated with Aβ₁-₄₂ (10 μM) for 24 hours at 37 °C in a humidified 5% CO₂ environment. Supernatants were removed for cytokine analysis by ELISA and cells were harvested for flow cytometry. Table 2.2 summarises the co-culture of Th1/Th17 cells with mixed glia and their treatment with either IFN-γ/IL-17 and Aβ₁₋₄₂.

Table 2.2. Co-culture of Th1 and Th17 cells with mixed glia and treatments.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Mixed glia co-culture with Th1 cells</th>
<th>Mixed glia co-culture with Th17 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (± Aβ)</td>
<td>Control (± Aβ)</td>
<td></td>
</tr>
<tr>
<td>Th1 cells (± Aβ)</td>
<td>Th17 cells (± Aβ)</td>
<td></td>
</tr>
<tr>
<td>IFN-γ (± Aβ)</td>
<td>IL-17 (± Aβ)</td>
<td></td>
</tr>
</tbody>
</table>

~ 54 ~
2.7.4. Generation of T cells for adoptive transfer APP/PS1 in vivo study

2.7.4.1. Generation of Aβ-specific T cell lines

C57BL/6 mice received a footpad immunisation of Aβ (75 µg/mouse) and CpG-containing oligodeoxynucleotide (CpG, 25 µg/mouse; Sigma-Aldrich, UK) into each paw (25 µl/paw). After 21 days the mice were boosted with Aβ and CpG. Mice were sacrificed 7 days after the booster injection and the spleens were harvested. The spleens were restimulated ex vivo with Aβ (25 µg/ml) and an array of cytokines and antibodies depending on the type of T cell line being developed. At the beginning of the culture Th1 cells were incubated with IL-12 (10 ng/ml), Th2 cell lines with dexamethasone (1 x 10⁻⁸ M, Sigma-Aldrich, UK), IL-4 (10 ng/ml), and anti-IFN-γ (5 µg/ml). Th17 cell lines were incubated with IL-1β (10 ng/ml), IL-23 (10 ng/ml), and anti-IFN-γ. After 4 days of incubation, Th1 and Th2 cell cultures received IL-2 (5 ng/ml R&D Systems), while Th17 cell cultures received Roswell Park memorial institute medium (RPMI; Gibco, US). After a further 7 days of incubation, surviving T cells (1 x 10⁶ cells/ml) were restimulated with irradiated APCs (2 x 10⁶ cells/ml), generated from spleens irradiated at a dose of 30 Gy in an irradiation chamber of a Nordian Gammcell 3000 irradiator, and Aβ (25 µg/ml) for 4 days followed by 7 days of incubation with IL-2.

Following generation of Aβ-specific T cell lines, Aβ-specific Th1, Th2, and Th17 cells (15 x 10⁶ cells/mouse), cells were washed and injected intravenously (i.v.) (15 x 10⁶ cells/mouse in 300 µl serum-free medium) into 6-7 month-old APP/PS1 mice. Control animals received 300 µl serum-free medium alone. After 2 weeks APP/PS1 mice were anaesthetised with sodium pentobarbital (40 µl; Euthatal, Merial Animal Health, UK) and perfused intracardially with ice-cold PBS (20 ml). The brains were rapidly removed, bisected and snap-frozen for later analysis by multi-spot ELISA and immunohistochemistry

2.7.4.2. Intravenous injection of Th1 cells for anti-IFN-γ study

APP/PS1 and littermate control mice received an i.p. injection of either anti-IFN-γ (600 µg/mouse) or a control antibody (β-galactosidase: 600 µg/mouse, R&D Systems, US). Twenty four hours later, Th1 cells (15 x 10⁶ cells/mouse) in
serum-free RPMI were injected (300 µl) into the lateral tail vein of the mice. Serum-free RPMI was injected into the tail vein of mice from other groups as a control. Anti-IFN-γ or β-galactosidase antibody injections were repeated on days 3, 7, 10, 14, 17, 21, 24, 28 and 31 after T cell transfer, in order to ensure that no IFN-γ was present in these animals. Mice were killed 34 days after T cell transfer by cervical dislocation and tissue was taken for analysis. The procedure described above was carried out by Dr Keith Mc Quillan. The tissue was gifted to me for analysis described hereafter.

2.8. Mononuclear cell isolation from CNS tissue

Animals were anaesthetised with sodium pentobarbital (40 µl) and perfused intracardially with sterile ice-cold PBS (20 ml). The brain was removed and placed into a 50 ml falcon tube containing Hank’s buffered saline solution (2 ml) containing 3 % FBS (HBSS/FBS; Sigma-Aldrich, UK). Tissue was dissociated through a sterile 70 µm nylon mesh filter, washed with HBSS/FBS and centrifuged at 170 x g for 10 minutes at RT. The supernatant was removed and the remaining pellet resuspended in HBSS/FBS (2 ml) containing collagenase D (1 mg/ml, Roche, Ireland) and DNAse I (10 μg/ml, Sigma-Aldrich, UK), and incubated for 1 hour at 37 °C. Cells were washed in HBSS/FBS and centrifuged at 1,200 rpm for 5 minutes. Supernatants were removed and cells were resuspended in 1.088 g/ml Percoll (9 ml; Sigma-Aldrich, UK). This was underlayed with 1.122 g/ml Percoll (5 ml), and overlayed with 1.072 g/ml Percoll (9 ml) followed by 1.030 g/ml Percoll (9 ml), and finally PBS (9 ml). Percoll gradients were centrifuged at 1,250 x g for 45 minutes at 18 °C. Mononuclear cells were removed from between the 1.088:1.072 and 1.072:1.030 g/ml interfaces and placed in 50 ml falcon tubes. Cells were washed twice in HBSS/FBS and counted to obtain absolute numbers before being transferred to FACS tubes for assessment by flow cytometry. Figure 2.3 illustrates the Percoll separation method and isolation of mononuclear cells.
Figure 2.3. Diagram of Percoll separation used to isolate mononuclear cells from the CNS.

Diagram illustrating Percoll separation of cells. Red blood cell and dead cells are found at the 1.123:1.088 g/ml interface. Myelin debris is found at the 1.030 g/ml:PBS interface. Mononuclear cells are found at the 1.088:1.072 and 1.072:1.030 g/ml interfaces.
2.9. Flow cytometry

2.9.1. Flow cytometry of T cells and glia

Expression of cell surface markers was assessed on T cells and glial cells from *in vitro* and *in vivo* studies by flow cytometry using a DAKO CyAN®ADP flow cytometer, calibrated using Flow-Check Fluorospheres (Beckman Coulter, Ireland). T cells were removed from glia by pipetting up and down several times and transferred to respective FACS tubes. Mixed glia, microglia and astrocytes were harvested by addition of Trypsin-EDTA (150-300 µl/well) to the wells, and incubated for 5 minutes. Cells were transferred to respective FACS tubes and centrifuged at 1,200 rpm for 5 minutes and resuspended in FACS buffer (100 µl). Cells were blocked by incubating with anti-CD16/CD32 FcγRIII block (1:100; BD Biosciences, US) for 15 minutes at RT. Cells were incubated with the appropriate FACS antibodies or isotype control antibodies (see Table 2.3 for details) for 30 minutes at RT in the dark. Cells were then washed with FACS buffer to remove excess antibody, centrifuged at 1,200 rpm for 5 minutes between washes, resuspended in FACS buffer (400 ml) and analysed using Summit software and FlowJo software.

2.9.2. Flow cytometry of mononuclear cells

Mononuclear cells prepared from CNS tissue were prepared for intracellular staining using a cell permeabilisation kit (Dako, Denmark). Cells were centrifuged at 1,200 rpm for 5 minutes before stimulation with x-vivo media (200 µl) containing phorbol myristate acetate (PMA; 10 ng/ml; Sigma-Aldrich, US), Ionomycin (1 µg/ml; Sigma-Aldrich, US) and brefeldin A (BFA; 5 µg/ml; Sigma-Aldrich, US) for 4/5 hours. Following stimulation, cells were centrifuged at 1,200 rpm for 5 minutes and resuspended. Low-affinity IgG receptors (FcγRIII) were blocked by incubating cells in FACS buffer (50 µl/sample) containing anti-CD16/CD32 FcγRIII (1:100) for 10 minutes at RT. Cells were then incubated in 50 µl/sample FACS buffer containing the appropriate FACS antibodies (see Table 2.3 for details) for 15 minutes at RT, and fixed in IntraStain Reagent A (50 µl/sample; Dako, Denmark) for 15 minutes at RT. Cells were washed twice with FACS buffer, centrifuged at 1,200 rpm for 5 minutes, and permeabilised with
IntraStain Reagent B (50 µl/sample; Dako, Denmark) plus intracellular antibodies (see Table 2.3 for details) for 15 minutes at RT in the dark. Cells were washed twice in FACS buffer and centrifuged at 1,200 rpm for 5 minutes. Immunofluorescence analysis was performed using Summit software (Dako, Denmark) and the results analysed using FlowJo software (Tree Star, US).

Table 2.3. Antibodies used in flow cytometry.

<table>
<thead>
<tr>
<th>Surface Marker</th>
<th>Fluorescent Label</th>
<th>Dilution Factor</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td>APC/PeCy7</td>
<td>1/100</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD3</td>
<td>V500/APC/A780</td>
<td>1/100-200</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD4</td>
<td>FITC/V450/PeCy7</td>
<td>1/100-200</td>
<td>BD Biosciences</td>
</tr>
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<td>A780</td>
<td>1/50</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD40</td>
<td>FITC/PE</td>
<td>1/100</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>MHCII</td>
<td>PE/PeCy5</td>
<td>1/100</td>
<td>BD Biosciences</td>
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<td>CD86</td>
<td>PerCPc5.5/FITC</td>
<td>1/100</td>
<td>BD Biosciences /</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Biolegend</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>PerCPc5.5/FITC</td>
<td>1/100</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>IL-17</td>
<td>V450/PE</td>
<td>1/100</td>
<td>BD Biosciences</td>
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<tr>
<td>TLR2</td>
<td>FITC</td>
<td>1/100</td>
<td>BD Biosciences</td>
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<tr>
<td>CD45</td>
<td>PeCy7</td>
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<tr>
<td>marker</td>
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</tr>
</tbody>
</table>
2.10. Protein quantification

Protein quantification was assessed using the bicinchoninic acid (BCA) assay. Protein standards were prepared from a stock solution of 2000 μg/ml BSA. A range of standards (0 to 2000 μg/ml) was generated by diluting the stock solution in sample buffer. Standards and samples (25 μl) were added to a 96-well plate in triplicate (Sarstedt, Ireland). BCA reagent was added and the samples were incubated at 37 °C for 30 minutes and absorbance assessed at 562 nm using a 96-well plate reader (Biotek, Mason Technology, Ireland). A regression line was plotted (GraphPad Prism, USA) to enable determination of the protein concentration.

2.11. Quantitative Polymerase Chain Reaction (QPCR)

2.11.1. RNA extraction

Messenger RNA (mRNA) was extracted from snap-frozen brain tissue from the in vivo studies and from cells from the in vitro studies. Tissue from the in vivo studies was homogenised in cell lysis mastermix (350 μl; Nucleospin RNA II, Macherey-Nagel, Germany) using a Polytron homogenizer (Kinematica, Switzerland). Cells from in vitro studies were harvested in lysis buffer as per section 2.1.4. The lysate from both in vitro and in vivo studies was filtered through a Nucleospin filter (Macherey-Nagel, Germany), and centrifuged at 11,000 x g for 1 minute. Lysates were mixed with ethanol (350 μl; 70 %) and loaded onto Nucleospin RNA II columns (Macherey-Nagel, Germany). Samples were centrifuged at 8,000 x g for 30 seconds allowing the RNA to bind to the columns. The silica membrane was desalted with membrane desalting buffer (350 μl; Macherey-Nagel, Germany) and centrifuged at 11,000 x g for 1 minute to dry the membrane. DNase reaction mixture (95 μl) was added directly onto the centre of the silica membrane column and incubated for 15 minutes at RT. The silica membrane was washed and dried 3 times. In the first wash, RA2 buffer (200 μl; Macherey-Nagel, Germany) was added to the Nucleospin RNA II column and centrifuged at 8,000 x g for 30 seconds. For the second wash, RA3 buffer (600 μl; Macherey-Nagel, Germany) was added to the Nucleospin RNA II column and
centrifuged at 8,000 x g for 30 seconds. In the final wash, RA3 buffer (250 µl)
was added to the Nucleospin RNA II column and centrifuged at 11,000 x g for 2
minutes to dry the membrane completely. The RNA was eluted in nuclease-free
water (60 µl; Macherey-Nagel, Germany) and centrifuged at 11,000 x g for 1
minute. The RNA concentration was quantified using a NanoDrop
Spectrophotometer.

2.11.2. cDNA synthesis

Extracted mRNA was reverse-transcribed into complementary DNA
cDNA) using the high capacity cDNA reverse transcription kit (Applied
Biosystems, Germany). RNA was equalised with nuclease-free water, the sample
was vortex-mixed and stored on ice. A 2X mastermix was prepared containing the
appropriate volumes of 10X RT buffer, 25X dNTPs, 10X random primers,
multiscribe reverse transcriptase (50 U/µl) and nuclease-free water and kept on
ice. Mastermix (25-30 µl) was added to equalised RNA samples (25-30 µl) to
make a total volume of 50-60 µl. Samples were incubated for 10 minutes at 25 °C
followed by 120 minutes at 37 °C on a thermocycler (PTC-200, Peltier Thermal
Cycler, MJ Research, Biosciences Ireland). cDNA was stored at -20 °C until
analysis.

2.11.3. QPCR

QPCR primers and probes were delivered as “TaqMan® Gene Expression
Assays” (see Table 2.4 for details; Applied Biosystems, Germany). Quantitative
Real-time PCR was performed on Applied Biosystems ABI Prism 7300 Sequence
Detection System v1.3.1 in 96 well format where there was 25 µl reaction volume
per well (3 µl cDNA and 22 µl PCR mastermix). The cDNA was mixed with
Taqman universal PCR mastermix (Applied Biosystems, Germany) and the
respective gene target assay. Mouse β-actin RNA primers (Applied Biosystems,
Germany) were added as the reference guide. Each sample was measured in
duplicate in a single RT-PCR run. Forty cycles were allowed run under the
following conditions: 2 minutes at 50 °C, 10 minutes at 95 °C, and for denaturing
15 seconds at 95 °C and 1 minute at 60 °C for transcription. Analysis of the gene
expression values was carried out using the efficiency-corrected comparative CT method determining target gene expression relative to the β-actin endogenous control expression and relative to a control samples.
Table 2.4. QPCR primers gene expression assay numbers.

<table>
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<tr>
<th>Gene name</th>
<th>Gene Description</th>
<th>Taqman Gene expression Assay number</th>
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<tbody>
<tr>
<td>TNFa</td>
<td>Tumour Necrosis Factor alpha</td>
<td>Mm00443258_ml</td>
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<td>Cluster of Differentiation 68</td>
<td>Mm03047340_ml</td>
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<td>CD200R</td>
<td>Cluster of Differentiation 200 receptor</td>
<td>Mm00491164_ml</td>
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<td>Chemokine Ligand 2</td>
<td>Mm00441242_ml</td>
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<td>CXCL10</td>
<td>C-X-C motif Chemokine 10</td>
<td>Mm00445235_ml</td>
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<td>Interferon-gamma</td>
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<td>CD11b</td>
<td>Cluster of Differentiation 11b</td>
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<td>Inter-Cellular-Adhesion-Molecule-1</td>
<td>Mm00516027_g1</td>
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<td>Interleukin-4</td>
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<td>Cluster of Differentiation 86</td>
<td>Mm01344638_ml</td>
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</table>

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2.12. Analysis of cytokine and chemokine expression by ELISA

The concentrations of the cytokines TNFα, IL-1β, IL-6, IL-17, IFN-γ and chemokines MCP-1, RANTES, MIP-1α and IP-10 were measured using commercially available ELISA kits (See Table 2.5 for details; R&D Systems, UK). Plates (Nunc-Immuno plate, Thermo Fisher Scientific, UK) were coated with 100 μl/well of rat anti-mouse TNFα (0.8 μg/ml), IL-1β (4 μg/ml), IL-6 (2 μg/ml), IL-17 (2 μg/ml), IFN-γ (4 μg/ml), MCP-1 (0.2 μg/ml), RANTES (2 μg/ml), MIP-1α (0.4 μg/ml) and IP-10 (2 μg/ml) capture antibody in PBS, and incubated at 4 °C overnight. Plates were washed several times with PBS containing 0.05 % Tween-20 (PBS-T), blocked for a minimum of 1 hour at RT with diluent (200 μl/well; 0.1 M PBS, pH 7.3, with 1 % BSA), and washed several times with PBS-T. Plates were incubated with 100 μl/well of recombinant mouse TNFα (0-2000 pg/ml), IL-1β (0-1000 pg/ml), IL-6 (0-5000 pg/ml), IL-17 (0-1000 pg/ml), IFN-γ (0-2000 pg/ml), MCP-1 (0-250 pg/ml), RANTES (0-2000 pg/ml), MIP-1α (0-500 pg/ml) and IP-10 (0-4000 pg/ml) in 1 % BSA/PBS or sample for 2 hours at RT. Plates were washed three times with PBS-T and incubated with 100 μl/well of secondary antibody, biotinylated goat anti-mouse, TNFα (200 ng/ml), IL-1β (100 ng/ml), IL-6 (200 ng/ml), IL-17 (400 ng/ml), IFN-γ (100 ng/ml), MCP-1 (50 ng/ml), RANTES (400 ng/ml), MIP-1α (100 ng/ml) and IP-10 (50 ng/ml) in 1 % BSA for 2 hours at RT. Plates were washed three times in PBS-T, incubated with horse radish peroxidase (HRP)-conjugated streptavidin (100 μl/well; 1:200 in 1 % BSA; R&D Systems, UK) for 20 minutes in the dark at RT, washed three times in PBS-T and substrate solution (100 μl/well; R&D Systems, UK) was then added to the plates. The reaction was stopped with 1 M H2SO4 (50 μl/well). Absorbance was read at 420 nm within 30 minutes of stopping the reaction. A standard curve was generated by plotting the standards and their absorbance values and the concentrations were corrected allowing for protein concentrations. Protein concentrations from tissue were expressed as pg/mg and for supernatants as pg/ml (GraphPad Prism v 4.0; GraphPad Software, US).
Table 2.5. Origin and specificities of antibodies for ELISA.

<table>
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<tr>
<th>Antibody specificity</th>
<th>Catalogue number</th>
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<td>R&amp;D Systems</td>
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<td>DY479</td>
<td>R&amp;D Systems</td>
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<tr>
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<tr>
<td>Mouse IL-17</td>
<td>DY421</td>
<td>R&amp;D Systems</td>
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</tbody>
</table>
2.13. Determination of Aβ concentrations in tissue samples using multi-spot ELISA

2.13.1. Extraction of soluble and insoluble Aβ from brain tissue

Snap-frozen cortical tissue from wildtype and APP/PS1 mice were homogenised in 5 volumes (w/v) of homogenising buffer (SDS/NaCl in dH₂O with proteases). The samples were centrifuged at 15,000 rpm for 40 minutes at 4 °C. The supernatant samples were removed to extract SDS-soluble Aβ and the pellets were kept for extraction of insoluble Aβ.

Supernatants were equalised (3 mg/ml) with homogenising buffer using a BCA protein assay as per section 2.10. Equalised supernatant samples were neutralised by the addition of 10 % (w/v) 0.5 M Tris-HCl (pH 6.8). Samples were stored at -20 °C for later detection of soluble Aβ.

Pellets were incubated in guanidine buffer (50 µl; 5 M guanidine-HCl/50 mM Tris-HCl, pH 8, Sigma-Aldrich, UK) for 4 hours on ice, and centrifuged at 15,000 rpm for 30 minutes at 4 °C. Supernatant samples were equalised (0.3 mg/ml) with guanidine buffer and stored at -20 °C for later detection of insoluble Aβ.

2.13.2. Detection of soluble and insoluble Aβ concentrations in cortical tissue by multi-spot ELISA

Concentrations of soluble and insoluble Aβ were determined using “MSD® 96-well multi-spot 4G8 Aβ triple ultra-sensitive assay” kits (Meso Scale Discovery, USA). All solutions and reagents used were supplied by Meso Scale Discovery. Multi-spot Aβ 3-plex plates were blocked with 1 % Blocker A (150 µl/well; 50 ml 1 X Tris wash buffer: 0.5 g Blocker A) for 1 hour at RT with vigorous shaking (300-1000 rpm). Plates were washed 3 times in 1 X Tris wash buffer (TWB) and detection antibody solution (25 µl/well; 60 µl 50 X SULFOTAG 4G8 detection antibody, 30 µl 100 X Blocker G in 2910 µl 1 % Blocker A solution) was added. SDS-solubilised samples were diluted in Blocker A (200 µg/ml) plus protease inhibitors (1: 100) and guanidine-treated samples were diluted in Blocker A (0.3 µg/ml) solution. Standards were prepared by serial
dilutions of recombinant human Aβ₁-38 (0-3,000 pg/ml), Aβ₁-40 (0-10,000 pg/ml) and Aβ₁-42 (0-3,000 pg/ml) in 1% Blocker A. Samples and standards (25 µl/well) were added in duplicate to the appropriate wells of the 96-well plates which were incubated for 2 hours at RT with vigorous shaking (300-1000 rpm). Plates were washed in wash buffer and 2 X MSD read buffer (150 µl/well; 10 ml dH₂O; 10 ml 4 X MSD read buffer) was added. Plates were read immediately using a Sector Imager plate reader (Meso Scale Discovery, US) and Aβ concentrations in test samples were evaluated with reference to the standard curve prepared using Aβ₁-38 (0-3,000 pg/ml), Aβ₁-40 (0-10,000 pg/ml) and Aβ₁-42 (0-3,000 pg/ml).

2.14. Immunohistochemical analysis

2.14.1. Subbing of slides

Glass slides were coated in a subbing solution prior to sectioning of tissue to ensure adhesion of the tissue to the slide. Gelatine (2.5g; Fluka, Switzerland) and chromium potassium sulphate (0.25g; Sigma-Aldrich, UK) was dissolved in 500 ml dH₂O and heated to 60 °C. Subbing solution was filtered through Whatman filter paper (Whatman International, UK) and clean glass slides (Fluka, Switzerland) were submerged into the solution for 1 minute. Slides were removed and allowed to dry overnight.

2.14.2. Preparation of tissue sections for immunohistochemistry

Sections were prepared from tissue obtained from wildtype mice and APP/PS1 mice treated with Th1, Th17, Th1 and anti-IFN-γ or anti-IFN-γ. The left hemisphere of each brain was placed onto cork discs and coated with optimum cooling temperature compound (OCT; Sakura Tissue-Tek, Netherlands), snap frozen in pre-chilled isopropanol and stored at -80 °C. Before sectioning the tissue was allowed to equilibrate to -20 °C for 30 minutes. Sagittal sections (10 µm thick) were cut using a cryostat (Leica, Meyer, UK), mounted on gelatine-coated glass slides, allowed to dry for 20 minutes and stored at -20 °C for later immunohistochemical analysis.
2.14.3. Immunohistochemical staining for CD11b

Frozen brain section from wildtype and APP/PS1 mice were thawed and brought to RT for approximately 30 minutes. Individual brain sections were surrounded in a hydrophobic well using a cytomation pen (Dako, Denmark). Sections were fixed in an acetone and ethanol mixture (1:1, Sigma-Aldrich, US) for 5-10 minutes and endogenous peroxidase activity was blocked with 0.3 % H$_2$O$_2$ (Sigma-Aldrich, US) in PBS for 5 minutes. Sections were washed with PBS (2 x 10 minute washes), and 10 % normal rabbit serum was added to block non-specific binding (NRS; Vector Laboratories, UK). Sections were incubated with rat anti-CD11b (1:100; clone 5C6, AbD Serotec, UK) in 5 % NRS in PBS overnight at RT. Negative controls were incubated in 5 % NRS in PBS. Sections were washed in PBS (2 x 10 minute washes) and incubated in secondary antibody, biotinylated rabbit anti-rat IgG (1:200; Vector, US) in 5 % NRS in PBS for 2 hours at RT. Sections were washed again and incubated with Vectostain Elite ABC reagent (2 drops of A/B in 5 ml PBS, Vector Laboratories, US) for 1 hour at RT. Sections were re-washed to remove excess ABC with PBS and developed using the substrate 3, 3 diaminobenzidine (DAB) enhanced liquid substrate system tetrahydrochloride (1 drop of solution B in 1 ml of solution A, Sigma-Aldrich, US) for approximately 10 minutes until the colour developed. Sections were immediately counterstained with 1 % methyl green (w/v, Sigma-Aldrich, UK) for 10 minutes and dehydrated by dipping for 5 seconds in 70 %, 80 %, 95 % and 2 minutes in 100 % ethanol followed by 2 x 4 minute incubations in xylene (VWR International Ltd., UK). Sections were mounted with xylene-based mountant dibutyl phthalate (DPX; VWR International Ltd., UK) and allowed to dry overnight in the fume hood. The sections were stored at RT until analysis. Sections were examined and analysed using a light microscope. The areas assessed for evidence of microglial activation were the dentate gyrus of the hippocampus and the cortex. The slides were visualized with an Olympus 1 x 51 light microscope (Tokyo, Japan) and micrographs were taken using an Olympus UCMAD3 (Japan) at 40 x magnification.
2.14.5. Quantification of CD11b immunohistochemical analysis using Image J

CD11b positive immunoreactivity was analysed using Image J software. Micrographs of cortical and hippocampal sections were converted to Tiff-tag images and opened in the Image J software. Images were then converted to 8 bit and the threshold adjusted. CD11b positive cells were then identified using the analyze particles tool. All images were analysed at the same threshold, cell circularity and pixel size.

2.14.6. Immunohistochemical staining for Aβ plaques using Congo red

Congo red solution was made by adding Congo red powder (0.1 g; Sigma-Aldrich, US) to saturated sodium chloride (NaCl; 500 ml; 50 grams in 80 % ethanol, Sigma-Aldrich, UK) and the solution was stirred overnight before use. Frozen brain sections from wildtype and APP/PS1 mice were stained for Aβ plaques using congo red. Sections were thawed and brought to RT for 30 minutes. Individual brain sections were surrounded in a hydrophobic well using a cytomatic pen. The brain sections were fixed using ice-cold ethanol and washed 3 x 5 minutes in PBS. During the washes, sodium hydroxide (NaOH 1M; 2 ml, Sigma-Aldrich, UK) was added to saturated NaCl (200 ml) to produce an alkaline solution. Sections were incubated for 20 minutes at RT in the alkaline solution. During the incubation, Congo red solution (200 ml) was filtered through a 50 ml syringe using a cellulose acetate membrane filter (0.2 μM Supor membrane Acrodisc syringe filters, Pall Corporation, US). The filtered Congo red solution (200 ml) was added to 1 M NaOH (2 ml) to generate an alkaline solution. The sections were incubated in the Congo red solution for 30 minutes at RT. Sections were rinsed in dH2O to remove excess Congo red stain and sections were counterstained in 1 % methyl green solution for 10 minutes. Sections were dehydrated by dipping 5/6 times in 80 %, 95 %, 100 % and 100 % ethanol and 3 x 5 minute incubations in xylene. Slides were mounted in DPX and allowed to dry in the fume hood overnight. The slides were visualized with an Olympus 1 x 51 light microscope (Tokyo, Japan) and micrographs were taken using an Olympus UCMAD3 (Japan) at 40 x magnification. Positively-stained Aβ plaques were counted in representative sections from each animal under the same light and
magnification settings and results were expressed as the average number of plaques per sections.

2.14.7. Double-immunofluorescence staining for Aβ and CD11b

Frozen brain section from wildtype and APP/PS1 mice were thawed and brought to RT for approximately 30 minutes. Individual brain sections were surrounded with a cytoming pen and fixed in ice-cold methanol for 10 minutes followed by 3 x 1 minute washes in PHEM (HEPES sodium salt 25 mM; EGTA 10 mM; PIPES 60 mM; MgCl₂ 2 mM; pH6.9; Sigma-Aldrich, UK). Sections were permeabilized in 0.1 % Triton (Sigma-Aldrich, UK) in PHEM for 10 minutes followed by 3 x 1 minute washes in PHEM. Non-specific binding was blocked by incubating sections in 10 % normal goat serum (NGS) for 2 hours at RT. Sections were incubated overnight with primary antibodies: Pan Aβ (1:1000, Calbiochem, US) and rat anti-CD11b (1:100, clone 5C6, AbD, Serotec, UK) in 5 % NGS in PHEM. Sections were washed 3 x 1 minute washes in PHEM and incubated in secondary antibody ALEXA 488 (1:4000, Invitrogen, US) and ALEXA 546 (1:1000, Invitrogen, US) in 5 % NGS for 90 minutes at RT. Sections were washed every 10 minutes for 90 minutes, mounted with dapi-containing mounting medium (Vector Labs, US) and analysed using a confocal microscope (Axioplan 2, Zeiss, Germany).

2.15. Statistical analysis

Statistical analysis was performed using GraphPad Prism. Data were analysed using student’s t test, two-way ANOVA or one-way ANOVA followed by Newman-Keuls post-hoc test. Data are expressed as means with standard errors (SEM) and deemed statistically significant when p<0.05.
Chapter 3
Chapter 3

3.1. Introduction

The immune system is crucial for the maintenance of tissue homeostasis, clearing pathogens and responding to injury. It has been well documented that microglia are the major resident CNS immune cell, constantly surveying their microenvironment and participating in innate and adaptive responses resulting in the release of pro-inflammatory cytokines and chemokines, phagocytosis and the regulation of T cell responses (Aloisi, 2001). Astrocytes have also been implicated in immune functions and the development of CNS inflammation (Dong & Benveniste, 2001). There is extensive literature describing the phenotypical transformation of ‘resting’ microglia into ‘activated’ microglia. These phenotypical changes include the upregulation of various cells surface markers including CD11b (Roy et al., 2008), CD40 and MHC class II (Benveniste et al., 2001). Furthermore, Cytokines become promptly unregulated as a consequence of microglial (Aloisi, 2001) and astrocytic activation (Dong & Benveniste, 2001; Carpentier et al., 2005), which in turn is associated with the expression of inflammatory chemokines.

It is imperative that activated microglia ultimately return to an inactivated state, when the insult is cleared to prevent unwanted tissue damage. In the normal brain, interaction of microglia with other glia and neurons can act to suppress ‘activation’ and dampen the pro-inflammatory response (Colton, 2009). The interaction of receptor-ligand pairs such as CD200, expressed on neurons and astrocytes, and CD200R, expressed on microglia, acts to inhibit the production of pro-inflammatory mediators by microglia (Lyons et al., 2007; Meuth et al., 2008; Masocha, 2009; Walker et al., 2009). The importance of the CD200-CD200R interaction has been demonstrated in several different models including the facial nerve transaction model, EAE and CIA (Hoek et al., 2000). The use of CD200 fusion protein (Gorczynski et al., 2002b) and CD200 antibodies (Copland et al., 2007) have confirmed the anti-inflammatory potential of CD200-CD200R interaction. Interestingly, both CD200 and CD200R levels are decreased in brain tissue of AD patients (Walker et al., 2009).
Microglia possess a number of receptors involved in immune function, most notably the TLRs, which allow them to rapidly detect pathogens and prevent unwanted tissue damage (Palm & Medzhitov, 2009). Similarly, astrocytes have also been found to express TLRs (Bowman et al., 2003). To date, thirteen mammalian TLRs (TLR1-13) have been identified, each with their own set of PAMPs and signalling pathways. The pattern of TLRs expressed by glial cells and the signalling pathways employed play a significant role in determining the inflammatory response occurring within the CNS (Jack et al., 2005). This study focuses on TLR2, which recognises the largest array of PAMPs including lipoteichoic acids, PGN, surface proteins on both gram-negative and gram-positive bacteria, mycobacteria and zymosan (Kielian, 2006). Previous studies have found that TLR2 works as a heterodimer with TLR1 and TLR6 to discriminate between lipopeptides (Ozinsky et al., 2000). Studies using TLR1 and TLR6 knockout animals have demonstrated that TLR2 uses TLR1 to recognise triacyl lipopeptides such as Pam\(_3\)CysSerLys\(_4\) (Pam\(_3\)Csk\(_4\)) a TLR2 agonist (Takeda et al., 2002). Further investigations of the TLR2/TLR1 relationship, using Pam\(_3\)Csk\(_4\), established that Pam\(_3\)Csk\(_4\) was needed to bridge the two TLRs together thus aiding in the formation of the heterodimer facilitating downstream signalling activation (O'Neill et al., 2009).

Although the activation of TLRs is crucial to mount an appropriate immune response, their activation comes at a cost and this is implicated in the pathogenesis of autoimmune, chronic inflammatory and infectious diseases (Chen et al., 2007; O'Neill et al., 2009) and in the pathogenesis of neurodegenerative diseases (Cameron & Landreth, 2010). There is a growing body of evidence suggesting that TLRs namely TLR2 and TLR4 and the TLR co-receptor, CD14, participate in microglial responses to A\(\beta\). It is now understood that TLRs can regulate microglial phenotypes and responses in the AD brain (Landreth & Reed-Geaghan, 2009). Recognition of A\(\beta\) through TLRs and other cell surface receptors results in the production and secretion of pro-inflammatory cytokines (Cameron & Landreth, 2010). Several studies have found increased expression of TLR2 and TLR4 in mouse models of AD and the AD brain (Fassbender et al., 2004; Walter et al., 2007; Letiembre et al., 2009), furthermore plaque-associated microglia have increased expression of TLRs (Frank et al., 2009). Previous studies emphasise the role of TLR2 in A\(\beta\)-induced microglial activation. It has been
found that blocking TLR2 through neutralising antibodies or siRNA knockdown prevents Aβ-induced secretion of cytokines IL-6 and TNFα (Jana et al., 2008; Udan et al., 2008).

The aims of this study were to:

• Investigate *in vitro* the role of CD200 on microglial activation following stimulation with a TLR2 agonist Pam3Csk4.

• Investigate *in vitro* the effect of Aβ on microglial activation and to establish whether Aβ-induced changes are dependent on activation of TLR2.
3.2. Methods

Mixed glia were prepared from neonatal C57BL/6 wildtype and CD200⁻/⁻ mice and cultured for 14 days before incubation with Pam₃Csk₄ (100 ng/ml) for 24 hours (see section 2.1. for details). Similarly, mixed glia were prepared from neonatal C57BL/6 mice and cultured for 14 days before incubation with Aβ₁⁻⁴₀/₁⁻⁴₂ (10 μM, 24 hours) or pre-incubation with anti-TLR2 (2.5 μg/ml), for 2 hours, and Aβ₁⁻⁴₀/₁⁻⁴₂ for 24 hours (see section 2.1.3 for details). Supernatant samples were taken to determine cytokine and chemokine concentrations by ELISA and cells were taken to determine mRNA expression of surface proteins by QPCR (see sections 2.1.1 and 2.1.2 for details). Data are expressed as means ± SEM. ANOVA and student’s t-test were performed to determine whether significant differences existed between treatment groups and Bonferroni post-hoc test was applied where appropriate.
3.3. Results

3.3.1. Pam₃Csk₄ increased CD11b and CD40 and decreased CD68 mRNA expression in mixed glia prepared from wildtype and CD200⁻/⁻ mice.

TLRs are expressed on cells of the immune system and play a critical role in pathogen recognition and tailoring of the adaptive immune response (Medzhitov & Janeway, 1997). Microglia and astrocytes are capable of recognising a wide array of pathogens through TLRs. Ligation of TLRs allows for the progressive transformation of glia into a more “activated” phenotype which is measured here by assessing expression of markers of activation. Pam₃Csk₄, a synthetic TLR2 agonist, induced a significant increase in mRNA expression of CD11b and CD40 in mixed glia prepared from wildtype and CD200⁻/⁻ mice (***p<0.001, ANOVA; Figures 3.1A and B). There was no genotype effect observed as the effect of Pam₃Csk₄ was similar in mixed glia prepared from wildtype and CD200⁻/⁻ mice. Basal mRNA expression of CD40 was significantly increased in glia prepared from CD200⁻/⁻, compared with wildtype, mice (⁺⁺⁺p<0.001, student’s t test; Figure 3.1b).

Pam₃Csk₄ induced a significant decrease in the mRNA expression of CD68 in mixed glia prepared from wildtype and CD200⁻/⁻ mice (***p<0.001, ANOVA; Figure 3.2). While no significant genotype effect was observed, there was a trend towards an increase in basal mRNA expression of CD68 in glia prepared from CD200⁻/⁻, compared with wildtype, mice.

3.3.2. Pam₃Csk₄ increased TNFα and IL-6 in mixed glia prepared from wildtype and CD200⁻/⁻ mice.

TLR activation, through interaction with different pathogen-associated molecules, results in activation of signalling cascades resulting in the production of pro-inflammatory cytokines tailored to the activating stimulus (Jack et al., 2005). Previously Pam₃Csk₄ has been found to increase pro-inflammatory cytokine production by microglia including TNFα and IL-6 (Ebert et al., 2005; Jack et al., 2005; Shah et al., 2009). The present data show that Pam₃Csk₄ significantly increased mRNA expression of TNFα and supernatant...
concentrations of TNFα in mixed glia prepared from wildtype and CD200+/− mice (**p<0.001, ANOVA; Figure 3.3A and B) and significantly enhanced TNFα supernatant concentration obtained from mixed glia prepared from CD200+/−, compared with wildtype, mice (+++p<0.001, ANOVA; Figure 3.3B).

Pam3Csk4 also induced a significant increase in mRNA expression of IL-6 and supernatant concentrations of IL-6 in mixed glia prepared from wildtype and CD200+/− mice (**p<0.001, ANOVA; Figure 3.4A and 3.4B). Both were further increased in mixed glia prepared from CD200+/−, compared with wildtype, mice (†p<0.01, +++p<0.001, ANOVA; Figure 3.4A and B).

3.3.3. Pam3Csk4 significantly increased MCP-1 and IP-10 in mixed glia prepared from wildtype and CD200+/− mice.

Expression and supernatant concentrations of the chemokines MCP-1 and IP-10, were assessed in mixed glia following Pam3Csk4 incubation. Pam3Csk4 significantly increased mRNA expression of MCP-1 and IP-10 (**p<0.001, ANOVA; Figure 3.5A and 3.6A) and supernatant concentrations of MCP-1 and IP-10 in mixed glia prepared from wildtype and CD200+/− mice (**p<0.001, ANOVA; Figure 3.5B and 3.6B). The data show that the effect of Pam3Csk4 on the mRNA expression of MCP-1 and supernatant concentration was significantly greater in mixed glia prepared from CD200+/−, compared with wildtype, mice (†p<0.01, +++p<0.001, ANOVA; Figure 3.5A and B). The effect of Pam3Csk4 on supernatant concentrations of IP-10 was significantly greater in cells prepared from CD200+/−, compared with wildtype, mice (†p<0.05, ANOVA; Figure 3.6B).

3.3.4. Pam3Csk4 increased TLR mRNA expression in mixed glia prepared from wildtype and CD200+/− mice.

A previous study demonstrated that TLR2 works in conjunction with TLR1 to recognise triacylated lipoproteins (Takeuchi et al., 1999). In this study, it was found that Pam3Csk4 significantly increased both mRNA expression of TLR2 and TLR1 in mixed glia prepared from wildtype and CD200+/− mice (***p<0.001, ANOVA; Figure 3.7A and B). No genotype effect on mRNA expression of TLR2 was observed following Pam3Csk4 incubation. Basal mRNA expression of TLR2
was greater in mixed glia prepared from CD200\(^{-/-}\), compared with wildtype, mice but the difference was not statistically significant (Figure 3.7A). To further assess whether expression of TLR2 on microglia was altered, FACS analysis was performed on mixed glia. The data, (kindly donated by Dr Anthony Lyons), revealed that expression of TLR2 on CD11b\(^{+}\) cells was significantly increased in cells prepared from CD200\(^{-/-}\), compared with wildtype, mice (\(^{**}p<0.01\), student’s \(t\)-test; Figure 3.8). Table 3.1 summarises the mRNA expression results and supernatant concentration results.

**3.3.5. Aβ-induced mRNA expression of markers of microglial activation were attenuated by anti-TLR2.**

Microglia and astrocytes are capable of responding to Aβ through TLRs resulting in an inflammatory immune response (Landreth & Reed-Geaghan, 2009). Previously it has been found that Aβ\(_{1-42}\) increases the expression of TLR2 in microglial cultures (Jana et al., 2008). Here the effect of Aβ\(_{1-40/1-42}\) on mRNA expression of TLRs in mixed glia, prepared from neonatal C57BL/6 mice, was investigated. It was found that incubation of mixed glia in the presence of Aβ\(_{1-40/1-42}\) significantly increased mRNA expression of TLR2 in mixed glia (\(^{*}p<0.05\), student’s \(t\)-test; Figure 3.9A). However mRNA expression of neither TLR1 (Figure 3.9B) nor TLR4 (Figure 3.9C) were affected by Aβ\(_{1-40/1-42}\).

Studies by Jana and colleagues (2008) found that increased TLR2 expression was accompanied by increased production of pro-inflammatory markers and that knockdown of TLR2 suppressed the induction of these pro-inflammatory molecules and expression of integrin markers on microglia. In this study, several markers of microglial activation were investigated. Incubation of mixed glia in the presence of Aβ\(_{1-40/1-42}\) significantly increased mRNA expression of CD86 and CD40 (\(^{*}p<0.05\), \(^{**}p<0.01\), ANOVA; Figure 3.10A and B) and pre-incubation of mixed glia with anti-TLR2 significantly attenuated the Aβ-induced increase in mRNA expression of CD86 and CD40 (\(^{*}p<0.05\), \(^{++}p<0.01\), ANOVA; Figure 3.10A and B). Incubation of mixed glia in the presence of Aβ\(_{1-40/1-42}\) significantly decreased mRNA expression of CD11b but had no effect on CD68 (\(^{**}p<0.01\), ANOVA; Figure 3.11A and B). No significant effect of anti-TLR2 was observed in either CD11b or CD68 (Figure 3.11A and B).
3.3.6. Aβ-induced production of pro-inflammatory cytokines and chemokines was attenuated by anti-TLR2.

Data from several studies have demonstrated that TLR2 deficiency or knockdown decreases the production of Aβ-induced pro-inflammatory cytokines and chemokines by microglia (Udan et al., 2008; Liu et al., 2012). Here the production of cytokines and chemokine by mixed glia was assessed and the role of TLR2 in their production was investigated. Incubation of mixed glia in the presence of Aβ\(_{1-40}\) significantly increased mRNA expression of TNFα and IL-6 (***p<0.001, *p<0.05, ANOVA; Figure 3.12 and 3.13A). Incubation of mixed glia with anti-TLR2 significantly decreased mRNA expression of TNFα and IL-6 in mixed glia (***p<0.001, *p<0.05, ANOVA; Figure 3.12 and 3.13A). Aβ\(_{1-40}\) incubation significantly increased supernatant concentrations of IL-6 in mixed glia (**p<0.01, ANOVA; Figure 3.13B) and this Aβ-induced increase was attenuated by pre-incubation with anti-TLR2 (**p<0.01, ANOVA; Figure 3.14B). Supernatant concentrations of TNFα were not detectable.

Incubation of mixed glia in the presence of Aβ\(_{1-40}\) significantly increased mRNA expression of IP-10, MCP-1, MIP-1α and RANTES (***p<0.01, **p<0.001; ANOVA; Figures 3.15A-3.17A). The Aβ-induced increases in mRNA expression of IP-10, MCP-1, MIP-1α and RANTES were significantly attenuated by pre-incubation with anti-TLR2 (***p<0.01, **p<0.001, ANOVA; Figures 3.14A-3.17A). Aβ\(_{1-40}\) incubation significantly increased supernatant concentrations of MCP-1 and RANTES in mixed glia (**p<0.01; ANOVA; Figure 3.15B and 3.17B). Incubation with anti-TLR2 significantly decreased supernatant concentrations of MCP-1 and RANTES (**p<0.05, ANOVA; Figure 3.15B and 3.17B). Supernatant concentrations of IP-10 and MIP-1α were not significantly changed by Aβ\(_{1-40}\) incubation or anti-TLR2 pre-incubation (Figure 3.14A and 3.16A).

3.3.7. Aβ- and anti-TLR2-treatment induced different mRNA expression of immunosuppressant receptors and ligands on glia.

Several receptors expressed on neurons and astrocytes play an important role in suppressing microglial activation. Interaction of fractalkine and CD200
with their respective ligands plays an important role in dampening the pro-
inflammatory response (Cameron & Landreth, 2010). In this study, both CD200 receptor and fractalkine receptor-ligand interactions were investigated. It was found that incubation of mixed glia in the presence of Aβ1-40/1-42 increases mRNA expression of CD200R (\#p<0.05, student’s t-test; Figure 3.18a) but not that of CD200L (Figure 3.18B). Incubation of mixed glia with anti-TLR2 had no significant effect on mRNA expression of CD200R (Figure 3.18A). CD200L mRNA expression was significantly increased in mixed glia incubated with anti-
TLR2 (\**p<0.01, ANOVA; Figure 3.18B) and this effect was attenuated in cells which were incubated in the presence of Aβ1-40/1-42 and anti-TLR2 (\++p<0.01, ANOVA; Figure 3.18B).

Incubation of mixed glia in the presence of Aβ1-40/1-42 significantly decreased mRNA expression of fractalkine receptor (\***p<0.001, ANOVA; Figure 3.19A), whereas anti-TLR2-incubation significantly increased mRNA expression of fractalkine receptor in mixed glia (\'p<0.05, ANOVA; Figure 3.19A). Incubation of mixed glia in the presence of Aβ1-40/1-42 significantly increased mRNA expression of fractalkine (\***p<0.001, ANOVA; Figure 3.20B), whereas anti-TLR2-incubation significantly decreased mRNA expression of fractalkine in mixed glia (\++p<0.001, ANOVA; Figure 3.19B).

Table 3.2 summarises the mRNA expression results and Table 3.3 summerises the supernatant concentration results.
Figure 3.1. Pam₃Csk₄ increased CD11b and CD40 mRNA expression.

Pam₃Csk₄ (100 ng/ml; 24 hours) significantly increased CD11b (A) and CD40 (B) mRNA expression in mixed glia prepared from wildtype and CD200⁻⁄ mice (**p<0.001, ANOVA). Basal CD40 mRNA expression was significantly increased in CD200⁻⁄, compared with wildtype, mice (b) (⁺p<0.05, student’s t-test). Values are expressed as means ± SEM (n=3-5).

(A) 2-way ANOVA: Pam₃Csk₄ effect F (1,13) = 15.25; p<0.0001, Genotype effect F (1,13) = 2.059; p= 0.175, Interaction effect F (1,13) = 0.7202; p=0.414

(B) 2-way ANOVA: Pam₃Csk₄ effect F (1,10) = 114.7; p<0.0001, Genotype effect F (1,10) = 0.02292; p= 0.8827, Interaction effect F (1,10) = 0.01606; p=0.9017

(b) ⁺p=0.0384; 0.8208 ± 0.1215; n=4 versus 1.447 ± 0.2037; n=4
Figure 3.2. Pam₃Csk₄ decreased CD68 mRNA expression.

Pam₃Csk₄ (100 ng/ml; 24 hours) significantly decreased CD68 mRNA expression in mixed glia prepared from wildtype and CD200⁻/⁻ mice (**p<0.001, ANOVA). Values are expressed as means ± SEM (n=4).

2-way ANOVA: Pam₃Csk₄ effect F (1,11) = 23.91; p=0.0005, Genotype effect F (1,11) = 1.874; p=0.1983, Interaction effect F (1,11) = 3.708; p=0.0804
Figure 3.3. Pam₃Csk₄ increased production of TNFα.

Pam₃Csk₄ (100 ng/ml; 24 hours) caused a significant increase in TNFα mRNA expression (A) and supernatant concentration (B) in mixed glia prepared from wildtype and CD200⁻/⁻ mice (***p<0.001, ANOVA). There was a significant increase in supernatant concentration (B) of TNFα in mixed glia prepared from CD200⁻/⁻, compared with wildtype, mice (+++p<0.001, ANOVA), and a significant interaction between Pam₃Csk₄ and genotype was observed (B) (++;p<0.01, ANOVA). Values are expressed as means ± SEM (n=3-4).

(A) 2-way ANOVA: Pam₃Csk₄ effect F (1,9) = 297.2; p<0.0001, Genotype effect F (1,9) = 1.072; p= 0.3275, Interaction effect F (1,9) = 0.1757; p=0.6850

(B) 2-way ANOVA: Pam₃Csk₄ effect F (1,10) = 123.2; p<0.0001, Genotype effect F (1,10) = 33.70; p= 0.0002, Interaction effect F (1,10) = 33.29; p=0.0002
Figure 3.4. Pam3Csk4 increased production of IL-6.

Pam3Csk4 (100 ng/ml; 24 hours) caused a significant increase in IL-6 mRNA expression (A) and supernatant concentration (B) in mixed glia prepared from wildtype and CD200^+ mice (***p<0.001, ANOVA). There was a significant increase in IL-6 mRNA expression (A) and supernatant concentration (B) in mixed glia prepared from CD200^+ compared with wildtype, mice (^+p<0.01, +++p<0.001, ANOVA). A significant interaction between Pam3Csk4 and genotype was observed (A and B) (^+p<0.01, +++p<0.001, ANOVA). Values are expressed as means ± SEM (n=3-4).

(A) 2-way ANOVA: Pam3Csk4 effect F (1,7) = 85.78; p<0.001, Genotype effect F (1,7) = 26.47; p = 0.0013, Interaction effect F (1,7) = 26.47; p=0.0013

(B) 2-way ANOVA: Pam3Csk4 effect F (1,10) = 236.4; p<0.0001, Genotype effect F (1,10) = 26.20; p= 0.0005, Interaction effect F (1,10) = 55.12; p=0.0005
Figure 3.5. Pam3Csk4 increased production of MCP-1.

Pam3Csk4 (100 ng/ml; 24 hours) caused a significant increase in MCP-1 mRNA expression (A) and supernatant concentration (B) in mixed glia prepared from wildtype and CD200−/− mice (***p<0.001, ANOVA). There was a significant increase in MCP-1 mRNA expression (A) and supernatant concentration (B) in mixed glia prepared from CD200−/−, compared with wildtype, mice (++p<0.01, +++p<0.001, ANOVA). A significant interaction between Pam3Csk4 and genotype was observed (A and B) (p<0.05, ++p<0.01, ANOVA). Values are expressed as means ± SEM (n=3-6).

(A) 2-way ANOVA: Pam3Csk4 effect F (1,9) = 109.7; p<0.0001, Genotype effect F (1,9) = 15.92; p= 0.0032, Interaction effect F (1,9) = 15.70; p=0.0033

(B) 2-way ANOVA: Pam3Csk4 effect F (1,17) = 1281; p<0.0001, Genotype effect F (1,17) = 21.33; p= 0.0002, Interaction effect F (1,17) = 5.752; p=0.0282
Figure 3.6. Pam$_3$Csk$_4$ increased production of IP-10.

Pam$_3$Csk$_4$ (100 ng/ml; 24 hours) caused a significant increase in IP-10 mRNA expression (A) and supernatant concentration (B) in mixed glia prepared from wildtype and CD200$^{-/-}$ mice (***p<0.001, ANOVA). There was a significant increase in IP-10 supernatant concentration (B) in mixed glia prepared from CD200$^{-/-}$, compared with wildtype, mice (**p<0.05, ANOVA). A significant interaction between Pam$_3$Csk$_4$ and genotype was observed (B) (*p<0.05, ANOVA). Values are expressed as means ± SEM (n=4-5).

(A) 2-way ANOVA: Pam$_3$Csk$_4$ effect F (1,12) = 47.56; p<0.0001, Genotype effect F (1,12) = 1.355; p= 0.2671, Interaction effect F (1,12) = 1.181; p=0.2985

(B) 2-way ANOVA: Pam$_3$Csk$_4$ effect F (1,15) = 134.8; p<0.0001, Genotype effect F (1,15) = 5.280; p= 0.0364, Interaction effect F (1,15) = 4.703; p=0.0466
Figure 3.7. Pam₃Csk₄ increased TLR mRNA expression.

Pam₃Csk₄ (100 ng/ml; 24 hours) significantly increased TLR2 (A) and TLR1 (B) mRNA expression in mixed glia prepared from wildtype and CD200⁻/⁻ mice (***p<0.001, ANOVA). Values are expressed as ± SEM (n=4-5).

(A) 2-way ANOVA: Pam₃Csk₄ effect F (1,12) = 68.61; p<0.0001, Genotype effect F (1,12) = 1.530; p= 0.2397, Interaction effect F (1,12) = 0.5228; p=0.4835

(B) 2-way ANOVA: Pam₃Csk₄ effect F (1,13) = 208.6; p<0.0001, Genotype effect F (1,13) = 2.118; p= 0.1693, Interaction effect F (1,13) = 2.854; p=0.115
Figure 3.8. Increased number of TLR2 expressing CD11b⁺ cells in CD200⁻/⁻, compared with wildtype, mice.

There was a significant increase in TLR2 expression on CD11b⁺ cells in mixed glia prepared from CD200⁻/⁻, compared with wildtype, mice (**p<0.01, student’s t-test). Values are expressed as ± SEM (n=3-5).

**p=0.006; 7.676 ± 1.634; n=5 versus 23.58 ± 4.318; n=4
Figure 3.9. TLR2 mRNA expression was increased by Aβ_{1-40/1-42}.

Aβ_{1-40/1-42} (10 μM; 24 hours) significantly increased TLR2 mRNA expression (A) in mixed glia prepared from neonatal C57BL/6 mice (*p<0.05, student’s t-test). TLR1 (B) and TLR4 (C) mRNA expression was not affected by Aβ_{1-40/1-42}. Values are expressed as means ± SEM (n=6).

(A) *p=0.0320; 1.281 ± 0.1333; n=6 versus 2.154 ± 0.3242; n=6
Figure 3.10. The Aβ-induced increase in CD86 and CD40 mRNA expression was attenuated by anti-TLR2.

Incubation of mixed glia in the presence of Aβ_{1-40/1-42} (10 μM; 24 hours) significantly increased CD86 and CD40 mRNA expression (A and B) (*p<0.05, **p<0.01, ANOVA). Incubation of mixed glia with anti-TLR2 (2.5 μg/ml; 2 hours) significantly decreased CD40 mRNA expression (B) (++p<0.01, ANOVA). A significant interaction between Aβ_{1-40/1-42} and anti-TLR2 pre-incubation was observed (A and B) (*p<0.05, **p<0.01, ANOVA). Values are expressed as means ± SEM (n=5-6).

(A) 2-way ANOVA: Aβ effect F (1,16) = 5.191; p=0.0368, anti-TLR2 effect F (1,16) = 1.641; p=0.2184, Interaction effect F (1,16) = 12.77; p=0.0025

(B) 2-way ANOVA: Aβ effect F (1,20) = 10.95; p=0.0035, anti-TLR2 effect F (1,20) = 9.241; p=0.0065, Interaction effect F (1,20) = 5.892; p=0.0248

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Figure 3.11. CD11b and CD68 mRNA expression was unaffected by Aβ_{1-40/1-42} or anti-TLR2.

Incubation of mixed glia in the presence of Aβ_{1-40/1-42} (10 μM; 24 hours) significantly decreased CD11b mRNA expression (A) (**p<0.01, ANOVA) whereas CD68 mRNA expression was unaffected (B). Anti-TLR2 incubation (2.5 μg/ml; 2 hours) had no significant effect on the mRNA expression of either CD11b or CD68 (A and B). Values are expressed as means ± SEM (n=5-6).

(A) 2-way ANOVA: Aβ effect F (1,120) = 14.19; p=0.0012, anti-TLR2 effect F (1,20) = 1.574; p=0.2240, Interaction effect F (1,19) = 2.878; p=0.1053

(B) 2-way ANOVA: Aβ effect F (1,17) = 2.108; p=0.1648, anti-TLR2 effect F (1,17) =0.5450; p=0.4704, Interaction effect F (1,17) = 1.104; p=0.3081
Figure 3.12. The Aβ-induced increase in TNFα mRNA expression was attenuated by anti-TLR2.

Incubation of mixed glia in the presence of Aβ_{1-40/1-42} (10 μM; 24 hours) significantly increased TNFα mRNA expression (***p<0.001, ANOVA). Incubation of mixed glia with anti-TLR2 (2.5 μg/ml; 2 hours) significantly decreased TNFα mRNA expression (***p<0.001, ANOVA). Values are expressed as means ± SEM (n=6).

2-way ANOVA: Aβ effect F (1,20) = 16.63; p=0.0006, anti-TLR2 effect F (1,20) = 45.58; p<0.0001, Interaction effect F (1,20) = 3.155; p=0.0909
Figure 3.13. The Aβ-induced increase in IL-6 production was attenuated by anti-TLR2.

Incubation of mixed glia in the presence of Aβ_{1-40/1-42} (10 μM; 24 hours) significantly increased IL-6 mRNA expression (A) and supernatant concentration (B) (\(^*p<0.05\), \(^{**}p<0.01\), ANOVA). Incubation of mixed glia with anti-TLR2 (2.5 μg/ml; 2 hours) significantly decreased IL-6 mRNA expression (A) and supernatant concentration (B) (\(^*p<0.05\), \(^{+++}p<0.001\), ANOVA). A significant interaction between Aβ_{1-40/1-42} and anti-TLR2 pre-incubation on IL-6 supernatant concentrations was observed (B) (\(^{++}p<0.01\), ANOVA). Values are expressed as means ± SEM (n=4-6).

(A) 2-way ANOVA: Aβ effect \(F(1,19) = 5.462; p=0.0305\), anti-TLR2 effect \(F(1,19) = 4.846; p=0.0403\), Interaction effect \(F(1,19) = 3.400; p=0.0809\)

(B) 2-way ANOVA: Aβ effect \(F(1,12) = 17.62; p=0.0012\), anti-TLR2 effect \(F(1,12) =30.38; p=0.0001\), Interaction effect \(F(1,12) = 9.540; p=0.0094\)
Figure 3.14. The Aβ-induced increase in IP-10 mRNA expression was attenuated by anti-TLR2.

Incubation of mixed glia in the presence of Aβ1-40/1-42 (10 μM; 24 hours) significantly increased IP-10 mRNA expression (A) (**p<0.01, ANOVA). Incubation of mixed glia with anti-TLR2 (2.5 μg/ml; 2 hours) significantly decreased IP-10 mRNA expression (A) (\(^{+}p<0.05\), ANOVA), and a significant interaction between Aβ1-40/1-42 and anti-TLR2 pre-incubation was observed (A) (\(^{+}p<0.05\), ANOVA). IP-10 supernatant concentrations (B) were unaltered following incubation of mixed glia in the presence of Aβ1-40/1-42 with or without anti-TLR2 pre-incubation. Values are expressed as means ± SEM (n=3-6).

(A) 2-way ANOVA: Aβ\_effect F (1,19) = 7.384; p=0.0014, anti-TLR2 effect F (1,19) = 5.975; p=0.0244, Interaction effect F (1,19) = 13.90; p=0.0137

(B) 2-way ANOVA: Aβ\_effect F (1,9) = 2.855; p=0.1254, anti-TLR2 effect F (1,9) =1.055; p=0.3311, Interaction effect F (1,9) = 1.941; p=0.1970
Figure 3.15. The Aβ-induced increase in MCP-1 production was attenuated by anti-TLR2.

Incubation of mixed glia in the presence of Aβ$_{1-40/1-42}$ (10 μM; 24 hours) significantly increased MCP-1 mRNA expression (A) and supernatant concentration (B) (*** p<0.001, ANOVA). Incubation of mixed glia with anti-TLR2 (2.5 μg/ml; 2 hours) significantly decreased MCP-1 mRNA expression (A) and supernatant concentration (B) (** p<0.01, * p<0.05, ANOVA). A significant interaction between Aβ$_{1-40/1-42}$ and anti-TLR2 pre-incubation on MCP-1 mRNA expression was observed (A) (** p<0.01, ANOVA). Values are expressed as means ± SEM (n=4-6).

(A) 2-way ANOVA: Aβ effect F (1,19) = 22.59; p=0.0001, anti-TLR2 effect F (1,19) = 14.28; p=0.0013, Interaction effect F (1,19) = 13.02; p=0.0019

(B) 2-way ANOVA: Aβ effect F (1,12) = 19.16; p=0.0009, anti-TLR2 effect F (1,12) =7.712; p=0.0167, Interaction effect F (1,12) = 0.1034; p=0.7533
Figure 3.16. The Aβ-induced increase in MIP-1α production was attenuated by anti-TLR2.

Incubation of mixed glia in the presence of Aβ\textsubscript{1-40/1-42} (10 μM; 24 hours) significantly increased MIP-1α mRNA expression (A) (**p<0.001, ANOVA). Incubation of mixed glia with anti-TLR2 (2.5 μg/ml; 2 hours) significantly decreased MIP-1α mRNA expression (A) (+++p<0.001, ANOVA) and a significant interaction between Aβ\textsubscript{1-40/1-42} and anti-TLR2 pre-incubation was observed (A) (+++p<0.001, ANOVA). MIP-1α supernatant concentrations (B) were unaffected by incubation of mixed glia in Aβ\textsubscript{1-40/1-42} with or without anti-TLR2. Values are expressed as means ± SEM (n=6-8).

(A) 2-way ANOVA: Aβ effect F (1,20) = 43.48; p<0.0001, anti-TLR2 effect F (1,20) = 22.59; p=0.0001, Interaction effect F (1,20) = 222.14; p=0.0001

(B) 2-way ANOVA: Aβ effect F (1,12) = 19.16; p=0.0009, anti-TLR2 effect F (1,12) =7.712; p=0.0167, Interaction effect F (1,12) = 0.1034; p=0.7533
Figure 3.17. The Aβ-induced increase in RANTES production was attenuated by anti-TLR2.

Incubation of mixed glia in the presence of Aβ_{1-40/1-42} (10 μM; 24 hours) significantly increased RANTES mRNA expression (A) and supernatant concentration (B) (**p<0.01, ***p<0.001, ANOVA). Incubation of mixed glia with anti-TLR2 (2.5 μg/ml; 2 hours) significantly decreased RANTES mRNA expression (A) and supernatant concentration (B) (**p<0.01, +p<0.05, ANOVA). A significant interaction between Aβ_{1-40/1-42} and anti-TLR2 pre-incubation on RANTES mRNA expression was observed (A) (^^p<0.01, ANOVA). Values are expressed as means ± SEM (n=4-6).

(A) 2-way ANOVA: Aβ effect F (1,19) = 20.05; p=0.0003, anti-TLR2 effect F (1,19) = 12.50; p=0.0022, Interaction effect F (1,19) = 8.397; p=0.0092

(B) 2-way ANOVA: Aβ effect F (1,11) = 16.24; p=0.0020, anti-TLR2 effect F (1,11) =5.598; p=0.0374, Interaction effect F (1,11) = 2.481; p=0.1436
Figure 3.18. Aβ$_{1-40/1-42}$ increased CD200R but not CD200L mRNA expression.

Incubation of mixed glia in the presence of Aβ$_{1-40/1-42}$ (10 µM; 24 hours) increased CD200R mRNA expression (A) (**p<0.01, ANOVA). Incubation of mixed glia with anti-TLR2 (2.5 µg/ml; 2 hours) had no effect on CD200R mRNA expression (A). Incubation of mixed glia in the presence of Aβ$_{1-40/1-42}$ (10 µM; 24 hours) had no effect on CD200L mRNA expression (B). CD200L mRNA expression was significantly increased in mixed glia incubated with anti-TLR2 (B) (**p<0.01, ANOVA). A significant interaction between Aβ$_{1-40/1-42}$ and anti-TLR2 pre-incubation was observed. Aβ$_{1-40/1-42}$ incubation attenuated the significant anti-TLR2-induced increase in CD200L mRNA expression (B) (**p<0.01, ANOVA). Values are expressed as means ± SEM (n=6).

(A) 2-way ANOVA: Aβ effect F (1,18) = 0.6840; p=0.4190, anti-TLR2 effect F (1,18) = 1.594; p=0.2229, Interaction effect F (1,18) = 0.2423; p=0.6285

(B) 2-way ANOVA: Aβ effect F (1,18) = 15.29; p=0.0010, anti-TLR2 effect F (1,18) =10.78; p=0.0041, Interaction effect F (1,18) =10.37; p=0.0047

(a) *p=0.0425; 0.7224 ± 0.08632; n=5 versus 2.525 ± 0.6870; n=6

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Figure 3.19. $\text{A} \beta_{1-40/1-42}$ exerted different effects on fractalkine receptor and fractalkine ligand mRNA expression.

Incubation of mixed glia in the presence of $\text{A} \beta_{1-40/1-42}$ (10 $\mu$M; 24 hours) significantly decreased mRNA expression of fractalkine receptor (A) ($^{***} p<0.001$, ANOVA). Incubation of mixed glia with anti-TLR2 (2.5 $\mu$g/ml for 2 hours) significantly increased fractalkine receptor mRNA expression in mixed glia compared with controls (A) ($^{*} p<0.05$, ANOVA). Incubation of mixed glia in the presence of $\text{A} \beta_{1-40/1-42}$ (10 $\mu$M; 24 hours) significantly increased fractalkine ligand (B) ($^{***} p<0.001$, ANOVA). Incubation of mixed glia with anti-TLR2 (2.5 $\mu$g/ml; 2 hours) significantly decreased fractalkine ligand mRNA expression (B) ($^{**} p<0.001$, ANOVA). Values are expressed as means ± SEM (n=6).

(A) 2-way ANOVA: $\text{A} \beta$ effect $F (1,19) = 38.39$; $p < 0.0001$, anti-TLR2 effect $F (1,19) = 6.886$; $p = 0.0167$, Interaction effect $F (1,19) = 1.616$; $p = 0.2191$

(B) 2-way ANOVA: $\text{A} \beta$ effect $F (1,20) = 24.24$; $p < 0.0001$, anti-TLR2 effect $F (1,20) = 26.12$; $p < 0.0001$, Interaction effect $F (1,20) = 1.689$; $p = 0.2085$

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3.4. Discussion

The aims of this study were to investigate changes induced by the TLR2 agonist Pam$_3$Csk$_4$ in mixed glia prepared from CD200$^{+/+}$, compared with wildtype, mice and to examine the role of the CD200-CD200R interaction in modulating microglial activation. To establish whether activation of TLR2 modulates Aβ-induced changes in these cells.

During neuroinflammation microglia become progressively more activated. This activation is associated with a morphological change and the upregulation of several cell surface markers including CD11b and CD40. These cell surface markers enable interaction with other cells like T cells to initiate an immune response but can also be used to measure the extent of microglial activation. Roy and colleagues (2008) reported that increased expression of CD11b correlates with the degree of microglial activation. Microglial stimulation by *S. aureus* and PGN, both of which activate TLR2, elevates expression of MHC class II, CD40, CD80 and CD86 (Kielian *et al.*, 2002). Furthermore, stimulation of microglial cultures with Pam$_3$Csk$_4$ leads to microglial morphological changes resulting in the more activated amoeboid form (Ebert *et al.*, 2005). In this study, Pam$_3$Csk$_4$ increased CD11b and CD40 mRNA expression in mixed glia prepared from wildtype and CD200$^{+/+}$ mice yet no exaggerated increase was observed in glia prepared from CD200$^{+/+}$ mice. Further, CD40 but not CD11b mRNA expression was increased in cells prepared from CD200$^{+/+}$, compared with wildtype, mice. These results are consistent with previous findings from this laboratory suggesting that CD200R engagement maintains microglia in a quiescent phenotype (Lyons *et al.*, 2007). CD68, a marker of phagocytic activity, has been found to be increased in CD200$^{+/+}$ mice infected with EAE (Hoek *et al.*, 2000). CD68 mRNA expression was found to be decreased in mixed glia prepared from wildtype and CD200$^{+/+}$ mice following Pam$_3$Csk$_4$ incubation. CD68 is a lysosomal protein and it appears to be cycled to the membrane when the cell is undergoing phagocytic activity. The expression of CD68 measured here was at the gene level and perhaps any changes in expression may have been observed at the protein level. Furthermore, the cells may be activated as measured by other markers (CD40 and CD11b) but simply not involved in phagocytosis.
TLR activation through differential pathogen-associated molecules leads to the activation of signalling cascades which result in the production of a tailored pro-inflammatory cytokine response (Jack et al., 2005). Both microglia (Applequist et al., 2002) and astrocytes (Bowman et al., 2003) are reported to express an array of TLRs including TLR2. Studies using human microglial and astrocyte cultures (Jack et al., 2005) and murine microglial cultures (Shah et al., 2009) found that TNFα and IL-6 concentrations were increased following Pam3Csk4 incubation. Du and co-authors (2011) demonstrated that Pam3Csk4 treatment significantly increased IL-6 production in brain homogenate samples from murine pups and this effect was significantly greater than that induced by LPS (Du et al., 2011). Additionally, PGN, another TLR2 agonist, induces astrocytes to produce TNFα and IL-6 (Esen et al., 2004). Here Pam3Csk4 increased the mRNA expression and release of TNFα and IL-6 from mixed glia prepared from wildtype and CD200<sup>-/-</sup> mice and the effects were enhanced in glia prepared from CD200<sup>-/-</sup>, compared with wildtype, mice. These results are consistent with previous studies from this laboratory and others which demonstrate an increase of IL-6 and TNFα in glial cultures in the absence of CD200 (Lyons et al., 2007; Lyons et al., 2009b). Studies using macrophages prepared from CD200<sup>-/-</sup> animals have demonstrated increased IL-6 and TNFα levels in response to LPS (Snelgrove et al., 2008). Furthermore, neutralizing the receptor-ligand interaction significantly amplifies IFN-γ induced IL-6 secretion in macrophages (Meuth et al., 2008) whereas the use of DX109, a CD200R agonist, can inhibit IL-6 production from IFN-γ-stimulated bone-marrow derived macrophages (Copland et al., 2007). In contrast it has been shown that macrophages from the colon of CD200R1<sup>-/-</sup> mice do not have exacerbated TNFα production in response to TLR ligands compared with wildtype macrophages (Bain & Mowat, 2012). The discrepancy between studies in macrophages suggests that perhaps the CD200-CD200R interaction may not be a universal inhibitor of macrophage function and may have differential control mechanisms based on the microenvironment of the tissue.

Chemokines and their receptors are upregulated in neurodegenerative diseases and this probably contributes to increased infiltration of leukocytes into the brain and exacerbated inflammation (Mennicken et al., 1999; Cartier et al., 2005). Both microglia and astrocytes upregulate chemokine production following
TLR activation and cytokine stimulation (Okun et al., 2009). There are several publications emphasizing the potency of Pam3Csk4 stimulation on chemokine secretion from various cell types. It has been demonstrated that human monocytes have increased secretion of MCP-1 following Pam3Csk4 stimulation (Dasu et al., 2009). These authors found a significant increase in NF-κB p65 dependent DNA binding activity in response to Pam3Csk4 which accounts for the large increase in cytokines and chemokines following TLR2 stimulation. A study by Gurley and colleagues (2008) investigating TLRs modulation by peroxisome proliferator-activated receptor (PPAR)-γ agonists revealed a similar increase in CXCL2 concentrations, a chemokine of the same family as IP-10, in murine astrocytes and microglia stimulated with Pam3Csk4 alone. This group also found an increase in MCP-1 concentrations in astrocytes and microglia treated with Pam3Csk4 compared with controls (Gurley et al., 2008). Currently there are few publications on the impact of CD200 on chemokine production by microglia and astrocytes however, here it was found that Pam3Csk4 induced a significant increase in both MCP-1 and IP-10 mRNA expression, with enhanced MCP-1 mRNA expression and MCP-1 and IP-10 release in mixed glia prepared from CD200''/'' compared with wildtype, mice. Previously it has been shown that a CD200R agonist can inhibit IFN-γ induced production of IP-10 and other chemokines in human monocytic cells (Jenmalm et al., 2006). Inhibition of chemokines such as IP-10 would reduce recruitment of peripheral inflammatory cells such as neutrophils and Th1 cells to sites of inflammation (Proudfoot, 2002). This suggests that the loss of the CD200-CD200R inhibitory signal allows for increased chemokine production which may, in turn, facilitate increased inflammatory cell infiltration to sites of tissue damage.

Pam3Csk4 is a synthetic tripalmitoylated lipopeptide that is specifically recognized by a heterodimer of TLR2 and TLR1 (Takeda et al., 2002; Du et al., 2011). Previous studies have established that microglia constitutively express TLR2 (Laflamme et al., 2001; Kielian et al., 2002; Olson & Miller, 2004). TLR2 expression has been observed on human (Jack et al., 2005) and murine (Kielian et al., 2002) microglia cells. Several studies have demonstrated enhanced TLR2 and TLR1 expression following administration of TLR agonists including LPS (Laflamme et al., 2001), S. aureus and PGN (Laflamme et al., 2001; Kielian et al., 2002). TLR2 and TLR1 mRNA expression was investigated in the present
study and the data shows that expression of both TLRs was increased in mixed glia prepared from wildtype and CD200<sup>−/−</sup> mice in response to Pam<sub>3</sub>Csk<sub>4</sub>. There was no difference in TLR1 or TLR2 expression in mixed glia prepared from CD200<sup>−/−</sup>, compared with wildtype, mice. However assessment of mRNA expression may not be a reflection of cell surface expression of the receptor therefore TLR2 surface expression on microglia prepared from wildtype and CD200<sup>−/−</sup> mice was investigated. Flow cytometric analysis revealed that there was significantly greater surface expression of TLR2 on CD11b<sup>+</sup> cells prepared from CD200<sup>−/−</sup>, compared with wildtype, mice.

These data suggest that the greater response in glia prepared from CD200<sup>−/−</sup>, compared with wildtype, mice to Pam<sub>3</sub>Csk<sub>4</sub> may be due, at least in part, to the increased expression of TLR2. Taken together, the in vitro data suggests that enhanced activation seen in glia prepared from CD200<sup>−/−</sup> mice may be due to both the absence of the CD200-CD200R interaction and also due to increased expression of TLR2 on CD200<sup>−/−</sup> microglia. It is possible that increased expression of TLR2 on CD200<sup>−/−</sup> microglia may allow for their over-activation and the increased inflammatory profile. CD200 is now implicated in the pathology of both neurodegenerative and autoimmune diseases. Thus the role of CD200 and its interaction with TLRs needs to be further investigated in an in vivo model as both these receptors may be targeted for therapeutic manipulation. The role of CD200-CD200R in an in vivo model is investigated in the next chapter.

A specific aim of this study was to investigate the role of TLR2 in mediating glial activation in responses to Aβ and development of a pro-inflammatory environment. While it is important for microglia to become “activated” resulting in an inflammatory environment for an effective response for elimination of pathogens, it is maladaptive when microglial activation is initiated by disease processes that originate within CNS and to date there is clear evidence implicating microglia in the pathogenesis of neurodegenerative diseases (Cameron & Landreth, 2010). It has been demonstrated that the extent of inflammation and the phenotype adopted by glial cells may rely on what receptors are activated and which inflammatory molecules are present at the time of stimulation. Increasing evidence now suggests that TLRs may play a significant role in the neuroinflammation seen in the pathogenesis of AD. Studies from mouse models of AD have found that TLR2 and TLR7 expression are upregulated (Letiembre et
al., 2009). TLR2 and TLR4 expression are increased in the post-mortem brain of AD patients (Walter et al., 2007). Evidence from other studies have implicated TLRs and their co-receptor, CD14, in neurodegeneration (Liu et al., 2005; Jana et al., 2008; Udan et al., 2008). Together, these studies suggest that the increase in TLRs may make them potential players in neurodegenerative mechanisms however, it is still unclear if TLRs activation is beneficial or detrimental. Several studies have established that microglia constitutively express TLR2 (Kielian, 2006) and that it becomes robustly upregulated on microglia during infection and disease (Glezer et al., 2007). Here it was found that incubation of mixed glia in the presence of Aβ significantly increased TLR2 expression but not that of TLR4 or TLR1. The increase in TLR2 expression is consistent with a study from Jana and co-workers (2008) who similarly found increased TLR2 expression in both microglial cultures and BV-2 cell lines in response to Aβ. It is interesting that there was no change seen in TLR1 expression as a recent study by Liu and colleagues (2012) found that TLR2 interacting with TLR1, rather than TLR6, enhanced Aβ-triggered inflammation.

Previous investigators have found that cultured microglia deficient in CD14, TLR2 and TLR4 exhibit significantly reduced Aβ-induced microglial activation (Walter et al., 2007; Jana et al., 2008; Udan et al., 2008; Reed-Geaghan et al., 2009). Furthermore it has been demonstrated that Aβ-induced TLR2 expression, in microglial cultures, is associated with increases in markers of microglial activation (Jana et al., 2008). Here it was found that Aβ increased the expression of CD40 and CD86 and this was attenuated by pre-treatment with anti-TLR2. CD68, a marker of phagocytosis, and CD11b, an integrin marker, were also investigated and it was found that CD68 expression was unchanged while CD11b was significantly decreased following Aβ-treatment. These results were a little surprising as both CD68 and CD11b have previously been found to be increased in microglial cultures in response to Aβ (Jana et al., 2008). However, previously it has been described that ligation of CD40 significantly decreases phagocytosis of Aβ by microglia (Townsend et al., 2005). It is possible that increases in CD40 and CD86 rather than CD68 suggest the cells have adopted an APC phenotype rather than a phagocytic phenotype. At present it still remains unclear under what circumstances and by what mechanism TLR activation may promote Aβ phagocytosis. Studies suggest that both TLR2 and TLR4 are involved

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in the phagocytosis of Aβ. Chen and colleagues (2006) describe the internalisation of Aβ through TLR2 and TLR9 activation via the activation of the murine G-protein-coupled formyl peptide receptor 1 (mFPRL1). Similarly TLR4-induced Aβ phagocytosis requires mFPRL1 and can be blocked by the G-protein inhibitor pertussis toxin (Tahara et al., 2006). Furthermore, investigators have found that murine microglial cultures (Chen et al., 2006) and BV-2 cell lines increase Aβ uptake when stimulated with TLR2, TLR4 and TLR9 agonists (Tahara et al., 2006). Conversely a recent study reports that microglia from TLR2-deficient mice phagocytose significantly more Aβ than cells from wildtype mice, (Liu et al., 2012), suggesting that TLR2 activation by Aβ leads to a pro-inflammatory response rather than phagocytic response. Importantly caution has been suggested when interpreting some results as the discrepancies seen between studies may be due to the contamination of the Aβ preparation with bacterial components (Kiellian, 2006). It is worth noting that previous reports suggest that both TLR2 and TLR4 are needed for Aβ-stimulated phagocytosis, (Reed-Geaghan et al., 2009), and in the cultures used here no significant increase in TLR4 expression was observed which may account for the lack of change in CD68 expression.

There is a wealth of literature describing the production of cytokines and chemokines by glial cells following TLR ligation by an array of ligands (Jack et al., 2005; Kiellian, 2006). The recent appreciation that Aβ also acts as a ligand for TLRs may account, in part, for microglial responses to Aβ and the production of pro-inflammatory cytokines. The production and secretion of two pro-inflammatory cytokines was investigated and it was found that the Aβ-induced increases in TNFα expression and IL-6 expression and production were attenuated by anti-TLR2 treatment. Consistent with the present findings, previous studies have found that blocking TLR2 through antibodies (Udan et al., 2008) and anti-sense knockdown (Jana et al., 2008) prevents Aβ-induced IL-6 and TNFα production.

Under normal circumstances, chemokine expression within the CNS is low however several chemokines including MCP-1, IP-10, MIP-1α and RANTES become unregulated during diverse pathological states including neurodegenerative diseases (Cartier et al., 2005). The implications of chemokines in CNS pathologies such as MS and HIV has already been widely investigated. Both IP-10 and RANTES have been detected in CSF samples taken from MS
patients (Sorensen et al., 1999) and increased MCP-1 (McManus et al., 1998), RANTES (Sorensen et al., 1999) and MIP-1α (Simpson et al., 1998) expression has been demonstrated in MS lesions. Furthermore, it has been found that antibodies against MIP-1α reduced inflammation and inhibited development of symptoms in an adoptively-transfer model EAE while antibodies against MCP-1 inhibited relapses (Karpus & Kennedy, 1997). Similarly MCP-1 and MIP-1α have been detected in the CNS of HIV patients (Conant et al., 1998; McManus et al., 2000). Although chemokines and their receptors were only thought to play a role in neurological disorders such as MS, there is now growing evidence to support the role of chemokines in AD (Cartier et al., 2005). Previously it has been found that there is a marked IP-10 upregulation in reactive astrocytes in post-mortem brain tissue from AD patients and a frequent association between IP-10 positive astrocytes and amyloid deposits has been observed (Xia et al., 2000). Furthermore, the presence of MCP-1 has been reported in mature senile plaques of brain tissue from AD patients (Ishizuka et al., 1997). More recently IP-10 and MCP-1 levels have been found to be increased in CSF samples from AD patients (Galimberti et al., 2003).

One aim was to investigate if TLR2 activation played a role in Aβ-induced production of chemokines. It was found that the Aβ-induced increase in the mRNA expression of the chemokines MCP-1, IP-10, MIP-1α and RANTES was attenuated by anti-TLR2. At the protein level, Aβ-induced an increase in MCP-1 and RANTES supernatant concentrations and this was also ameliorated by anti-TLR2 although concentrations of IP-10 and MIP1-α were unchanged. These data are consistent with previous in vitro studies in astrocytes, oligodendrocytes and human monocytes which demonstrated an Aβ-induced increase in MCP-1, RANTES and MIP-1α (Cartier et al., 2005). However the finding that the Aβ-induced increases in chemokine expression was attenuated by anti-TLR2 treatment has not been demonstrated before. It is worth noting that Aβ increased CD40; CD40 has been associated with promoting increased chemokine secretion by microglia which in turn facilitates leukocyte infiltration and an amplified inflammatory process in HIV post-mortem brain tissue (D'Aversa et al., 2002). One possibility is that the increase in CD40 and chemokine production would allow for peripheral cell infiltration in an in vivo setting resulting in exacerbated inflammation.
Numerous inhibitory receptors have been described on microglia and are therefore likely to be involved in immune responses (Mukhopadhyay et al., 2010). Receptor-ligand interaction such as the CD200-CD200 receptor and the fractalkine-fractalkine receptor interactions are both implicated in “dampening” down the inflammatory response and aiding the return to homeostasis (Cameron & Landreth, 2010). Previous studies from this laboratory have found that CD200R is decreased in the brain with age (Lyons et al., 2007) and also in APP/PS1 mice (unpublished observation). Walker and colleagues (2009) reported a decrease in both CD200 receptor and ligand in the AD brain (Walker et al., 2009). The data presented here found that Aβ increased CD200R expression which was unchanged with anti-TLR2. A recent study using macrophages has suggested that TLRs induce the expression of CD200 receptor and CD200 ligand which in turn act as a feedback loop to control expression of TLRs (Mukhopadhyay et al., 2010).

Interestingly treatment of mixed glia with anti-TLR2 increased the expression of CD200L and this increase in CD200L was decreased in glia treated with anti-TLR2 and Aβ together.

Fractalkine is a transmembrane chemokine that exists in both soluble and membrane-bound forms and acts as a neuroimmune regulatory protein. Fractalkine acts *in vitro* as an anti-inflammatory molecule by downregulating the production of pro-inflammatory cytokines (Gemma et al., 2010). Previous studies from this laboratory have reported that both soluble and membrane-bound fractalkine attenuates LPS-induced microglial activation. Furthermore age-related increases in microglial activation are accompanied by a decrease in fractalkine expression, and age-related microglial activation is attenuated in rats treated with fractalkine (Lyons *et al*., 2009a). Here the effect of Aβ and anti-TLR2 on fractalkine and fractalkine receptor expression in mixed glia was investigated. Fractalkine receptor was found to be significantly decreased in response to Aβ-treatment and significantly increased by anti-TLR2. The opposite was seen for fractalkine ligand as it was increased in response to Aβ-stimulation and significantly decreased by anti-TLR2 treatment.

Together these data show that a significant part of the inflammatory response induced by Aβ in glia was due to TLR2 activation and blocking TLR2 dampens the development of a pro-inflammatory environment. At present it remains unclear if the activation of TLRs by Aβ contributes to, or inhibits AD
progression. It has been suggested that mild TLR activation may be beneficial in promoting Aβ uptake and clearance whereas excessive TLR activation may lead to accumulation of cytotoxic molecules like cytokines and reactive oxygen species (Okun et al., 2009). The present data favour the latter proposal as anti-TLR2 attenuated the Aβ-induced cytokine and chemokine production in cultured cells. Furthermore, glia appeared to have adopted an APC phenotype rather than a phagocytic phenotype. Together the present findings and those of other research groups implicate TLR2 as a major player in neuroinflammation and suggest that targeting these receptors may have potential therapeutic benefits for the treatment of neuroinflammation in neurodegenerative diseases.
Table 3.1. Results summary of inflammatory markers assessed in mixed glia prepared from wildtype and CD200−/− mice.

<table>
<thead>
<tr>
<th>mRNA expression</th>
<th>Genotype effect</th>
<th>Interaction effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IP-10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR1</td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Supernatant concentrations</th>
<th>Genotype Effect</th>
<th>Interaction effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IP-10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2. Results summary of mRNA expression of inflammatory markers assessed in mixed glia treated with Aβ1-40/1-42 and anti-TLR2.

<table>
<thead>
<tr>
<th></th>
<th>Aβ1-40/1-42 effect</th>
<th>Anti-TLR2 effect</th>
<th>Interaction effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD86</td>
<td>↑</td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>CD40</td>
<td>↑↑</td>
<td>↓↓</td>
<td>↓</td>
</tr>
<tr>
<td>CD11b</td>
<td>↓↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>↑↑↑</td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>↑</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>IP-10</td>
<td>↑↑</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>MCP1</td>
<td>↑↑</td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>MIP-1β</td>
<td>↑↑</td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>RANTES</td>
<td>↑↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD200R</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD200L</td>
<td>↑↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fractalkine receptor</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fractalkine ligand</td>
<td>↑↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
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<tr>
<td>TLR4</td>
<td>Not assessed</td>
<td>Not assessed</td>
<td></td>
</tr>
<tr>
<td>TLR1</td>
<td>Not assessed</td>
<td>Not assessed</td>
<td></td>
</tr>
</tbody>
</table>

~ 110 ~
Table 3.3. Results summary of supernatant concentrations of cytokines and chemokines assessed in mixed glia treated with Aβ1-40/1-42 and anti-TLR2.

<table>
<thead>
<tr>
<th></th>
<th>Aβ1-40/42 effect</th>
<th>Anti-TLR2 effect</th>
<th>Interaction effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>↑↑</td>
<td>↓↓</td>
<td>↓↓</td>
</tr>
<tr>
<td>IP-10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP1</td>
<td>↑↑</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>MIP-1α</td>
<td>↑↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANTES</td>
<td>↑↑</td>
<td></td>
<td>↓</td>
</tr>
</tbody>
</table>
Chapter 4
Chapter 4

4.1. Introduction

Evidence from the previous chapter in vitro, which examined the effect of CD200-deficiency on Pam$_3$Csk$_4$-induced changes in glia and recent reports, have highlighted the importance of CD200-CD200R engagement in limiting the magnitude of inflammation and promoting inflammatory resolution. Markers of microglial activation, cytokine and chemokine expression and production have been shown to be increased in mixed glia prepared from CD200$^{-/-}$, compared with wildtype, mice. Indeed recent evidence from this laboratory has identified an exaggerated inflammatory response in mixed glia prepared from CD200$^{-/-}$, compared with wildtype, mice following both LPS and Pam$_3$Csk$_4$ stimulation (Costello et al., 2011). Consistent with studies in vitro several studies in CD200$^{-/-}$ mice (Hoek et al., 2000; Snelgrove et al., 2008) have demonstrated the importance of the CD200R as an inhibitory receptor in inflammation. The data has been consolidated by analysis of the effect of CD200R antibodies (Meuth et al., 2008).

The aims of this study were to:

- Investigate in vivo the role of CD200 on inflammation and microglial activation following stimulation with a TLR2 agonist Pam$_3$Csk$_4$.

- Assess whether changes in TLR2 expression may be accountable for increased inflammation in vivo as seen in vitro.
4.2. Methods

Male and female C57BL/6 wildtype and CD200" mice (2-3 months old) were randomly assigned to control or treatment groups (n=8). Mice were injected i.p. with either saline or Pam\textsubscript{3}Csk\textsubscript{4} (100 µg/100 µl) and 5 hours later animals were anaesthetised with urethane (1.5 g/kg) and perfused intracardially with sterile ice-cold PBS (see section 2.5 for details). Brains were removed and hippocampal and cortical tissue was taken to determine mRNA expression of surface proteins by QPCR (see section 2.11 for details). The remainder of the brain tissue was harvested for microglial isolation by the 5 layer Percoll method (see section 2.8 for details) and markers of microglial activation were assessed by FACS (see section 2.9 for details). Data are expressed as means ± SEM. ANOVA was performed to determine whether significant differences existed between treatment groups and Bonferroni post-hoc test was applied where appropriate.
4.3. Results

4.3.1. Pam₃Csk₄ increased cytokine mRNA expression in hippocampal and cortical tissue prepared from wildtype and CD200⁻/⁻ mice.

The immunosuppressive role of the CD200-CD200R interaction was investigated *in vitro* in the previous chapter, here the same parameters were investigated *in vivo*. The results from the previous chapter demonstrated that mixed glia generated from CD200⁻/⁻ mice have an exaggerated inflammatory phenotype in response to Pam₃Csk₄ compared with mixed glia from wildtype mice. Thus it was hypothesised that i.p. injection of Pam₃Csk₄ would initiate an exaggerated cytokine response in CD200⁻/⁻, compared with wildtype, mice. Pam₃Csk₄ treatment significantly increased mRNA expression of TNFα (**p<0.001, ANOVA; Figure 4.1) and IL-1β (**p<0.001, ANOVA; Figure 4.2) in both hippocampal (Figure 4.1A and 4.2A) and cortical (Figure 4.1B and 4.2B) tissue prepared from wildtype and CD200⁻/⁻ mice. TNFα (\(^{+}p<0.01\), ANOVA; Figure 4.1A) and IL-1β (\(^{p}<0.05\), ANOVA; Figure 4.2A) mRNA expression was significantly increased in hippocampal tissue prepared from CD200⁻/⁻, compared with wildtype, mice. Further analysis of the data revealed a significant interaction between Pam₃Csk₄ treatment and genotype (\(^{+}p<0.01\), \(^{+}p<0.05\), ANOVA; Figure 4.1A and 4.2A). In contrast, in the cortex there was no significant change in mRNA expression of TNFα (Figure 4.1B) or IL-1β (Figure 4.2B) in tissue prepared from CD200⁻/⁻, compared with wildtype, mice and no interaction was observed.

Pam₃Csk₄ induced a significant increase in the mRNA expression of IL-6 in both hippocampal (**p<0.001, ANOVA; Figure 4.3A) and cortical (\(^{p}<0.05\), ANOVA; Figure 4.3B) tissue prepared from wildtype and CD200⁻/⁻ mice. IL-6 mRNA expression was not significantly changed in hippocampal (Figure 4.3A) tissue prepared from CD200⁻/⁻, compared with wildtype, mice. IL-6 mRNA expression was significantly decreased in cortical tissue prepared from CD200⁻/⁻, compared with wildtype, mice (\(^{p}<0.05\), ANOVA; Figure 4.3B). There was no significant interaction observed between Pam₃Csk₄ treatment and genotype in either hippocampal (Figure 4.3A) or cortical (Figure 4.3B) tissue.
4.3.2. Pam₃Csk₄ increased chemokine mRNA expression in hippocampal and cortical tissue prepared from wildtype and CD200°/⁻ mice.

Expression of chemokines, IP-10, MIP-1α, MCP-1 and RANTES was assessed in hippocampal and cortical tissue harvested from wildtype and CD200°/⁻ mice following Pam₃Csk₄ treatment. Pam₃Csk₄ treatment significantly increased mRNA expression of IP-10 (***p<0.001, ANOVA; Figure 4.4) and MIP-1α (***p<0.001, ANOVA; Figure 4.5) in both hippocampal (Figure 4.4A and 4.5A) and cortical (Figure 4.4B and 4.5B) tissue prepared from wildtype and CD200°/⁻ mice. IP-10 mRNA expression was increased in hippocampal (†p<0.05, ANOVA; Figure 4.4A) and cortical (†p<0.05, ANOVA; Figure 4.5B) tissue prepared from CD200°/⁻, compared with wildtype, mice. MIP-1α mRNA expression was increased in hippocampal (†p<0.05, ANOVA; Figure 4.5A), but not cortical (Figure 4.5B), tissue prepared from CD200°/⁻, compared with wildtype, mice. Further analysis of the data revealed a significant interaction between Pam₃Csk₄ treatment and genotype on mRNA expression of IP-10 (†p<0.05, ANOVA; Figure 4.4A) and MIP-1α (†p<0.05, ANOVA; Figure 4.5A) in hippocampal tissue. In cortical tissue there was no significant interaction observed between Pam₃Csk₄ treatment and genotype on mRNA expression of either IP-10 (Figure 4.4) or MIP-1α (Figure 4.5).

Pam₃Csk₄ treatment significantly increased mRNA expression of MCP-1 (Figure 4.6) and RANTES (Figure 4.7) in both hippocampal (***p<0.001, ANOVA; Figure 4.6A and 4.7A) and cortical (***p<0.001, **p<0.01, ANOVA Figure 4.6B and 4.7B) tissue prepared from wildtype and CD200°/⁻ mice. Neither mRNA expression of MCP-1 (Figure 4.6) nor RANTES (Figure 4.7) was further increased in hippocampal (Figure 4.6A and 4.7A) or cortical (Figure 4.6B and 4.7B) tissue prepared from CD200°/⁻, compared with wildtype, mice. No significant interaction was observed between Pam₃Csk₄ treatment and genotype on mRNA expression of either MCP-1 (Figure 4.6) or RANTES (Figure 4.7) in hippocampal (Figure 4.6A and 4.7A) and cortical (Figure 4.6B and 4.7B) tissue.
4.3.3. Pam$_3$Csk$_4$ increased TLR mRNA expression in hippocampal and cortical tissue prepared from wildtype and CD200$^{+/}$ mice.

Evidence from the literature suggests that TLR2 works in conjunction with TLR1 to recognise triacylated lipoproteins, such as Pam$_3$Csk$_4$, to exert a pro-inflammatory response. In the previous chapter it was found that that expression of TLR2 on CD11b$^+$ cells was significantly increased in cells prepared from CD200$^{+/}$, compared with wildtype, mice. Furthermore Pam$_3$Csk$_4$ treatment was found to increase both mRNA expression of TLR2 and TLR1 in mixed glia prepared from both wildtype and CD200$^{+/}$ mice. Here expression of TLR2 was assessed in whole brain tissue by FACS analysis and QPCR in hippocampal and cortical tissue harvested from wildtype and CD200$^{+/}$ mice following Pam$_3$Csk$_4$ treatment. FACS analysis revealed that Pam$_3$Csk$_4$ treatment had no significant effect on TLR2 expression on CD11b$^+$ cells prepared from wildtype and CD200$^{+/}$ mice (Figure 4.8B). There was an apparent, but not significant, increase in TLR2 expression on CD11b$^+$ cells prepared from CD200$^{+/}$ mice (Figure 4.8B). Further analysis of TLR2 expression by QPCR revealed that Pam$_3$Csk$_4$ treatment significantly increased mRNA expression of TLR2 in both hippocampal (***p<0.001, ANOVA; Figure 4.9A) and cortical (**p<0.001, ANOVA; Figure 4.9B) tissue prepared from wildtype and CD200$^{+/}$ mice. TLR2 mRNA expression was not further increased in hippocampal (Figure 4.9A) or cortical (Figure 4.9B) tissue prepared from CD200$^{+/}$, compared with wildtype, mice and no significant interaction was observed between Pam$_3$Csk$_4$ treatment and genotype in either hippocampal (Figure 4.9A) or cortical (Figure 4.9B) tissue.

Pam$_3$Csk$_4$ treatment significantly increased mRNA expression of TLR1 in both hippocampal (***p<0.001, ANOVA; Figure 4.10A) and cortical (**p<0.001, ANOVA; Figure 4.10B) tissue prepared from wildtype and CD200$^{+/}$ mice. TLR1 mRNA expression was unaltered in hippocampal tissue (Figure 4.10A) but significantly decreased in cortical (*p<0.05, ANOVA; Figure 4.10B) tissue prepared from CD200$^{+/}$, compared with wildtype, mice. No significant interaction was observed between Pam$_3$Csk$_4$ treatment and genotype in either hippocampal (Figure 4.10A) or cortical (Figure 4.10B) tissue.
4.3.4. Markers of microglial activation were differentially altered in hippocampal and cortical tissue prepared from wildtype and CD200<sup>−/−</sup> mice.

There is a wealth of literature demonstrating that CD200 contributes to maintaining microglia in a quiescent state (Barclay <i>et al.</i>, 2002; Lyons <i>et al.</i>, 2007) and it has been established that CD200<sup>−/−</sup> mice have increased microglial activation in models of disease (Hoek <i>et al.</i>, 2000). Expression of markers of microglial activation were assessed in whole brain tissue by FACS analysis and QPCR in hippocampal and cortical tissue harvested from wildtype and CD200<sup>−/−</sup> mice following Pam<sub>3</sub>Csk<sub>4</sub> treatment. FACS analysis revealed that Pam<sub>3</sub>Csk<sub>4</sub> treatment had no significant effect on MHC class II (Figure 4.11B) and CD40 (Figure 4.11C) expression on CD11b<sup>+</sup> cells prepared from wildtype and CD200<sup>−/−</sup> mice (Figure 4.8B). There was a significant increase in MHC class II expression on CD11b<sup>+</sup> cells prepared from CD200<sup>−/−</sup> mice compared with wildtype mice (**p<0.0001, ANOVA; Figure 4.11B). There was no significant change reported in CD40 expression on CD11b<sup>+</sup> cells prepared from CD200<sup>−/−</sup> mice compared with wildtype mice (Figure 4.11C). No significant interaction was observed between Pam<sub>3</sub>Csk<sub>4</sub> treatment and genotype on the percentage expression of either MHC class II (Figure 4.11B) or CD40 (Figure 4.11C) on CD11b<sup>+</sup> cells. CD40 expression was also assessed in hippocampal and cortical tissue by QPCR. Pam<sub>3</sub>Csk<sub>4</sub> treatment had no significant effect on mRNA expression of CD40 in either hippocampal (Figure 4.12A) or cortical (Figure 4.12B) tissue prepared from wildtype and CD200<sup>−/−</sup> mice. In contrast to the findings <i>in vitro</i> in the previous chapter, mRNA expression of CD40 was significantly decreased in both hippocampal (**p<0.01, ANOVA; Figure 4.12A) and cortical (**p<0.01, ANOVA; Figure 4.12B) tissue prepared from CD200<sup>−/−</sup>, compared with wildtype, mice. No significant interaction was observed between Pam<sub>3</sub>Csk<sub>4</sub> treatment and genotype in either hippocampal (Figure 4.12A) or cortical (Figure 4.12B) tissue.

Pam<sub>3</sub>Csk<sub>4</sub> treatment significantly increased mRNA expression of CD86 in hippocampal (**p<0.001, ANOVA; Figure 4.13A), but not cortical (Figure 4.13B), tissue prepared from wildtype and CD200<sup>−/−</sup> mice. Pam<sub>3</sub>Csk<sub>4</sub> treatment had no effect on mRNA expression of CD68 in hippocampal and cortical tissue prepared from wildtype and CD200<sup>−/−</sup> mice (Figure 4.14A and B). However both mRNA expression of CD86 (Figure 4.13) and CD68 (Figure 4.14) was
significantly decreased in hippocampal (++p<0.01, *p<0.05, ANOVA; Figure 4.13A and 4.14A) and cortical (***p<0.001, ANOVA; Figure 4.13B and 4.14B) tissue prepared from CD200−/−, compared with wildtype, mice. No significant interaction was observed between Pam3Csk4 treatment and genotype on either mRNA expression of CD86 (Figure 4.13) or CD68 (Figure 4.14) in hippocampal (Figure 4.13A and 4.14A) or cortical (Figure 4.13B and 4.14B) tissue.

Table 4.1 summarises the mRNA expression results and Table 4.2 summarises the FACS results.
Figure 4.1. Pam₃Csk₄ increased TNFα mRNA expression in hippocampal and cortical tissue prepared from wildtype and CD200⁻/⁻ mice.

Wildtype and CD200⁻/⁻ mice received an i.p. injection of sterile saline (100 μl) or Pam₃Csk₄ (100 μg/ml). After 5 hours mice were perfused intracardially and sacrificed. Cortex and hippocampus was harvested for QPCR analysis. Pam₃Csk₄ treatment significantly increased TNFα mRNA expression in both hippocampal (A) and cortical (B) tissue prepared from wildtype and CD200⁻/⁻ mice (***p<0.001, ANOVA). TNFα mRNA expression (A) was significantly increased in hippocampal tissue prepared from CD200⁻/⁻, compared with wildtype, mice (**p<0.01, ANOVA), and a significant interaction between Pam₃Csk₄ treatment and genotype was observed (A) (***p<0.01, ANOVA). Values are expressed as means ± SEM (n=7-8).

(A) 2-way ANOVA: Pam₃Csk₄ effect F (1,25) = 52.48; p<0.0001, Genotype effect F (1,25) = 8.117; p= 0.0087, Interaction effect F (1,25) = 8.381; p=0.0078

(B) 2-way ANOVA: Pam₃Csk₄ effect F (1,24) = 36.34; p<0.0001, Genotype effect F (1,24) = 0.1261; p=0.7256, Interaction effect F (1,24) = 0.1563; p=0.6961
Figure 4.2. Pam₃Csk₄ increased IL-1β mRNA expression in hippocampal and cortical tissue prepared from wildtype and CD20₀⁺/⁻ mice.

Hippocampal and cortical tissue was prepared from wildtype and CD20₀⁺/⁻ mice for QPCR analysis as described in Figure 4.1. Pam₃Csk₄ treatment significantly increased IL-1β mRNA expression in both hippocampal (A) and cortical (B) tissue prepared from wildtype and CD20₀⁺/⁻ mice (**p<0.001, ANOVA). IL-1β mRNA expression was significantly increased in hippocampal (A) tissue prepared from CD20₀⁺/⁻, compared with wildtype, mice (′p<0.05, ANOVA), and a significant interaction between Pam₃Csk₄ treatment and genotype was observed (A) (′p<0.05, ANOVA). Values are expressed as means ± SEM (n=6-8).

(A) 2-way ANOVA: Pam₃Csk₄ effect F (1,25) = 47.30; p<0.0001, Genotype effect F (1,25) = 5.162; p= 0.0319, Interaction effect F (1,25) = 5.630; p=0.0257

(B) 2-way ANOVA: Pam₃Csk₄ effect F (1,25) = 39.93; p<0.0001, Genotype effect F (1,25) = 2.234; p=0.1475, Interaction effect F (1,25) = 2.985; p=0.0964
Figure 4.3. Pam$_3$Csk$_4$ increased IL-6 mRNA expression in hippocampal and cortical tissue prepared from wildtype and CD200$^{-/-}$ mice.

Hippocampal and cortical tissue was prepared from wildtype and CD200$^{-/-}$ mice for QPCR analysis as described in Figure 4.1. Pam$_3$Csk$_4$ treatment significantly increased IL-6 mRNA expression in both hippocampal (A) and cortical (B) tissue prepared from wildtype and CD200$^{-/-}$ mice (***p<0.001, *p<0.05, ANOVA). IL-6 mRNA expression was significantly decreased in cortical (B) tissue prepared from CD200$^{-/-}$, compared with wildtype, mice (p<0.05, ANOVA). Values are expressed as means ± SEM (n=6-8).

(A) 2-way ANOVA: Pam$_3$Csk$_4$ effect F (1,25) = 16.25; p=0.0005, Genotype effect F (1,25) = 0.4842; p= 0.4929, Interaction effect F (1,25) = 1.087; p = 0.3072

(B) 2-way ANOVA: Pam$_3$Csk$_4$ effect F (1,23) = 6.452; p=0.0183, Genotype effect F (1,23) = 5.135; p=0.00332, Interaction effect F (1,23) = 0.5557; p=0.4635
Figure 4.4. Pam$_3$Csk$_4$ increased IP-10 mRNA expression in hippocampal and cortical tissue prepared from wildtype and CD200$^{+/c}$ mice.

Hippocampal and cortical tissue was prepared from wildtype and CD200$^{+/c}$ mice for QPCR analysis as described in Figure 4.1. Pam$_3$Csk$_4$ treatment significantly increased IP-10 mRNA expression in both hippocampal (A) and cortical (B) tissue prepared from wildtype and CD200$^{+/c}$ mice (***p<0.001, ANOVA). IP-10 mRNA expression was significantly increased in hippocampal (A) and cortical (B) tissue prepared from CD200$^{+/c}$, compared with wildtype, mice (^p<0.05, ANOVA). A significant interaction between Pam$_3$Csk$_4$ treatment and genotype in hippocampal tissue was observed (A) (^p<0.05, ANOVA). Values are expressed as means ± SEM (n=6-8).

(A) 2-way ANOVA: Pam$_3$Csk$_4$ effect F (1,25) = 31.38; p<0.0001, Genotype effect F (1,25) = 4.052; p= 0.0550, Interaction effect F (1,25) = 4.459; p=0.0449

(B) 2-way ANOVA: Pam$_3$Csk$_4$ effect F (1,24) = 33.15; p<0.0001, Genotype effect F (1,24) = 5.733; p=0.0248, Interaction effect F (1,24) = 3.414; p=0.0770
Figure 4.5. Pam3Csk4 increased MIP-1α mRNA expression in hippocampal and cortical tissue prepared from wildtype and CD200^-/- mice.

Hippocampal and cortical tissue was prepared from wildtype and CD200^-/- mice for QPCR analysis as described in Figure 4.1. Pam3Csk4 treatment significantly increased MIP-1α mRNA expression in both hippocampal (A) and cortical (B) tissue prepared from wildtype and CD200^-/- mice (**p<0.001, ANOVA). MIP-1α mRNA expression was significantly increased in hippocampal (A) tissue prepared from CD200^-/-, compared with wildtype, mice (^p<0.05, ANOVA), and a significant interaction between Pam3Csk4 treatment and genotype was observed (A) (^p<0.05, ANOVA). Values are expressed as means ± SEM (n=7-8).

(A) 2-way ANOVA: Pam3Csk4 effect F (1,25) = 24.44; p<0.0001, Genotype effect F (1,25) = 4.206; p= 0.0509, Interaction effect F (1,25) = 4.672; p=0.0404

(B) 2-way ANOVA: Pam3Csk4 effect F (1,25) = 36.96; p<0.0001, Genotype effect F (1,25) = 1.516; p=0.2297, Interaction effect F (1,25) = 1.624; p=0.2143
Figure 4.6. Pam₃Csk₄ increased MCP-1 mRNA expression in hippocampal and cortical tissue prepared from wildtype and CD200⁻/⁻ mice.

Hippocampal and cortical tissue was prepared from wildtype and CD200⁻/⁻ mice for QPCR analysis as described in Figure 4.1. Pam₃Csk₄ treatment significantly increased MCP-1 mRNA expression in hippocampal (A) and cortical (B) tissue prepared from wildtype and CD200⁻/⁻ mice (**p<0.001, ANOVA). Values are expressed as means ± SEM (n=6-8).

(A) 2-way ANOVA: Pam₃Csk₄ effect F (1,24) = 21.82; p<0.0001, Genotype effect F (1,24) = 1.607; p=0.2171, Interaction effect F (1,24) = 1.826; p=0.1892

(B) 2-way ANOVA: Pam₃Csk₄ effect F (1,24) = 19.45; p=0.0002, Genotype effect F (1,24) = 0.06390; p=0.8026, Interaction effect F (1,24) = 0.1939; p=0.6636
Figure 4.7. Pam₃Csk₄ increased RANTES mRNA expression in hippocampal and cortical tissue prepared from wildtype and CD20₀⁻/⁻ mice.

Hippocampal and cortical tissue was prepared from wildtype and CD20₀⁻/⁻ mice for QPCR analysis as described in Figure 4.1. Pam₃Csk₄ treatment significantly increased RANTES mRNA expression in hippocampal (A) and cortical (B) tissue prepared from wildtype and CD20₀⁻/⁻ mice (**p<0.01, ***p<0.001, ANOVA). Values are expressed as means ± SEM (n=7-8).

(A) 2-way ANOVA: Pam₃Csk₄ effect F (1,24) = 18.95; p=0.0002, Genotype effect F (1,24) = 0.9015; p=0.3519, Interaction effect F (1,24) = 3.729; p=0.0654

(B) 2-way ANOVA: Pam₃Csk₄ effect F (1,24) = 10.88; p=0.0030, Genotype effect F (1,24) = 2.728; p=0.1116, Interaction effect F (1,24) = 2.169; p=0.1538
Figure 4.8. Pam$_3$Csk$_4$ treatment had no effect on the number of TLR2 expressing CD11b$^+$ cells in wildtype and CD200$^{-/-}$ mice.

Wildtype and CD200$^{-/-}$ mice received an i.p. injection of sterile saline (100 μl) or Pam$_3$Csk$_4$ (100 μg/ml). After 5 hours mice were perfused intracardially and sacrificed. Over half the brain was harvested for percoll separation and microglial isolation for FACS analysis. TLR2 expression was assessed on CD11b$^+$ cells. (A) Representative FACS plots. Pam$_3$Csk$_4$ treatment had no significant effect on TLR2 expression on CD11b$^+$ cells prepared from wildtype and CD200$^{-/-}$ mice (B). Values are expressed as means ± SEM (n=7).

(B) 2-way ANOVA: Pam$_3$Csk$_4$ effect F (1,23) = 1.010; p=0.3253, Genotype effect, F (1,23) = 2.839; p= 0.1055, Interaction effect, F (1,23) = 0.1569; p=0.6957
Figure 4.9. Pam$_3$Csk$_4$ increased TLR2 mRNA expression in hippocampal and cortical tissue prepared from wildtype and CD200$^{-/-}$ mice.

Hippocampal and cortical tissue was prepared from wildtype and CD200$^{-/-}$ mice for QPCR analysis as described in Figure 4.1. Pam$_3$Csk$_4$ treatment significantly increased TLR2 mRNA expression in hippocampal (A) and cortical (B) tissue prepared from wildtype and CD200$^{-/-}$ mice (**p<0.001, ANOVA). Values are expressed as means ± SEM (n=7-8).

(A) 2-way ANOVA: Pam$_3$Csk$_4$ effect F (1,24) = 27.51; p=0.0001, Genotype effect F (1,24) = 0.1575; p=0.6949, Interaction effect F (1,24) = 0.3265; p=0.5731

(B) 2-way ANOVA: Pam$_3$Csk$_4$ effect F (1,26) = 66.26; p=0.0001, Genotype effect F (1,26) = 0.3617; p=0.5528, Interaction effect F (1,26) = 1.260; p=0.2718
Figure 4.10. Pam$_3$Csk$_4$ increased TLR1 mRNA expression in hippocampal and cortical tissue prepared from wildtype and CD200$^{-/-}$ mice.

Hippocampal and cortical tissue was prepared from wildtype and CD200$^{-/-}$ mice for QPCR analysis as described in Figure 4.1. Pam$_3$Csk$_4$ treatment significantly increased TLR1 mRNA expression in both hippocampal (A) and cortical (B) tissue prepared from wildtype and CD200$^{-/-}$ mice (**p<0.001, ANOVA). TLR1 mRNA expression was significantly decreased in cortical (B) tissue prepared from CD200$^{-/-}$, compared with wildtype, mice (p<0.05, ANOVA). Values are expressed as means ± SEM (n=7-8).

(A) 2-way ANOVA: Pam$_3$Csk$_4$ effect F (1,23) = 19.70; p=0.0002, Genotype effect F (1,23) = 0.1513; p= 0.7009, Interaction effect F (1,23) = 3.635; p=0.0692

(B) 2-way ANOVA: Pam$_3$Csk$_4$ effect F (1,25) = 24.56; p=<0.0001, Genotype effect F (1,25) = 6.118; p= 0.0205, Interaction effect F (1,25) = 0.8640; p=0.3615
Figure 4.11. Pam$_3$Csk$_4$ treatment had no effect on the number of MHC class II and CD40 expressing CD11b$^+$ cells in wildtype and CD200$^{-/-}$ mice.

Brain tissue was prepared for FACS analysis from wildtype and CD200$^{-/-}$ mice as described in Figure 4.8. (A) Representative FACS plots. Pam$_3$Csk$_4$ treatment had no significant effect on either MHC class II (B) or CD40 (C) expression on CD11b$^+$ cells prepared from wildtype and CD200$^{-/-}$ mice. There was a significant increase in MHC class II expression on CD11b$^+$ cells prepared from CD200$^{-/-}$ mice compared with wildtype mice (B) (**p<0.0001, ANOVA). Values are expressed as means ± SEM (n=7).

(A) 2-way ANOVA: Pam$_3$Csk$_4$ effect F (1,20) = 0.2674; p=0.6107, Genotype effect F (1,20) = 23.05; p=0.0001, Interaction effect F (1,20) = 0.004691; p=0.9461

(B) 2-way ANOVA: Pam$_3$Csk$_4$ effect F (1,20) = 1.4447; p=0.2430, Genotype effect F (1,20) = 1.483; p=0.2374, Interaction effect F (1,20) = 0.4682; p=0.5017
Figure 4.12. CD40 mRNA expression was decreased in hippocampal and cortical tissue prepared from CD200"/" mice.

Hippocampal and cortical tissue was prepared from wildtype and CD200"/" mice for QPCR analysis as described in Figure 4.1. Pam3Csk4 treatment had no significant effect on CD40 mRNA expression in either hippocampal (A) or cortical (B) tissue prepared from wildtype and CD200"/" mice. CD40 mRNA expression was significantly decreased in both hippocampal (A) and cortical (B) tissue prepared from CD200"/", compared with wildtype, mice (**p<0.01, ANOVA). Values are expressed as means ± SEM (n=7-8).

(A) 2-way ANOVA: Pam3Csk4 effect F (1,28) = 1.299; p=0.2640, Genotype effect F (1,28) = 12.50; p= 0.0014, Interaction effect F (1,28) = 1.956; p=0.1729

(B) 2-way ANOVA: Pam3Csk4 effect F (1,24) = 1.497; p=0.2330, Genotype effect F (1,24) = 11.21; p= 0.0027, Interaction effect F (1,24) = 0.0003998; p=0.9842
Figure 4.13. CD86 mRNA expression was decreased in hippocampal and cortical tissue prepared from CD200^{−/−} mice.

Hippocampal and cortical tissue was prepared from wildtype and CD200^{−/−} mice for QPCR analysis as described in Figure 4.1. Pam\textsubscript{3}Csk\textsubscript{4} treatment significantly increased CD86 mRNA expression in hippocampal (A) tissue prepared from wildtype and CD200^{−/−} mice (**p<0.01, ANOVA). CD86 mRNA expression was significantly decreased in hippocampal (A) and cortical (B) tissue prepared from CD200^{−/−}, compared with wildtype, mice (***p<0.001, ANOVA). Values are expressed as means ± SEM (n=7-8).

(A) 2-way ANOVA: Pam\textsubscript{3}Csk\textsubscript{4} effect F (1,28) = 11.83; p=0.0018, Genotype effect F (1,28) = 8.411; p= 0.0072, Interaction effect F (1,28) = 0.0008686; p=0.9767

(B) 2-way ANOVA: Pam\textsubscript{3}Csk\textsubscript{4} effect F (1,27) = 1.510; p=0.2297, Genotype effect F (1,27) = 20.55; p= 0.0001, Interaction effect F (1,27) = 2.987; p=0.0954
Figure 4.14. CD68 mRNA expression was decreased in hippocampal and cortical tissue prepared from CD200^−/− mice.

Hippocampal and cortical tissue was prepared from wildtype and CD200^−/− mice for QPCR analysis as described in Figure 4.1. Pam3Csk4 treatment had no significant effect on CD68 mRNA expression in either hippocampal (A) or cortical (B) tissue prepared from wildtype and CD200^−/− mice. CD68 mRNA expression was significantly decreased in both hippocampal (A) and cortical (B) tissue prepared from CD200^−/−, compared with wildtype, mice (*p<0.05, ***p<0.001, ANOVA). Values are expressed as means ± SEM (n=7-8).

(A) 2-way ANOVA: Pam3Csk4 effect F (1,25) = 0.9145; p=0.3481, Genotype effect F (1,25) = 4.962; p= 0.0352, Interaction effect F (1,25) = 0.1402; p=0.7112

(B) 2-way ANOVA: Pam3Csk4 effect F (1,24) = 0.0002139; p=0.9885, Genotype effect F (1,24) = 29.95; p<0.0001, Interaction effect F (1,24) = 3.583; p=0.0705
4.4. Discussion

One of the fundamental aims of this study was to determine the role of CD200 in the maintenance of microglial responses in an in vivo environment in response to a TLR stimulus Pam3Csk4. It is well documented that the activation of TLRs may have a major role to play in immune and inflammatory diseases (O'Neill et al., 2009). TLR activation results in a robust inflammatory response characterised by increased production of cytokines, chemokines and the upregulation of cell surface receptors (Kielian et al., 2001; O'Neill et al., 2009). Disruption of the CD200-CD200R partnership has already been widely investigated in several models of disease (Hoek et al., 2000; Broderick et al., 2002; Meuth et al., 2008; Snelgrove et al., 2008) however, the effect of Pam3Csk4-induced neurotoxicity in CD200^−/− mice has not yet been evaluated. The hypothesis proposed here is that loss of CD200, coupled with Pam3Csk4 challenge, will lead to an exaggerated neuroinflammatory response in CD200^−/−, compared with wildtype, mice.

Previous studies, using both CD200 knockdown and an increase in the availability of CD200, have documented the role of the CD200-CD200R interaction in regulating cytokine production. The use of CD200Fc in studies has been correlated with a switching of cytokine production away from the production of type-1 cytokines, namely IFN-γ, IL-2 and TNFα, towards the production of type-2 cytokines (Chen et al., 2005). The data here indicate that TNFα and IL-1β mRNA expression was significantly increased in response to TLR2 activation by Pam3Csk4 and was significantly more exaggerated in hippocampal tissue prepared from CD200^−/−, compared with wildtype, mice. This is consistent with published data demonstrating that loss of CD200 results in increased TNFα production in a mouse model of Toxoplasma encephalitis (TE) (Deckert et al., 2006) and a mouse model of arthritis (Gorzynski et al., 2001). Additionally Gorczynski and colleagues (2002) demonstrated that replacement of CD200 through infusion of CD200Fc or of anti-CD200R could not merely suppress the development of CIA, which occurred in association with decreased serum levels of TNFα, but also ameliorates pre-existing disease in a mouse model of arthritis. Interestingly studies investigating stress which is known to induce a pro-inflammatory response in the CNS, with IL-1β acting as the main cytokine (Nguyen et al., 1998;
O'Connor et al., 2003), have demonstrated that stress downregulates hippocampal CD200 expression and that this is accompanied by an inflammatory microenvironment in the CNS (Frank et al., 2007).

IL-6 expression was investigated and the results demonstrated an increase in IL-6 mRNA expression following Pam3Csk4 treatment in both hippocampal and cortical tissue prepared from wildtype and CD200−/− mice. However, IL-6 mRNA expression was not exacerbated in tissue prepared from CD200−/−, compared with wildtype, mice conversely IL-6 mRNA expression was significantly decreased in cortical tissue prepared from CD200−/−, compared with wildtype, mice. This result was unexpected as previous studies have found that loss of CD200 leads to increased IL-6 production while elevated levels of CD200 reduce IL-6 production. Chitnis and colleagues (2007) demonstrated that elevated expression of CD200 results in reduced IL-6 expression accompanied by diminished accumulation of microglia and macrophages in the CNS in an EAE model (Chitnis et al., 2007). Indeed bone-marrow derived macrophages treated with DX109, a CD200R agonist, decrease the production of IL-6 following IFN-γ stimulation (Copland et al., 2007), while macrophages extracted from anti-CD200R Ab-treated rats exhibited a profound increase in IL-6 secretion (Meuth et al., 2008). Ex-vivo studies have shown that alveolar macrophages from CD200−/− mice have enhanced TNFα and IL-6 production in response to another TLR agonist, LPS (Snelgrove et al., 2008). Nonetheless it is difficult to interpret this result as IL-6 is a pluripotent cytokine having both beneficial and destructive effects within the CNS (Dong & Benveniste, 2001).

Previous data from this laboratory has reported an increase in BBB permeability in CD200−/− mice compared with wildtype counterparts and a further increase in BBB breakdown as a consequence of LPS treatment (Kelly et al., unpublished data). It has been established that tight junctions, which modulate the BBB, can be disrupted by cytokines such as TNFα and IL-1β (Kebir et al., 2007) both of which were found to be exaggerated, in this study, in tissue prepared from CD200−/− mice. Thus with this in mind the expression of chemo attractant mediator’s chemokines, which allow for the migration of peripheral immune cells into the CNS, was investigated. These experiments found a significant increase in IP-10, MIP-1α, MCP-1 and RANTES mRNA expression in response to Pam3Csk4 treatment in wildtype and CD200−/− mice. Furthermore IP-10 and MIP-1α mRNA
expression was exacerbated in tissue prepared from CD200⁻/⁻, compared with wildtype, mice. To date, there is not much published data examining chemokines in the CD200-deficient mouse model but some evidence points to a correlation between the loss of CD200 and increased peripheral cell infiltration. Accelerated and increased leukocyte infiltration has been observed in CD200⁻/⁻ mice undergoing EAU (Broderick et al., 2002). Furthermore, the presence of CD200 on endothelium, and the increase in retinal macrophage infiltrate after depletion of CD200, infers a possible control of macrophage trafficking via CD200-CD200R interactions (Broderick et al., 2002). It might be hypothesised that part of the inflammation seen in these CD200⁻/⁻ mice might be attributed to increased chemokine circulation and a leaky BBB allowing for increased peripheral cell infiltration into the CNS.

It is widely acknowledged that TLRs act to mobilise a robust immune reaction in response to pathogens and a number of studies have reported an association between TLRs and neurodegenerative diseases, that have a large inflammatory component, such as AD (Fassbender et al., 2004; Liu et al., 2005; Jana et al., 2008). Furthermore, it has been found that CD200R expression is decreased in AD (Walker et al., 2009). Recent evidence from this lab in vitro has suggested that loss of CD200 and the resulting exacerbated microglial response may be attributed to an increase in basal TLR2 and TLR4 expression on microglia in CD200⁻/⁻ mice (Costello et al., 2011). Considering this evidence, the next aim was to investigate if the exaggerated cytokine and chemokine production in the CD200⁻/⁻ mice was as a result of altered TLR expression in these animals. Here it was found that there was an apparent but not significant increase in basal TLR2 expression on CD11b⁺ cells prepared from CD200⁻/⁻, compared with wildtype, mice. Interestingly Pam₃Csk₄ significantly increased TLR2 mRNA expression in hippocampal and cortical tissue prepared from both wildtype and CD200⁻/⁻ mice but this effect was not seen at the protein level. It is well established that TLR2 recognises Pam₃Csk₄ through interaction with TLR1 to elicit an inflammatory response (Takeda et al., 2002). The present data show that Pam₃Csk₄ significantly increased TLR1 mRNA expression in hippocampal and cortical tissue prepared from both wildtype, and CD200⁻/⁻, mice. In contrast, a significant decrease in TLR1 mRNA expression was seen in cortical tissue prepared from CD200⁻/⁻, compared with wildtype, mice.
Having established that there was a profound increase in pro-inflammatory cytokine and chemokine production in CD200\(^{−/−}\), compared with wildtype, mice the extent and phenotype of microglial activation was assessed. There is extensive literature describing the various cell surface markers on microglia and their upregulation in response to pathogenic inflammatory stimuli such as LPS, IL-1\(β\) and Aβ (Roy et al., 2008). Previous authors have correlated a decrease in CD200 with chronic microglial activation and suggested that disease or endotoxin-induced disruption of the CD200-CD200R axis may contribute to and maintain chronic microglial activation in the brain (Koning et al., 2007; Walker et al., 2009). Increased microglia/macrophage activation in CD200\(^{−/−}\) mice has been described in several models of inflammation including; facial nerve transfection, EAE, CIA (Hoek et al., 2000) and EAU (Broderick et al., 2002). Hoek and colleagues (2000) demonstrated that microglia from CD200\(^{−/−}\) mice exhibit many features of activation including decreased ramification and increased CD11b and CD45 expression. Furthermore this group showed that microglia from CD200\(^{−/−}\) mice were found aggregated together in the spinal cord of EAE animals, a feature that is associated with inflammation and neurodegeneration (Hoek et al., 2000). In this study, it was found that MHC class II expression was significantly increased on CD11b\(^{+}\) cells prepared from CD200\(^{−/−}\) mice compared with wildtype mice. Unusually it was found that Pam\(_{3}\)Csk\(_{4}\) treatment had no effect on MHC class II expression on CD11b\(^{+}\) cells prepared from wildtype and CD200\(^{−/−}\) mice. Both CD40 and CD86, two co-stimulatory molecules, were found to be significantly decreased in tissue prepared from CD200\(^{−/−}\) mice compared with wildtype mice and CD86 expression was only significantly increased following Pam\(_{3}\)Csk\(_{4}\) treatment in hippocampal tissue prepared from wildtype mice. This result is in agreement with studies from Broderick and colleagues (2002) using CD200\(^{−/−}\) mice to investigate EAU. These authors found that despite increased numbers of retinal microglia/macrophages in CD200\(^{−/−}\) mice compared with wildtype mice, there was no significant difference in the expression of MHC class II, CD86 and CD40 but found that expression of NOS2 was significantly greater in CD200\(^{−/−}\), compared with wildtype, mice (Broderick et al., 2002). This is important as under normal circumstances microglia in the retina are NOS2-negative, with NOS2 expression associated with peak EAU, and NOS2 inhibitors have been found to protect against photoreceptor destruction (Hoey et al., 1997).
It may be interesting to investigate NOS2 expression in hippocampal and cortical tissue from CD200\(^{-/-}\) mice challenged with Pam\(_3\)Csk\(_4\) in a future study. Furthermore, a study by Deckert and colleagues (2006) examining *Toxoplasma* encephalitis (TE) found no changes in basal levels of the cell surface molecules CD45, CD80, CD86 and MHC class I and II following acute TE in CD200\(^{-/-}\), compared with wildtype, mice. This group demonstrated that these markers of activation only become significantly more enhanced in the CD200\(^{-/-}\) mice compared with wildtype mice following chronic TE (Deckert *et al.*, 2006). This evidence raises the question that chronic rather than acute administration of Pam\(_3\)Csk\(_4\) is required to see a greater increase in these markers of activation in CD200\(^{-/-}\), compared with wildtype, mice.

CD68 expression was also investigated and the results revealed a significant decrease in tissue prepared from CD200\(^{-/-}\), compared with wildtype, mice and no effect of Pam\(_3\)Csk\(_4\) treatment in tissue prepared from wildtype and CD200\(^{-/-}\) mice. In contrast, Hoek and colleagues (2000) found increased CD68 staining in CD200\(^{-/-}\) mice undergoing EAE. However, it is worth noting that these authors found increased macrophage numbers in spleens from CD200-deficient, compared to wildtype, mice (Hoek *et al.*, 2000). It has been suggested that the increase in CD68 staining seen in the EAE model may not reflect increased phagocytosis but rather an increase in circulating macrophages in the tissue (Nathan & Muller, 2001). Evidence from the literature also suggests that the phagocytic activity of microglia is attenuated by pro-inflammatory cytokines (Hanisch & Kettenmann, 2007). The exaggerated cytokine response seen in tissue prepared from CD200\(^{-/-}\) mice, in this study, may account for the decrease in CD68 expression. The discrepancy between the results in the present study and those from Hoek and colleagues needs further investigation.

In conclusion, the data presented here and the data from the previous chapter indicate that loss of CD200, in the presence of Pam\(_3\)Csk\(_4\) challenge, results in an exaggerated immune response as measured by cytokine and chemokine expression accompanied by increased MHC class II expression on microglia. The possibility that CD200-CD200R interaction is involved in both cytokine and chemokine regulation in the CNS provides an area for future investigation. Especially targeting modulation of TNF\(\alpha\) which, alongside IL-1\(\beta\) and IL-6 has been found to be elevated during both neurodegenerative and
autoimmune diseases all of which have an inflammatory component as a driving force behind disease progression. TNFα has already been addressed as a therapeutic approach in the treatment of inflammatory disorders. Both decoy TNFα receptor molecules and antibodies to TNFα have been used successfully as treatment (Maini et al., 1999; Moreland et al., 1999) however these treatment are not without significant side effects (Finch & Ruvkun, 2001). Consequently the potential to use CD200 to manipulate pro-inflammatory cytokine production may be of therapeutic benefit. The finding that loss of CD200-CD200R axis can alter chemokine production is a more novel finding and further research will need to be addressed to fully investigate the impact of their regulation by CD200. It is worth noting that a profoundly exaggerated inflammatory response was observed in hippocampal tissue, rather than cortical tissue, prepared from CD200" mice. This difference may be accounted for by a regional difference in microglial activation states as already seen in studies using macrophages from different organs. Previous studies have found that the CD200-CD200R axis plays a role in controlling inflammatory reactions in lung macrophages during influenza (Snelgrove et al., 2008) whereas intestinal macrophages are unaffected by the loss of CD200-CD200R axis during dextran sodium sulphate-induced colitis (Bain & Mowat, 2012). These findings may indicate a differential role for the CD200-CD200R interaction in diverse forms of immunopathologies based on the stimulus and the resident myeloid cells present in the affected tissue.
Table 4.1. Results summary of mRNA expression of inflammatory markers assessed in hippocampal and cortical tissue prepared from wildtype and CD200^- mice.

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Table 4.2. Results summary of cell surface receptor expression on microglia prepared from wildtype and CD200<sup>−/−</sup> mice.

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<th>Pam&lt;sub&gt;3&lt;/sub&gt;Csk&lt;sub&gt;ε&lt;/sub&gt; effect</th>
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Chapter 5
Chapter 5

5.1. Introduction

Microglia are the resident brain mononuclear phagocytes and have functions similar to macrophages such as antigen presentation, phagocytosis, production of complement components, oxidative radicals, nitric oxide, cytokines and chemokines (Benveniste et al., 2001). Although microglia are involved in maintaining homeostasis and secreting neurotrophic factors required for neuronal survival it is believed that exacerbated activation of microglia causes a robust inflammatory response involved in neuronal death and brain injury (Roy et al., 2008). Under normal circumstances it is thought that microglia survey the microenvironment with relevantly low expression of cell surface markers and production of cytokines and chemokines. However during an immune response, resulting in microglial activation, both cytokines and chemokines are rapidly produced and secreted allowing for the activation of other immune cells and importantly the infiltration of peripheral immune cells into the CNS (Lynch, 2009). Activation is identified by the upregulation of several cell surface markers. These markers include MHC class II, CD40 and CD86 all of which are required for effective peripheral T cell activation and proliferation. The role of microglia in neurodegenerative conditions such as AD has received extensive attention in recent years and it is becoming increasingly evident that microglial activation is a fundamental characteristic of AD.

There is compelling evidence from the literature that chronic inflammatory processes participate in the pathophysiology of AD (McGeer & McGeer, 2001; McGeer & McGeer, 2003). These inflammatory changes include microgliosis, astrocytosis, as well as complement activation and increased cytokine and chemokine production. Presently there is extensive literature describing microglial activation both in mouse models of AD and in the AD brain. Activated microglia and astrocytes are found in close proximity to senile plaques (Haga et al., 1989; Itagaki et al., 1989). It has been reported that pro-inflammatory cytokine production is increased in post-mortem brain tissue from AD patients compared with non-demented controls (Griffin et al., 1989) and isolated neuritic plaques and
Aβ have been shown to induce microglia to release neurotoxins (Giulian et al., 1995; Giulian et al., 1996). It has been suggested that the presence of Aβ plaques may keep microglia consistently activated resulting in chronic inflammation within the CNS. Indeed it has been proposed that one of the neuropathogenic mechanisms occurring in AD is that Aβ maintains microglia in an activated state resulting in the production of pro-inflammatory cytokines and neurotoxic mediators resulting in neuronal damage (Benveniste et al., 2001). The release of these neurotoxic factors and cytokines can in turn activate surrounding astrocytes which in turn are capable of damaging neurons through the release of NO (Minagar et al., 2002). It has been suggested that Aβ activates microglia through an ensemble of cell surface receptors, (Bamberger et al., 2003), resulting in the activation of signalling cascades that results in the activation of microglia into different phenotypes (Combs et al., 2001). Indeed the results presented in Chapter 3 and those of other authors emphasise the production of a range of cytokines and chemokines including IL-1β, TNFα, IL-6, MCP-1 and MIP-1α (Meda et al., 1995; Walker et al., 1995) by microglia in response to Aβ challenge. Furthermore, the upregulation of the co-stimulatory molecule CD40 has been reported to be increased on microglia following Aβ stimulation in vitro while in vivo studies have demonstrated enhanced CD40 expression on microglia in a mouse model of AD (Tan et al., 1999). Interaction of CD40 with its ligand CD154 (found predominately on T cells) is critical for an effective immune response and the activation of T cells and APC (Benveniste et al., 2001). Despite the fact that microglia are competent phagocytes there is conflicting evidence over the ability of microglia to phagocytose Aβ. Indeed it appears that microglia in close proximity to plaques seem unable to internalise Aβ (Stalder et al., 2001). On the other hand it has been demonstrated that microglia are capable of phagocytosing fibrillar Aβ through an integrin receptor-dependent manner (Koenigsknecht & Landreth, 2004). Nonetheless it is still not clear if microglial activation and associated inflammatory changes trigger the pathogenesis of AD or whether microglial activation occurs in response to the early changes associated with the disease (Lynch, 2009).

Several studies suggest that inflammation within the CNS arises principally from glia, however with evidence that microglia can act as APCs for peripheral cells it is now thought that peripheral T cells can contribute and
maintain this inflammatory environment (Town et al., 2005). The role of T cells in CNS tissue damage has been widely studied in recent years. The role of T cells, particularly Th1 and Th17 in neurodegenerative pathologies such as MS is clearly demonstrated in studies using an animal model of MS, EAE. Adoptive transfer of MOG-specific Th1 cells can induce EAE (Kroenke et al., 2008). Furthermore, previous studies from this laboratory using the EAE model have demonstrated IL-17+ and IL-17+IFN-γ+ T cells infiltrate the brains of these mice before onset of clinical symptoms and this is associated with enhanced IL-1β and IL-6 expression. It was also reported that upon onset of clinical symptoms there was a significant increase in IFN-γ producing T cells and upregulation of the expression of CD40, CD86 and CD80 on activated microglia and macrophages (Murphy et al., 2010). There is clear evidence of the detrimental role of T cells in MS through their interaction with glia, however their role in AD is not as clear and there is still much debate if T cells play a beneficial or detrimental role in disease pathology.

It has been proposed that under normal circumstances T cells visit the healthy brain but during AD these visits become more frequent and T cells are thought to begin to accumulate in the brain (Town et al., 2005). Rogers and colleagues (1988) demonstrated that the human leukocyte antigen-DR+ could be seen in AD brain tissue. Togo and co-workers (2002), using immunohistochemical analysis, found T cells present in brain tissue taken from individuals with non-AD degenerative dementias, AD and controls but T cell numbers were far greater in brain sections taken from the AD group compared with controls. Several investigators have also addressed whether or not a peripheral T cell response might exist in AD and how this response may or may not contribute to the pathology of the disease. It has been shown that peripheral T cells in AD patients have a switched CD45 isotype from a naive phenotype (as measured by the expression of CD45RA+) to a memory phenotype (as measured by the expression of CD45RO+) compared with normal aged-matched controls or patients with other forms of dementias (Tan et al., 2002). This finding was corroborated with a finding by Togo and colleagues (2002) that CD45RO+-T cells were present in brains of AD patients. Furthermore, it has been shown that T cells isolated from AD patients had increased CD45RO expression compared with controls (Lombardi et al., 2004). This group also reported an increase in CD4+ T cells and T-regulatory subsets isolated from AD patients compared with age-
matched controls. Probably the most damning evidence suggesting T cells are more reactive in AD comes from studies examining the presence of Aβ-reactive T cells in AD patients. Monsonego and colleagues (2003) report the presence of Aβ-reactive T cells in AD patients and showed that these T cells increase with age. There is clear evidence that T cells can exacerbate inflammation however several studies have implicated T cells in decreasing plaque burden. A clinical trial in which a synthetic Aβ1-42 peptide (AN-1792) was peripherally administered to AD patients resulted in aseptic T cell meningoencephalitis. It has been suggested that this occurred as a result of an immune response to Aβ mediated by infiltrating T cells (Pfeifer et al., 2002). Interestingly some of the patients in this study were found to have increased clearance of Aβ plaque load (Nicoll et al., 2003) and a recent study by Monsonego and co-workers (2006) found increased Aβ clearance in mice overexpressing IFN-γ immunised with Aβ. Thus it is clear that T cells are present in the AD brain however the role of T cells in AD pathology is still debatable.

With both activated microglia and T cells present in the AD brain it has imperative to investigate how these cells may interact with each other and contribute to inflammation. Currently the interaction of T cells and glia and the potential modulatory effects of different T cell subsets on glial activation is not well characterised in relation to AD (McQuillan et al., 2010). However microglia in active MS lesions exhibit an activated phenotype with enhanced expression of MHC class II and co-stimulatory molecules CD40, CD86 and CD80 (Cannella et al., 1995; Cannella & Raine, 1995). Moreover it has been shown that microglia produce pro-inflammatory cytokines in response to T cells (Dasgupta et al., 2005). Microglia isolated from the CNS are able to present antigens to T cells resulting in the production of the pro-inflammatory cytokines IFN-γ and IL-2 (Aloisi et al., 2000b), while IFN-γ can upregulate co-stimulatory molecule expression on microglia (Meda et al., 1995; Nguyen & Benveniste, 2000). On the other hand it has been reported that Th2 cells may have beneficial effects through inhibition of CNS inflammation by the secretion of anti-inflammatory cytokines. Indeed the Th2 cytokine profile is typified by the production of cytokines such as IL-4 and IL-10 which decrease microglial activation, and increase phagocytosis (Koenigsknecht-Talboo & Landreth, 2005). Th2-type cytokines can attenuate Aβ-induced pro-inflammatory cytokine production by microglia (Szczepanik et al.,
While Th17 cells have a pathogenic potential similar to that of Th1 cells, and have been implicated in tissue specific autoimmunity, their role in host defence is not as well defined (Harrington et al., 2005). However adoptive transfer of IL-17-secreting cells induces EAE (Langrish et al., 2005) and IL-17 expressing T cells have been observed in active MS lesions (Kebir et al., 2007). Furthermore, studies have demonstrated that Th1/Th17 cells significantly enhance MHC class II and co-stimulatory molecule expression on microglia (Murphy et al., 2010). It is still uncertain if microglia contribute towards directing infiltrating T cells to a neuroprotective or a neurotoxic function (Melchior et al., 2006).

The aims of this study were to:

- Investigate the consequence of interaction of non antigen-specific and Aβ-specific Th1, Th2 and Th17 cells with microglia and astrocytes with a focus on investigating their modulation of co-stimulatory molecule expression and cytokine production by glia.

- To establish if the interaction of glia and T cell lines generated from wildtype mice, differed to those generated from APP/PS1 mice.
5.2. Methods

Spleens were harvested from 12 month old APP/PS1 mice and littermate controls and CD4\(^+\) T cells were isolated using a MACS and a CD4\(^+\) T cell isolation kit (see section 2.7.1.1 for details). CD4\(^+\) T cells were plated in 24-well plates and polarized into Th1, Th2 and Th17 cells by incubation of the cells in the presence of specific polarising cytokines for 5 days (see section 2.7.1.2 for details). In addition Aβ-specific T cell lines were plated in 24-well plates and polarised into Aβ-specific Th1, Th2 and Th17 cells by incubation of cells in the presence of polarising cytokines and Aβ\(_{1-42}\) for 5 days (see section 2.7.1.3 for details). On the 5\(^{th}\) day of the culture, supernatants were taken for cytokine analysis of IFN-γ, IL-10 and IL-17 by ELISA and cells were harvested for co-culture with microglia and astrocytes.

Mixed glia were prepared from 1-day old C57BL/6 mice and cultured for 12 days before shaking and separation into microglia and astrocytes. Microglia were plated in 24-well plates (1 \(\times\) 10\(^5\) cells/well) and astrocytes were plated in 6-well plates (3 \(\times\) 10\(^5\) cells/well) and both cell types were incubated for 24 hours prior to T cell co-culture (see section 2.1.2 for details). One group of microglia and astrocytes were co-cultured with non-specific T cell lines and incubated for 24 hours. A second group of microglia and astrocytes were co-cultured with Aβ-specific T cell lines and treated with Aβ\(_{1-42}\) (15 µg/ml) and incubated for 24 hours (see section 2.7.2.2 for details). T cells were co-cultured with microglia and astrocytes at a ratio of 1:2 which has previously been determined to be the optimal ratio for co-culture of these cell types. Following co-culture, supernatants were removed for cytokine analysis of IL-6 and TNFα by ELISA (see section 2.12 for details) and cells were harvested for analysis of cell surface expression of CD40 and CD86 by flow cytometry (see section 2.9.1 for details). Data are expressed as means ± SEM. ANOVA and Newman’s Keuls post-hoc test was applied where appropriate.
5.3. Results

One of the primary aims of this study was to investigate consequences of the interaction between T cell lines and Aβ-specific T cell lines with microglia and astrocytes by assessing markers of microglial and astrocytic activation and cytokine production. A second aim was to establish if T cells generated from APP/PS1 mice exerted different effects on microglia and astrocytes than T cells generated from littermate controls.

5.3.1. Generation of TH1, TH2 and TH17 T cell lines.

The cytokine environment at the time of T cell activation is crucial for determining the subsequent polarisation into different T cell subsets (Kaiko et al., 2008). Once CD4⁺ T cells are differentiated, specific T cell subtypes secrete an array of cytokines. IFN-γ is primarily released from Th1 cells and NK cells and is strongly associated with a Th1 immune response. IL-10 is an anti-inflammatory cytokine produced by a wide range of cells including Th2, whereas Th17 cells are characterised by their production of IL-17 (Park et al., 2005). In an effort to ensure that the CD4⁺ T cells were correctly polarised into specific Th1, Th2 and Th17 cell lines, supernatants from the specific T cell lines were assessed for the expression of IFN-γ, IL-10 and IL-17. Figure 5.1 demonstrates that cells polarised under Th1-inducing conditions were found to secrete high levels of IFN-γ and IL-10 and low levels of IL-17. Cells polarised under Th2-inducing conditions produced high levels of IL-10 and low levels of IFN-γ and IL-17. Cells polarised under Th17-inducing conditions produced low levels of IFN-γ and high levels IL-10 and IL-17.

5.3.2. TH1 cells increase the expression of co-stimulatory molecules and production of cytokines by microglia and astrocytes.

To examine the effect of TH1 cells on microglial and astrocytic activation, the expression of the co-stimulatory molecules CD40 and CD86 were assessed by FACS and the production of the pro-inflammatory cytokines IL-6 and TNFα were assessed by ELISA. The data show that co-culture of microglia and astrocytes
with Th1 cells generated from wildtype and APP/PS1 mice had no significant effect on CD86 expression (Figure 5.2A and B). Co-culture of microglia with Th1 cells, generated from APP/PS1 mice, significantly increased the expression of CD40 on microglia (\(^*p<0.01\), ANOVA; Figure 5.2C). There was a significant increase in the expression of CD40 on microglia co-cultured with Th1 cells generated from APP/PS1 mice compared with Th1 cells generated from wildtype mice (\( \dagger p<0.05 \), ANOVA; Figure 5.2C). Co-culture of astrocytes with Th1 cells, generated from wildtype and APP/PS1 mice, significantly increased the expression of CD40 on astrocytes compared (\( \ddagger p<0.05 \), ANOVA; Figure 5.2D).

Co-culture of microglia with Th1 cells, generated from APP/PS1 mice, but not wildtype mice significantly increased supernatant concentrations of TNF\(\alpha\) (\( \dagger p<0.05 \), ANOVA; Figure 5.3A). Conversely, co-culture of astrocytes with Th1 cells, generated from wildtype mice but not APP/PS1 mice, significantly increased TNF\(\alpha\) supernatant concentrations compared with controls (\( \dagger p<0.05 \), ANOVA; Figure 5.3B). Co-culture of microglia with Th1 cells from wildtype and APP/PS1 mice had no significant effect on IL-6 concentrations (Figure 5.3C) whereas co-culture of astrocytes with Th1 cells generated from both wildtype and APP/PS1 mice significantly increased supernatant concentrations of IL-6 (\( \ddagger p<0.05 \), ANOVA; Figure 5.3D).

### 5.3.3. Th2 cells had no effect on the expression of co-stimulatory molecules and production of cytokines by microglia and astrocytes.

Co-culture of microglia and astrocytes with Th2 cells generated from wildtype and APP/PS1 mice had no significant effect on the expression of CD86 (Figure 5.4A and B) and CD40 on microglia and astrocytes (Figure 5.4C and D). Co-culture of microglia and astrocytes with Th2 cells generated from wildtype and APP/PS1 mice had no significant effect on supernatant concentrations of TNF\(\alpha\) (Figure 5.5A and B) or IL-6 (Figure 5.5C and D).
5.3.4. **Th17 cells increase the expression of co-stimulatory molecules and production of cytokines by astrocytes but not by microglia.**

Co-culture of microglia and astrocytes with Th17 cells from wildtype and APP/PS1 mice had no significant effect on CD86 (Figure 5.6A and B) expression. Co-culture of Th17 cells, generated from wildtype and APP/PS1 mice had no effect on the expression of CD40 on microglia (Figure 5.6C) but co-culture of astrocytes with Th17 cells generated from APP/PS1 mice, but not wildtype mice, significantly increased expression of CD40 on astrocytes (***p<0.001, ANOVA; Figure 5.6D).

Co-culture of microglia with Th17 cells from wildtype and APP/PS1 mice had no significant effect on supernatant concentrations of TNFα and IL-6 (Figure 5.7A and B). Co-culture of astrocytes with Th17 cells from wildtype and APP/PS1 mice significantly increased supernatant concentrations of IL-6 (*p<0.05, **p<0.01, ANOVA; Figure 5.7C). Supernatant concentrations of TNFα were undetectable following co-culture of Th17 cells with astrocytes. Table 5.1 summarises the expression of co-stimulatory molecules and production of pro-inflammatory cytokines following co-culture of T cell lines with microglia and astrocytes.

5.3.5. **Generation of Aβ-specific Th1, Th2 and Th17 T cell lines.**

Having established that co-culture of T cell subsets with glia increased the expression of co-stimulatory molecules and production of pro-inflammatory cytokines, irrespective of whether they were generated from wildtype or APP/PS1 mice, the effect of Aβ-specific T cells on glial function was assessed. Aβ-specific T cell lines were generated and assessed for the production of IFN-γ, IL-10 and IL-17 to ensure that they were correctly polarised into Aβ-specific T cell subtypes. Figure 5.8 demonstrates that cells polarised under Aβ-specific Th1-inducing conditions produced high levels of IFN-γ and IL-10 and low levels of IL-17. Cells polarised under Aβ-specific Th2-inducing conditions produced high levels of IL-10 and low levels of IFN-γ and IL-17. Cells polarised under Aβ-specific Th17-inducing conditions produced low levels of IFN-γ and IL-10 and high levels of IL-17.
5.3.6. Aβ-specific Th1 cells did not effect the expression of co-stimulatory molecules on microglia and astrocytes but increased production of TNFα.

Co-culture of microglia and astrocytes with Aβ-specific Th1 cells generated from wildtype and APP/PS1 mice had no significant effect on the expression of CD86 or CD40 on microglia and astrocytes (Figure 5.9A-D). Co-culture of microglia with Aβ-specific Th1 cells, generated from wildtype and APP/PS1 mice, significantly increased supernatant concentrations of TNFα (*p<0.05, **p<0.01, ANOVA; Figure 5.10A). Co-culture of astrocytes with Aβ-specific Th1 cells, from wildtype mice significantly increased supernatant concentrations of TNFα but co-culture of astrocytes with Aβ-specific Th1 cells generated from APP/PS1 mice significantly decreased supernatant concentration of TNFα (**p<0.001, +++p<0.001, ANOVA; Figure 5.10B). Co-culture of astrocytes (Figure 5.10D) but not microglia (Figure 5.10C) with Aβ-specific Th1 cells from wildtype but not APP/PS1 mice significantly increased supernatant concentrations of IL-6 (*p<0.05, ANOVA; Figure 5.10D).

5.3.7. Aβ-specific Th2 and Th17 cells had no effect on the expression of co-stimulatory molecules and production of cytokines by microglia and astrocytes.

Co-culture of microglia and astrocytes with Aβ-specific Th2 (Figure 5.11 and 5.12) and Th17 (Figure 5.13 and 5.14) cells generated from wildtype and APP/PS1 mice had no significant effect on the expression of CD86 and CD40 on microglia and astrocytes (Figure 5.11A-D and 5.13A-D). Co-culture of microglia and astrocytes with Aβ-specific Th2 cells generated from wildtype and APP/PS1 mice had no significant effect on supernatant concentrations of TNFα and IL-6 (Figure 5.12A-C and 5.14A-C). TNFα was undetectable following co-culture of Aβ-specific Th2 and Th17 cells with astrocytes.

Table 5.2 summarises the expression of co-stimulatory molecules and the production of pro-inflammatory cytokines, following co-culture of Aβ-specific T cell lines with microglia and astrocytes.
Figure 5.1. Generation of non-specific Th1, Th2 and Th17 T cell lines.

Spleens were harvested from APP/PS1 and wildtype littermate control mice (12 months old), and cells were stimulated into T cell lines under polarising conditions. Th1 polarising conditions with IL-12 (50 ng/ml), Th2 polarising conditions with IL-4 (10 ng/ml) and anti-IFN-γ (5 μg/ml) and Th17 polarising conditions with TGF-β (5 ng/ml), IL-1β (25 ng/ml), IL-23 (50 ng/ml) and anti-IFN-γ. On the 4th day of the culture supernatants were removed for analysis of IFN-γ (A), IL-10 (B) and IL-17 (C) concentrations by ELISA. Th1 cells have high IFN-γ and IL-10 and low IL-17 production (A-C). Th2 cells have high IL-10 and low IFN-γ and IL-17 production (A-C). Th17 cells have low IFN-γ and high IL-10 and IL-17 production (A-C). Values are expressed as means ± SEM (n=10).
Figure 5.2. Increased CD40 expression on microglia and astrocytes co-cultured with Th1 cells.

Th1 cell were generated from wildtype and APP/PS1 mice as described in Figure 5.1. After 4 days of polarisation Th1 cell lines were co-cultured with microglia (CD11b⁺) and astrocytes (CD11b⁻) prepared from neonatal C57BL/6 mice, for 24 hours. Expression of CD86 and CD40 on CD11b⁺ and CD11b⁻ cells was quantified by FACS. Co-culture of microglia with Th1 cells from wildtype and APP/PS1 mice had no significant effect on the expression of CD86 on microglia or astrocytes (A and B). Co-culture of microglia with Th1 cells, generated from APP/PS1 mice, significantly increased the expression of CD40 on microglia (C) (**p<0.01, ANOVA). There was a significant increase in the expression of CD40 on microglia co-cultured with Th1 cells generated from APP/PS1 mice compared with Th1 cells generated from wildtype mice (C) ('p<0.05, ANOVA). Co-culture of astrocytes with Th1 cells, generated from wildtype and APP/PS1 mice, significantly increased expression of CD40 on astrocytes (D) ('p<0.05, ANOVA). Values are expressed as means ± SEM (n=3-10).

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Figure 5.3. Increased cytokine production by microglia and astrocytes following co-culture with Th1 cells.

Th1 cells were generated from wildtype and APP/PS1 mice and co-cultured with microglia and astrocytes as described in Figure 5.1 and 5.2. Supernatants were harvested for analysis of TNFα and IL-6 concentrations by ELISA. Co-culture of microglia with Th1 cells, generated from APP/PS1 mice but not wildtype mice, significantly increased supernatant concentrations of TNFα (A) (*p<0.05, ANOVA). Co-culture of astrocytes with specific Th1 cells, generated from wildtype mice but not APP/PS1 mice, significantly increased supernatant concentrations of TNFα (B) (*p<0.05, ANOVA). Co-culture of microglia with Th1 cells generated from wildtype and APP/PS1 mice had no significant effect on IL-6 concentrations (C) whereas co-culture of astrocytes with Th1 cells generated from wildtype and APP/PS1 mice significantly increased supernatant concentrations of IL-6 (D) (*p<0.05, ANOVA). Values are expressed as means ± SEM (n=4).
Figure 5.4. CD40 and CD86 expression is unaltered on microglia and astrocytes following co-culture with Th2 cells.

Th2 cells were generated from wildtype and APP/PS1 mice and co-cultured with microglia (CD11b⁺) and astrocytes (CD11b⁻) as described in Figure 5.1 and 5.2. Expression of CD86 and CD40 on CD11b⁺ and CD11b⁻ cells was quantified by FACS. Co-culture of microglia and astrocytes with Th2 cells from wildtype and APP/PS1 mice had no significant effect on the expression of CD86 and CD40 on microglia and astrocytes (A- D). Values are expressed as means ± SEM (n=4-10).
Figure 5.5. Cytokine production by microglia and astrocytes is unaltered following co-culture with Th2 cells.

Th2 cells were generated from wildtype and APP/PS1 mice and co-cultured with microglia and astrocytes as described in Figure 5.1 and 5.2. Supernatants were harvested for analysis of TNFα and IL-6 concentrations by ELISA. Co-culture of microglia and astrocytes with Th2 cells from wildtype and APP/PS1 mice had no significant effect on supernatant concentrations of TNFα or IL-6 (A-D). Values are expressed as means ± SEM (n=4-5).
Figure 5.6. Increased CD40 expression on astrocytes following co-culture with Th17 cells.

Th17 cells were generated from wildtype and APP/PS1 mice and co-cultured with microglia (CD11b⁺) and astrocytes (CD11b⁻) as described in Figure 5.1 and 5.2. Expression of CD86 and CD40 on CD11b⁺ and CD11b⁻ cells was quantified by FACS. Co-culture of microglia and astrocytes with Th17 cells generated from wildtype and APP/PS1 mice had no significant effect on expression of CD86 (A and B). Co-culture of microglia with Th17 cells, generated from wildtype and APP/PS1 mice had no effect on CD40 expression (C). Co-culture of astrocytes with Th17 cells, generated from APP/PS1 mice but not wildtype mice, significantly increased the expression of CD40 on astrocytes (D) (***p<0.001, ANOVA). Values are expressed as means ± SEM (n=4-10).
Figure 5.7. IL-6 production is increased by astrocytes following co-culture with Th17 cells

Th17 cells were generated from wildtype and APP/PS1 mice and co-cultured with microglia and astrocytes as described in Figure 5.1 and 5.2. Supernatants were harvested for analysis of TNFα and IL-6 concentrations by ELISA. Co-culture of microglia with Th17 cells generated from wildtype and APP/PS1 mice had no significant effect on supernatant concentration of either TNFα or IL-6 (A and B). Co-culture of astrocytes with Th17 cells generated from wildtype and APP/PS1 mice significantly increased supernatant concentrations of IL-6 (C) (*p<0.05, **p<0.01, ANOVA). Supernatant concentrations of TNFα were undetectable following co-culture of Th17 cells with astrocytes. Values are expressed as means ± SEM (n=4).
Figure 5.8. Generation of Aβ-specific Th1, Th2 and Th17 T cell lines.

Spleens were harvested from APP/PS1 and wildtype littermate control mice and cells were stimulated into Aβ-specific T cell lines under polarising conditions and in the presence of Aβ1-42 (15 µg/ml). Th1 polarising conditions were IL-12 (50 ng/ml), Th2 polarising conditions were IL-4 (10 ng/ml) and anti-IFN-γ (5 µg/ml) and Th17 polarising conditions were TGF-β (5 ng/ml), IL-1β (25 ng/ml), IL-23 (50 ng/ml) and anti-IFN-γ. On the 4th day of the culture supernatants were removed for analysis of IFN-γ (A), IL-10 (B) and IL-17 (C) concentrations by ELISA. Th1 cells have high IFN-γ and IL-10 and low IL-17 production (A-C). Th2 cells have high IL-10 and low IFN-γ and IL-17 production (A-C). Th17 cells have high IL-17 production and low IFN-γ and IL-10 (A-C). Values are expressed as means ± SEM (n=10).
Figure 5.9. CD40 and CD86 expression is unaltered on microglia and astrocytes following co-culture with Aβ-specific Th1 cells.

Aβ-specific Th1 cells were generated from wildtype and APP/PS1 mice as described in Figure 5.8. After 4 days of polarisation Aβ-specific Th1 cell lines were co-cultured with microglia (CD11b⁺) and astrocytes (CD11b⁺) prepared from neonatal C57BL/6 mice, treated with Aβ1-42 (15 μg/ml), for 24 hours. Expression of CD86 and CD40 on CD11b⁺ (A and C) and CD11b⁺ (B and D) cells was quantified by FACS. Co-culture of microglia and astrocytes with Aβ-specific Th1 cells generated from wildtype and APP/PS1 mice had no significant effect on the expression of CD86 or CD40 on microglia and astrocytes (A-D). Values are expressed as means ± SEM (n=5-10).
Figure 5.10. TNFα production was increased by microglia and astrocytes following co-culture with Aβ-specific Th1 cells.

Aβ-specific Th1 cells were generated from wildtype and APP/PS1 mice and cocultured with microglia and astrocytes treated with Aβ1-42 as described in Figure 5.8 and 5.9. Supernatants were harvested for analysis of TNFα and IL-6 concentrations by ELISA. Co-culture of microglia with Aβ-specific Th1 cells, generated from wildtype and APP/PS1 mice, significantly increased supernatant concentrations of TNFα (A) (*p<0.05, ***p<0.01, ANOVA). Co-culture of astrocytes with Aβ-specific Th1 cells generated from wildtype mice significantly increased supernatant concentrations of TNFα (B) (**p<0.001, ANOVA), and this increase was significantly attenuated by co-culture of astrocytes with Aβ-specific Th1 cells generated from APP/PS1 mice (B) (++p<0.001, ANOVA). Co-culture of astrocytes (D), but not microglia (C), with Aβ-specific Th1 cells generated from wildtype mice significantly increased IL-6 supernatant concentrations (D) (*p<0.05, ANOVA). Values are expressed as means ± SEM (n=4-8).
Figure 5.11. CD40 and CD86 expression is unaltered on microglia and astrocytes following co-culture with Aβ-specific Th2 cells.

Aβ-specific Th2 cells were generated from wildtype and APP/PS1 mice and co-cultured with microglia (CD11b⁺) and astrocytes (CD11b⁻) treated with Aβ1-42 as described in Figure 5.8 and 5.9. Expression of CD86 and CD40 on CD11b⁺ (A and C) and CD11b⁻ (B and D) cells was quantified by FACS. Co-culture of microglia and astrocytes with Aβ-specific Th2 cells generated from wildtype and APP/PS1 mice had no significant effect on the expression of CD86 and CD40 on microglia and astrocytes (A-D). Values are expressed as means ± SEM (n=5-10).
Figure 5.12. Cytokine production by microglia and astrocytes is unaltered following co-culture with Aβ-specific Th2 cells.

Aβ-specific Th2 cells were generated from wildtype and APP/PS1 mice and co-cultured with microglia and astrocytes treated with Aβ1-42 as described in Figure 5.8 and 5.9. Supernatants were harvested for analysis of TNFα and IL-6 concentrations by ELISA. Co-culture of microglia (A and B) and astrocytes (C) with Aβ-specific Th2 cells generated from wildtype and APP/PS1 mice had no significant effect on supernatant concentrations of TNFα and IL-6 (A-C). Supernatant concentrations of TNFα were undetectable following co-culture of Aβ-specific Th2 cells with astrocytes. Values are expressed as means ± SEM (n=4-7).
Figure 5.13. CD40 and CD86 expression is unaltered on microglia and astrocytes following co-culture with Aβ-specific Th17 cells.

Aβ-specific Th17 cells were generated from wildtype and APP/PS1 mice and co-cultured with microglia (CD11b⁺) and astrocytes (CD11b⁻) treated with Aβ1-42 as described in Figure 5.8 and 5.9. Expression of CD86 and CD40 on CD11b⁺ (A and C) and CD11b⁻ (B and D) cells was quantified by FACS. Co-culture of microglia and astrocytes with Aβ-specific Th17 cells generated from wildtype and APP/PS1 mice had no significant effect on the expression of CD86 and CD40 on microglia and astrocytes (A-D). Values are expressed as means ± SEM (n=5-10).
Figure 5.14. Cytokine production by microglia and astrocytes is unaltered following co-culture with Aβ-specific Th17 cells.

Aβ-specific Th17 cells were generated from wildtype and APP/PS1 mice and co-cultured with microglia and astrocytes treated with Aβ1-42 as described in Figure 5.8 and 5.9. Supernatants were harvested for analysis of TNFα and IL-6 concentrations by ELISA. Co-culture of microglia (A and B) and astrocytes (C) with Aβ-specific Th17 cells generated from wildtype and APP/PS1 mice had no significant effect on supernatant concentrations of TNFα and IL-6 (A-C). Supernatant concentrations of TNFα were undetectable following co-culture of Aβ-specific Th17 cells with astrocytes. Values are expressed as means ± SEM (n=4-8).
5.4. Discussion

In this chapter the interaction of glia and distinct T cell subsets, including those that were Aβ-specific was investigated. Specifically the effect of Th1, Th2 and Th17 cells in modulating the expression of CD40 and CD86 and the production of the pro-inflammatory cytokines TNFα and IL-6 by glia was investigated. The effect of T cell subtypes generated from the spleens of wildtype mice were compared with cells generated from APP/PS1 mice. The data revealed that co-culturing of Th1 and Th17 cells with glia markedly enhanced microglial and astrocytic activation and, in some cases, this was enhanced if glia were co-cultured with T cells generated from APP/PS1 mice. In contrast it was found that co-culturing Th2 cells with glia exerted no effect. Aβ-specific Th1 cells induced glial activation but neither Aβ-specific Th2 or Th17 cells exerted any significant effect.

The process by which naive CD4^+ T cells differentiate into specific effector Th cells is well understood. It has been established that once naive T cells migrate from the thymus they can become differentiated into appropriate effector cells following activation by two signals. The first signal is delivered through interaction of MHC class II on cells of the innate immune system with their specific TCRs (Huang et al., 1999; Sharpe & Freeman, 2002). This process is accompanied by a secondary signal from co-stimulatory molecules CD80 and CD86 on innate immune cells, to CD28 on T cells. Once both signals are present, naive CD4^+ T cells will rapidly divide into a subpopulation of T cells with effector functions (Sharpe & Freeman, 2002). The phenotype of the activated CD4^+ T cells generated depends, not only on the antigen presented, but on the cytokine environment present at the time of clonal expansion (Mosmann et al., 1986). Naive T cells differentiate into Th1 cells in the presence of IL-12, Th2 cells in the presence of IL-4 and Th17 cells in the presence of TGF-β, IL-1β and IL-23 (Schluns & Lefrancois, 2003; Korn et al., 2009; McQuillan et al., 2010). In this study CD4^+ T cells were polarised into non-specific and Aβ-specific Th1 cells by incubating cells in the presence of IL-12, Th2 cells by incubating cells in the presence of IL-4 and anti-IFN-γ and Th17 cells by incubating cells in the presence of TGF-β, IL-1β, IL-23 and anti-IFN-γ. Predictably non-specific and Aβ-specific
Th1 cells secreted predominantly IFN-γ, Th2 cells secreted predominantly IL-10 and Th17 cells secreted predominantly IL-17. The secretion of a pattern of cytokines by T cells has already been described in both CD4^+ clones, human and mouse T cells and the function of specific T cell subgroups correlates well with their distinctive cytokine profiles. For example Th1 cytokines are involved in cell mediated-inflammatory reactions and play a role in autoimmune diseases while Th2 promote antibody production and are involved in strong antibody and allergic responses (Mosmann & Sad, 1996). Th17 cells are implicated in pathogen clearance and also play a role in autoimmune disease (Korn et al., 2009).

Previous studies have found that IFN-γ increases the expression of co-stimulatory molecules on human microglia and the secretion of cytokines by microglia (Becher & Antel, 1996). Priming of microglia in vitro with IFN-γ upregulates MHC class II expression and co-stimulatory molecules on microglia allowing for antigen processing and T cell restimulation (Aloisi et al., 2000b). The role of astrocytes as APCs is unclear. It has been noted that the type of T cell present renders astrocytes as either efficient or inefficient APCs (Aloisi et al., 2000b). Aloisi and colleagues (1999) demonstrated that astrocytes are more efficient in restimulating Th2 cells compared with Th1 cells and fail to prime naïve T cells. However astrocytes have been found to upregulate MHC class II expression following IFN-γ exposure (Shrikant & Benveniste, 1996). The data presented here indicate that co-culture of glia with non-specific Th1 cells significantly increased the expression of CD40 on both microglia and astrocytes, and the effect on microglia was greater following co-culture with Th1 cells generated from APP/PS1. Co-culture of Th17 cells with glia significantly enhanced expression of CD40 on astrocytes. These results are consistent with previous studies from this lab which have found that co-culture of MOG-specific Th1 and Th17 cells with glia increases the expression of CD40 on microglia (Murphy et al., 2010). It has been proposed that increased expression of CD40 and CD80 is a consequence of cell-cell contact (Wolf et al., 2001). Although Seguin and colleagues (2003) demonstrated that supernatants taken from Th1 cells could induce both CD86 and CD40 expression on microglia. It has been suggested that Th2 cells are inefficient at stimulating microglia due to their secretion of microglia-deactivating factors, IL-4 and IL-10 (Mosmann & Sad, 1996). Consistently no significant change in the expression of CD40 by glia was
observed following co-culture with Th2 cells which supports previous findings that Th2 cells cultured with adult murine microglia failed to increase expression of APC related molecules and in turn microglia failed to restimulate Th2 cells (Aloisi et al., 2000a). Although a study by Gimsa and colleagues (2001) using entorhinal-hippocampal slices demonstrated that prestimulated Th2 cells were able to invade slices to the same degree as Th1 cells and that both Th1 and Th2 cells increased the expression of CD40 on microglia compared to control cultures however total CD40 staining was somewhat higher in Th1 co-cultures. These authors also found that Th2 cells were unable to increase the expression of ICAM-1, an important adhesion and co-stimulatory molecule, on microglial but were able to diminish the Th1-induced increase in microglial expression of ICAM-1 (Gimsa et al., 2001).

It has been suggested that CD40 is fundamental marker of certain disease pathologies and inflammation. A role for CD40-CD40L pathway in MS has been suggested; it has been shown that CD40+ microglia and macrophages are present in MS lesions (Gerritse et al., 1996). Indeed studies have shown that myelin basic protein (MBP)-specific TCR mice lacking CD40L have T cell priming deficiencies and EAE could not be provoked in these animals following immunisation with encephalitogenic peptide (Grewal & Flavell, 1996). Whereas blockade of the CD40-CD40L interaction reduces disease severity in experimental autoimmune diseases such as EAE (Gerritse et al., 1996; Grewal & Flavell, 1996). Furthermore it has been shown that CD40-CD40L interaction in the presence of IFN-γ is required for IL-12 production by microglia which in turn promotes polarisation of T cells into Th1 cells and that blockade of this pathway could have the additional advantage of inhibiting IL-12 production without affecting the ability of CNS APCs to produce PGE2, which has an inhibitory influence on Th1 responses. (Aloisi et al., 1999).

Previously it has been suggested that interaction of CD28 on T cells and CD80/CD86 on APCs is crucial for the onset and course of EAE (Wolf et al., 2001). Both CD80 and CD86 are upregulated on microglia following co-culture with MOG-specific Th1 and Th17 cells (Murphy et al., 2010). However no significant change in CD86 expression on microglia and astrocytes following co-culture with Th1, Th2 and Th17 cells was observed in the present study. However, it has previously been suggested that initial CD86 stimulation can induce the
expression of IL-4, and favour a Th2 response, while CD80 stimulation has been reported to favour a Th1 response (Freeman et al., 1995). Furthermore, Wolf and colleagues (2001) demonstrated that Th1 cells independent of antigen specificity, can switch the expression of the co-stimulatory molecules on microglia from CD86 to CD80. Taking this evidence into account further experiments should assess expression of CD80 as well as CD86.

Activation of microglia during CNS disease leads to the production of the pro-inflammatory cytokines such as TNFα and IL-6, which have been found to contribute to myelin damage and CNS inflammation (Benveniste, 1997). There is extensive literature implicating the production of TNFα specifically as a critical mediator of autoimmune inflammatory pathologies such as MS and EAE. TNFα is detectable in lesions of MS and in EAE and is increased in the serum and CSF of patients with MS. TNFα can induce cytotoxicity in rodent and human primary oligodendrocytes. (Probert et al., 2000) and can influence lymphocyte trafficking across the endothelium and upregulate many of the adhesion molecules involved in this process (Chabot et al., 1997). It has been demonstrated that T cell-microglia interactions in vitro significantly increase the production of TNFα by microglia (Chabot et al., 1997). Dasgupta and colleagues (2005) demonstrated that neuroantigen-specific T cells interacting with microglia increase the expression of IL-1β and IL-6 as well as TNFα. Furthermore supernatants from Th1 cells can induce the secretion of pro-inflammatory cytokines, TNFα and IL-6, and the chemokine IP-10 from microglia (Seguin et al., 2003). While studies have found that in addition to the effect of Th1 cells, Th17 cells co-cultured with glia enhance the production of TNFα and IL-6 (Murphy et al., 2010). The results of the present studies show that Th1 cells increased secretion of TNFα and IL-6 in microglial and astrocytic cultures and Th17 cells increased IL-6 production by astrocytes; no significant change was observed following co-culture of glia with Th2 cells. The increased production of cytokines by glia co-cultured with Th1 and Th17 cells is concomitant with the increase in CD40 expression on microglia and astrocytes which concurs with the finding that ligation of CD40 with CD40L activates NF-κB and enhances cytokine production. This may also account for the lack of cytokine production observed in Th2 co-cultures as no significant increase in CD40 expression was observed in these cultures. Th17 cells, surprisingly, had no affect on cytokine production from microglia but the difference in cytokine

~ 170 ~
production by glial cells in response to Th1 and Th17 cells could be explained by a recent study from Kroenke and colleagues (2008). These authors suggest that although Th1 and Th17 cells are involved in the pathology of MS, the form of inflammation generated by the T cells differs between the two subsets. This group reported that Th1-driven EAE favoured the upregulation of monocyte- and lymphocyte-attracting chemokines while Th17-driven EAE favoured the upregulation of neutrophil chemokines (Kroenke et al., 2008). Together the data suggest that co-culture of glia with Th1 and Th17 cells but not Th2 cells significantly exaggerates pro-inflammatory cytokine production as measured by TNFα and IL-6, suggesting that interaction of these cells facilitates a detrimental inflammatory environment. However there is some debate over the role of TNFα in EAE with some evidence suggesting that disease progression is exacerbated in TNFα−/− mice immunised with MOG (Liu et al., 1998) and other evidence indicating that TNFα stimulates development and progression of the disease (Probert et al., 2000). The present results might suggest that the production of TNFα by glia has a detrimental role as it was only produced in response to Th1 cells. The production of anti-inflammatory cytokines by glia in response to Th2 cells was not assessed in this study and should be addressed in the future.

Infiltrating T cells are not as common in AD as in conditions such as meningitis and MS (Bitsch et al., 2000) none the less their presence, although low, has been described in several studies (Rogers et al., 1988; Togo et al., 2002). A study from Monsonego and colleagues (2003) described the presence of Aβ-reactive T cells in the brains of healthy young and elderly subjects and in AD patients, with the crucial finding that these T cells, although present in all subjects, were significantly increased in elderly patients and patients with AD. These authors postulated that capture of Aβ by APCs results in the migration of APCs to lymph nodes in turn inducing T cell activation (Monsonego et al., 2003). However it still remains unclear if the role of T cells is beneficial or detrimental. Evidence suggests that glia act as effective APCs for Aβ-specific Th1 and Th17 cells and enhance Aβ-induced pro-inflammatory cytokine production and co-stimulatory molecule expression on glia (McQuillan et al., 2010) and one evidence shows that MHC class II and T cells are co-localised with plaques (McGeer et al., 1987). Furthermore it has been reported that IFN-γ which is derived from Th1 cells is required for Aβ-induced neuronal toxicity (Li et al.,
2004). In contrast Th2-type cytokines can attenuate Aβ-induced cytokine production by microglia (Szczepeanik et al., 2001).

In contrast to the effects of non-specific T cells there was no significant change in either the expression of CD40 or CD86 on microglia and astrocytes following co-culture with Aβ-specific Th1, Th2 and Th17 cells. This was surprising given the effect of non-specific Th1 and Th17 cells and in view of the findings from another study that both Aβ-specific Th1 and Aβ-specific Th17 cells enhanced Aβ-induced expression of CD40 and CD86 as well as MHC class II and CD80 on microglia (McQuillan et al., 2010). It is worth noting that some of the experimental conditions were different between these two studies and may account for the discrepancies seen. Firstly the concentration of Aβ used here to stimulate microglia and astrocytes was 15 μg/ml versus 40 μg/ml in the published report. Furthermore the Aβ-specific T cells were generated ex-vivo by the addition of Aβ1-42 to the T cell cultures where as McQuillan and colleagues (2010) generated their Aβ-specific T cells through the immunisation of C57BL/6 mice with Aβ and CpG and then restimulated T cells ex-vivo with Aβ. The lack of effect of Th2 cells concurs with my earlier findings and previous published data from this lab (McQuillan et al., 2010). Interestingly McQuillan and colleagues (2010) reported that Th2 cells were found to decrease Th1-induced CD40 expression.

Both TNFα and IL-6 are secreted by microglia and astrocytes in the AD brain (Lue et al., 2001a). It has been well documented that Aβ induces the production of pro-inflammatory cytokines in cultured microglia (Davis et al., 1992; Meda et al., 1995). In the present study the role of Aβ-specific Th1, Th2 and Th17 cells on TNFα and IL-6 production by microglia and astrocytes treated with Aβ was investigated. Here it was found that both TNFα and IL-6 were produced by glia following co-culture with Aβ-specific Th1 cells however neither Aβ-specific Th17 nor Aβ-specific Th2 cells had any affect on cytokine production by glia. These results are consistent with others suggesting that augmenting factors such IFN-γ act synergistically with Aβ to enhance this inflammatory response; previously it has been demonstrated that IFN-γ enhances Aβ-induced production of TNFα and NO by glia (Goodwin et al., 1995; Meda et al., 1995). A more recent study from Gasic-Milenkovic and colleagues (2003) found that Aβ synergised with IFN-γ, LPS and advanced glycation endproducts to enhance
TNFα and IL-6 production from microglia. Similarly a study from McQuillan and colleagues (2010) also observed that Aβ-specific Th1 cells induced the production of TNFα and IL-6 by glia and that Aβ-specific Th2 cells had no influence on the production of either cytokine by glia. These results implicate the interaction between glia and Aβ-specific Th1 cells as detrimental in response to Aβ, while the absence of a Th2 cell response further supports their potential anti-inflammatory role in AD. Furthermore it has been found that both IFN-γ and IL-12 (Th1 cytokines) are enhanced in the cerebral cortex of APP transgenic mice whereas, IL-4 (Th2 cytokine) is decreased (Abbas et al., 2002). Previously it has been suggested that Aβ-specific Th2 cells could be beneficial by secreting anti-inflammatory cytokines and downregulating and inflammatory environment (Monsonego et al., 2003). Aβ-specific Th2 cells can reduce plaque associated microglia in APP/PS1 mice and this is associated with an improvement in cognition in these mice (Cao et al., 2009). The Th2 cytokine IL-4 has been shown to reduce microglia activation in response to IFN-γ (Nguyen & Benveniste, 2000) and modulate co-stimulatory molecule expression on glia in response to Th1 and Th17 cells (McQuillan et al., 2010).

Interestingly in these studies TNFα production was significantly decreased in astrocytes following co-culture with Aβ-specific Th1 cells generated from APP/PS1 mice compared with Aβ-specific Th1 cells generated from wildtype mice. This result may be explained by a previous observation that increased T cell reactivity to Aβ is not observed in APP transgenic mice possibly as a consequence of T cell tolerance (Monsonego et al., 2001). These authors suggested that the chronic expression of Aβ increased T cell hyporesponsiveness and tolerance. Thus it might be hypothesised that the decreased responsiveness in glia cytokine production to Aβ-specific Th1 cells generated from APP/PS1 mice is due to the tolerance of these T cells.

The results from these studies implicate a role for T cells specifically, Th1 and Th17, in inducing and/or exacerbating inflammation as a result of their interaction with microglia and astrocytes. Here it was observed that Th1 cells induced the expression of CD40 on microglia and astrocytes and the production of TNFα and IL-6 by microglia and astrocytes. Th2 cells were found to have no effect on either the expression of co-stimulatory molecules on glia or the production of cytokines by glia. Th17 cells induced the expression of CD40 and
the production of IL-6 in astrocytes. Furthermore it was observed that Aβ-specific Th1 cells induced the production of TNFα and IL-6 by glia yet Aβ-specific Th2 and Th17 cells had no effect. Together the data suggest that Th1 cells have the greatest effect on glial activation and this topic needs further investigation in an in vivo model which will be addressed in the next chapter.
Table 5.1. Results summary of the expression of co-stimulatory molecules and production of cytokines following co-culture of non-specific T cells with microglia and astrocytes.

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<td>Wildtype mice</td>
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- **Microglia**
  - CD86
  - CD40
  - TNFα
  - IL-6

- **Astrocytes**
  - CD86
  - CD40
  - TNFα
  - IL-6

- **Th1**
- **Th2**
- **Th17**
Table 5.2. Results summary of the expression of co-stimulatory molecules and production of cytokines following co-culture of Aβ-specific T cells with microglia and astrocytes.

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- **Microglia**
  - CD86
  - CD40
  - TNFα
  - IL-6

- **Astrocytes**
  - CD86
  - CD40
  - TNFα
  - IL-6
Chapter 6
Chapter 6

6.1. Introduction

AD is a progressive neurodegenerative disease that is characterized by profound neuronal loss and development of neurofibrillary tau tangles and neuritic plaques. The Aβ cascade hypothesis is one of the main theories which has been suggested to explain the development of neurodegeneration in AD. This theory suggests that an imbalance between the production and clearance of Aβ in the brain gives rise to the accumulation and the formation of Aβ-plaques, exacerbated glial cell activation and neuronal loss which ultimately causes cognitive decline (Karran et al., 2011). There is strong evidence in the literature which supports the Aβ hypothesis. Of note is the fact that neuritic plaques, which are composed of the Aβ-protein, are associated with activated microglia whilst diffuse plaques, which have been termed clinically benign Aβ deposits, are not associated with activated microglia (Benveniste et al., 2001). It has been suggested that interaction of Aβ with microglia gives rise to chronic inflammation which in turn contributes to both neuronal death and plaque formation (Benveniste et al., 2001; McGeer & McGeer, 2003). One proposal is that the chronic inflammatory environment is not causative but plays a large role in the pathogenesis of AD (McGeer & McGeer, 2003). As a result, research has focused on the development of therapies that could allow Aβ clearance from the brain, and therapies that could have an anti-inflammatory effect.

The removal of Aβ from the brain has received substantial attention as a therapeutic strategy and Aβ clearance after anti-Aβ immunotherapy was first reported in the PDAPP transgenic mouse model of AD following Aβ1-42 vaccination by Schenk and colleagues (1999). Since then other investigators, using the same vaccination protocol and other transgenic models of AD, have shown similarly-reduced Aβ levels which is associated with a decrease in cognitive deficits in the brains of these mice (Janus et al., 2000; Morgan et al., 2000). It is not clear by what mechanism immunotherapy acts although Wilcock and colleagues (2003) have reported that diffuse Aβ is removed independent of microglial activation whereas removal of compact Aβ required microglial
activation. In a later study these authors found that vaccination of transgenic mice, over a 3 month period, similarly decreased Aβ load and this was accompanied by microglial activation and a dramatic increase in circulating Aβ levels in the plasma (Wilcock et al., 2004). Thus it has been hypothesized that anti-Aβ immunotherapy and the resultant plaque clearance may due to the activation of microglia to a phagocytic phenotype, via opsonization of Aβ (Schenk et al., 1999). It is possible that Aβ may move down the concentration gradient from the brain into the plasma; this has been termed the peripheral sink mechanism (DeMattos et al., 2001). Following animal trials in which no significant unwanted side effects were observed, an anti-Aβ immunotherapy clinical trial was initiated. In this clinical trial, a synthetic Aβ1-42 peptide (AN-1792), with adjuvant QS21, was peripherally administered to AD patients. The trial passed Phase I but was rapidly halted in Phase II when patients developed meningoencephalitis, which was thought to be as a result of an immune response to Aβ mediated by infiltrating Th1 cells (Pfeifer et al., 2002). However active immunisation promoted plaque clearance in the brains of these AD patients (Nicoll et al., 2003). Despite the negative side effects of the current Aβ-based immunotherapeutic approaches, it was established that both mouse models of AD and AD patients were capable of developing Aβ-specific adaptive immune responses suggesting that successful therapeutic intervention may lie in limiting or altering the Aβ-specific immune response (Ethell et al., 2006).

Previously the CNS was described as an immunologically privileged site but more recently it is more correctly viewed as an immunologically-specialized site (Ransohoff et al., 2003). Extensive literature describes the potential mechanisms of T cell entry into the CNS during AD. It is acknowledged that the BBB may become more permeable during ageing and AD allowing for the invasion of peripheral immune cells into the CNS (Farrall & Wardlaw, 2009). Furthermore, the results presented in chapter 3 suggest that, Aβ can stimulate the production of a range of cytokines and chemokines in vitro, while others have found similar cytokine and chemokine production from human post-mortem microglia stimulated with Aβ (Lue et al., 2001b). The significance of this is that it has been suggested that the production of different cytokines and chemokines may direct selective peripheral immune cell recruitment into the CNS (Tran et al., 2000b; Stalder et al., 2005). It is also known that Aβ1-42 can freely cross the BBB
and stimulate naïve T cells outside of the CNS (DeMattos et al., 2002). Indeed there is evidence documenting the presence of T cells in the AD brain (Rogers et al., 1988; Togo et al., 2002; Monsonego et al., 2003), although not to the same degree as seen in autoimmune diseases like MS. None the less, their presence has prompted investigators to assess the role they may have in the pathogenesis of AD and it remains uncertain if the presence of T cells in the brain is beneficial or destructive. A destructive role for T cells specifically Aβ-specific Th1 cells was implicated in the AN-1792 clinical trial and therefore a newer vaccine, CAD106, designed to avoid the activation of Aβ-specific T cells has shown reduced Aβ-accumulation in APP transgenic mice and is now in Phase II trials in AD patients (Wiessner et al., 2011).

It has been shown in vitro that both Th1 cells (McQuillan et al., 2010) and Th1 cell-secreted cytokines (Seguin et al., 2003; Li et al., 2004) exacerbate microglial activation and inhibit Aβ degradation (Yamamoto et al., 2008). A beneficial role for T cells has been implicated in studies from the laboratory of Ethell and colleagues (2006) who found that adoptive transfer of a mixed population of Aβ-specific T cells into APP/PS1 mice reversed synaptic loss and cognitive decline. These authors observed that the cognitive improvement was attributed to Th2 cells and not Th1 cells (Ethell et al., 2006). A more recent study using APP transgenic mice expressing low levels of IFN-γ showed decreased plaque burden which the authors attributed to the infiltration of Aβ-specific T cells (Fisher et al., 2010). Correspondingly in vitro studies suggest that both Th2 cells (McQuillan et al., 2010) and Th2 cell-secreted cytokines (Szczepanik et al., 2001) decrease microglial activation and thus could have a beneficial effect by downregulating the pro-inflammatory environment. It has been shown that Th2 cell cytokines enhance Aβ degradation (Yamamoto et al., 2008). Not much is known about the role of Th17 cells in the pathogenesis of AD although their role in the pathogenesis of EAE is well established, (Langrish et al., 2005), and the evidence indicates that they upregulate expression of co-stimulatory molecules and pro-inflammatory cytokine production in vitro (McQuillan et al., 2010; Murphy et al., 2010).

The mechanism by which T cells have beneficial or detrimental roles may involve their modulatory effect on microglial activation both through cell-cell contacts and as a consequence of their ability to secrete an array of pro- and anti-
inflammatory cytokines. The results discussed in the previous chapter and evidence from others have established that T cells, specifically Th1 and Th17 cells can induce expression of co-stimulatory molecules on glia and alter the production of pro-inflammatory cytokines, while it was found that Th2 cells exerted no effect on glial activation. It has been established that T cell-induced or Aβ-induced activation of microglia can be enhanced when these two factors are present together. Specifically, the Th1 cell-secreted cytokine IFN-γ has a profound effect on microglial activation in vitro, and it synergises with Aβ to induce microglia-mediated neurotoxicity (Goodwin et al., 1995; Meda et al., 1995; Li et al., 2004). While it inhibits microglial phagocytosis of Aβ (Koenigsknecht-Talboo & Landreth, 2005; Yamamoto et al., 2008). Furthermore, microglia stimulated with IFN-γ can present Aβ to T cells and trigger proliferation of Aβ-specific T cells (Town et al., 2005), while IFN-γ upregulates β-secretase resulting in increased Aβ production (Yamamoto et al., 2007).

Interestingly it has been found that IFN-γ is significantly enhanced in the cerebral cortex of APP transgenic mice (Abbas et al., 2002) and higher levels of IFN-γ have been found in the post-mortem brain tissue of AD patients compared with age-matched controls (Huberman et al., 1994). Together these studies suggest that the interaction of Th1 cells with microglia and Aβ may have a profound effect on the progression of AD, suggesting that perhaps targeting Th1 cells and the secretion of their signature cytokine, IFN-γ, may have beneficial effects.
The aims of this study were to:

• Establish if Th1 and Th17 cells were present in the brains of aged APP/PS1 mice.

• Investigate the effects of Th1 and Th17 cells, and their signature cytokines IFN-γ and IL-17, respectively on microglial activation in mixed glia in the presence or absence of Aβ1-42.

• Investigate the effect of adoptive Aβ-specific T cell transfer on microglial activation in APP/PS1 mice.

• Investigate the effect of Aβ-specific Th1 cell transfer on plaque load and microglial activation and to examine what effect blocking IFN-γ might have on microglial activation and plaque burden in APP/PS1 mice.
6.2. Methods

Male and female APP/PS1, and wildtype littermate control, mice (18 months old) were anaesthetised with sodium pentobarbital (40 μl) and perfused intracardially with sterile ice-cold PBS (20 ml). Brains were harvested for mononuclear cell isolation by the 5 layer percoll method (see section 2.8 for details). Following separation of the cells into layers based on relative densities using percoll gradients, the mononuclear cell layer was transferred into FACS tubes. Cells were washed and prepared for intracellular staining using a cell permeabilisation kit and stimulated with PMA, ionomycin and BFA for 4 hours. Following stimulation, cells were incubated in the presence of anti-CD16/CD32 to block non-specific binding, incubated with appropriate FACS antibodies and fixed to allow analysis using FlowJo software (see section 2.9.2 for details).

In a separate set of experiments, spleens were harvested from C57BL/6 mice (2 months old) and CD4+ T cells were isolated using a MACS and a CD4+ T cell isolation kit. CD4+ T cells were plated in 24-well plates and polarized into Th1 and Th17 cells in the presence of specific polarising cytokines for 4 days (see section 2.7.3.1 for details). On the 4th day of the culture, supernatants were taken for cytokine analysis of IFN-γ and IL-17 concentrations by ELISA (see section 2.12 for details), and cells were harvested for co-culture with mixed glia. Mixed glia were prepared from 1-day old C57BL/6 mice and cultured for 14 days prior to T cell co-culture (see section 2.1.2 for details). Th1/Th17 cell lines were co-cultured with mixed glia at a ratio of 1:2 and, after 1 hour of incubation, cells were stimulated with Aβ1-42 (10 μM) and incubated for 24 hours. Mixed glia were also treated in the presence or absence of IFN-γ (20 ng/ml) or IL-17 (20 ng/ml) (see section 2.7.3.2 for details). Cells were harvested for cell surface marker expression of MHC class II, CD86 and CD40 by flow cytometry (see section 2.9.1 for details).

In a separate series of experiments, Aβ-specific T cells were generated from wildtype mice which received a footpad immunisation of Aβ (75 μg/mouse) and CpG (25 μg/mouse) and, after 21 days, the mice were boosted with Aβ and CpG. Mice were sacrificed 7 days after the booster injection and the spleens were harvested. The cell preparation was restimulated ex vivo with Aβ (25 μg/ml) and appropriate cytokines and/or antibodies depending on the type of T cell line being
developed (see section 2.7.4.1 for details). Following generation of Aβ-specific T cell lines, Aβ-specific Th1 and Th17 cells were injected i.v. into APP/PS1 mice (6-7 months old; 15 x 10^6 cells/mouse; see section 2.7.4.1 for details). After 2 weeks APP/PS1 mice were anaesthetised with sodium pentobarbital (40 µl) and perfused intracardially with ice-cold PBS (20 ml). The brains were rapidly removed, bisected and snap-frozen for later immunohistochemical analysis (see section 2.14 for details).

In another series of experiments, APP/PS1 and wildtype littermates received an i.p. injection of either anti-IFN-γ or β-galactosidase (600 µg/mouse). Twenty four hours later, Th1 cells (15 x 10^6 cells/mouse) were injected (300 µl) into the lateral tail vein of the mice. Anti-IFN-γ or β-galactosidase antibody injections were repeated several days after adoptive Aβ-specific T cell transfer (see section 2.7.4.2 for details). Mice were killed 34 days after adoptive Aβ-specific T cell transfer by cervical dislocation and tissue was taken for later analysis of Aβ concentration by multi-spot ELISA (see section 2.13 for details) and microglial activation and Aβ-plaque load by immunohistochemistry (see section 2.14 for details).
6.3. Results

6.3.1. Th1 and Th17 cell are present in the periphery and infiltrate the brains of APP/PS1 mice.

Flow cytometry was used to investigate the presence of T cells in the brains of wildtype and APP/PS1 mice. The data revealed that there were very few CD3^CD4^ cells in the brain of wildtype mice but a significantly greater number in brain tissue prepared from APP/PS1 mice (**p<0.001; student's t-test; Figure 6.1A and C). Intracellular staining revealed that a proportion of these CD4^ cells stained positively for IFN-γ and for IL-17, but there was no significant difference between the numbers of CD4^ IFN-γ^ cells and CD4^ IL-17^ cells, suggesting that equal numbers of Th1 and Th17 cells were present in the brains of APP/PS1 mice (Figure 6.1B and D). The presence of CD3^CD8^ cells was also assessed and it was found that there was no genotype-related difference in the number of these cells in brain tissue (Figure 6.2A and B), although intracellular staining indicated that a greater proportion of these CD8^ cells stained positively for IFN-γ compared with IL-17 (**p<0.01; student's t-test; Figure 6.2C).

6.3.2. Generation of Th1 and Th17 T cell lines.

To assess that CD4^ T cells were correctly polarised into Th1 and Th17 cell lines, supernatants from the T cell lines were assessed for the production of IFN-γ and IL-17. Figure 6.3A and B demonstrates that cells polarised under Th1-inducing conditions were found to produce high levels of IFN-γ and low concentrations of IL-17. Cells polarised under Th17-inducing conditions produced low concentrations of IFN-γ and high concentrations of IL-17.

6.3.3. Th1 and Th17 cells and their signature cytokines, IFN-γ and IL-17, alter the expression of MHC class II and co-stimulatory molecules on microglia.

T cells were co-cultured with mixed glia in the presence or absence of Aβ1-42. Mixed glia were also incubated with IFN-γ or IL-17 in the presence or
absence of Aβ1-42. To examine the effect of Th1 and Th17 cells and their signature cytokines IFN-γ and IL-17 on microglial activation, the expression of MHC class II and co-stimulatory molecules CD40 and CD86 were assessed by FACS. The data revealed that the percentage of CD11b⁺CD40⁺ cells was not affected by incubating microglia in the presence of Th1 cells or IFN-γ. However Aβ1-42 markedly increased the percentage of CD11b⁺CD40⁺ cells and this was not affected by the presence of Th1 cells or IFN-γ (**p<0.01, ***p<0.001, ANOVA; Figure 6.4A and B).

The percentage of CD11b⁺CD86⁺ cells was significantly increased by incubating microglia in the presence of Th1 cells or IFN-γ (*p<0.05, ***p<0.001, ANOVA; Figure 6.4A and C). The percentage of CD11b⁺CD86⁺ cells was significantly enhanced by incubating microglia in the presence of IFN-γ compared with Th1 cells (**p<0.01, ANOVA; Figure 6.4A and C). However the percentage of CD11b⁺CD86⁺ cells was not altered by incubating microglia in the presence of Aβ1-42.

The percentage of CD11b⁺MHC class II⁺ cells was significantly increased by incubating microglia in the presence of IFN-γ (**p<0.01, ANOVA; Figure 6.5A and B). The percentage of CD11b⁺MHC class II⁺ cells was significantly enhanced by incubating microglia in the presence of IFN-γ compared with Th1 cells (**p<0.01, ANOVA; Figure 6.5A and C). However the percentage of CD11b⁺MHC class II⁺ cells was not altered by incubating microglia in the presence of Th1 cells or Aβ1-42.

The same parameters were investigated following co-culture of mixed glia with Th17 cells and incubation with IL-17 in the presence or absence of Aβ1-42. The data revealed that the percentage of CD11b⁺CD40⁺ cells was not affected by incubating microglia in the presence of Th17 cells or IL-17. However Aβ1-42 markedly increased the percentage of CD11b⁺CD40⁺ cells and this was not affected by the presence of Th17 cells or IL-17 (p<0.05, **p<0.01, ***p<0.001, ANOVA; Figure 6.6A and B).

The percentage of CD11b⁺CD86⁺ cells and CD11b⁺MHC class II⁺ cells was not affected by incubating microglia in the presence of Th17 cells, IL-17 or Aβ1-42 (Figure 6.6A and C and 6.7A and B).
6.3.4. Aβ-specific Th1 and Th17 cells increase microglial activation in the brains of APP/PS1 mice.

Having established, both in this chapter and the previous chapter, that Th1 and Th17 cells increase microglial activation in vitro as measured by the expression of co-stimulatory molecules, the effect of adoptive transfer of Aβ-specific Th1 and Th17 cells was investigated in APP/PS1 mice. The micrographs revealed that CD11b-positive immunoreactivity was low in cortical and hippocampal sections taken from wildtype mice (Figure 6.8A and 6.9A) and there was an apparent increase in CD11b-positive immunoreactivity in cortical and hippocampal sections taken from control APP/PS1 mice (Figure 6.8B and 6.9B). A further increase in CD11b-positive immunoreactivity in cortical and hippocampal sections taken from APP/PS1 mice which received Aβ-specific Th1 and Th17 cells was observed (Figure 6.8C-D and 6.9C-D). Quantitative analysis of the micrographs by Image J revealed that CD11b expression was significantly increased in cortical sections prepared from APP/PS1 mice which received Aβ-specific Th1 cells (*p<0.05, ANOVA; Figure 6.8E); an increase in CD11b expression in cortical sections prepared from APP/PS1 mice which received Aβ-specific Th17 cells was also observed but this did not reach statistical significance (Figure 6.8E). The increase in CD11b expression in hippocampal sections prepared from APP/PS1 mice which received Aβ-specific Th1 cells did not reach statistical significance (Figure 6.9E).

6.3.5. Aβ-specific Th1 cells enhance Aβ load and plaque burden in APP/PS1 mice.

The effect of Aβ-specific Th1 cells on Aβ load and plaque burden in APP/PS1 mice was investigated. The concentration of insoluble Aβ1-38, Aβ1-40 and Aβ1-42 were significantly increased in cortical tissue prepared from APP/PS1 mice compared to wildtype littermates (**p< 0.01, ***p<0.001, ANOVA; Figure 6.10A-C). Aβ1-38, Aβ1-40 and Aβ1-42 concentrations were significantly enhanced in APP/PS1 mice which received Aβ-specific Th1 cells compared with control APP/PS1 mice (‘p<0.01, ‘’’p<0.001, ANOVA; Figure 6.10A-C). The effect that neutralizing IFN-γ might have on Aβ-specific Th1-induced Aβ load was
investigated and the data revealed that the Aβ-specific Th1-induced increase in Aβ1-38, Aβ1-40 and Aβ1-42 concentrations in APP/PS1 mice was attenuated in APP/PS1 mice that received Aβ-specific Th1 cells and anti-IFN-γ antibody ($$p<0.05$$, $$$p<0.001$$, ANOVA; Figure 6.10A-C). The effect of Aβ-specific Th1 cells and neutralizing IFN-γ on plaque burden in APP/PS1 mice was also investigated. There was a significant increase in positively-stained Aβ-plaques in control APP/PS1 sections compared with wildtype sections (**p<0.01, ANOVA; Figure 6.11A) but there was no significant increase in the number of Aβ-plaques in APP/PS1 mice which received Aβ-specific Th1 cells compared with control APP/PS1 mice (Figure 6.11A). However further analysis revealed a significant increase in the number of positively-stained Aβ-plaques in APP/PS1 mice which received Aβ-specific Th1 cells compared with control APP/PS1 mice (**p<0.01, student’s t test; Figure 6.11B). The data revealed that this Aβ-specific Th1-induced increase in Aβ-plaque load was significantly attenuated in APP/PS1 mice which received Aβ-specific Th1 cells together with anti-IFN-γ treatment (’p<0.05, ANOVA; Figure 6.11A).

6.3.6. Aβ-specific Th1 cells enhance microglial activation in APP/PS1 mice.

Microglial activation in cortical and hippocampal sections was assessed following adoptive Aβ-specific Th1 cell transfer and IFN-γ neutralization in APP/PS1 mice. The micrographs revealed that CD11b-positive immunoreactivity was low in cortical and hippocampal sections prepared from wildtype mice and there was an apparent increase in sections prepared from all APP/PS1 groups (Figure 6.12A-E and 6.13A-E). There was an apparent increase in CD11b immunoreactivity in cortical and hippocampal sections prepared from APP/PS1 mice which received Aβ-specific Th1 cells compared with sections prepared from control APP/PS1 mice (Figure 6.12C and 6.13C) and an attenuation of the Aβ-specific Th1-induced CD11b-positive immunoreactivity in sections prepared from APP/PS1 mice that received Aβ-specific Th1 cells and anti-IFN-γ treatment (Figure 6.12D-E and 6.13D-E). Quantitative analysis of the micrographs by Image J revealed that CD11b expression was not significantly increased in cortical sections prepared from any of the APP/PS1 groups (Figure 6.12F).
Quantitative analysis of hippocampal sections revealed that CD11b expression was significantly increased in APP/PS1 mice which received Aβ-specific Th1 cells (*p<0.05, ANOVA; Figure 6.13F). The Th1-induced increase in CD11b expression was significantly attenuated in APP/PS1 mice that received Aβ-specific Th1 cells and anti-IFN-γ treatment (*p<0.05, ANOVA; Figure 6.13F).

6.3.7. Co-localisation of Aβ and CD11b expression in cortical and hippocampal tissue prepared from APP/PS1 mice.

Having established that adoptive transfer of Aβ-specific Th1 cells into APP/PS1 mice increased plaque burden and microglial activation in these animals it was investigated if microglia were co-localised to Aβ-plaques in cortical and hippocampal sections taken from these animals. The micrographs revealed that pan Aβ-positive immunoreactivity was increased in cortical and hippocampal sections prepared from all APP/PS1 groups (Figure 6.14B-E and 6.15B-E). There was an apparent increase in pan Aβ-positive immunoreactivity in sections prepared from APP/PS1 mice which received Aβ-specific Th1 cells and an apparent attenuation in sections prepared from APP/PS1 mice that received Aβ-specific Th1 cells and anti-IFN-γ treatment and anti-IFN-γ treatment (Figure 6.14C-E and 6.15C-E). CD11b-positive immunoreactivity was found to be co-localised with Aβ-positive immunoreactivity in all APP/PS1 groups (Figure 6.14B-E and 6.15B-E).
Figure 6.1. CD3^CD4^ cells infiltrate the brains of wildtype and APP/PS1 mice.

Mononuclear cells were isolated from the brains of APP/PS1 and wildtype littermate control mice and cells were surface stained with antibodies specific for CD3, CD4 and intracellularly for IFN-γ and IL-17 and FACS was performed. (A) Representative FACS plots of CD4^ cells in brains of wildtype and APP/PS1 mice. There was a significant increase in the percentage of CD3^CD4^ cells in brains of APP/PS1 mice compared to wildtype mice (C) (**p<0.001, student’s t-test). (B) Representative FACS plots of CD4^IFN-γ^ and CD4^IL-17^ cells in brains of APP/PS1 mice. CD4^ cells stained equally for both IFN-γ and IL-17 with no significant difference between the presence of the two cytokines (D). Values are expressed as means ± SEM (n=4).

(C) ***p=0.0008; 2.723 ± 0.09733; n=4 versus 26.48 ± 3.857; n=4
Figure 6.2. CD3^CD8^ cells infiltrate the brains of wildtype and APP/PS1 mice.

Mononuclear cells were isolated from the brains of APP/PS1 and wildtype littermate control mice and cells were surface stained with antibodies specific for CD8 and intracellularly for IFN-γ and IL-17 and FACS was performed. (A) Representative FACS plots of CD8^+ cells in brains of wildtype and APP/PS1 mice. There was no significant difference in the percentage of CD8^+ cells infiltrating the brains of wildtype and APP/PS1 mice (B). There was a significant increase in the percentage of CD8^+ cells stained positively for IFN-γ compared with the number stained positively for IL-17 in brain tissue prepared from APP/PS1 mice (C) (**p<0.01, student’s t-test). Values are expressed as means ± SEM (n=4).

(C) **p=0.0014; 52.65 ± 8.9111; n=4 versus 2.257 ± 1.053; n=4
Figure 6.3. Generation of Th1 and Th17 cell lines.

Spleens were harvested from C57BL/6 mice and cells were stimulated under Th1 polarising conditions with IL-12 (50 ng/ml) and Th17 polarising conditions with TGF-β (5 ng/ml), IL-1β (25 ng/ml), IL-23 (50 ng/ml) and anti-IFN-γ (5 μg/ml). On the 4th day of the culture supernatants were removed for analysis of IFN-γ and IL-17 concentrations by ELISA. Th1 cells have high IFN-γ, and low IL-17, concentrations (A and B). Th17 cells have low IFN-γ, and high IL-17, concentrations (A and B). Values are expressed as means ± SEM (n=4).
Figure 6.4. Expression of CD40 and CD86 on microglia was assessed following co-culture with Th1 cells and incubation with IFN-γ and Aβ_{1-42}.

Th1 cell lines were co-cultured with mixed glia, prepared from neonatal C57BL/6 mice, for 24 hours. Mixed glia were also treated with IFN-γ (20 ng) and Aβ_{1-42} (10 μM) for 24 hours. Expression of CD86 and CD40 on microglia (CD11b^{+}) was quantified by FACS. (A) Representative FACS plots of CD40 and CD86 expression on CD11b^{+} cells. Aβ_{1-42} significantly increased the expression of CD40 on microglia (B) (**p<0.01, ***p<0.001, ANOVA). Co-culture of mixed glia with Th1 cells and IFN-γ treatment significantly increased the expression of CD86 on microglia (C) (*p<0.05, ***p<0.001, ANOVA). There was a significant increase in the expression of CD86 on microglia treated with IFN-γ compared with Th1 co-culture (C) (*p<0.05, ANOVA). Values are expressed as means ± SEM (n=6).
Figure 6.5. Expression of MHC class II on microglia was assessed following co-culture with Th1 cells and incubation with IFN-γ and Aβ₁₋₄₂.

Th1 cell were generated from C57BL/6 mice and co-cultured with mixed glia treated with IFN-γ (20 ng) and Aβ₁₋₄₂ (10 μM) as described in Figure 6.3 and 6.4. Expression of MHC class II on microglia (CD11b⁺) was quantified by FACS. (A) Representative FACS plots of MHC class II expression on CD11b⁺ cells. Incubation of mixed glia with IFN-γ significantly increased the expression of MHC class II on microglia (B) (**p<0.01, ANOVA). There was a significant increase in the expression of MHC class II on microglia incubated with IFN-γ compared with Th1 co-culture (B) (^p<0.05, ANOVA). Values are expressed as means ± SEM (n=6).
Figure 6.6. Expression of CD40 and CD86 on microglia was assessed following co-culture with Th17 cells and incubation with IL-17 and Aβ1-42.

Th17 cell were generated from C57BL/6 mice as described in Figure 6.3. After 4 days of polarisation Th1 cell lines were co-cultured with mixed glia, prepared from neonatal C57BL/6 mice, for 24 hours. Mixed glia were also treated with IL-17 (20 ng) and Aβ1-42 (10 μM) for 24 hours. Expression of CD86 and CD40 on microglia (CD11b⁺) was quantified by FACS. (A) Representative FACS plots of CD40 and CD86 expression on CD11b⁺ cells. Aβ1-42 significantly increased the expression of CD40 on microglia (B) (*p<0.05, **p<0.01, ***p<0.001, ANOVA). Expression of CD86 on microglia was not significantly altered following co-culture with Th17 cells or incubation with IL-17 or Aβ1-42 (C). Values are expressed as means ± SEM (n=6).
Figure 6.7. Expression of MHC class II on microglia was assessed following co-culture with Th17 cells and incubation with IL-17 and Aβ1-42.

Th17 cell were generated from C57BL/6 mice and co-cultured with mixed glia which were also treated with IL-17 (20 ng) and Aβ1-42 (10 μM) as described in Figure 6.3 and 6.6. Expression of MHC class II on microglia (CD11b⁺) was quantified by FACS. (A) Representative FACS plots of MHC class II expression on CD11b⁺ cells. MHC class II expression on microglia was not significantly altered following co-culture with Th17 cells and incubation with IL-17 or Aβ1-42. Values are expressed as means ± SEM (n=6).
Figure 6.8. CD11b expression is increased in cortical sections prepared from APP/PS1, compared to wildtype, mice.

Aβ-specific Th1 and Th17 cells were generated in C57BL/6 mice and cells were injected i.v. (15 x 10^6 cells/mouse) into APP/PS1 mice. Control animals received serum-free medium alone (300 μl). Animals were sacrificed 2 weeks after adoptive Aβ-specific Th1 and Th17 cell transfer and tissue was taken for CD11b immunohistochemical analysis of microglial activation. CD11b-positive immunoreactivity was low in cortical sections prepared from wildtype mice (A). There was an apparent increase in CD11b-positive immunoreactivity in sections prepared from all APP/PS1 groups (B-D). CD11b expression was analysed using Image J software and the results were expressed as a fold change. CD11b expression was significantly increased in APP/PS1 mice which received Aβ-specific Th1 cells (E) (*p<0.05, ANOVA). Values are expressed as means ± SEM (n=3-5).

(40X magnification)
Figure 6.9. CD11b expression is increased in hippocampal sections prepared from APP/PS1, compared with wildtype, mice.

Aβ-specific Th1 and Th17 cells were generated and adoptively transferred into APP/PS1 mice and sections were stained for CD11b-positive immunoreactivity as described in figure 6.8. CD11b-positive immunoreactivity was low in hippocampal sections prepared from wildtype mice (A). There was an apparent increase in CD11b-positive immunoreactivity in sections prepared from all APP/PS1 groups (B-D). CD11b expression was quantified using Image J software and the results were expressed as a fold change. CD11b expression was not significantly changed between groups (E). Values are expressed as means ± SEM (n=3-5).

(40X magnification)
Figure 6.10. Anti-IFN-γ antibody attenuates the Aβ-specific Th1-induced increase in Aβ1-38, Aβ1-40 and Aβ1-42 concentrations in APP/PS1 mice.

Wildtype and APP/PS1 mice were injected i.p. with anti-IFN-γ antibody or β-galactosidase (600 μg) and after 24 hours mice were injected i.v. with Aβ-specific Th1 cells (15 x 10^6 cells/mouse) as described in Figure 6.8. Anti-IFN-γ or β-galactosidase antibody injections were repeated several days after adoptive Th1 cell transfer. Animals were sacrificed 34 days after adoptive T cell transfer and cortical tissue was taken for analysis of Aβ load by multi-spot ELISA. Aβ1-38, Aβ1-40 and Aβ1-42 concentrations were significantly increased in control APP/PS1 mice (A-C) (**p<0.01, ***p<0.001, ANOVA). Aβ1-38, Aβ1-40 and Aβ1-42 concentrations were significantly enhanced in APP/PS1 mice which received Aβ-specific Th1 cells (+++p<0.01, ++++p<0.001, ANOVA). The Aβ-specific Th1-induced increase in Aβ1-38, Aβ1-40 and Aβ1-42 concentrations was attenuated in APP/PS1 mice that received Aβ-specific Th1 cells and anti-IFN-γ antibody (A-C) (##p<0.05, §§§p<0.001, ANOVA). Values are expressed as means ± SEM (n=4-13).
Figure 6.11. Aβ-specific Th1 cells increase the number of Aβ-plaques in APP/PS1 mice and this effect is attenuated by anti-IFN-γ treatment.

Wildtype and APP/PS1 mice received Aβ-specific Th1 cells and an anti-IFN-γ antibody as described in figure 6.10 and tissue was taken for immunohistochemical analysis of Aβ-plaque load. Sections were stained with congo red to detect compact Aβ-plaques and plaque numbers were counted across the whole section. There was a significant increase in positively stained Aβ-plaques in sections prepared from control APP/PS1 mice (A) (**p<0.01, ANOVA). There was a significant increase in the number of positively stained Aβ-plaques in APP/PS1 mice which received Aβ-specific Th1 cells compared to control APP/PS1 mice (B) (**p<0.01, student’s t test). The Aβ-specific Th1-induced increase in plaque load was significantly attenuated in APP/PS1 mice which received Aβ-specific Th1 and anti-IFN-γ treatment (A) (*p<0.05, ANOVA). The micrograph represents congo red positive staining in a section taken from an APP/PS1 (C). Values are expressed as means ± SEM (n=3-5).

(B) **p=0.0021; 11.25 ± 0.3227; n=4 versus 16.94 ± 1.053; n=4
Figure 6.12. CD11b expression was assessed in cortical sections prepared from wildtype and APP/PS1 mice.

Wildtype and APP/PS1 mice received Aβ-specific Th1 cells and an anti-IFN-γ antibody as described in figure 6.10 and cortical sections were stained for CD11b-positive immunoreactivity as a measure of microglial activation. CD11b-positive immunoreactivity was low in cortical sections prepared from wildtype mice (A). There was an apparent increase in CD11b-positive immunoreactivity in cortical sections prepared from all APP/PS1 groups (B-E). CD11b expression was quantified using Image J software and the results were expressed as a fold change. CD11b expression was not significantly changed between groups (F). Values are expressed as means ± SEM (n=3-7).

(40X magnification)
Figure 6.13. CD11b expression was assessed in hippocampal sections prepared from wildtype and APP/PS1 mice.

Wildtype and APP/PS1 mice received Aβ-specific Th1 cells and an anti-IFN-γ antibody as described in figure 6.10 and hippocampal sections were stained for CD11b-positive immunoreactivity as a measure of microglial activation. CD11b-positive immunoreactivity was low in hippocampal sections prepared from wildtype mice (A). There was an apparent increase in CD11b-positive immunoreactivity in hippocampal sections prepared from all APP/PS1 groups (B-E). CD11b expression was quantified using Image J software and the results were expressed as a fold change. CD11b expression was significantly increased in APP/PS1 mice which received Aβ-specific Th1 cells (F) (*p<0.05, ANOVA). The Th1-induced increase in CD11b expression was significantly attenuated in APP/PS1 mice that received Aβ-specific Th1 and anti-IFN-γ treatment (F) (†p<0.05, ANOVA). Values are expressed as means ± SEM (n=3-7).

(40X magnification)
Figure 6.14. Pan Aβ and CD11b expression was co-localised in cortical sections prepared APP/PS1 mice.

Wildtype and APP/PS1 mice received Aβ-specific Th1 and an anti-IFN-γ antibody as described in figure 6.10 and sections were stained for CD11b- and Aβ-positive immunoreactivity. Pan Aβ-positive immunoreactivity was increased in cortical sections prepared from all APP/PS1 groups compared with sections prepared from wildtype mice (A-E). There was an apparent increase in pan Aβ-positive immunoreactivity in sections prepared from APP/PS1 mice which received Aβ-specific Th1 cells and there was an apparent attenuation in sections prepared from APP/PS1 mice that received Aβ-specific Th1 and anti-IFN-γ treatment and anti-IFN-γ treatment (B-E). CD11b-positive immunoreactivity was found co-localised with pan Aβ-positive immunoreactivity in all APP/PS1 groups (B-E).

(Pan Aβ immunoreactivity (red), CD11b immunoreactivity (green), 40X magnification)
Figure 6.15. Pan Aβ and CD11b expression was co-localised in hippocampal sections prepared APP/PS1 mice.

Wildtype and APP/PS1 mice received Aβ-specific Th1 cells and an anti-IFN-γ antibody as described in figure 6.10 and sections were stained for CD11b- and Aβ-positive immunoreactivity. Pan Aβ-positive immunoreactivity was increased in hippocampal sections prepared from all APP/PS1 groups compared with sections prepared from wildtype mice (A-E). There was an apparent increase in pan Aβ-positive immunoreactivity in sections prepared from APP/PS1 mice which received Aβ-specific Th1 cells and there was an apparent attenuation in sections prepared from APP/PS1 mice that received Aβ-specific Th1 and anti-IFN-γ treatment and anti-IFN-γ treatment (C-E). CD11b-positive immunoreactivity was found co-localised with pan Aβ-positive immunoreactivity in all APP/PS1 groups (B-E).

(Pan Aβ immunoreactivity (red), CD11b immunoreactivity (green), 40X magnification)
6.4. Discussion

In the previous chapter it was established that Th1 and Th17 cells induced a greater effect on glial activation than Th2 cells *in vitro*. Specifically, Aβ-specific Th1 cells induced an exacerbated inflammatory response in glia compared with Aβ-specific Th2 and Th17 cells. The focus of this chapter was to investigate if Th1 and Th17 cells were present in the CNS in a mouse model of AD and to establish if Aβ-specific Th1 and Th17 cells could influence inflammation. A specific aim was to examine if Aβ-specific Th1-induced inflammation was mediated through the secretion of IFN-γ. The data revealed that T cells which stained intracellularly for IFN-γ and IL-17 were present in greater numbers in the brains of APP/PS1 mice compared with wildtype mice. Furthermore it was found that adoptive transfer of Aβ-specific Th1 cells enhanced microglial activation and plaque burden in brains of APP/PS1 mice and this effect was attenuated in animals which received an anti-IFN-γ treatment.

The CNS can only deploy a limited range of immune-defence components and, for this reason, peripheral immune cells can gain entry to the CNS as part of normal immune surveillance (Ransohoff *et al.*, 2003). Indeed it has been shown that activated CNS-irrelevant T cells can invade the CNS and promote alterations in the permeability of the BBB without causing any glial pathology while, encephalitogenic T cells similarly enter the CNS and disrupt the BBB; crucially these cells trigger microglial activation, astrogliosis and demyelination (Smorodchenko *et al.*, 2007). It has already been established that disruption of the BBB and trafficking of autoreactive T cells are early events in MS; both MOG-specific Th1 and Th17 cells have been found to expand in the periphery and cross the BBB in mouse models of MS (Kebir *et al.*, 2007). Evidence suggests that, with ageing and during AD, the BBB becomes leakier which could allow for the infiltration of peripheral immune cells which could in turn contribute to the chronic uncontrolled inflammatory environment observed in AD (Farrall & Wardlaw, 2009). Consistent with previous reports that T cells can infiltrate the brains of mice older than 12 months (Stichel & Luebbert, 2007), here CD3⁺CD4⁺ cells were observed in the brains of 18 month-old wildtype and APP/PS1 mice. The data presented here indicate that there is a significant increase in the number of CD3⁺CD4⁺ cells infiltrating the brains of APP/PS1, compared with wildtype,
mice and that these cells stained positively for IFN-γ and IL-17, suggesting the presence of both Th1 and Th17 cells. These results are consistent with previous studies from this laboratory where increased BBB permeability was observed in APP/PS1 mice compared with aged matched controls (Kelly et al., personal communications). Further, a previous study from Stalder and colleagues (2005) using APP23 transgenic mice injected with green fluorescent protein-positive donor cells, found significant invasion of T cells into the brains of transgenic mice, although they found no indication of T cell-mediated inflammation (Stalder et al., 2005). CD3<sup>+</sup>CD8<sup>+</sup> cells were also observed in the brains of wildtype and APP/PS1 mice, and these cells produced a greater amount of IFN-γ compared with IL-17 in the brains of APP/PS1 mice. Previously it has been observed that CD8<sup>+</sup> cells, along with CD4<sup>+</sup> cells, invade the CNS of transgenic mouse models of AD with the greater portion of these cells being CD4<sup>+</sup> T cells (Fisher et al., 2010). Similarly here it was found that there was a greater portion of CD4<sup>+</sup> T cells versus CD8<sup>+</sup> T cells in the brains of APP/PS1 mice. Consistent with this, it has been reported that there is an increase in CD4<sup>+</sup> T cells while CD8<sup>+</sup> T cells are decreased in AD and interestingly the CD4/CD8-ratio correlated with cognitive deterioration as measured by MMSE (Schindowski et al., 2007).

It has been suggested that the extent of microglial activation and the degree of co-stimulatory molecule expression in experimental and pathological conditions may affect the outcome of microglia-T cell interactions (Aloisi, 2001). In the previous chapter it was established that Th1 and Th17 cells enhanced the expression of co-stimulatory molecules on microglia. The previous study was extended here with an investigation of the role of Th1 and Th17 cell signature cytokines IFN-γ and IL-17. The data revealed that Th1 cells increased expression of CD86 whilst IFN-γ caused a greater increase in the expression of both CD86 and MHC class II compared with Th1 cells, while Th17 cells and IL-17 had no significant effect on the expression of either CD86 or MHC class II. Interestingly, a previous study from this laboratory observed that Th17 cells enhanced the expression of MHC class II and CD86 whereas the cytokine IL-17 had no effect on their expression suggesting that perhaps the Th17 cell cytokine IL-17 may mediate effects on glia via other receptors (McQuillan et al., 2010). No exaggerated Th1- or Th17-induction of the expression of CD86 and MHC class II in the presence of Aβ<sub>1-42</sub> was observed. This result was a little surprising as...
McQuillan and colleagues (2010) observed an increase in the expression of CD86 and MHC class II following co-culture of microglia with Th1 and Th17 cells and Aβ-stimulation. These results are consistent with the previous chapter and highlight that Th1 cells rather than Th17 cells induce the greatest expression of markers microglial activation although here it was also established that IFN-γ had a more profound effect than Th1 cells. These results suggest IFN-γ may be a major mediator of Th1 cell-induced microglial activation. Incubation of glia with Aβ1-42 significantly increased the expression of CD40 on microglia however neither Th1 nor Th17 or their signature cytokines IFN-γ or IL-17 had any additional effect on expression of CD40; previously it has been shown that CD40 leads to the synergistic activation of microglia by Aβ (Townsend et al., 2005). Together the *in vitro* results demonstrated an increase in the expression of CD40 in response to Aβ1-42 which was accompanied by an increase in the expression of CD86 and MHC class II in response to Th1 cells and IFN-γ. This is consistent with previous suggestions that CD40 ligation in the presence of Aβ directs microglia away from a phagocytic phenotype towards an MHC class II presentation phenotype (Townsend et al., 2005). These authors suggest that therapeutic intervention in AD might favour selective promotion of a phagocytic-phenotype while downregulating APC function.

Invasion of T cells into the CNS results in microglial and astrocytic activation (Probert et al., 2000). Here the objective was to evaluate the effect of Aβ-specific Th1 and Th17 cells on microglial activation in APP/PS1 mice and therefore these cells were adoptively transferred into APP/PS1 mice, and CD11b was used as a marker of microglial activation. Previously it has been reported that CD11b expression is increased during neuroinflammation and its expression corresponds with the severity of microglial activation (Roy et al., 2008). The data revealed that microglial activation was low in control APP/PS1 mice and markedly increased in cortical and hippocampal regions in the brains of APP/PS1 mice which received Aβ-specific Th1 cells. In contrast, microglial activation in the brains of APP/PS1 mice which received Aβ-specific Th17 cells was similar to control APP/PS1 mice. These data are consistent with the results *in vitro* in both this chapter and the previous chapter which established that Th1 cells, rather than Th17 cells, have a greater impact on microglial activation. Furthermore, previous unpublished data from this laboratory have shown that adoptively transferring Aβ-
specific Th17 into APP/PS1 mice induces a modest reduction in soluble $A\beta_{1-40}$ concentration (Quillan, 2009). Although both Th1 cells (Kroenke et al., 2008) and Th17 cells (Langrish et al., 2005) are pathogenic in EAE, the in vitro and in vivo data presented here imply that Th1, and not Th17, cells may have a potential pathogenic role in AD.

Activated Th1 cells produce and secrete IFN-$\gamma$ however the presence of IFN-$\gamma$ and its role in AD is still not clear (Li et al., 2004). Earlier it was established that IFN-$\gamma$ has a profound effect on microglial activation in vitro thus one of the primary aims of this study was to investigate if neutralizing IFN-$\gamma$ could attenuate $A\beta$-specific Th1 cell-induced inflammation in APP/PS1 mice. Thus $A\beta$-specific Th1 cells were adoptively transferred into APP/PS1 mice which were also treated with an anti-IFN-$\gamma$ antibody at the same time and $A\beta$-load and plaque burden was investigated. It was observed that $A\beta_{1-38}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ cortical concentrations were significantly increased in APP/PS1 mice and this was further enhanced in APP/PS1 mice which received $A\beta$-specific Th1 cells compared with control APP/PS1 mice. The $A\beta$-specific Th1-induced increase in $A\beta_{1-38}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ concentrations was attenuated in APP/PS1 mice that received $A\beta$-specific Th1 cells and anti-IFN-$\gamma$ antibody. These changes were paralleled by changes in plaque burden. Previously it has been suggested that IFN-$\gamma$ inhibits microglial phagocytosis of $A\beta$ (Koenigsknecht-Talboo & Landreth, 2005; Yamamoto et al., 2008) and that IFN-$\gamma$ upregulates $\beta$-secretase resulting in increased $A\beta$-production (Yamamoto et al., 2007). These results are consistent with a previous study from Yamamoto and colleagues (2007) which demonstrated that IFN-$\gamma$ receptor-deficient Tg2576 transgenic mice had reduced plaque burden which was accompanied by reduced gliosis. These authors postulated that glial activation mediated $A\beta$ deposition and reduced $A\beta$ clearance through IFN-$\gamma$, TNF$\alpha$ and increased BACE1 activity (Yamamoto et al., 2007). Interestingly reduced glial-induced inflammation has been demonstrated in IFN-$\gamma$-deficient mice undergoing EAE (Tran et al., 2000b). Thus it might be hypothesised that Th1 cells contribute to plaque burden through the production of IFN-$\gamma$ which increases microglial activation and inhibits phagocytosis. In contrast, a study from Fisher and colleagues (2010) observed a reduction in $A\beta$-load and plaque number in the brains of transgenic mice injected with $A\beta_{1-42}$, in CFA, which they concluded was due to the occurrence of activated T cells displaying a Th1
phenotype at sites of plaque burden. Monsengo and colleagues (2006) similarly reported reduced plaque burden in APP mice over expressing IFN-\(\gamma\), and enhanced microglia uptake of A\(\beta\) when stimulated with IFN-\(\gamma\). It has been suggested that the discrepancies relating to the effect of IFN-\(\gamma\) on plaque burden might be due to the differential mechanisms by which IFN-\(\gamma\) regulates A\(\beta\) uptake and degradation by microglia (Yamamoto et al., 2007).

There is a wealth of literature documenting microglial activation following a range of insults and it has been shown that A\(\beta\) maintains microglia in an activated state both in vitro and in vivo (Clarke et al., 2007; Lyons et al., 2007). It is well established that microglia can interact with T cells resulting in the production of cytokines, which in turn, cause further activation of both microglia and T cells (Aloisi et al., 2000b; Dasgupta et al., 2005). In parallel with the observed increase in A\(\beta\)-load and plaque burden described here it was found that A\(\beta\)-specific Th1 cells induced a significant increase in microglial activation in hippocampal brain sections, and to a lesser extent cortical sections, of APP/PS1 mice compared with control APP/PS1. This Th1-induced increase in microglial activation was accompanied by a significant attenuation of CD11b-positive microglia in APP/PS1 mice that were simultaneously treated with anti-IFN-\(\gamma\) antibody. Studies from Seguin and colleagues (2003) using human microglia have demonstrated that addition of supernatants from Th1 cells to microglial cultures significantly enhances APC function. This group also showed that incubation of microglia with anti-IFN-\(\gamma\) antibody could attenuate the Th1-induced effects, suggesting that the secretion of IFN-\(\gamma\) by these cells may contribute to the inflammatory response (Seguin et al., 2003). The differences between the cortex and the hippocampus may be as a result of differences between site specific microglia and/or the numbers of microglia present in different brain regions. It has been shown that microglia more densely populate the hippocampus than the cortex in the adult mouse brain (Lawson et al., 1990). It is worth noting that A\(\beta\) enhances microglial activation in vivo without the presence of IFN-\(\gamma\) (Muehlhauser et al., 2001), thus suggesting that in the case of microglial activation, as seen in the cortex, cell-cell contacts alone may be enough to mediate an exacerbated inflammatory response and hence the anti-IFN-\(\gamma\) treatment did not have a significant effect.
Inflammatory processes associated with Aβ-plaques consist of local CNS immune cells including activated microglia and reactive astrocytes. There is extensive literature documenting the presence of microglia immediately adjacent to Aβ-deposits in both the AD brain (McGeer et al., 1993) and transgenic models of AD (Bornemann et al., 2001). Here the data supported the previous findings that microglia were found co-localised to Aβ-positive immunoreactivity in both cortical and hippocampal regions of APP/PS1 mice and there was an apparent increase in Aβ-positive reactivity in APP/PS1 mice which received Aβ-specific Th1 cells and an apparent attenuation in APP/PS1 mice which received anti-IFN-γ antibody. These results are consistent with previous publications in which plaque-associated microglia have been widely observed in a range of transgenic mouse models of AD (Morgan et al., 2005). A previous study from Bornemann and colleagues (2001) using CD11b, as a microglia marker and a different mouse model of AD, observed the presence of activated microglia in association with Aβ-plaques. It has also been demonstrated that CD4^+ T cells and CD11b^+ microglia can be found co-localised with Aβ-plaques especially in the hippocampus (Fisher et al., 2010).

Immunogenic responses to Aβ have received ample attention in past few years as a potential therapeutic avenue for the treatment of AD. The role of T cells in the pathogenesis of AD remains speculative. The data here suggests that Aβ-specific Th1 cells increase microglial activation and potentially influence the development of an APC-phenotype rather than a phagocytic phenotype which would result in a greater inflammatory environment as seen by increased Aβ-load and microglial activation. The data further imply that Aβ-specific Th1-induced microglial activation may, to some extent, be due to the production of IFN-γ and perhaps inhibiting this cytokine may have therapeutic advantages although caution must be exercised as low levels of IFN-γ has been noted to have beneficial effects through mediating neurogenesis. Butovsky and colleagues (2006) showed in vitro that microglia activated by IFN-γ induced neuronal differentiation, while it has been shown in both wildtype, and APP-transgenic, mice that IFN-γ can induce neurogenesis (Baron et al., 2008). The data presented here suggests that Aβ-specific Th1 cells are detrimental and that targeting their ability to induce inflammation may be beneficial in therapeutic approaches to AD.
Chapter 7
General Discussion
Chapter 7

7.1. General Discussion

Given the acknowledged importance of inflammation and microglial activation in the pathogenesis of neurodegenerative disorders such as AD the overall objective of this study was to investigate microglial activation in two models of inflammation, the CD200⁻/⁻ mouse model and the APP/PS1 mouse model, when challenged with immune stimuli. Both of these models have previously been shown to exhibit signs of neuroinflammation. Several investigators have established that CD200⁻/⁻ mice display an inflammatory phenotype under resting and stress-induced conditions. Hoek and colleagues (2000) established that microglia from CD200⁻/⁻ mice were localized in clumps and constitutively expressed markers of microglial activation. Specifically, microglia from CD200⁻/⁻ animals have been found to be strongly CD11b and CD45 positive where normally microglia have low expression of both these molecules (Minas & Liversidge, 2006). Others have suggested that CD200⁻/⁻ mice are more likely to develop an autoimmune disease and display dysregulated macrophage function (Feuer, 2007). The second model used in this study investigated neuroinflammatory changes in a mouse model of AD, the APP/PS1 mouse model. There is extensive literature describing the activation of immune and inflammatory pathways in this mouse model (Morgan et al., 2005; Wyss-Coray, 2006). Previous studies from this laboratory and my own studies have observed increased expression of markers of microglial activation (CD11b, CD40, CD68 and TLR2) and production of pro-inflammatory cytokines (TNFα and IL-1β) in the brains of APP/PS1 mice (data not shown).

CD200 is expressed on neurons and oligodendrocytes (Koning et al., 2009), while microglia express the CD200R (Minas & Liversidge, 2006). More recent research has established that CD200 is expressed on astrocytes in post-mortem tissue from MS patients (Koning et al., 2009) and recent work from this laboratory has observed CD200 expression on astrocytes in vitro (Costello et al., 2011). Considering that the ligand is knocked out in CD200⁻/⁻ mice (Minas & Liversidge, 2006), mixed glial cultures which consist of approximately 85%
astrocytes and 15% microglia were used in the *in vitro* study rather than isolated microglia. The data revealed that mixed glia cultured from CD200<sup>−/−</sup> mice had increased basal expression of CD40 but, surprisingly, none of the other markers of glial activation assessed were increased at basal levels. Interestingly, while the expression of CD11b and CD40 was increased following Pam<sub>3</sub>Csk<sub>4</sub> stimulation in CD200<sup>−/−</sup>, compared with wildtype, mice, expression of CD68 was decreased following Pam<sub>3</sub>Csk<sub>4</sub> treatment. These results are consistent with studies from Kielian and colleagues (2002) who using *S. aureus*, another TLR2 agonist, observed enhanced MHC class II, CD40, CD80 and CD86 on microglia.

The CD200 signalling cascade results in the inhibition of signalling cascades involved in cytokine production such as ERK, JNK and p38 MAPK activation (Mihrshahi *et al.*, 2009). The data found that the expression and concentration of pro-inflammatory cytokines TNFa and IL-6 and chemokines MCP-1 and IP-10 were enhanced in glia cultured from CD200<sup>−/−</sup> mice following Pam<sub>3</sub>Csk<sub>4</sub> stimulation, but there was no difference in cytokine and chemokine expression and release when glia were unstimulated. The increase in cytokines and chemokines may be as a result of TLR stimulation. It has been suggested that TLR stimulation results in a positive feedback loop allowing for the increased expression of TLRs in response to stimuli. It has been shown that TLR2 and TLR1/TLR6 levels are enhanced on microglia following bacterial stimulation; this may act as a mechanism to potentate the antibacterial immune response (Kielian *et al.*, 2002). Consistent with this idea both the expression of TLR2 and TLR1 were increased in response to TLR2 stimulation in glia prepared from wildtype and CD200<sup>−/−</sup> mice.

The data revealed that there was increased expression of TLR2 on microglia cultured from CD200<sup>−/−</sup> mice. This may provide the mechanism by which Pam<sub>3</sub>Csk<sub>4</sub> stimulation had a greater effect in glia cultured from CD200<sup>−/−</sup>, compared with wildtype, mice. Extensive work from Mukhopadhyay and colleagues (2010) has proposed a mechanism by which CD200 acts as an inhibitory receptor on myeloid cells in response to TLR activation. These authors demonstrated that TLR activation induces CD200 to initiate a negative feedback loop to regulate TLR responses and prevent uncontrolled inflammatory responses without compromising pathogen killing. It has been suggested that this negative feedback loop works by limiting NF-κB nuclear translocation (Mukhopadhyay *et al.*).
Thus it is possible that in the present study that the Pam$_3$Csk$_4$-induced increase in TLR2 expression induces an inflammatory environment which is exaggerated in the CD200$^{-/-}$ mice due to the lack of the negative feedback loop that in wildtype animals downregulates the production of pro-inflammatory cytokines and chemokines.

The same parameters were investigated in vivo. Here the data revealed that loss of CD200 resulted in an increase in MHC class II expression. Previous studies from this laboratory have observed that interaction of neuronal CD200 and CD200R on microglia can significantly decrease MHC class II expression (Lyons et al., 2007). Unexpectedly loss of CD200 resulted in a decrease in co-stimulatory molecule expression. However as MHC class II expression is upregulated in virtually all inflammatory conditions, as a marker of activation, it would suggest that these cells are activated. The increase in expression of MHC class II accompanied by a decrease in co-stimulatory molecules could be an indication of how these APCs interact with T cells and suggests that this situation would induce T cell anergy rather than T cell stimulation and proliferation due to the lack of co-stimulatory molecules (Becher et al., 2000b).

The data revealed that the expression of pro-inflammatory cytokines TNFα, IL-1β and IL-6 and chemokines IP-10, MCP-1, RANTES and MIP-1α were exacerbated in cortical and hippocampal tissue prepared from wildtype and CD200$^{-/-}$ mice following Pam$_3$Csk$_4$ stimulation. Interestingly, it was found that the expression of TNFα, IL-1β and MIP-1α were enhanced in hippocampal, but not cortical, tissue following Pam$_3$Csk$_4$ stimulation in CD200$^{-/-}$, compared with wildtype, mice. These results are of particular interest as they suggest that the hippocampus is more susceptible to microglia-induced inflammatory changes following the loss of CD200. Moreover this laboratory has reported that loss of CD200 affects synaptic function; LTP is reduced in CA1 synapses of hippocampal slices prepared from CD200$^{-/-}$, compared with wildtype, mice (Costello et al., 2011). Furthermore studies have shown decreased CD200 expression is coupled with increased microglial activation in the hippocampus of aged and Aβ-treated rats (Lyons et al., 2007; Downer et al., 2010).

Activation of TLR2 in vitro and in vivo consistently increased expression of pro-inflammatory cytokines and chemokines but these changes were not accompanied by upregulation of markers of microglial activation which is
generally reported. This could be because astrocytes, as well as microglia, contribute to the release of these inflammatory mediators which implies that there is a separation between the secretory and APC function of microglia which is uncovered by loss of CD200. It is possible that microglia from CD200^−/− animals are in a state of tonic activation at basal levels and that the manner in which they respond to an insult/stimuli is governed by the CD200-CD200R interaction. It is possible that no enhanced increase in the expression of glial markers was observed in CD200^−/− animals as Pam\textsuperscript{3}Csk\textsubscript{4} primarily induces a cytokine and chemokine response rather than the upregulation of the markers that were examined. A future study should investigate a broader range of cell surface markers.

It has been suggested that chronic TLR2 expression leads to an exaggerated inflammatory response and is part of the mechanisms underlying neurodegeneration (Laflamme et al., 2001). Increased expression of TLR2, TLR4 and the co-receptor CD14 in the brains of mouse models of AD and AD patients (Fassbender et al., 2004; Walter et al., 2007; Letiembre et al., 2009) has initiated extensive research into their role in inflammation. The ability of TLR2 to modulate Aβ-induced microglial activation has been demonstrated in vitro and in vivo (Jana et al., 2008; Liu et al., 2012). The data from the present study corresponds with those of others and showed that expression of TLR2 is upregulated in response to Aβ\textsubscript{1-40}/1-42 and that TLR2 activation was having a profound effect on Aβ-induced glial activation. Pre-treatment of glia with anti-TLR2 attenuated Aβ-induced expression of co-stimulatory molecules CD40 and CD86 and attenuated the production of pro-inflammatory cytokines, TNFα and IL-6, and chemokines IP-10, MCP-1 and RANTES. These pro-inflammatory molecules have been shown to play a role in neurodegeneration and their upregulation has been correlated with neurodegenerative changes (Lynch, 2009). Microglia from the brains of Parkinson’s diseased individuals have been shown to be TNFα and IL-6-positive in areas of the brain associated with dramatic neuronal loss (Sawada et al., 2006) while, IP-10-positive astrocytes are found co-localised to Aβ-plaques (Xia et al., 2000). In the EAE model it has been suggested that RANTES and MCP-1 are the predominant chemokines which recruit peripheral immune cells to produce a nonlethal remitting EAE while other chemokines like MIP-2 produce acute lethal EAE (Tran et al., 2000b).
Interestingly anti-TLR2 treatment had no effect on CD68 expression. This was somewhat surprising since CD68 expression is considered to be a marker of phagocytosis and since it has been reported that TLR2-deficiency enhances Aβ-phagocytosis (Liu et al., 2012). It has been observed that TLR2 forms a heterodimer with TLR1 to recognise Aβ (Liu et al., 2012); in a future study it would be interesting to assess the effect of anti-TLR1 and anti-TLR2 combined on glial activation.

Although the evidence presented here suggests that blocking or removing TLR2 may have beneficial effects by reducing Aβ-induced inflammation on the other hand, a role for stimulating TLR2 activation has been suggested in several studies. For example a role for TLRs, including TLR2, in phagocytosis of Aβ has been described. The stimulation of TLR2, TLR4, and TLR9 in BV-2 cell lines causes significant uptake of Aβ1-42 whereas TLR4-deficient mice exhibit increased Aβ-load (Tahara et al., 2006). Similarly Aβ uptake was observed in primary murine microglia following activation of TLR2 by PGN (Chen et al., 2006). It has been proposed that the efficacy of TLRs in the phagocytosis of Aβ occurs in an age-dependent manner. Studies from Tahara and colleagues (2006), demonstrate an increase in Aβ-load in 14-16 month old APP/PS1 mice lacking TLR4. Whereas Richard and colleagues (2008), found that TLR2-deficient APP/PS1 mice had delayed plaque burden at 6 months of age but not at 9 months of age. These studies suggest that TLR-independent mechanism are more effective in the early stages of AD due to the absence of TLR-mediated inflammation but as the disease progresses the potential lack of TLRs enhances Aβ deposition (Reed-Geaghan et al., 2009). The mechanism through which Aβ mediates microglial activation/phagocytosis is complex. It has been shown that fibrillar Aβ binds to a receptor complex consisting of α6β1 integrin, CD47, CD36 and the scavenger receptor A (Bamberger et al., 2003); importantly, CD36 and CD47 associate with TLRs (Reed-Geaghan et al., 2009). Immune cells employ a wide range of receptors to recognize an array of pathogens and tailor immune responses; it is known that Aβ binds to different receptors and receptor complexes and that, in the absence of one, it will bind to another to initiate a response. Therefore it may be the combination of receptors that Aβ binds to or the types of Aβ present (i.e. oligomeric or fibrillar) that will govern the exact immune response elicited.
There is still much dispute in the literature regarding the role of TLRs as potential targets for modulating the pathogenesis of AD. The data presented here suggest a significant role for TLR2 in mediating Aβ-induced inflammation by activating microglia into an APC phenotype and/or upregulating the production of cytokines and chemokines. The data suggests that blocking TLR2 may have therapeutic advantages, especially give recent evidence that an anti-TLR2 antibody (OPN-301) inhibits detrimental NF-κB-mediated inflammation, cytokine production and leukocyte infiltration in a mouse model of myocardial ischemia reperfusion injury (Arslan et al., 2010), and a humanized version has now entered phase II clinical trials for reperfusion injury post kidney transplantation. However this topic needs further investigation particularly in an in vivo model as the limitations of an in vitro model are widely recognised; it has been suggested that microglia in culture have a more reactive phenotype as they have been released from their normal microenvironment where neuronal factors contribute to their quiescence (Ransohoff & Perry, 2009).

The second model of inflammation in which microglial activation was investigated was the APP/PS1 mouse model of AD. The fact that inflammation plays an important role in AD was suggested over 20 year ago, and since then our understanding of the role of microglia in the diseased brain has grown (Cameron & Landreth, 2010). However despite the extensive research, it still remains unclear why these cells which are supposed to provide protection to the CNS become detrimental during disease progression. The literature is replete with evidence that the production of Aβ and its interaction with microglia results in the production of the characteristic chronic inflammatory environment. As a result research in recent times has focused on Aβ-immunotherapies to aid the removal of Aβ (Schenk et al., 1999; Morgan et al., 2000). However following the abrupt termination of an anti-Aβ vaccination clinical trial due to a small percentage of AD patients developing an unexpected T cell mediated meningoencephalitis (Orgogozo et al., 2003), attention has moved towards the investigation of adaptive immune cell interactions with microglia in the pathogenesis of AD.

In this study the initial findings indicated that Th1 and Th17 cells induced glial cells to adopt an APC phenotype and to secrete cytokines, while Th2 cells were having no effect on glial activation. Of note are the findings that Th1 cells induced expression of CD40 and MHC class II and production of TNFα.
are consistent with observations that Th1 and Th17 cells propagate inflammation whilst Th2 cells have anti-inflammatory effects (Benveniste et al., 2001; McQuillan et al., 2010). A role for CD40 in AD pathology has been demonstrated in previous studies. Tan and colleagues (2002) reported decreased microgliosis and astrocytosis in an APP mouse model lacking CD40 and this was associated with decreased plaque burden. Neurons express CD40 and treatment with CD40L has been shown to increase the amyloidogenic processing of APP suggesting that interaction of CD40 with CD40L on T cells may contribute to the inflammatory environment (Wyss-Coray, 2006). It has been suggested that ligation of CD40 and CD40L directs microglia away from a phagocytic phenotype towards an APC phenotype (Townsend et al., 2005) and ligation enhances the expression of cytokines and chemokines (Grewal & Flavell, 1996).

The present data also shows that Th1 cells increased MHC class II expression on microglia and this was further increased by IFN-γ. IFN-γ is known to be the most potent inducer of MHC class II expression and MHC class II engagement leads to the production of TNFα and IL-1β (Benveniste et al., 2001). Thus it is possible that interaction of Th1 cells with microglia alters their phenotype towards a pro-inflammatory APC form which may contribute to the progression of AD. However it has been noted in a facial axotomy model of motoneuron degeneration that T cell mediated neuroprotection is actually dependent on antigen presentation by microglia (Byram et al., 2004).

The finding that the production of TNFα in microglia was increased following the addition of Th1 cells is a significant observation as studies have suggested that TNFα and IFN-γ work together to increase iNOS expression in microglia and enhance NF-κB nuclear translocation thus enhancing the upregulation of genes involved in inflammation (Mir et al., 2008). Further TNFα has been shown to increase the infiltration of T cells into the brain in an age-dependent manner and this was associated with increased ICAM-1 upregulation (Xu et al., 2010). The observation that both TNFα and CD40 expression were upregulated in glial cultures in response to Th1 cells is interesting since it has been show that neither TNFα or IFN-γ can induce IL-12 secretion, which allows for further T cells stimulation, without CD40 signalling (Becher et al., 2000a).

A possible mechanism by which T cells induce microglial activation has been proposed by Aloisi and colleagues (1999). These authors suggested that
peripherally-activated T cells may cross the BBB and stimulate microglia to adopt an APC phenotype; the mechanism proposed was that IFN-\(\gamma\) induced the upregulation of MHC class II and CD40 which triggered the interaction of MHC class II and CD40 with TCR and CD40L on T cells, resulting in the secretion of cytokines. Since these cytokines enhance T cell activation, a positive feedback loop is created. These authors also postulated that this loop was downregulated by the secretion of PGE\(_2\) (Aloisi et al., 1999). However more recently knocking out the PGE\(_2\) receptor EP2, has been shown to decrease plaque burden in transgenic animals, leading to the proposal that EP2 signalling promotes BACE cleavage in an age-dependent manner (Liang et al., 2005). Additional experiments investigating the modulatory role of PGE\(_2\) in this situation would be interesting.

T cells were found to infiltrate the brains of aged wildtype and APP/PS1 mice with a significantly greater number infiltrating the brains of APP/PS1 mice. On closer analysis these cells were found to secrete IFN-\(\gamma\) and IL-17, suggesting the presence of both Th1 and Th17 cells. The consequences of the infiltration of T cells into the brain, and the outcome, depends on the manner of interactions of T cells with glia and neurons (Carson et al., 2006). It is interesting that both Th1 and Th17 cells have been implicated in the progression of MS in mouse models (Kebir et al., 2007; Murphy et al., 2010), but their role in AD is not as well examined.

The data described here that Th1 cells significantly impacted on both microglial activation and A\(\beta\)-load and burden in the brains of APP/PS1 mice, which is consistent with other authors. Studies from Stalder and colleagues (2005) have suggested a role for peripheral immune cells in cerebral amyloidogenesis and they found that some of these invading peripheral immune cells were targeted to plaques. In this study, it was found that anti-IFN-\(\gamma\) treatment significantly attenuated Th1-induced A\(\beta\)-load and burden. Furthermore it was found that anti-IFN-\(\gamma\) treatment significantly attenuated Th1-induced microglial activation in the hippocampus of APP/PS1 mice. The decrease in plaque burden and A\(\beta\)-load in APP/PS1 mice treated with anti-IFN-\(\gamma\) may be due several possible mechanisms. Firstly it could lead to increased phagocytosis, as \textit{in vitro} studies have established that IFN-\(\gamma\) inhibits effective phagocytosis of A\(\beta\) by microglia, (Koenigsknecht-Talboo & Landreth, 2005). Secondly anti-IFN-\(\gamma\) treatment could reduce microglial activation as IFN-\(\gamma\) has been shown to synergise with pro-inflammatory cytokines, such as TNF\(\alpha\) as described earlier, and with A\(\beta\), to increase microglia activation.
and neurotoxicity (Goodwin et al., 1995; Meda et al., 1995). Finally the treatment could alter Aβ accumulation since IFN-γ has been observed to increase Aβ production in the presence of TNFα through increasing β-secretase activity (Yamamoto et al., 2007). The present results suggest that Aβ accumulation in this model is a consequence of infiltrating Th1 cells which release IFN-γ, activating microglia, and inducing an inflammatory phenotype which triggers APP proteolysis. A future study should include more extensive analysis of microglial activation and focus on evaluating changes in phagocytic ability to make a more conclusive determination of the mechanism by which Aβ load and burden was reduced.

In conclusion, the results of these studies have provided important insights into the potential roles that CD200, TLR2 and T cells have in modulating glial activation. How these factors interact with one and another to enhance inflammation in neurodegeneration such as AD is not yet clear. AD is associated with altered basal microglial and astrocytic activation, these changes are thought to be in part inherent to the glia although changes may also be due to changes in neuronal health such as the expression of CD200 (Carson et al., 2006). Changes in the expression of both CD200 and TLR have been reported in AD. Indeed CD200 and CD200R has been found to be downregulated in AD (Walker et al., 2009), and CD200R is decreased in brains of APP/PS1 mice (unpublished observation). Previous evidence from this laboratory has identified an inverse relationship between neuronal expression of CD200 and microglial activation, and has established that the anti-inflammatory cytokine IL-4, which is secreted by Th2 cells, can increase the expression of CD200 (Lyons et al., 2007). TLR expression has been found to be upregulated in AD and in mouse models of AD (Fassbender et al., 2004; Walter et al., 2007; Letiembre et al., 2009).

Interestingly the chemokine, RANTES, that was induced by Aβ1-40/1-42 and attenuated by anti-TLR2 treatment in the in vitro studies has been shown to preferentially recruit Th1 cells to sites of inflammation (Siveke & Hamann, 1998). The definitive roles of chemokines in AD have not been as well established as those in EAE (Tran et al., 2000b), none the less their presence must raise the question of the possibility that they attract peripheral immune cells to sites of inflammation in the CNS during AD. The data highlight the fact that T cells can enter the brains of APP/PS1 mice and that the interaction of Th1 cells with
microglia may contribute to the progression of the disease through glial activation via cell-cell contacts or the secretion of IFN-γ. As described earlier IFN-γ has a potent effect not only on glial activation and the production of inflammatory mediators but also through its potential to increase Aβ-load and more importantly it has been found that both IFN-γ and IL-12 are increased in the AD brain (Huberman et al., 1994).

Together the data suggest that during AD the loss of CD200 and increased TLR expression, which is a receptor for Aβ, may result in a pro-inflammatory environment that favours the recruitment of peripheral Th1 cells, rather than Th2 cells, into the CNS. In turn these Th1 cells exacerbate inflammation and plaque development through their interaction with glia and the production of IFN-γ. The data suggest that microglia in an APC phenotype have a detrimental impact and that potential therapeutic targets lie in the switching of microglia to a phagocytic phenotype. Additional experiments are required to unveil the precise mechanisms by which T cells and TLRs, possibly in a joint manner, induce a phenotypic state resulting in a disadvantageous inflammatory environment, and question the role played by different glial phenotypic states in neurodegeneration.
Chapter 8

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Morgan D, Gordon MN, Tan J, Wilcock D & Rojiani AM. (2005). Dynamic complexity of the microglial activation response in transgenic models of amyloid deposition:

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implications for Alzheimer therapeutics. *Journal of neuropathology and experimental neurology* 64, 743-753.


Mosmann TR & Sad S. (1996). The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunology today* 17, 138-146.


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Appendix I

List of Publications

Long-term potentiation is impaired in membrane glycoprotein CD200-deficient
mice: a role for Toll-like receptor activation. *Journal of Biological Chemistry*,
286, 34722-32.

Blau CW, Cowley TR, O'Sullivan J, Grehan B, Browne TC, Kelly L, Birch A,
Murphy N, Kelly AM, Kerskens CM, Lynch MA. (2011)
The age-related deficit in LTP is associated with changes in perfusion and blood-

Browne TC, McQuillan K, McManus RM, O'Reilly JA, Mills KHG, Lynch
MA.
IFN-γ production by amyloid β-specific Th1 cells promotes microglial activation
and increases plaque burden in a mouse model of Alzheimer's disease. *The
Journal of Immunology*.
Appendix II

List of Materials

Acetone
Acrodisc syringe filters
Ammonium chloride
Anti-CD3/CD28
Anti-CD11b
Anti-CD16/CD32
Anti-IFN-γ/IL-17
Anti-TLR2
Aβ
BFA
BSA
β-galactosidase
β-mercaptoethanol
cDNA kit
Chromium potassium sulphate
Collagenase D
Confocal secondary antibodies
Congo red
CpG
Cryostat
Cytokines (recombinant)
Cytomation pen
DAB
Dapi mounting medium
Dexamethasone
Dulbecco’s modified eagles medium
DNeasy® blood and tissue kit
DNA ladder
DNase-RNase free water

Sigma-Aldrich
Pall Corporation
Sigma-Aldrich
BD Biosciences
AbD Serotec
BD Biosciences
BD Biosciences
Hycult Biotech
Invitrogen
Sigma-Aldrich
Sigma-Aldrich
R&D Systems
Sigma-Aldrich
Applied Biosystems
Sigma-Aldrich
Roche
Invitrogen
Sigma-Aldrich
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Leica
R&D Systems
Dako
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Vector
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Gibco
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Sigma-Aldrich

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MSD 96-well multi-spot 4G8 Aβ  Meso Scale Discovery
Triple ultra-sensitive assay kit  NanoDrop Technologies Inc
NanoDrop spectrophotometer  Jencons
Nuaire Flow CO2 incubator  Macherey-Nagel
NucleoSpin® RNA extraction kit  Vector
NRS/NGS  Sakura
OCT compound  Invivogen
Pam3Csk4  Calbiochem
Pan Aβ  Gibco
Penicillin/streptomycin  Fisher Scientific
Petri-dish  Sigma-Aldrich
Percoll  Sigma-Aldrich
Phosphate buffered saline  Sigma-Aldrich
PIPS  Fisher Scientific
Plates (6/24 well)  Sigma-Aldrich
PMA  Sigma-Aldrich
Polytron homogeniser  Kinematica
Primers for genotyping  MWG Biotech
RA1 buffer  Macherey-Nagel
Reaction buffer (10X)  Promega
Scalpel  Swann-Morton
Sodium Hydroxide  Sigma-Aldrich
Sodium chloride  Sigma-Aldrich
Sulphuric acid  Lennox
Taq polymerase  Sigma-Aldrich
Taqman® gene expression assays  Applied Biosystems
Thioflavin T  Sigma-Aldrich
Tris-HCl  Sigma-Aldrich
Trypan Blue  Sigma-Aldrich
Trypsin-EDTA  Invitrogen
Tubes (1.5 ml)  Sarstedt
Tween-20  Sigma-Aldrich
Vectorstain Elite ABC reagent  Vector

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Appendix III

List of Solutions

**Calcium Solution**
2.94g CaCl$_2$ in 20 ml dH$_2$O

**Cell culture media**
Dulbecco’s Modified Eagles Medium (DMEM) (GIBCO, UK) was supplemented with 10% heat activated (56°C for 60 minutes) foetal bovine serum (FBS), 100 μg/ml penicillin/streptomycin.

**Congo red staining solution**
2.5 g Congo red
500 ml Saturated sodium chloride solution
Stir overnight before use

**ELISA wash buffer**
500 ml 20X PBS
9.5 L dH$_2$O
5 ml Tween 20

**ELISA stopping solution (1 M)**
26.6 ml 18.8M (H$_2$SO$_4$)
473.4 ml dH$_2$O

**ELISA substrate solution**
3,3',5,5'-Tetramethylbenzidine (TMB)

**FACS buffer**
500 ml PBS
10 ml FBS
Glycine stock solution (100 mM)
7.51 g Glycine
1 L dH₂O
pH 8.5

Krebs Solution
3.975 g NaCl
0.095 g KCl
0.08 g KH₂PO₄
0.135 g MgSO₄
0.67 g NaHCO₃
0.9 g Glucose
Make up to 500 ml with dH₂O and pH 7.3

Krebs Calcium solution
For short term use or storage CaCl₂ is added to Krebs buffer (1:500 dilution).
For long term storage 10% DMSO is added to the above Krebs/ CaCl₂ buffer.

PHEM Buffer
18.14 g PIPES
6.5 g HEPES
3.8 g EGTA
0.99 g MgSO₄

Saturated sodium chloride solution
50 g Sodium chloride (NaCl)
500 ml 80% ethanol
Stir overnight before use.

Thioflavin T stock solution (2 mM)
500 ml dH₂O
0.32 g ThT
Filter before use.
Appendix IV

List of Company Addresses

Alexis
Axxora (UK) Ltd.
P.O. Box 6757
Bingham
Nottingham NG13 8LS
UK

AbD Serotec
Medical Supply Ltd.,
Damastown
Mulhuddart
Dublin 15
Ireland

Applied Biosystems
Applied Biosystems
Frankfurter Str. 129b
64293 Darmstadt
Germany

BD Biosciences
BD Pharmingen
10975 Torreyana Road
San Diego
CA 92121
US

Beckman Coulter
Beckman Coulter Inc.
4300 N Harbor Boulevard
PO Box 3100
Fullerton
CA 92834
US

~ 253 ~
Biologend
Medical Supply Ltd.,
Damastown
Mulhuddart
Dublin 15
Ireland

Bioimaging Systems
UVP
2066 W 11th Street
Upland
CA 91786
US

Biosciences
Biosciences Ltd.
3 Charlemont Terrace
Crofton Road
Dun Laoghaire
Co Dublin
Ireland

Calbiochem
Merck Biosciences
Boulevard Industrial Park
Padge Road
Beeston
Nottingham
UK

Dako
Alere Ltd
Pepper Road Hazel Grove
Stockport SK7 SBW
UK
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<tr>
<td>Fisher Scientific</td>
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<td>Hycult Biotech</td>
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Invitrogen

Invitrogen Ltd
3 Fountain drive
Linchinnan Drive
Paisley PA4 9RF
Scotland
UK

Invivogen

5, Rue Jean Rodier
F-31400
Toulouse
France

Jencons

Jencons
Unit 15
The Birches
Willard Way
Imberhome Industrial Estate
East Grinstead
West Sussex RH19 1XZ
UK

Kinematica

Kinematica AG
Luzernerstrasse 147a
Littau-Lucerne
6014
Switzerland

Labsystems

Labsystems OY
Sorvaajankatu 15
SF-FIN-00811
Helsinki
Finland

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Pierce

Pierce Biotechnology Inc.
3747 N Meridian Road
PO Box 117
Rockford
IL 61105
US

Promega

Promega US
2800 Woods Hollow Road
Madison
WI 53711
US

Qiagen

Qiagen House
Fleming Way
Crawley
West Sussex RH10 9NQ
UK

R & D Systems

R & D Systems
19 Barton Lane
Abingdon Science Park
Abingdon OX143N3
UK

Roche

Roche Ireland Ltd.
Clarecastle
Co.Clare
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The age-related deficit in LTP is associated with changes in perfusion and blood-brain barrier permeability

Christoph W. Blau, Thelma R. Cowley*, Joan O’Sullivan, Belinda Grehan, Tara C. Browne, Laura Kelly, Amy Birch, Niamh Murphy, Aine M. Kelly, Christian M. Kerskens, Marina A. Lynch

Trinity College, Institute of Neuroscience, Trinity College, Dublin, Ireland

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Abstract

In view of the increase in the aging population and the unavoidable parallel increase in the incidence of age-related neurodegenerative diseases, a key challenge in neuroscience is the identification of clinical signatures which change with age and impact on neuronal and cognitive function. Early diagnosis offers the possibility of early therapeutic intervention, thus magnetic resonance imaging (MRI) is potentially a powerful diagnostic tool. We evaluated age-related changes in relaxometry, blood flow, and blood-brain barrier (BBB) permeability in the rat by magnetic resonance imaging and assessed these changes in the context of the age-related decrease in synaptic plasticity. We report that T2 relaxation time was decreased with age; this was coupled with a decrease in gray matter perfusion, suggesting that the observed microglial activation, as identified by increased expression of CD11b, MHCII, and CD68 by immunohistochemistry, flow cytometry, or polymerase chain reaction (PCR), might be a downstream consequence of these changes. Increased permeability of the blood-brain barrier was observed in the perivascular area and the hippocampus of aged, compared with young, rats. Similarly there was an age-related increase in CD45-positive cells by flow cytometry, which are most likely infiltrating macrophages, with a parallel increase in the messenger mRNA expression of chemokines IP-10 and MCP-1. These combined changes may contribute to the deficit in long-term potentiation (LTP) in perforant path-granule cell synapses of aged animals.

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Keywords: Microglial activation; Long-term potentiation (LTP); Age; magnetic resonance imaging (MRI); blood-brain barrier (BBB); Hippocampus; T2 relaxation time; Perfusion imaging

1. Introduction

The age-related deterioration in synaptic function is associated with neuroinflammatory changes which are typified by activation of both microglia and astrocytes (Lynch, 2010). Emerging evidence suggests that these changes may be evaluated noninvasively by magnetic resonance imaging (MRI). Thus local increases in brain T1 relaxation time have been linked with acute astrocyte activation (Anderson et al., 2006; Sibson et al., 2008), while the age-related increase in glial fibrillary acidic protein (GFAP) expression and immunoreactivity was also accompanied by increased T1 relaxation time (Cowley et al., 2010). Decreased T2 relaxation time has been correlated with microglial activation in a rat model of ischemic stroke (Justicia et al., 2008). T2 also decreases with age in humans (Siemonsen et al., 2008) although the evidence indicates that its effect is more profound in advanced age. However, T1 and T2 relaxation times are also affected by several factors including localized edema, altered vascular dynamics, and axonal loss (Fabene et al., 2003; Grohn et al., 2000; van Walderveen et al., 1998).

An age-related decrease in cerebral blood flow has been reported using arterial spin labeling (ASL) whereby the...
mean transit time (MTT) and capillary transit time (CTT) of magnetically labeled blood water can be measured by MRI (Kelly et al., 2010; Mitschelen et al., 2009). Consistent with this, Small and colleagues reported an age-related decrease in brain oxygenation, particularly in the hippocampus (Small et al., 2004). These changes are likely to have a significant effect on synaptic function (Reitz et al., 2009).

The factors which trigger the age-related activation of glia are not known, although a change in the balance between pro- and anti-inflammatory cytokines, as well as dysregulation of antioxidative processes, which are likely to promote activation, have been reported (Nolan et al., 2005; Roy et al., 2008). In addition, glial cell activation by infiltrating peripheral cells has been reported recently (McQuillan et al., 2010; Murphy et al., 2010). The evidence suggests that there is minimal infiltration of peripheral cells in the healthy brain; this is probably primarily due to the fact that expression of chemotactic agents is low, because migration of cells appears to be controlled mainly by expression of chemokines and adhesion molecules and their receptors. However, there is a marked increase in cell infiltration following trauma (Shichita et al., 2009) and in neurodegenerative conditions (Stone et al., 2009; Togo et al., 2002) when the blood-brain barrier (BBB) is breached and when expression of chemotactic agents is increased.

Here we set out to assess whether the age-related increase in microglial activation, as indicated by cell surface markers CD11b, MHCI (OX6), and CD68, was accompanied by changes in T2 relaxation time. Furthermore, we aimed to evaluate whether these changes were associated with evidence of altered perfusion (assessed by ASL) and BBB permeability (using the MRI contrast agent meglumine gadopentate). These measures may provide a further understanding of the factors which contribute to the age-related decrease in hippocampal synaptic plasticity, assessed by the ability to sustain long-term potentiation (LTP). The evidence suggests that perfusion and BBB permeability are altered in aged rats compared with young rats, particularly in the hippocampus, and that these modifications are linked with a decrease in T2 relaxation time. It is proposed that one consequence of these combined age-related changes is a decrease in the ability of aged rats to sustain LTP.

2. Methods

2.1. Animals

Groups of young (3–5 months old) and aged (20–26 months old) male Wistar rats (B&K Universal, Hull, UK) were housed in the BioResources Unit, Trinity College Dublin under standard conditions (2–3 per cage; 12-hour light-dark cycle; 22 °C–23 °C; food and water ad libitum). For some experiments, male Wistar rats of intermediate ages (14–26 months old and 18–20 months old) were also used. Experiments were performed under license and in accordance with ethical guidelines.

2.2. Magnetic resonance imaging

MRI was carried out on a dedicated rodent Bruker Biospec system (Bruker Biospin, Germany) with a 7 Tesla magnet and a 30 cm diameter core, equipped with a 20 cm actively-shielded gradient system. A pair of actively decoupled 12 cm Helmholtz transmitters, and 3 cm surface quadrature receive coils (Bruker Biospin), were used for signal transmission and reception respectively. The machine was connected to a workstation running ParaVision 4.0 software (Bruker Biospin) for data reconstruction and analysis.

Animals were anesthetized with 4% isoflurane (Isoflo; Abbott Animal Health, Maidenhead, UK) in 100% oxygen and maintained with 15.5%–2% isoflurane administered by facemask in either 100% oxygen, or, in the case of the perfusion imaging, a 30%:70% oxygen:nitrogen mix; the depth of anesthesia was monitored using respiratory rate and heart rate and controlled by altering isoflurane concentration. To carry out MRI, animals were placed in a custom-built magnetic resonance (MR)-compatible Perspex rat cradle, positioned using a bite bar and ear supports with the receive coil placed over the skull. Accurate positioning was ensured by acquiring an initial pilot image using a fast gradient echo scan and a single-slice high contrast scan taken at the isocenter of the B0 magnetic field to ensure that the imaging region was centered at bregma −3.60 mm and allow consistent imaging of the cortical and hippocampal regions of interest (ROIs).

2.2. Arterial spin labeling

The ASL sequence consisted of a 5-second preparation interval containing the inversion pulse, followed by a snapshot fast low-angle shot (FLASH) image acquisition (echo time [TE] = 3.5 ms, repetition time [TR] = 11 ms, flip angle [FA] = 30°, bandwidth [BW] = 25 kHz, number of repetitions [NR] = 6, slice thickness = 1.6 mm, number of slices = 1, field of view [FOV] = 3.0 × 3.0 cm, matrix = 128 × 64, acquisition time = 2 minutes, 34 seconds per repetition [Kerskens et al., 1996]). Flow-induced fast adiabatic passage of inflowing inverted arterial spins was performed using a rectangular pulse, inverting arterial spins that then travel to the imaging plane (Dixon et al., 1986; Kelly et al., 2009). The inversion pulse radio frequency power (~22 dB) and offset frequency (~12 kHz) were determined to give optimal perfusion contrast by achieving inversion 20 mm proximal to the imaging plane. Control images with the offset frequency reversed (12 kHz), in which inflowing spins were left undisturbed, were also acquired, in an interleaved fashion. Six repetitions of each image type were acquired for signal averaging, and corresponding pairs of labeled and control images were subtracted to provide perfusion-weighted maps. A theoretical
model to facilitate the quantification of cerebral perfusion with ASL (Kelly et al., 2010) was applied to the data. The model uses a bolus-tracking ASL sequence to provide 11 data points on a signal-time curve of the passage of a 3-second bolus through the imaging slice, and yields values for the MTT, CTT, and relative cerebral blood volume of labeled water (rCBV) in a user-defined region of interest.

2.3. T1 and T2 relaxometry

A rapid acquisition with relaxation enhancement (RARE) and with variable repetition time (RARE-VTR) scan was used to calculate T1 relaxation times (TE = 12.637 ms, TR = 300, 589.116, 942.255, 1396.084, 2031.981, 3103.081, and 8000 ms, FA = 180°, BW = 100 kHz, RARE factor = 4, slice thickness = 1.5 mm, number of slices = 1, FOV = 3.0 × 3.0 cm, matrix = 128 × 128; giving a resolution of 234 × 234 × 1500 μm; and an acquisition time of 8 minutes, 43 seconds). A multislice multiecho (MSME) scan was used to calculate T2 relaxation times (echo spacing = 8.06 ms, number of echoes = 12, TR = 2000 ms, FA = 180°, BW = 100 kHz, slice thickness = 1.5 mm, number of slices = 5, FOV = 3.0 × 3.0 cm, matrix = 128 × 128, acquisition time = 4 minutes, 16 seconds). A fast imaging with steady-state precession (FISP) scan (TE = 1.5 ms, TR = 3.0 ms, FA = 60°, BW = 150 kHz, NR = 60, inversion time = 6 ms, slice thickness = 1.5 mm, number of slices = 5, FOV = 3.0 × 3.0 cm, matrix = 128 × 128, acquisition time = 5 minutes, 20 seconds) was performed from which T1 and T2 relaxation times were derived using an in-built macro in the scan acquisition software (Schmitt et al., 2004). To allow colocalization of relaxation times, the 3 scans shared the same geometry. Additionally, a “slice scout” anatomical RARE scan (TE = 12 ms, TR = 6250 ms, RARE partitions = 8, acquisition time 55 seconds) using the same matrix, field of view, and slice thickness as in the relaxometry experiments. This was used as the template for selection of the ROI from which local mean T1 and T2 relaxation times were calculated.

2.4. Contrast agent MRI

For analysis of BBB permeability, using the contrast agent meglumine gadopentate (Magnevist, HE Clissmann, Dublin, Ireland), tail veins were bilaterally cannulated to allow intravenous administration of the contrast agent and the propofol anesthetic. Animals were allowed to recover from isoflurane anesthesia until spontaneous movement occurred, and then reanesthetized with a single bolus of intravenous propofol (7.5 mg; Rapinovet, MSD Animal Health, Tallaght, Ireland) and, after 6 minutes, continuously infused with propofol, 45 mg kg⁻¹ hour⁻¹ while breathing room air (Griffin et al., 2010). Propofol was used to maintain anesthesia in these experiments because it has been reported that isoflurane opens the BBB (Tétrault et al., 2008).

A fast T1-weighted fast low-angle shot sequence (TE = 2.5 ms, TR = 4.5 ms, FA = 30°, BW = 100 kHz, NR = 60, slice thickness = 1.5 mm, number of slices = 11, FOV = 3.0 × 3.0 cm, matrix = 128 × 128, acquisition time = 15 minutes [Kerskens et al., 1996]) with T2*-crusher gradients was developed to assess blood brain barrier permeability to the contrast agent meglumine gadopentate. The tail vein catheter was flushed with saline, and the cannula and tubing were loaded with the contrast agent. Five repetitions of the scan were acquired prior to delivery of a bolus of contrast agent (0.2 mmol/kg) and scanning continued for 15 minutes. Following scanning, animals were allowed to recover on a heat pad, breathing room air, until they were sufficiently awake to be reintroduced to their home cage.

2.5. MRI analysis

MRI data were analyzed using the data acquisition and analysis software, ParaVision (Bruker Biospin), and scripts written in Interactive Data Language (IDL: Exelis Visual Information Solutions UK, Bracknell, UK) software. For T2 relaxation time analysis, a voxel-wise linear fitting to the logarithm of the echo train decay was performed using a region of interest mask superimposed on a high resolution scan. For T1 relaxation time analysis, a voxelwise least squares curve fit of the signal recovery curve was performed and the same region of interest mask was used. ASL data were analyzed in IDL: the MTT and CTT were quantified using the previously described bolus tracking ASL method (Kelly et al., 2010). Briefly, a noncompartmental model of cerebral perfusion was fitted to the bolus tracking ASL data. The MTT and CTT were calculated from the first and second statistical moments of the signal-time curves, respectively. The rCBV was also calculated from the amplitude of the fitted curve, which is directly proportional to the area under the curve (Kelly et al., 2010). This parameter was therefore used to estimate the rCBV in the ROI. Contrast MRI data were analyzed in IDL: the average signal intensity change in the whole dataset was plotted against time, to determine the iteration at which the bolus first became detectable on the dataset. ROI were drawn on the first image of the dataset, and the progression of the logarithm of the contrast change, compared with baseline levels, was extracted and normalized to the average of the precontrast agent signal intensity in that region. Because the animals received the bolus of contrast agent in situ, the positioning of the pre- and postcontrast scans was identical enabling anatomically identical ROIs to be evaluated over time. A standard stereotaxic atlas (Paxinos and Watson, 2004) was used to ensure that the same ROIs were assessed in young and aged rats and all ROIs were drawn on high resolution scans and transferred to the contrast-enhanced datasets of the same animals.

2.6. Analysis of LTP

Rats were allowed to recover for at least 48 hours after MRI scanning before LTP was assessed in vivo. Animals were anesthetized by intraperitoneal injection of urethane.
(1.5 g/kg) and, if necessary, a top-up dose of urethane (maximum dose 2.5 g/kg) was given to achieve deep anesthesia as indicated by the absence of a pedal reflex. LTP was assessed in perforant path-granule cell synapses as previously described (Loane et al., 2009). Briefly, a bipolar stimulating electrode and a unipolar recording electrode were positioned in the perforant path (4.4 mm lateral to lambda) and in the dorsal cell body region of the dentate gyrus (2.5 mm lateral and 3.9 mm posterior to bregma). Test shocks were delivered at 30-second intervals, and after a stabilization period, responses were recorded for 10 minutes before and 45 minutes after tetanic stimulation (3 trains of stimuli; 250 Hz for 200 ms; 30 seconds intertrain interval).

Rats were killed by decapitation at the end of the period of electrophysiological recording and the brains were rapidly removed. One portion of the brain was stored in Tissue Tek OCT (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) at −80 °C and used for preparation of cryostat sections and subsequent immunohistochemical analysis of CD11b and CD68. The hippocampus and cortex were dissected from the remaining brain tissue and placed in RNALater (Applied Biosystems, Warrington, UK) to ensure that the integrity of the ribonucleic acid (RNA) was maintained for later gene expression analysis.

2.7. Analysis of CD11b, CD68, IP-10, and MCP-1 mRNA

We also assessed age-related changes in microglial activation by evaluating messenger (m) mRNA expression of markers of activation, CD11b and CD68, and the chemokines IP-10 and MCP-1. RNA was extracted from hippocampal tissue using a NucleoSpin RNAII isolation kit (Macherey-Nagel Inc., Düren, Germany) and concentrations were equalized to 1 μg prior to cDNA synthesis using a high capacity cDNA RT Kit (Applied Biosystems), according to the manufacturer’s instructions. Equal concentrations of cDNA were used for RT-polymerase chain reaction (PCR) amplification. Real-time polymerase chain reaction primers were delivered as “Taqman Gene Expression Assays” containing forward and reverse primers, and a FAM-labeled MGB TaqMan probe for each gene (Applied Biosystems, UK) as described previously (Downer et al., 2010). The assay IDs were as follows: CD11b (Rn00709342_m1), CD68 (Rn014995631_m1), IP-10 (Rn00594648_m1), and MCP-1 (Rn00580555_m1). Gene expression was calculated relative to the endogenous control samples (β-actin) to give a relative quantification (RQ) value (2^ΔΔCT, where CT is threshold cycle).

2.8. CD11b and CD68 immunohistochemical staining

To assess CD11b immunohistochemistry by light microscopy brain sections were thawed, kept at room temperature for 30 minutes, fixed using ice-cold alcohol and washed in Tris-buffered saline (TBS, pH 7.5). The sections were incubated in blocking solution (10% normal horse serum (Vector Laboratories Ltd., Peterborough, UK) and 4% bovine serum albumin, (BSA: Sigma-Aldrich Ireland Ltd., Arklow, Ireland), in TBS) for 30 minutes at room temperature and overnight in anti-CD11b primary antibody solution (1/100 antibody in 2% BSA/TBS; AbD Serotec, Kidlington, UK). Negative controls were incubated in 2% BSA in TBS. The sections were washed, incubated in the secondary antibody solution (1/200 in 2% BSA/TBS; IgG antimouse biotinylated; Vector Laboratories Ltd., Peterborough, UK; 1 hour; room temperature). The sections were washed, endogenous peroxidases were blocked by incubating in 0.3% hydrogen peroxide in TBS for 15 minutes and sections were washed again and incubated in ABC reagent (2 drops of A/Ab in 5 mL TBS; Vector Laboratories Ltd.) for 1 hour at room temperature. Sections were washed, incubated in DAB stain (Dako, USA) for 10 minutes and this reaction was stopped by washing the slides with double deionized water. The sections were counterstained by incubating in the presence of methyl green (Sigma-Aldrich Ireland Ltd.) for 10 minutes, dehydrated by submerging the slides in a series of alcohols, cleared in xylene, mounted in DPX (p-xylene-bis-pyridinium bromide) and stored for later examination.

To assess CD68 immunohistochemistry, sections were permeabilized for 5 minutes with Triton X-100 surfactant, (Sigma-Aldrich Ireland Ltd.) and nonspecific interactions were blocked (10% normal goat serum (Vector Laboratories Ltd.), in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl2, pH 6.9; Sigma-Aldrich Ireland Ltd., Arklow, Ireland) containing 1% BSA for 4 hours at room temperature. Sections were incubated overnight in primary antibody solution (1/200 anti-CD68 antibody raised in mouse in 1% normal goat serum in PHEM). Negative controls were incubated in 1% normal goat serum in PHEM alone in the presence of a concentration-matched control anti-IgG antibody. Sections were rinsed 3 times in PHEM, incubated in fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 2 hours at room temperature in the dark and washed. Slides were mounted with DAPI nucleic counterstain enhanced mountant (Vector Laboratories Ltd.) and coverslipped. A thin line of nail polish was applied around the coverslip to further stabilize it, and slides were stored at 4 °C.

2.9. Preparation of cells from young and aged rats and flow cytometric analysis

In one series of experiments, we used flow cytometry to assess age-related changes in expression of MHCII on CD11b+ cells as an indicator of microglial activation and expression of CD45 on CD11b+ cells as an indicator of macrophage infiltration. To do so, young rats (4–6 months; n = 8) and aged rats (24–26 months; n = 6) were transcardially perfused with 200–300 mL saline before tissue was harvested. In this case, brain tissue was cross-chopped and enzymatically digested in 5 mL Krebs buffer containing 10% collagenase D and 1% DNase, in a humidified neu-
was flushed through a 70-μm cell strainer using Dulbecco’s Modified Eagles Medium (DMEM; Gibco, Invitrogen, Ireland) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (Invitrogen). The resultant single cell suspension was centrifuged (300g, 5 minutes, room temperature) and the pellet was resuspended and incubated for 30 minutes in 5 mL complete DMEM containing 0.3 M sucrose and 10% wt/vol PEG-1000 (Fluka, UK). Samples were centrifuged at 300g for 5 minutes at 4°C and resuspended in flow cytometry buffer (phosphate-buffered saline, 137 mM NaCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, 2.7 mM KCl; pH 7.4) containing 2% FCS and 0.1% sodium azide; hereafter referred to as ECM buffer) and washed 3 times to remove DMEM. Cell suspensions were blocked in ECM buffer containing 50% ECS for 15 minutes at 4°C, washed and the number of CD11b+ cells expressing CD45 or OX6 (MHC II) was analyzed using a DakoCytomation (Dublin, Ireland) CyAn ADP flow cytometer and Summit software V4.3.

2.10. Statistical analysis

The data were assessed using Student t tests for independent means, 1-way analysis of variance (ANOVA) for more than 2 groups or where time and age were factors a 2-way ANOVA was used. Post hoc Newmann-Keuls tests were used to compare means. Correlations were performed and Pearson’s r is reported.

3. Results

Analysis of MTT in perfusate-aneurthesized rats revealed that it was significantly increased in the hippocampus and cortex of aged, compared with young, rats (* p < 0.05; ** p < 0.01; Student t test for independent means; Fig. 1a and c). Similarly, analysis of CTT revealed an age-related increase in both brain areas (*** p < 0.001; Student t test for independent means; Fig. 1b and d). These data concur with previous findings indicating a generalized decrease in cerebral perfusion with age (Mitschelen et al., 2009), which has been associated with cognitive decline (Murphy et al., 2008) and with impairment in synaptic plasticity (Lin et al., 2010). To evaluate whether the changes in MTT and CTT described here were associated with any deficits in synaptic plasticity, we assessed LTP in perforant path-granule cell synapses and show that the mean amplitude of the population spike was significantly decreased following the tetanic stimulation in aged, compared with young, rats (p < 0.001; ANOVA; Fig. 2a). Analysis of the amplitude in the last 10 minutes of the experiment confirmed that there was a significant age-related deficit in synaptic plasticity (** p < 0.001; ANOVA; Fig. 2b).

Several factors have been associated with decreased LTP, 1 of which is glial activation which is considered to significantly contribute to the age-related inflammatory changes which have been consistently reported (Lynch, 2010). Here we assessed expression of CD11b mRNA, CD11b immunoreactivity, and the number of CD11b+ OX6+ cells prepared from whole brain of young and aged rats using FCM. CD11b mRNA was significantly higher, and immunoreactivity for CD11b was markedly greater in hippocampal (Fig. 3a and b) and cortical (Fig. 3c and d) tissue prepared from aged, compared with young, rats (* p < 0.05; *** p < 0.001; Student t test for independent means). The number of CD11b+ OX6+ cells, which is indicative of microglial activation, was also significantly greater in dissociated cells prepared from the whole brain of aged, compared with young, rats (*** p < 0.001; Student t test for independent means; Fig. 1e). The changes in CD11b mRNA and CD11b immunoreactivity were mirrored by changes in CD68; CD68 mRNA was significantly greater in tissue prepared from hippocampus (Fig. 3f) and cortex (Fig. 1h) of aged, compared with young rats (* p < 0.05; ** p < 0.01; Student t test for independent means) and an age-related increase in CD68 immunoreactivity was similarly observed in hippocampus (Fig. 1g) and cortex (Fig. 1i).
There was a significant correlation between LTP and hippocampal expression of CD11b mRNA \((r = -0.77, p < 0.01; n = 11; \text{Fig. 3j})\) and CD68 mRNA \((r = -0.75, p < 0.01, n = 11; \text{Fig. 3k})\). A similar, though quantitatively different, age-related change was observed when T2 was assessed by the FISP method; in this case the mean values \((\pm \text{standard error of the mean [SEM]})\) in hippocampus were 69.85 \(\pm\) 1.02 and 63.08 \(\pm\) 2.14 in young and aged animals respectively \((p < 0.05)\) and the mean values in the cortex were 74.37 \(\pm\) 0.78 and 66.40 \(\pm\) 1.03 in young and aged animals respectively \((p < 0.001)\). There was a significant negative correlation between LTP and hippocampal expression of CD11b mRNA \((r = -0.51, p < 0.01; \text{Fig. 4d})\) and cortex \((r = -0.53, p < 0.05; \text{Fig. 4e})\). Furthermore, T2 relaxation in the hippocampus significantly correlated with LTP \((r = 0.78, p < 0.01, n = 11; \text{Fig. 4f})\) and negatively correlated with hippocampal CD11b \((r = -0.72, p < 0.01, n = 11; \text{Fig. 4g})\) and CD68 \((r = -0.82, p < 0.01, n = 11; \text{Fig. 4h})\) mRNA expression. The data presented were obtained from isoflurane-anesthetized rats and similar data were obtained from propofol-anesthetized rats (data not shown).

In contrast to the change in T2 relaxation time, analysis of T1 relaxation time using RARE revealed no significant age-related changes in either hippocampus (Fig. 5a) or cortex (Fig. 5c). However previous data indicated that T1 relaxation time was higher in 20-month-old rats than in younger animals (Cowley et al., 2010) and it has been reported by others that T1 relaxation time peaks at middle age (Aquino et al., 2009). To further evaluate this, data from several cohorts of rats were pooled and the findings support the contention that T1 relaxation time is age-dependent in hippocampus (Fig. 5b) and cortex (Fig. 5d).

Contrast-enhanced imaging was performed under propofol anesthesia because it has been reported that isoflurane opens the BBB (Tétrault et al., 2008). The periventricular region, hippocampus, and cortex of young and aged rats were analyzed for pre- versus postcontrast signal intensity. The images presented (Fig. 6a) are exemplary and suggest that signal intensity was significantly higher in the brain of an aged rat compared with a young rat 2 minutes after injection of contrast agent. Analysis of the periventricular region (Fig. 6b) indicated that there was a marked age-related and time-associated difference in diffusion and clearance of the contrast agent \((p < 0.001; \text{ANOVA}; \text{Fig. 6b})\). The periventricular region sampled comprises the ventricle (and therefore cerebrospinal fluid [CSF]) and also tissue adjacent to the ventricle which did not contain any major blood vessels (established by making reference to the ASL scans). The presence of the CSF explains the greater impact of the contrast agent on the signal in this, compared with hippocampal and cortical, ROIs. The data may be indicative of an age-related increase in permeability in the blood-CSF barrier \((\text{time} \times \text{age interaction}, p < 0.05; \text{repeated measures 2-way ANOVA})\). A significant age-related decrease in clearance of the contrast agent from the hippocampus was observed \((p < 0.001; \text{ANOVA}; \text{Fig. 6c})\) and the interaction between time and age was significant \((p < 0.05; \text{repeated measures 2-way ANOVA})\). It should be noted that an age-related difference in signal enhancement was evident about 2 minutes following intravenous administration of the contrast agent in the periventricular area compared with about 7 minutes after administration in the hippocampus. The delay in contrast enhancement in the hippocampal tissue probably reflects the slower diffusion of contrast agent deeper into the tissue, which is particularly evident in aged animals. A similar gradual increase in signal intensity has also been reported following temporary breakdown of the BBB by claudin 5 knockdown (Campbell et al.,...
Fig. 3. CD11b messenger Ribonucleic Acid (mRNA) was significantly increased in hippocampal (a) and cortical (c) tissue prepared from aged, compared with young, rats (* p < 0.05; *** p < 0.001; Student t test for independent means; n = 6). CD11b immunoreactivity in hippocampal (b) and cortical (d) tissue was also markedly increased with age. (e) The number of CD11b+ cells which also stained positively for OX6 was significantly increased in cells prepared from cortical tissue obtained from aged, compared with young, rats (*** p < 0.001; Student t test for independent means; n = 14). CD68 mRNA was significantly increased in hippocampal (f) and cortical (b) tissue prepared from aged, compared with young, rats (* p < 0.05; ** p < 0.01; Student t test for independent means; n = 6) and similarly CD68 immunoreactivity in hippocampal (g) and cortical (i) tissue were also markedly increased with age. There was a significant negative correlation between LTP and (j) hippocampal expression of CD11b mRNA (r = −0.77; p < 0.01; n = 11) and (k) CD68 mRNA (r = −0.75; p < 0.01; n = 11).

2008); poor junctional localization of claudin-5 with loss of integrity of the BBB in the hippocampus have previously been reported in female middle-aged rats (Bake et al., 2009). In contrast to the change in hippocampus, there was no significant age-related change in signal intensity in the cortex (Fig. 6d). The possibility exists that changes in hippocampal volume may impact on measurement of BBB permeability. In this study, there was no evidence of a change in hippocampal volume (4.18 ± 0.34% and 3.93 ± 0.49% of intracranial volume in young and aged rats respectively; p > 0.1) although a decrease in cortical thickness at the level of the dorsal hippocampus (bregma −3.6 mm) was observed (1.78 ± 0.13 mm and 1.45 ± 0.24 mm in young and aged rats respectively; p < 0.01; Student t test for independent means).

Although the blood-brain barrier is considered primarily to be a barrier to the movement of solutes and molecules, evidence suggests that an increase in its permeability may
Fig. 4. T2 relaxation time was decreased in the brains of aged, compared with young, rats (a) and analysis of the data in hippocampal (b) and cortical (c) tissue revealed a significant age-related decrease (** p < 0.01; Student t test for independent means; n = 6). There was a significant correlation between capillary transit time (CTT) and T2 relaxation in hippocampus (r = 0.51; n = 12; p < 0.01; d) and cortex (r = 0.53; n = 12; p < 0.05; e). T2 relaxation in the hippocampus significantly correlated with (f) long-term potentiation (LTP) (r = 0.78; p < 0.01; n = 11) and (g) hippocampal CD11b (r = —0.72; p < 0.01; n = 11) and (h) CD68 (r = —0.82; p < 0.01; n = 11) mRNA expression.

also permit infiltration of cells (Popescu et al., 2009), although this mainly occurs under the influence of chemotactic molecules. Here we show that expression of IP-10 and MCP-1 which play a significant role in chemotaxis of peripheral cells (Babcock et al., 2003) were increased in brain tissue prepared from aged, compared with young, rats (** p < 0.05; *** p < 0.001; Student t test for independent means; Fig. 7a and b). These changes were accompanied by an increase in the number of CD11b^+ CD45^+ cells (which are probably macrophages [Carson et al., 1998]) in whole
different ages were assessed, it was evident that T1 relaxation time was increased in aged and young rats. However, when data from other studies in which rats of different ages were assessed, it was evident that T1 relaxation time was increased in 18-month-old compared with 24-month-old rats in hippocampus and cortex (p < 0.001; analysis of variance [ANOVA]; n = 12; b and d).

4. Discussion

A key challenge in neuroscience is to understand the mechanisms underlying the age-related neuroinflammatory changes which contribute to the characteristic deficit in neuronal function. The ability to noninvasively detect age-related changes in vivo to which neuroinflammation may be contributing, and to link these changes to well-established ex vivo markers of neuroinflammation has the potential to provide valuable insights into the mechanisms behind age-related cognitive decline. In this study, we set out to evaluate whether the increase in microglial activation and the associated deficit in LTP in aged animals was linked with changes in perfusion or BBB permeability as assessed by MRI. In addition, we also investigated T1 and T2 relaxation times because previous findings suggested that these parameters are altered in several neuroinflammatory conditions and changes in these parameters may reflect changes in glial activation.

The evidence indicates that MTT and CTT were increased in hippocampus and cortex of aged, compared with young, rats and that these changes were accompanied by increased BBB permeability. An increase in the number of CD11b+CD45+ cells were observed in the brain of aged rats and this was associated with upregulation of MCP-1 and IP-10. Interestingly the data show that there was an age-related decrease in T2 relaxation time and this, as previously reported, was accompanied by evidence of microglial activation (Falangola et al., 2005; Justicia et al., 2008; Teipel et al., 2011). Overall the evidence suggests that multiple changes occur in the brain with age which might contribute to the deficit in LTP observed here and elsewhere.

The data demonstrate an age-related increase in both MTT and CTT in hippocampus and cortex, although similar changes were observed in most areas of the brain and also in a whole brain slice (data not shown). This suggests a generalized decrease in cerebral perfusion with age and concurs with the observations that cerebral blood flow is decreased in rat and humans with age (Mitschelen et al., 2009; van Es et al., 2010) and that cerebral blood flow changes in hippocampal subregions are inversely correlated with age in nonhuman primates and rats (Small et al., 2004). The increases in MTT and CTT are probably due to a combination of decreased blood flow and altered perfusion coefficient of the tissue (Kelly et al., 2009). MTT has been shown to correlate with accumulation of lactate following stroke suggesting that it may provide a marker of ischemia (Cvero et al., 2009) and, in this context, it is interesting that there is evidence of oxidative changes in the brain of aged rats (Kelly et al., 2011; O‘Donnell et al., 2000). These changes cannot be attributed to any age-related difference in hippocampal volume because, although a decrease in cortical thickness was observed, we found no evidence of any difference in hippocampal volume in aged, compared with young, rats. However, analysis using a 9.4 Tesla magnet revealed an age-dependent decrease in hippocampal volume of Fisher 344 X Brown Norway hybrid rats (Driscoll et al., 2006), suggesting that there may be strain differences between these rats and the Wistar rats used here, or that the field strength used in this study may not be strong enough to highlight subtle volumetric differences.

Chronic hypoperfusion has been associated with a decrease in cognitive function and neuronal plasticity, specifically LTP (Lin et al., 2010) and similar changes were observed following ischemia which is also associated with decreased perfusion (Li et al., 2010). Here we show that the age-related decrease in MTT and CTT in the hippocampus is associated with a decrease in LTP, which is reported here and elsewhere (Cowley et al., 2010; Kelly et al., 2010), indicating that a relatively modest decrease in cerebral perfusion may impact on synaptic function.

Among the several factors which accompany, and probably contribute to, the age-related decrease in synaptic plasticity is microglial activation (Lynch, 2010), probably because these cells are the main source of inflammatory...
Several factors contribute to the deterioration of synaptic plasticity with age and 1 of these factors appears to be a heightened level of activation of microglia, which may reflect impairment in the homeostatic ability of these cells with age, or an increase in responsiveness to modulatory molecules. The evidence presented here indicates that the age-related decrease in tissue perfusion, together with the increase in BBB permeability may alter the microenvironment in the brain; this, combined with the proposed age-related compromised homeostatic capability of microglia, may be a significant factor in maintaining the neuroinflammatory changes which have been described in the aged brain and which exert a negative impact on synaptic function.

Disclosure statement

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References


Long Term Potentiation Is Impaired in Membrane Glycoprotein CD200-deficient Mice

A ROLE FOR Toll-LIKE RECEPTOR ACTIVATION

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The membrane glycoprotein CD200 is expressed on several cell types, including neurons, whereas expression of its receptor, CD200R, is restricted principally to cells of the myeloid lineage, including microglia. The interaction between CD200 and CD200R maintains microglia and macrophages in a quiescent state; therefore, CD200-deficient mice express an inflammatory phenotype exhibiting increased macrophage or microglial activation in models of arthritis, encephalitis, and uveoretinitis. Here, we report that lipopolysaccharide (LPS) and Pam3CysSerLeu, exerted more profound effects on release of the proinflammatory cytokines, interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNFα), in glia prepared from CD200−/− mice compared with wild type mice. This effect is explained by the loss of CD200 on astrocytes, which modulates microglial activation. Expression of Toll-like receptors 4 and 2 (TLR4 and -2) was increased in glia prepared from CD200−/− mice, and the evidence indicates that microglial activation, assessed by the increased numbers of CD11b+ cells that stained positively for both MHC II and CD40, was enhanced in CD200−/− mice compared with wild type mice. These neuroinflammatory changes were associated with impaired long term potentiation (LTP) in CA1 of hippocampal slices prepared from CD200−/− mice. One possible explanation for this is the increase in TNFα in hippocampal tissue prepared from CD200−/− mice because TNFα application inhibited LTP in CA1. Significantly, LPS and Pam3CysSerLeu3, at concentrations that did not affect LTP in wild type mice, inhibited LTP in slices prepared from CD200−/− mice, probably due to the accompanying increase in TLR2 and TLR4. Thus, the neuroinflammatory changes that result from CD200 deficiency have a negative impact on synaptic plasticity.

CD200 is a type-1 membrane glycoprotein which has been identified as an immunosuppressive molecule, consistent with its expression on cells of the immune system, including dendritic cells, T and B cells, and endothelial and epithelial cells (1). Diverse immunomodulatory roles for CD200 have been reported; these include antigen-specific T cell responses, suppression of regulatory T cell responses, suppression of regulatory T cells (2), cytotoxic T cell-mediated tumor suppression (3), graft survival (4), and apoptosis-associated immune tolerance (5).

In the brain, CD200 is expressed on neurons (6) and oligodendrocytes (7) but not on microglia (8). A recent report has indicated that CD200 is expressed on reactive astrocytes in lesions from post-mortem multiple sclerosis brains (7). Expression of CD200R is mainly restricted to cells of the myeloid lineage and therefore, in the brain, has been identified on microglia (6, 7) but not on neurons (8). The complementary expression of ligand and receptor on neurons and microglia, respectively, suggests that their interaction may play a role in modulating microglial activation, and recent evidence has supported this contention. The LPS-induced increases in expression of cell surface markers of microglial activation and inflammatory cytokine production were inhibited by the addition of neurons, and this attenuating effect of neurons was blocked by an anti-CD200 antibody (8). This finding suggests that interaction of CD200 with its receptor has the capacity to modulate microglial activation.

In CD200-deficient mice, increased microglial and/or macrophage activation has been described in several models of inflammation (e.g. facial nerve transection, experimental autoimmune encephalomyelitis, an animal model of arthritis (9), and experimental autoimmune uveoretinitis (10)). Conversely, administration of a CD200 fusion protein ameliorates the inflammatory changes observed in collagen-induced arthritis (11, 12), whereas the decrease in experimental autoimmune encephalomyelitis-like symptoms in wild-type mice has been attributed to increased expression of CD200 on spinal cord neurons (13).

Reduced expression of CD200 is coupled with increased microglial activation in hippocampus of aged and β-amyloid (Aβ)3-treated rats (8, 14), and synaptic plasticity, specifically long term potentiation (LTP), is impaired when microglial activation is increased (15, 16). Therefore, we predicted that glia prepared from CD200-deficient mice would respond more profoundly to LPS and that this would be coupled with evidence of impaired LTP. The data show that LPS and Pam3CysSerLeu3 exert a greater effect on glia prepared from CD200−/− mice, presumably due to the observed increase in expression of TLR4 and TLR2 on these cells. In addition, LTP was markedly reduced at
CA1 synapses of hippocampal slices prepared from CD200−/−, compared with wild type, mice. LPS and Pam3CSk, further attenuated LTP in slices prepared from CD200−/− mice. The data provide further evidence for an important immunomodulatory role for CD200 and couple the loss of CD200 with a deficit in synaptic function and with increased expression of TLR2 and -4.

**EXPERIMENTAL PROCEDURES**

**Animals**—1-day-old and 2–6-month-old C57BL/6 or CD200−/− mice were used for preparation of glial cultures or for preparation of hippocampal slices, respectively. Tissue from 2–6-month-old mice was also used for analysis of expression of TLR2 and -4. All experiments were performed under license (Department of Health and Children, Ireland) and with ethical approval (BioResources, Trinity College, Dublin) in accordance with local guidelines. Animals were housed under controlled conditions (20–22 °C, food and water ad libitum) and maintained under veterinary supervision.

**Preparation and Treatment of Primary Glial Cultures**—Mixed glial cultures were prepared from 1-day-old C57BL/6 mice or CD200−/− mice as described previously (8). These cultures contained ~70% astrocytes and 30% microglia as assessed by expression of CD11b using FACS. We used mixed glia because CD200 is expressed on astrocytes but not microglia. This means that knocking out CD200 will have no impact on microglia unless they are in culture with astrocytes, and, in this case, the effect can be attributed to the loss of signaling through CD200R. In the context of this study, isolated microglia prepared from wild type and CD200−/− are essentially the same.

In one series of experiments, cells were harvested for flow cytometric analysis to evaluate expression of cell surface markers of microglial activation, for GLAST to identify astrocytes, or for PCR to evaluate expression of TLR2 and -4. In a second series of experiments, cells were incubated in the presence or absence of LPS (100 ng/ml; Alexis Biochemical) or Pam3CSK4 (100 ng/ml; InvivoGen), and, 24 h later, supernatant was collected and assayed for concentration of IL-1α, IL-1β, IL-6, and TNFα.

Purified astrocytes were prepared as described previously, using the shaking method to remove microgila (17), and membranes were isolated using a subcellular protein fractionation kit (Thermo Scientific). Cells were incubated in trypsin-EDTA (1 ml, 15 min, 37 °C), centrifuged (500 × g, 5 min), and washed with ice-cold PBS, resuspended in PBS, and centrifuged (500 × g, 5 min). The pellet was resuspended in ice-cold Cytoplasmic Extraction Buffer containing protease inhibitors (Thermo Scientific), incubated (4 °C, 10 min), and centrifuged (3,000 × g, 5 min); the supernatant provided the cytosolic fraction, whereas the pellet, which contained the membrane fraction, was resuspended in ice-cold Membrane Extraction Buffer containing protease inhibitors (Thermo Scientific), incubated (4 °C, 10 min), and centrifuged (3,000 × g, 5 min). The resultant supernatant provided the membrane fraction.

To prepare microglia, cells were initially seeded onto 25-cm² flasks, and, after 24 h, medium was replaced with cDMEM containing GM-CSF (10 ng/ml) and M-CSF (20 ng/ml). After 10–14 days in culture, non-adherent microglia were harvested by shaking (110 rpm, 2 h, room temperature), tapping, and centrifuging (2,000 rpm, 5 min). The pellet was resuspended in CDMEM, and the microglia were plated onto 24-well plates at a density of 1 × 10⁵ cells/ml and maintained at 37 °C in a 5% CO₂ humidified atmosphere for up to 3 days prior to treatment.

**Flow Cytometry**—Glia cells were trypsinized (0.25% trypsin-EDTA; Sigma) and washed three times in FACS buffer (2%BS, 0.1% Na₃ in PBS). Whole brain tissue was harvested and passed through a cell strainer (70 μm) and centrifuged (17× g, 10 min). The pellet was resuspended in PBS containing collagenase D (1 mg/ml) and DNase I (200 μg/ml), incubated at 37 °C for 30 min, and centrifuged (170 × g, 5 min). Pellets were resuspended in 1.088 g/ml Percoll (9 ml), underlayered with 0.122 g/ml Percoll (5 ml), and overlaid with 1.072 and 1.030 g/ml each Percoll and PBS (9 ml) and centrifuged (1,250 × g, 45 min). The mononuclear cells (between 1.088:1.072 g/ml and between 1.072:1.030 g/ml) were centrifuged, and the pellets were washed. All cells were blocked for 15 min at room temperature in FACS block (Mouse BD Fc Block (BD Pharmingen); 1:500 in FACS buffer). Cells were incubated with PE-Cy5- or allophycocyanin-rat anti-mouse CD11b (BD Biosciences), FITC-rat anti-mouse CD40 (BD Biosciences), PE-anti-mouse MHCI (BD Biosciences), aliphycocyanin-rat anti-mouse CD200 (BD Biosciences), PE-anti-mouse CD200R (Serotec), FITC-rat anti-mouse TLR2 (Cambridge Biosciences), FITC-rat anti-mouse TLR4 (Cambridge Biosciences), PE-Cy7-anti-mouse CD45 (BD Biosciences), and aliphycocyanin-rat anti-mouse GLAST (BD Biosciences). Antibodies were diluted (1:400) in FACS buffer. Immunofluorescence analysis was performed on a DAKO CyaN ADP 7 color flow cytometer (DAKO Cytomation) with Summit version 4.3 software.

**Real-time PCR Analysis of CD11b, TLR2, and TLR4**—Total RNA was extracted from snap-frozen hippocampal tissue and harvested mixed glial cells using a NucleoSpin® RNAII isolation kit (Macherey-Nagel Inc.), and cDNA synthesis was performed on 1 μg of total RNA using a High Capacity cDNA RT kit (Applied Biosystems); the protocols used were according to the manufacturer’s instructions. Real-time PCR was performed as described previously (8) using an ABI Prism 7300 instrument (Applied Biosystems). The primer IDs were as follows: CD11b, Mm01271265_m1; CD40, Mm00441895_m1; TLR2, Mm00442346_m1; TLR4, Mm00445273_m1 (Applied Biosystems). Samples were assayed in duplicate, and gene expression was calculated relative to the endogenous control samples (β-actin) to give a relative quantity value (2−ΔΔCt, where Ct is the threshold cycle).

**Analysis of IL-1β, IL-6, and TNFα**—The concentrations of IL-1β, IL-6, and TNFα were analyzed by ELISA in samples of supernatant obtained from in vitro experiments as described previously (8).

**Analysis of CD200, GFAP, pltBa, IL-1α, IL-1β, and TLR4**—Hippocampal lysate was assessed for expression of IL-1α, IL-1β, and TNFα; glial cell lysate was evaluated for expression of pltBa; and membrane and cytosolic preparations obtained from purified astrocytes were evaluated for expression of CD200 and GFAP using standard Western immunoblotting methods (8, 18). Primary antibodies directed against CD200 (anti-goat; 1:500; Santa Cruz Biotechnology,
Increased TLR Enhances Susceptibility in CD200^-/^- Mice

RESULTS

Loss of CD200 has been associated with evidence of increased inflammatory changes in hippocampal tissue prepared from aged animals as well as LPS- and β- treated animals (8, 21). In this study, the effects of the TLR4 and TLR2 agonists, LPS and PamCsk4, were assessed on cytokine production in mixed glia prepared from wild type and CD200^-/^- mice. The data indicate that incubation of cells prepared from wild type mice in the presence of LPS (100 ng/ml) increased release of the proinflammatory cytokines, IL-1β, IL-6, and TNFa, and the effect was significant in the case of IL-6 and TNFa (**, p < 0.001; ANOVA; Fig. 1, a–c). The effect of LPS was significantly greater in cells prepared from CD200^-/^- mice (+ +, p < 0.001; ANOVA; Fig. 1). Incubation of mixed glia prepared from wild type mice in the presence of PamCsk4 (100 ng/ml) also significantly increased release of inflammatory cytokines (**, p < 0.01; ***, p < 0.001; ANOVA; Fig. 1, d–f). The effect of PamCsk4 was greater in mixed glia prepared from CD200^-/^- mice, and this was statistically significant in the case of IL-6 and TNFa (+ +, p < 0.001; wild type versus CD200^-/^-; ANOVA). Both LPS and PamCsk4 also increased mRNA expression of these inflammatory cytokines, and the effect was greater in cells prepared from CD200^-/^- mice (data not shown). These data indicate that tonic activation by CD200 modulates cytokine release from glia. Analysis of the effect of LPS on cytokine release prepared from purified microglia obtained from wild type and CD200^-/^- mice revealed no genotype-related change for IL-1β (99.64 ± 26.52 pg/ml versus 67.80 ± 8.03 pg/ml for wild type and CD200^-/^- cells, respectively), IL-6 (3,873 ± 171.8 versus 3,875 ± 144.8), and TNFa (1,559 ± 88.31 versus 1,533 ± 204.5). This is consistent with the view that isolated microglia prepared from CD200^-/^- mice are unaffected, whereas when cultured with astrocytes that are deficient in CD200, an activated phenotype is evident.

Using CD11b as a marker of microglia, we show that the number of CD11b^- MHCI^ cells and CD11b^+ CD40^+ cells was increased in a mixed glial population prepared from CD200^-/^- compared with wild type mice (Fig. 2). These data suggest that CD200 contributes to maintenance of microglia (in a mixed glial preparation) in a quiescent state and therefore suggest that CD200 is expressed on astrocytes. To date, its expression on astrocytes has been reported only on reactive astrocytes in lesions from post-mortem brains of individuals with multiple sclerosis (7). Here, flow cytometry was used to evaluate CD200 expression on GLAST^+ cells in a purified culture of astrocytes prepared from wild type and CD200^-/^- mice (Fig. 3, a and b). Although CD200 expression was evident on GLAST^+ cells prepared from wild type mice, expression was absent on GLAST^+ cells prepared from CD200^-/^- mice. To confirm astrocytic expression of CD200, purified astrocytes were used to prepare membrane and cytosolic fractions for analysis by Western immunoblotting. CD200 was evident in...
membrane, but not cytosolic, fractions (Fig. 3c), whereas GFAP expression was, predictably, largely confined to the cytosolic fractions.

A possible explanation for the increase in responsiveness of cells from CD200−/− mice to LPS and Pam₃Csl₄ is the significant increase in expression of both TLR2 and TLR4 mRNA in mixed glia prepared from CD200−/− compared with wild type mice (*, p < 0.05; Student’s t test for independent means; Fig. 4, a and d). Flow cytometric analysis demonstrated that cell surface expression of both receptors was increased on CD11b+ cells obtained from CD200−/− compared with wild type mice, but the increase was significant only in the case of TLR2 (**, p < 0.01; Student’s t test for independent means; Fig. 4, b, c, e, and f). The significant increase in phosphorylated IkBα in cells prepared from CD200−/− compared with wild type mice (*, p < 0.05; Student’s t test for independent means; Fig. 4g) indicates that signaling through TLR is up-regulated in cells prepared from CD200−/− mice.

CD200 deficiency is accompanied by inflammatory changes (9, 10), and, in the brain, microglial activation is coupled with decreased CD200 in brains of aged animals and also in LPS-treated and Aβ-treated animals (8, 21). To investigate this correlation further, we evaluated expression of surface markers of microglial activation on cells prepared from CD200−/− and wild type mice using PCR and flow cytometry and show that CD40 mRNA, but not CD11b mRNA, was significantly increased in tissue prepared from CD200−/− compared with wild type mice (*, p < 0.05; Student’s t test for independent means; Fig. 5, a and b). Analysis by flow cytometry indicated that there was no genotype-related change in CD40+ cells (Fig. 5c), but the percentage of CD11b+ cells that were positive for MHCII and CD40 was significantly increased (**, p < 0.01; Student’s t test for independent means; Fig. 5, d–g).

CD45 has been used as a means of discriminating between macrophages (which express high levels of CD45) and microglial (which express low levels of CD45 (22)). Flow cytometric analysis revealed that the numbers of CD11b+ CD45low cells were significantly increased in hippocampus of CD200−/− compared with wild type mice (***, p < 0.001; Student’s t test for independent means; Fig. 6a) and that CD200R expression (b), CD40 (c), TLR2 (d), and TLR4 (f) on these cells was greater in tissue prepared from CD200−/− compared with wild type mice. The numbers of macrophages in the brain (i.e. CD11b+ CD45high cells) were negligible in CD200−/− and wild type mice. Analysis of expression of TLR in hippocampus revealed
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**Figure 2.** MHCII^+ CD11b^+ and CD40^+ CD11b^+ cells are increased in glia prepared from CD200^-/- mice. Shown is the mean percentage of CD11b^+ cells that also stained positively for MHCII (top panels) and CD40 (bottom panels). Data are presented as target proteins versus side scatter (SSC). The right-hand panels illustrate representative overlays.

**Figure 3.** CD200 is expressed on astrocytes. CD200 expression was observed on GLAST* cells from purified astrocytic cultures obtained from wild type (a) but not CD200^-/- (b) mice. CD200 was observed in membrane, but not cytosolic, fractions prepared from purified astrocytes obtained from wild type mice. GFAP expression was observed in the cytosolic fraction (c).

that both TLR2 and -4 were increased in CD200^-/- compared with wild type mice (*, p < 0.05; **, p < 0.01; Student's t test for independent means; Fig. 6, e and g). These changes indicate that microglial activation occurs in brain tissue of CD200^-/- mice, and therefore the changes in vivo are reflected in vitro, although the increase in expression of TLR2 mRNA in hippocampus is markedly greater than the change observed in cultured cells. Significantly, this was accompanied by a deficit in LTP in CA1 synapses where the response, 60 min following application of TBS, was markedly reduced in slices prepared from CD200^-/- mice (12 slices from seven mice) compared with wild type mice (15 slices from 11 mice; p < 0.001; unpaired Student's t test; Fig. 6b). Although a number of inflammatory cytokines released from activated microglia might exert this effect (17–21), here we show that whereas expression of IL-1α and IL-1β were similar in hippocampal tissue prepared from wild type and CD200^-/- mice (Fig. 7, a and b), TNFα was increased (p < 0.05; Student's t test for independent means; Fig. 7c). As previously demonstrated in hippocampal slices prepared from rats (19, 20, 23), application of TNFα (3 ng/ml) to mouse hippocampal slices significantly impaired LTP relative to vehicle controls (p < 0.05; unpaired Student's t test; three slices from two mice; Fig. 7d).

Because cells prepared from CD200^-/- mice showed increased susceptibility to LPS, we predicted that concentrations of LPS that exerted no effect on LTP in wild type mice may attenuate it in CD200^-/- mice. Application of LPS (20 μg/ml) to hippocampal slices from wild type mice for 60 min prior to TBS inhibited LTP (five slices from five mice) compared with controls (15 slices from 11 mice; p < 0.001; Fig. 8a). In contrast, a lower concentration of LPS (10 μg/ml; 20-min pretreatment), which exerted no effect on LTP in slices prepared from wild type mice (seven slices from six mice; Fig. 8b), significantly decreased LTP in slices from CD200^-/- mice (13 slices from nine mice) relative to control (12 slices from seven mice; p < 0.05; Fig. 8c).

Like LPS, Pam3Csk4 exerted a greater effect on inflammatory markers in cells prepared from CD200^-/- mice, and therefore we predicted that its effect on LTP would be genotype-specific. Application of Pam3Csk4 (20 μg/ml) to hippocampal slices pre-
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**FIGURE 4.** Expression of TLR2 and TLR4 is increased in glia prepared from CD200\(^{--}\) mice. TLR4 mRNA (p) and TLR2 mRNA (d) and the number of CD11b\(^{+}\) cells that stained positively for TLR4 (b and c) and TLR2 (e and f) were increased in glia prepared from CD200\(^{--}\) compared with wild type mice (*, p < 0.05; Student's t test for independent means; n = 5). Mean data from densitometric analysis (g) and a sample immunoblot (h) reveal that phosphorylated IkBa is increased in cells prepared from CD200\(^{--}\) compared with wild type mice (*, p < 0.05; Student's t test for independent means; n = 4 - 6). Error bars, S.E.

DISCUSSION

The loss of CD200 has a significant impact on activation of microglia in response to inflammatory stimuli, probably because of increased expression of TLR4 and TLR2 in vitro and in vivo. Whereas LTP in Schaffer collateral-CAl synapses was markedly impaired in slices prepared from CD200-deficient mice under control conditions, activation of TLR4 and TLR2, by LPS and Pam\(_3\)Csk\(_4\), respectively, exerted a more profound effect on LTP in slices prepared from CD200\(^{--}\) mice. We propose that the increased expression of TLR4 and TLR2 provides a plausible explanation for the increased responsiveness of CD200\(^{--}\) mice to inflammatory stimuli.

LPS and Pam\(_3\)Csk\(_4\) increased the release of proinflammatory cytokines, IL-1\(\beta\), IL-6, and TNF\(\alpha\), from mixed glial cultures, confirming previously described effects of TLR4 and TLR2 (21, 24, 25). Both agonists exerted a greater effect on release of proinflammatory cytokines in mixed glia prepared from CD200\(^{--}\) mice, compared with wild type mice. Thus tonic activation of CD200 receptor by CD200 is required to modulate inflammatory cytokine production. This concurs with data indicating that the interaction of neurons and microglia by means of CD200 receptor engagement by CD200 deceased microglial activation and production of IL-1\(\beta\) (8). In the current study, in which a mixed glial preparation was used, we propose that the modulating effect is a consequence of the interaction between microglia and astrocytes, which we demonstrate express CD200. It is known that CD200 is widely expressed on numerous cell types, although, in the case of astrocytes, expres-
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![Diagram](image)

**FIGURE 5.** Markers of microglial activation are increased in cells prepared from CD200\textsuperscript{\textasciitilde\textasciitilde} mice. a and b, expression of CD40 mRNA, but not CD11b mRNA, was significantly greater in mixed glia prepared from CD200\textsuperscript{\textasciitilde\textasciitilde} compared with wild type mice (*, p < 0.05; Student's t test for independent means; n = 4 - 5). c - f, flow cytometric analysis revealed that the percentage of CD11b\textsuperscript{+} cells was similar in wild type and CD200\textsuperscript{\textasciitilde\textasciitilde} (d), but the percentage of CD11b\textsuperscript{+} cells that also stained positively for CD40 (d and e) and MHCII (f and g) was significantly greater in mixed glia obtained from CD200\textsuperscript{\textasciitilde\textasciitilde} compared with wild type mice (*, p < 0.05; ***, p < 0.001; Student's t test for independent means; n = 4 - 8). Error bars, S.E.

The present findings in glia mirror those observed in peritoneal macrophages; thus, stimulation with LPS and peptidoglycan and also poly(I:C) increased release of TNF\textalpha and IL-6 to a greater extent in macrophages prepared from CD200\textsuperscript{\textasciitilde\textasciitilde} mice compared with wild type mice (26). Similarly, alveolar macrophages prepared from CD200\textsuperscript{\textasciitilde\textasciitilde} mice, when stimulated ex vivo with LPS or IFN\gamma, expressed more MHCII and released more inflammatory cytokines than macrophages from wild type mice (27). It has been known for many years that astrocytes are capable of modulating microglial/macroage function. They have been shown to modulate LPS-induced changes in inducible nitric oxide synthase and NO production (28, 29) and expression of MHCII (30), effects that have been attributed to astrocytic release of soluble factors like transforming growth factor TGF\beta. The present findings uncover another mechanism by which astrocytes can modulate microglial activation.

Several studies have established that responses to insults that induce inflammatory changes are exacerbated in CD200\textsuperscript{\textasciitilde\textasciitilde} mice. Thus, the symptoms and inflammation associated with experimental autoimmune encephalomyelitis, *Toxoplasma* encephalitis, experimental autoimmune uveoretinitis, collagen-induced arthritis, and facial nerve transaction are more profound in CD200-deficient mice (9, 10, 31). In addition, the response to an influenza dose of hemagglutinin was much more severe (inducing some fatalities) in CD200-deficient compared with wild type mice (27). Although it has been shown that CD200R activation by a CD200Fc ameliorates the symptoms associated with these conditions and although CD200R-mediated regulation of macrophages relies on the binding of Dok2 to the PTB binding motif in the cytoplasmic region of CD200R and
the subsequent recruitment and activation of RasGAP (32), the mechanism by which these changes lead to dampening the activation of macrophage/microglia remains to be fully explained. In this study, we show that increased expression of both TLR4 and TLR2 was observed in glia prepared from CD200<sup>−/−</sup> mice, and this may, at least in part, provide an explanation for the susceptibility of CD200<sup>−/−</sup> mice to inflammatory stimuli. Both TLR2 and TLR4 ultimately lead to activation of NFκB, and, in this study, the increased receptor expression in glia prepared from CD200<sup>−/−</sup> mice is coupled with increased expression of phosphorylated IκB,
which is indicative of NFkB activation. These changes clearly provide one possible explanation for the increased responsiveness of these cells to LPS and Pam3CSK4 in the present study and perhaps also in other models.

Loss of CD200 increases expression of markers of microglial activation in mixed glial cultures; CD200 deficiency was associated with enhanced expression of CD40 mRNA but not CD11b mRNA. In parallel, flow cytometry revealed that these markers and also MHCI were increased on CD11b-positive cells prepared from CD200−/− mice. Previous studies have highlighted the importance of the interaction between CD200 and CD200R in maintaining the quiescent state of microglia and have revealed that the age-related and Aβ-induced increases in microglial activation are coupled with decreased CD200 expression on neurons (8, 14, 21). The present observations also concur with the findings that under resting conditions, spinal cord microglia adopt an inflammatory morphology expressing more CD11b (9), and the number of CD45+CD11b+ cells prepared from retina of CD200−/− mice was increased (10).

In the past decade, it has become clear that neuroinflammatory changes, coupled with increased microglial activation, negatively affect synaptic plasticity in aged, LPS-treated, and Aβ-treated rats (15, 33–35). These observations are corroborated in this study, where we directly associate the loss of CD200 with microglial activation and a deficit in LTP. The evidence indicates that slices prepared from CD200−/− mice do not display LTP to the same degree as slices prepared from wild type mice. One possible explanation for this is that TNFα, which is increased in hippocampal tissue prepared from these mice, is released from activated microglia and inhibits LTP. We demonstrate that TNFα inhibits TBS-induced LTP in mouse Schaffer collateral-CAl synapses, which concurs with previous evidence indicating that it exerts a similar effect on tetanus-induced LTP in rats in vitro and in vivo (17, 19, 23).

In addition to the decrease in LTP observed in untreated slices prepared from CD200−/− mice, the data indicate that a subthreshold concentration of LPS or Pam3CSK4, which exerts minimal effects on LTP in wild type mice, markedly impairs LTP in slices prepared from CD200−/− mice. These findings show for the first time that activation of TLR2 leads to inhibition of LTP and further emphasize the protective effect of CD200-CD200R interaction, such that a deficit in CD200 leads to increased susceptibility to inflammatory stimuli. At this
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FIGURE 8. LTP is attenuated by LPS in CD200$$^{--}$$ mice. a, perfusion of LPS (20 $\mu$g/ml) for 60 min prior to TBS (arrow) decreased LTP in slices prepared from wild type mice (five slices from five mice), and the mean percentage EPSP slope in the last 5 min of the experiment was significantly decreased compared with control slices (p < 0.001); 15 slices from 11 mice). b and c, LTP in slices prepared from wild type mice was unaffected by perfusion of 10 $\mu$g/ml LPS for 20 min prior to TBS (b; seven slices from six mice relative to control 15 slices from 11 mice), but LTP was attenuated in slices from CD200$$^{--}$$ mice (c; p < 0.05; 13 slices from nine mice relative to control 12 slices from seven mice). Sample EPSP traces immediately prior to and 60 min following TBS are presented (scale bars, 1 mV/20 ms). Error bars, S.E.

FIGURE 9. LTP is attenuated by Pam$_3$Csk$_4$ in CD200$$^{--}$$ mice. a, perfusion of Pam$_3$Csk$_4$ (20 $\mu$g/ml) for 60 min prior to TBS (arrow) decreased LTP in slices prepared from wild type mice (three slices from three mice), and the mean percentage EPSP slope in the last 5 min of the experiment was significantly decreased compared with control slices (15 slices from 11 mice; p < 0.001). b and c, LTP in slices prepared from wild type mice was unaffected by perfusion of 10 $\mu$g/ml Pam$_3$Csk$_4$ for 20 min prior to TBS (b; four slices from three mice relative to control 15 slices from 11 mice). However, LTP was attenuated in slices from CD200$$^{--}$$ mice following treatment with 10 $\mu$g/ml Pam$_3$Csk$_4$ (c; six slices from five mice relative to control 12 slices from seven mice; p < 0.05). Sample EPSP traces immediately prior to and 60 min following TBS are presented (scale bars, 1 mV/20 ms). Error bars, S.E.

point, it is unclear whether the effects of LPS or Pam$_3$Csk$_4$ on LTP are secondary to changes in glia or are a consequence of a direct effect on neuronal TLR4 and TLR2. In this regard, it is important to note that although some groups have reported neuronal expression of most TLRs both in vitro and in vivo (36, 37), others have been unable to demonstrate expression of TLR2 on neurons (38). The implication of this finding for the present study is that the mechanism underlying the Pam$_3$Csk$_4$-induced depression in LTP may result from its ability to release IL-1$\beta$, IL-6, and TNF$\alpha$ from glia; each of these inflammatory cytokines has been shown to inhibit LTP (17, 39, 40).

Although there is an accumulating body of evidence indicating that CD200 deficiency also exerts a negative effect on LTP. A key factor underlying these changes is increased expression of these receptors. The findings highlight the importance of CD200 as a potential therapeutic target in disorders that are characterized by neuroinflammatory changes, coupled with loss of synaptic function.

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IFN-γ Production by Amyloid β–Specific Th1 Cells Promotes Microglial Activation and Increases Plaque Burden in a Mouse Model of Alzheimer’s Disease

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Alzheimer’s disease (AD) is characterized by the presence of amyloid-β (Aβ)–containing plaques, neurofibrillary tangles, and neuronal loss in the brain. Inflammatory changes, typified by activated microglia, particularly adjacent to Aβ plaques, are also a characteristic of the disease, but it is unclear whether these contribute to the pathogenesis of AD or are a consequence of the progressive neurodegenerative processes. Furthermore, the factors that drive the inflammation and neurodegeneration remain poorly understood. CNS-infiltrating T cells play a pivotal role in the pathogenesis of multiple sclerosis, but their role in the progression of AD is still unclear. In this study, we examined the role of Aβ-specific T cells on Aβ accumulation in transgenic mice that overexpress amyloid precursor protein and presenilin 1 (APP/PS1). We found significant infiltration of T cells in the brains of APP/PS1 mice, and a proportion of these cells secreted IFN-γ or IL-17. Aβ-specific CD4 T cells generated by immunization with Aβ and a TLR agonist and polarized in vitro to Th1-, Th2-, or IL-17-producing CD4+ T cells, were adoptively transferred to APP/PS1 mice at 6 to 7 mo of age. Assessment of animals 5 wk later revealed that Th1 cells, but not Th2 or IL-17-producing CD4+ T cells, increased microglial activation and Aβ deposition, and that these changes were associated with impaired cognitive function. The effects of Th1 cells were attenuated by treatment of the APP/PS1 mice with an anti–IFN-γ Ab. Our study suggests that release of IFN-γ from infiltrating Th1 cells significantly accelerates markers of diseases in an animal model of AD. The Journal of Immunology, 2013, 190: 2241–2251.

A role for inflammation in the pathogenesis of Alzheimer’s disease (AD) is suggested by epidemiological studies that have reported a decreased incidence of AD in patients treated with nonsteroidal anti-inflammatory drugs (1, 2); these findings are supported by evidence of preventative effects of these drugs in animal models of AD (3). Whereas the classical characteristics of AD are the presence of amyloid-β (Aβ) plaques and neurofibrillary tangles, together with selective neuronal loss, there is also evidence of innate immune activation in AD, with activation of microglia, the primary resident immune cell of the CNS. Activated microglia are found in the brain of AD patients with mild to moderate dementia (4) and in a significant proportion of cases with mild cognitive impairment (5). Microglia secrete inflammatory cytokines like IL-1β and TNF-α, which increase activity and expression of secretases (6, 7), contributing to Aβ deposition and the early pathogenic changes in AD (8). Inflammatory cytokines released from activated microglia are known to be potentially cytotoxic, but there is evidence indicating a positive effect of an inflammatory environment on Aβ clearance (9–11). Microglia demonstrate significant plasticity and also adopt other phenotypes that are associated with tissue repair (12). Furthermore, immune cells in the AD brain can have an alternative activated state as well as the classical proinflammatory phenotype (13). Cell-surface expression of MHC class II and costimulatory molecules is enhanced on activated microglia (14, 15), enabling them to act as APC. However, circulating cells, including T cells, are infrequently observed in the normal CNS, although there is a population of peripheral macrophages, distinct from microglia (16–18), and these cells may play an important anti-inflammatory function, perhaps mediated by a change in hypothalamic–pituitary–adrenal axis function (19).

The blood–brain barrier plays a key role in protecting the brain, restricting the entry of pathogens and macromolecules. An intact blood–brain barrier is also important in restricting entry of circulating cells, and increased blood–brain barrier permeability, which is a characteristic of several neurodegenerative conditions including multiple sclerosis, AD, and Parkinson’s disease (20–22), is associated with infiltration of circulating immune cells. Studies in multiple sclerosis and experimental allergic encephalomyelitis (EAE), a mouse model of multiple sclerosis, have shown that T cells, particularly IL-17–producing CD4+ T cells (Th17) cells, infiltrate the brain and spinal cord and are central to the pathogenesis of the disease (23). The role of Th1 cells in CNS inflammation associated with EAE is more controversial, with some studies suggesting that Th1 cells contribute to pathology and...
others suggesting a protective role for IFN-γ through inhibition of Th17 cells. As well as their role in demyelination, the interaction of T cells with microglia contributes to the inflammatory changes observed in EAE (24).

T cells are also present in the brain of patients with AD (25–28), and infiltration may result from increased expression of CXCR2 and MIP-1α on the T cells (29). Although T cells, in particular Th2 or regulatory T cells, can have a protective role in the brain (30, 31), the entry of activated effector T cells, particularly Th1 or Th17 cells, into the brain in which inflammatory changes are ongoing, is likely to escalate the inflammatory cascade. Consistent with this is the finding that Aβ-induced release of inflammatory cytokines from glia was exacerbated by Th1 and Th17 cells (32), and this effect was attenuated by Th2 cells. Immunization with Aβ peptides, formulated with various adjuvants, is being evaluated both in preclinical models and in the clinic as a potential therapy for AD-based on Ab-mediated reduction of Aβ plaque burden (33). However, a proportion of AD patients who received i.v. vaccine containing Aβ peptide formulated with the adjuvant QS21 (AN1792) developed meningoencephalitis (34). It is possible that the generation of certain subtypes of Aβ-specific T cell may contribute to inflammatory pathology in AD.

In this study, we used a transgenic mouse model of AD that overexpresses amyloid precursor protein (APP) with the Swedish mutation and exon-9-deleted presenilin 1 (PS1; APP/PS1 mice) to determine whether Aβ-specific T cell subsets can modulate Aβ burden and affect microglial activation. Aβ-specific effector T cells were generated by immunization with Aβ and CpG, polarized in vitro to Th1, Th2, and Th17 cells, and adoptively transferred to 6- to 7-mo-old APP/PS1 mice. We found that Aβ-specific Th1 cells increased Aβ deposition and microglial activation in APP/PS1 mice and negatively impacted on spatial

**FIGURE 1.** Th1 and Th17 infiltrate the brain of APP/PS1 mice. Mononuclear cells were prepared from the brain of APP/PS1 and WT mice, and cells were surface stained with Abs specific for CD3, CD4, CD8, intracellular IL-17, and IFN-γ, and flow cytometric analysis was performed. Mean frequency (A) and representative dot plots (B) of CD4+ and CD8+ cells in brain of WT and APP/PS1 mice. Mean frequency (C) and representative dot plots (D) of CD4+ cells stained positively for IFN-γ and IL-17 in brain of APP/PS1 mice. (E) Mean frequency of CD8+ cells stained positively for IFN-γ and IL-17 in brain tissue prepared from APP/PS1 mice. **p < 0.01, Student t test for independent means (n = 4), ***p < 0.001, Student t test for independent means. Representative of three experiments.

**FIGURE 2.** Cytokine production by in vitro polarized Aβ-specific T cells. Popliteal lymph nodes harvested from mice immunized with Aβ and CpG were cultured with Aβ in the presence of IL-12 to generate Th1 cells, dexamethasone, IL-4, and anti-IFN-γ to generate Th2 cells, or IL-23 and anti-IFN-γ to generate Th17 cells. IFN-γ, IL-4, and IL-17 concentrations were determined by ELISA on supernatants removed 3 d after stimulation with Ag and APC. Values are expressed as means ± SEM (n = 4); representative of four experiments.
learning. Treatment of mice with anti–IFN-γ Ab ameliorated these changes, suggesting that release of IFN-γ from infiltrating Th1 cells accelerates the pathology in these animals.

Materials and Methods

Animals

APP/PS1 mice and wild-type (WT) littermates (6 to 7 mo old) were obtained from The Jackson Laboratory and subsequently bred in a specific pathogen-free unit in the Bioresources Unit in Trinity College Dublin. GFP mice were a gift from Matthew Campbell, School of Genetics and Microbiology, Trinity College Dublin. Mice used were transgenic animals on a C37/B6J background expressing eGFP cDNA under the control of a chicken β-actin promoter and CMV enhancer. All mice were maintained in controlled conditions (temperature 22 to 23 °C, 12-h light-dark cycle, and food and water ad libitum) under veterinary supervision, and experimentation was carried out under a license granted by the Minister for Health and Children (Ireland) and with the appropriate ethical approval.

Isolation and FACS analysis on mononuclear cell isolation from CNS tissue

APP/PS1 mice and nontransgenic littermates were anesthetized with sodium pentobarbital (40 μl) and perfused intracardially with sterile ice-cold PBS (20 ml). The brain was removed and placed in HBSS (2 ml) containing 3% FBS (HBSS/FBS; Sigma-Aldrich). Tissue was dissociated through a sterile 70-μm nylon mesh filter, washed with HBSS/FBS, and centrifuged at 170 × g for 10 min at room temperature (RT). The supernatant was removed and the remaining pellet resuspended in HBSS/FBS (2 ml) containing collagenase D (1 mg/ml; Roche) and DNase I (10 μg/ml; Sigma-Aldrich) and incubated for 1 h at 37°C. Cells were washed in HBSS/FBS and centrifuged at 1200 rpm for 5 min. Supernatants were removed, and cells were resuspended in 1.088 g/ml Percoll (9 ml; Sigma-Aldrich). This was underlaid with 1.122 g/ml Percoll (5 ml) and overlaid with 1.072 g/ml Percoll (9 ml) followed by 1.030 g/ml Percoll (9 ml) and finally PBS (9 ml). Percoll gradients were centrifuged at 1250 × g for 45 min at 18°C. Mononuclear cells were removed from between the 1.088/1.072 and 1.072/1.030 g/ml interfaces, washed twice in HBSS/FBS, and counted.

Mononuclear cells prepared from CNS tissue were prepared for intracellular staining using a cell permeabilization kit (DakoCytomation). Cells were centrifuged at 1200 rpm for 5 min before stimulation with X-Vivo media (200 μl) containing PMA (10 ng/ml; Sigma-Aldrich), ionomycin (1 μg/ml; Sigma-Aldrich), and brefeldin A (5 μg/ml; Sigma-Aldrich) for 5 h. Following stimulation, cells were centrifuged at 1200 rpm for 5 min and resuspended. Low-affinity IgG receptors (FcγRII) were blocked by incubating cells in FACS buffer (50 μl/sample) containing CD16/CD32 FcγRIII (1:100) for 10 min at RT. Cells were incubated in 50 μl/sample FACS buffer containing the appropriate FACS Abs for 15 min at RT and fixed in IntraStain Reagent A (50 μl/sample; DakoCytomation) for 15 min at RT. Cells were washed twice with FACS buffer and centrifuged at 1200 rpm for 5 min, permeabilized with IntraStain Reagent B (50 μl/sample; DakoCytomation) plus intracellular Abs for 15 min at RT in the dark, washed twice in FACS buffer, and centrifuged at 1200 rpm for 5 min. Immunofluorescence analysis was performed on a DakoCytomation Cyan.

FIGURE 3. Spatial learning is impaired in APP/PS1 mice that received Aβ-specific Th1 cells. Aβ-specific Th1, Th2, and Th17 cells, generated as described in Fig. 2, were injected i.v. into APP/PS1 mice at 6 to 7 mo of age. Two weeks after injection, cognitive function was assessed.

(A) The latency to reach the platform of all groups. (B) Mean latency on day 5 of training. (C) Sample paths for individual mice in each treatment group. (D) The path length taken to reach the platform. (E) The path length taken to reach the platform. (F and G) In the probe test, the percentage of the total time and distance (i.e., path length) each animal spent swimming in the quadrant was measured. *p < 0.05, ANOVA; n ≥ 5; representative of two experiments. Con, Control; Tg, transgenic.
data acquired using Summit software (DakoCytomation), and the results analyzed using FlowJo software (Tree Star).

**Generation of Aβ-specific T cell lines and in vivo transfer**

WT mice were immunized in the footpad with Aβ42 (75 μg/mouse) and CpG (25 μg/mouse) and boosted after 21 d. Mice were sacrificed 7 d later, and the spleens and popliteal lymph nodes were harvested and restimulated with Aβ42 (25 μg/ml) in the presence of IL-12 (10 ng/ml) to generate Th1 cells, dexamethasone (1 × 10^{-8} M), IL-4 (10 ng/ml), and anti-IFN-γ (5 μg/ml) to generate Th2 cells, or IL-23 (10 ng/ml) and anti-IFN-γ (5 μg/ml) to generate Th17 cells. After 4 d, IL-2 (5 ng/ml) was added to the Th1 and Th2 cell preparations, RPMI-1640 culture medium only was added to the Th17 cells cultures, and incubation continued for a further 7 d. Cells were washed and injected i.v. (15 × 10^6 cells/mouse) into 3- to 7-mo-old APP/PS1 mice. Control animals received in 300 μl serum-free medium alone. Behavior analysis was assessed 2 wk after T cell transfer. Samples of supernatant were assessed by ELISA (see below) for IFN-γ, IL-4, IL-10, IL-17, and IL-5 production.

In a separate series of experiments, 6- to 7-mo-old APP/PS1 and WT control mice were injected i.p. with anti-IFN-γ Ab (600 μg) or a control Ab (anti-β-galactosidase: 600 μg; R&D Systems) and after 24 h were injected i.v. with Th1 cells (15 × 10^6 cells/mouse) as described above. Anti-IFN-γ or anti-β-galactosidase Ab injections were repeated 3, 7, 10, 14, 17, 21, 24, 28, and 31 d after T cell transfer. Behavioral analysis was assessed 21 d after T cell transfer.

**Tracking of Aβ-specific Th1 cells into the brain**

Aβ-specific Th1 were generated from GFP mice immunized with Aβ42 and CpG, restimulated in vitro with Aβ42 and IL-12, and expanded with IL-2 as described above. Cells were washed and injected i.v. (15 × 10^6 cells/mouse) into 6- to 7-mo-old APP/PS1 mice or WT mice. Mice were sacrificed 14 d later and mononuclear cells prepared from CNS tissue. Cells were stimulated with PMA and ionomycin and stained for surface CD3, CD4, CD8, and intracellular IFN-γ. Immunofluorescence analysis was performed on a DakoCytomation Cyan as described above.

**Behavioral analysis**

Gait was analyzed in WT and APP/PS1 mice using the footprint test to assess stride length and hind and front limb base widths. Muscular strength and coordination were assessed using the inverted screen and wing-hang tests. Two days later, 2 wk after administration of Aβ-specific T cells, mice were tested for spatial memory in the Morris water maze. The pool (1.2 m diameter; 0.6 m high; 0.24 m water depth; 0.15 m platform diameter placed in the northwest quadrant of the pool; 0.13 m from the edge of the pool) was sited in a well-lit room (22 to 25°C), and distinct visual cues were placed on the curtains that encircled the pool. Training commenced after 1 d of habituation and continued for 5 consecutive days on which the mice underwent four 1-min trials with an intertrial interval of 5 min. Each trial ended when mice located the platform or after 60 s when mice that failed to locate the platform were led to it; animals remained on the platform for 20 s. The day after the final day of training, the platform was removed, and mice were given a single 60-s probe trial. The percentage of time each animal spent swimming in the quadrant previously containing the platform was measured. Path length was also assessed.

**Preparation of tissue**

In the first study, in which the effect of transfer of Th1, Th2, and Th17 cells was assessed, mice were killed 24 h after the last behavioral analysis. In the second study, in which the effect of anti-IFN-γ Ab was assessed, mice were killed 34 d after the first injection of Ab. They were anesthetized with sodium pentobarbital (40 μl; Euthatal; Merial Animal Health) and perfused intracardially with ice-cold PBS (20 ml). The brains were rapidly removed, and one half of the brain was stored for later extraction and analysis of Aβ. The second half of the brain, which was used for immunohistochemical analysis, was placed onto cork discs, coated with optimum cooling temperature compound (Sakura Tissue-Tek), snap-frozen in prechilled isopropanol, and stored at −80°C. Before sectioning, the tissue was allowed to equilibrate to −20°C for 2 h. Sagittal sections (10-μm thick) were prepared using a cryostat (Leica, Meyer, U.K.), mounted on gelatin-coated (Fluka/Sarland) glass slides, allowed to dry for 20 min, and stored at −20°C for later immunohistochemical analysis.

**Detection of Ab**

Snap-frozen cortical tissue was homogenized in five volumes (w/v) of homogenizing buffer (SDS/NaCl in distilled H2O [dH2O] with proteases) and centrifuged (15,000 rpm, 40 min, 4°C). The supernatant samples were removed to extract SDS-soluble Aβ, and the pellets were kept for extraction of insoluble Aβ. Supernatants were equalized (3 mg/ml) with homogenizing buffer using a BCA protein assay, and samples were neutralized by the addition of 10% (w/v) 0.5 M Tris-HCl (pH 6.8). Samples were stored at −20°C for later detection of soluble Aβ. Pellets were incubated in guanine buffer (50 μl; 5 M guanine-HCl in 50 mM Tris-HCl, pH 8; Sigma-Aldrich) for 4 h on ice. Samples were centrifuged (15,000 rpm, 30 min, 4°C), and the supernatant samples were equalized (0.5 mg/ml) with guanine buffer and stored at −20°C for later detection of insoluble Aβ using MSD 96-well multi-spot 4GB Aβ triple ultra-sensitive assay kits according to the manufacturer’s instructions (Meso Scale Discovery). Standards (Aβ1-40, 0-3,000 pg/ml; Aβ1-42, 0-10,000 pg/ml; Aβ1-42, 0-3,000 pg/ml) and samples were added to the 96-well plates, incubated

FIGURE 4. Transferred Aβ-specific Th1 cells migrate into the brain of APP/PS1 mice. Aβ-specific Th1 cells, generated from GFP mice were injected i.v. into 6- to 7-mo-old WT or APP/PS1 mice. Two weeks after injection, mice were sacrificed, and mononuclear cells were prepared from the brain. Cells were surface-stained with Abs specific for CD3, CD4, and CD8 and intracellularly stained for IFN-γ, and flow cytometric analysis was performed to quantify GFP-expressing and IFN-γ-secreting T cells in the brain. (A) Results are mean absolute number of the indicated cells in the brain. (B) Sample FACS plots of GFP⁺ T cells (gated on CD3), IFN-γ⁺CD8⁺, and IFN-γ⁺ CD4⁺ cells; number represent percentage positive. Data in (A) represent mean ± SEM from four animals per experimental group from one experiment, data in (B) representative of four mice. **p < 0.01, ***p < 0.001, Student t test for independent means.
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(2 h, RT), washed, and read in a Sector Imager plate reader (Meso Scale Discovery) immediately after addition of the MSD read buffer. Aβ concentrations were calculated with reference to the standard curves and expressed as picograms per milliliter.

**Immunohistochemistry**

Cryostat sections were assessed for Aβ plaque deposition by staining with Congo red. Sections, equilibrated to RT, were fixed in ice-cold methanol for 5 min, washed in PBS, and incubated at room temperature for 20 min in an alkaline solution prepared by adding NaOH (2 ml; 1 M) to saturated NaCl (200 ml; 80% ethanol in H2O). Thereafter, sections were incubated in filtered Congo red solution (0.2% Congo red dye in the same alkaline solution) for 30 min, rinsed in dH2O, incubated in methyl green solution (1% in dH2O) for 30 s, washed, and dehydrated by dipping in 80, 95, and then 100% ethanol. Sections were dried, incubated in 100% xylene (3 × 5 min), mounted onto slides using dibutyl phthalate in xylene (RA Lamb), and allowed to dry overnight.

To assess CD11b, sections were fixed in an acetone/ethanol mixture (1:1) for 5–10 min, and endogenous peroxidase activity was blocked by incubating in 0.3% H2O2 in PBS for 5 min. Sections were washed, blocked in 10% rabbit serum (Vector Laboratories), incubated overnight at 25°C in rat anti-CD11b Ab (1:100 in 5% rabbit serum in PBS; clone 5C6; Serotec), washed, and incubated for 2 h at RT in biotinylated rabbit anti-rat IgG (1:200 in 5% rabbit serum in PBS; Vector Laboratories). Sections were washed, incubated in Vectastain Elite ABC reagent (two drops of A/B in 5 ml PBS; Vector Laboratories) for 1 h at RT, washed, and developed using the substrate 3,3′-diaminobenzidine-enhanced liquid substrate system tetrahydrochloride (one drop solution B in 1 ml solution A) for ~10 min until the color developed and counterstained with 1% methyl green for 10 min. Samples were dehydrated by dipping in graded ethanol (70, 80, 95, and 100%) and incubating in xylene (VWR International). Sections were mounted with dibutyl phthalate in xylene, dried overnight, and stored at RT. The sections were examined using an Olympus kx51 light microscope (Olympus, Tokyo, Japan), and micrographs were taken using an Olympus UCMAD3 (Olympus) at ×40 magnification. Data were quantified using the Immunoratio plugin (http://immicroscope.uta.fi/immunoratio/) available for the ImageJ software package (National Institutes of Health) (35). Colocalization of Aβ and CD11b was examined by confocal microscopy. Frozen brain sections brought to RT, fixed in ice-cold methanol, washed, permeabilized in 0.1% Triton (Sigma-Aldrich) in PHEM buffer, and washed. Nonspecific binding was blocked by incubating sections in 10% normal goat serum (2 h, RT), and sections were incubated overnight with pan-Aβ Ab (1:1000; Calbiochem) and rat anti-CD11b Ab (1:100, clone 5C6; AbD; Serotec) in 5% normal goat serum in PHEM buffer. Sections were washed, incubated in secondary Ab ALEXA 488 (1:4000; Invitrogen) and Alexa 546 (1:1000; Invitrogen; 90 min, RT), washed, mounted, and analyzed using confocal microscopy (Axioplan 2; Zeiss).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism (GraphPad). Data were analyzed using Student t test, two-way ANOVA, or one-way ANOVA followed by Newman–Keuls post hoc test. Data are expressed as means with SEM and deemed statistically significant when p < 0.05.

**Results**

**Th1 and Th17 cell are present in the periphery and infiltrate the brains of APP/PSI mice**

We used flow cytometry to assess the presence of T cells in the brain of WT and APP/PSI mice. We found that there were very few CD3+CD4+ cells in the brain of WT mice but a significantly

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**FIGURE 5.** Transfer of Aβ-specific Th1 cells enhanced Aβ deposition in brains of APP/PSI mice. APP/PSI mice mice were injected with Aβ-specific Th1, Th2, or Th17 cells as described in Fig. 3. (A) Cryostat sections were stained with Congo red to assess Aβ-containing plaques in hippocampus and cortex; the mean number of plaques was recorded (B). (C) The concentrations of insoluble Aβ1-42, Aβ1-40, and Aβ1-38 in the cortical tissue were quantified by ELISA. (D) The concentrations of soluble Aβ1-42 and Aβ1-40 in cortical tissue were quantified by ELISA. *p < 0.05, **p < 0.01, ***p < 0.001, ANOVA, APP/PSI versus WT, ***p < 0.01, **p < 0.05, *p < 0.05, ANOVA versus control untreated APP/PSI mice (n = 5 to 6); representative of two experiments, Con, Control.
greater number in brain tissue prepared from APP/PS1 mice (***p < 0.001; Student t test for independent means; Fig. 1A). Intracellular staining revealed that a proportion of CD4^+ cells stained positively for IFN-γ and also for IL-17 (Fig. 1B–D). There was no genotype-related difference in the number of CD3^+CD8^+ cells in the brain (Fig. 1E), although intracellular staining indicated that a greater proportion of these cells stained positively for IFN-γ compared with IL-17 (**p < 0.01; Student t test for independent means; Fig. 1F).

**Aβ-specific Th1 cells impair cognitive function in APP/PS1 mice**

Having demonstrated the presence of Th1 and Th17 cells in the brain of APP/PS1 mice, we set out to evaluate the effect of administration of Aβ-specific T cells on cognitive function, Aβ accumulation, and microglial activation in 6- to 7-mo-old APP/PS1 mice in which early pathological changes have been reported (36). To amplify Aβ-specific T cells, WT mice were immunized twice (0, 21 d) with Aβ and CpG, an adjuvant known to promote Thl and Th17 responses. Short-term Aβ-specific Th1, Th2, and Th17 cell lines were generated by restimulation with Ag and APC in the presence of polarizing mixture described in the Materials and Methods section. This protocol resulted in the generation of highly polarized populations of Th1, Th2, and Th17 cells; Th1 cells produced high levels of IFN-γ and low IL-4 and IL-17, Th2 cells secreted high levels of IL-4 and low IL-17 and IFN-γ, and Th17 cells produced high levels of IL-17 and no IL-4 or IFN-γ (Fig. 2). After one round of Ag-stimulation, surviving T cells were washed and injected i.v. (15 × 10^6 cells/mouse) into 6- to 7-mo-old APP/PS1 or WT mice. Mice were tested for spatial memory in the Morris water maze 2 wk after administration of Aβ-specific T cells. The latency to reach the platform decreased over the 5-d training period but changes were similar in WT mice and control-treated APP/PS1 mice or APP/PS1 mice that received T cells (Fig. 3A), and no treatment effect was observed on day 5 of training (Fig. 3B). The path length taken to reach the platform decreased with training, except in APP/PS1 mice, which received Th1 cells (Fig. 3D) as shown by the representative traces obtained on day 5 (Fig. 3C). The mean path length on day 5 was significantly increased in these mice compared with untreated APP/PS1 mice (*p < 0.05; ANOVA; Fig. 3E). In contrast, transfer of Th1 cells into WT mice had no significant effect on path length taken to reach the platform or mean path length on day 5 (Supplemental Fig. 1). The day after the final day of training, the platform was removed, and mice underwent a single 60-s probe trial. The percentage of the total time and distance (i.e., path length) each animal spent swimming in the quadrant that previously contained the platform was significantly decreased in APP/PS1 mice that received Th1 cells compared with untreated APP/PS1 mice (*p < 0.05; ANOVA; n ≥ 5; Fig. 3F, 3G). Therefore, Th1 cell transfer induces a deficit in spatial learning in APP/PS1 mice at an age at which such deficits are not generally observed. Importantly, no motor deficits were observed in these animals; stride length, hind limb base width, and front limb base width were similar in all groups of mice, and, on the hangwire task, there were no differences in the latency to fall between groups (data not shown). These findings suggest that transfer of Th1, but not Th2 or Th17 cells, around the time of onset of Aβ plaque formation impairs cognitive function in APP/PS1 mice.

We tracked the migration of transferred T cells into the CNS using Aβ-specific Th1 cells generated from GFP mice immunized with Aβ and CpG and polarized with IL-12. We found a higher proportion of CD3^+ T cells in the brain of APP/PS1, compared with WT, mice after transfer of Aβ-specific Th1 cells (Fig. 4). Furthermore, we detected GFP^+ cells in the brain 14 d following transfer of Th1 cells, and this was significantly greater in APP/PS1 mice. Finally, we found that CD8^+ as well as CD4^+ cells infiltrated...
the brain, and a significant number of these secreted IFN-γ (Fig. 4). These findings suggested that at least a proportion of Aβ-specific Th1 cells migrate into the brain following systemic de-

livery, and this is more pronounced in APP/PS1 when compared with WT mice. In addition, IFN-γ-secreting CD8 T cells are detected in higher numbers in the brains of APP/PS1 compared with WT mice.

Aβ-specific Th1 cells enhance Aβ plaque burden and enhance microglial activation in APP/PS1 mice

Aβ deposition has been reported in the brain of APP/PS1 mice as early as 6 mo of age (37). Cryostat sections prepared from the 6- to 7-mo-old APP/PS1 mice used in this study confirm the presence of Aβ-containing plaques in cortex and hippocampus. Adoptive transfer of Aβ-specific Th1 cells markedly increased Aβ load, particularly in cortex, whereas transfer of Th2 or Th17 cells had little effect (Fig. 5A). Mean plaque number was significantly increased in sections prepared from mice that received Th1 cells (*p < 0.05; ANOVA; Fig. 5B). Insoluble Aβ1-40, Aβ1-42, and Aβ1-42 were all significantly increased in tissue prepared from APP/PS1, compared with WT, mice (*p < 0.05; **p < 0.01; ANOVA; Fig. 5C). Injection of Th1 cells induced a further increase in the concentration of the three Aβ species (*p < 0.05; **p < 0.01, ANOVA, versus control APP/PS1 mice). Furthermore, soluble Aβ1-40 and Aβ1-42 were also significantly increased in tissue prepared from APP/PS1 following transfer of Th1 cells (Fig. 5D), although soluble Aβ1-38 was unchanged between treatment groups (data not shown). Neither Th2 nor Th17 cells exerted any significant effect on soluble or insoluble Aβ.

FIGURE 7. Anti–IFN-γ Ab attenuated the effect of Th1 cells on behavioral deficits. APP/PS1 mice were injected with Aβ-specific Th1 cells as described in Fig. 3, and mice were treated with anti–IFN-γ Ab or anti-β-galactosidase as a control Ab before and after injection of the cells. Three weeks after injection, cognitive function was analyzed in the Morris Water Maze test as described in Fig. 3. (A) The path length taken to reach the platform. (B) Mean path length on day 5 of training. (C and D) In the probe test, the time and path length in the quadrant that previously contained the platform (expressed as a percentage of the total) was measured. Data represent mean ± SEM from four to five animals per experimental group from two experiments. *p < 0.05, ANOVA, APP/PS1+Th1 cells versus control APP/PS1 mice, *p < 0.05, ANOVA, APP/PS1+Th1 cells versus APP/PS1 plus Th1 cells plus anti–IFN-γ Ab. Tg, Transgenic.

FIGURE 8. Anti–IFN-γ Ab attenuated the effect of Th1 cells on Aβ accumulation. APP/PS1 mice were injected with Aβ-specific Th1 cells and treated with anti–IFN-γ Ab or a control Ab as described in Fig. 6. Mice were sacrificed 5 wk after cell transfer. Cryostat sections were stained with Congo red to assess Aβ-containing plaques in hippocampus and cortex; the mean number of plaques was recorded (A), and the concentrations of insoluble Aβ1-42 (B), Aβ1-40 (C), and Aβ1-38 (D) were quantified by ELISA in brain tissue prepared from APP/PS1 and WT mice. Data represent mean ± SEM from four to five animals per experimental group from two experiments. *p < 0.01, **p < 0.001, ANOVA; (n = 4–6); *p < 0.05, **p < 0.01, ***p < 0.001, ANOVA; control APP/PS1 mice versus APP/PS1 mice that received Th1 cells; *p < 0.05; **p < 0.001, ANOVA, APP/PS1+Th1 cells versus APP/PS1+Th1 cells+anti–IFN-γ Ab. Con, Control.
Neutralization of IFN-γ attenuates the effect of Th1 cells on plaque burden

Anti-IFN-γ Ab attenuated the effects of Th1 cells on plaque number and concentration of insoluble Aβ₁₋₃₈, Aβ₁₋₄₀, and Aβ₁₋₄₂ in tissue prepared from APP/PS1 mice (Fig. 6). These measures were increased in tissue prepared from APP/PS1 mice compared with WT mice (***p < 0.01, ANOVA; Fig. 8), and these were significantly increased by administration of Th1 cells (*p < 0.05, ***p < 0.001, ANOVA; control APP/PS1 mice versus APP/PS1 mice that received Th1 cells). The increase in Aβ₁₋₃₈, Aβ₁₋₄₀, and Aβ₁₋₄₂ induced by Th1 cells was attenuated when mice were treated with anti-IFN-γ Ab (†p < 0.05, §§§p < 0.001, ANOVA, APP/PS1 mice that received Th1 cells versus Ab-treated APP/PS1 mice that received Th1 cells).

CD11b immunoreactivity was negligible in sections prepared from hippocampus and cortex of WT mice (Fig. 9), whereas some staining was observed in both areas in APP/PS1 mice. This was greater in APP/PS1 mice that received Th1 cells, but this effect was ameliorated to some degree in sections prepared from APP/PS1 mice that received Th1 cells and anti-IFN-γ Ab. Immunoreactivity was similar in sections prepared from control APP/PS1 mice and APP/PS1 mice, which received anti-IFN-γ Ab. Analysis of staining using confocal microscopy indicated that CD11b immunoreactivity (green staining; Fig. 10) was colocalized with Aβ deposition (red staining) in hippocampus and cortex. As shown in Figs. 6 and 9, Aβ accumulation was increased in sections prepared from APP/PS1 mice, which received Th1 cells, and this effect was attenuated by anti-IFN-γ Ab treatment (Fig. 10). These findings demonstrate that the impact of Th1 cells on Aβ plaque burden and microglial activation was mediated through IFN-γ.

Discussion

The significant finding of this study is that adoptive transfer of Th1 cells increases Aβ accumulation and microglial activation in
the brain of 6- to 7-mo-old APP/PS1 mice and impairs performance in a Morris water maze; these effects are attenuated by treatment of mice with anti-IFN-γ Ab.

It has been recognized for some time that T cells can infiltrate the brain (38, 39). T cell infiltration is significantly enhanced under pathological conditions (for example, in multiple sclerosis and EAE), and this is due, at least to some extent, to an increase in blood-brain barrier permeability (23, 40). In this study, we report that there is a significant increase in the number of CD3⁺CD4⁺ cells in the brain of APP/PS1 mice compared with WT mice and that a proportion of these are Th1 and Th17 cells. Consistent with this is the observation that significant numbers of peripheral T cells are present in the postmortem brain of AD patients compared with the relatively low numbers of cells in other degenerative dementia cases and, importantly, that these cells are clustered in areas of the brain in which pathology is more marked, such as the hippocampus and limbic regions (25). However, the role of T cells in the pathogenesis of AD is not clear, with circumstantial evidence of both host protective and damaging roles for Ab-specific T cells. Peripheral T cells specific for Ab1-42 have been detected in healthy individuals, but were absent in patients with AD (41), possibly suggesting that Ab1-40-specific T cells may prevent the development of Ab plaques. It has also been reported that Th1 cells directed against Ab1-42 are present in young individuals but decline with age and are lost in patients with AD, in whom IL-10-producing regulatory T cells predominate (42).

Vaccine studies in mouse models have shown that immunization with Ab1-42 in CFA prevented the development of Ab plaques and reduced the development of AD-like neuropathology (33, 43). The protection was associated with Ab and could be mimicked by passive transfer of Ab-specific Abs (44). There is also evidence from a clinical trial that active immunization with Ab1-42, formulated with the adjuvant QS21 (AN 1792), can reduce plaque burden in AD patients (45), though a number of patients developed meningoencephalitis, and the trial was halted. Although the cause of the meningoencephalitis is not clear, it has been suggested that it could result from the induction of inflammatory T cell responses (46). Interestingly, QS-21, the adjuvant used in AD vaccine, has been shown to promote Th1 responses to co-administered foreign Ag in mice (47). Our findings are consistent with a pathogenic role for Th1 cells, at least in a mouse model of AD.

To evaluate the impact of different T cell subtypes on plaque burden in the brain, we adoptively transferred Ab-specific Th1, Th2, and Th17 cells into 6- to 7-mo-old APP/PS1 mice. Consistent with previous findings (36), we found that there was some Ab accumulation in the brain of the 6-mo-old APP/PS1 mice. This was accompanied by increased concentrations of Ab1-42, Ab1-40, and Ab1-38 in cortical tissue. However, transfer of Th1 cells increased deposition of Ab (determined by Congo red staining) and markedly increased cortical Ab concentration. This suggests that Ab-specific Th1 cells may play a role in the development of Ab plaques in the brain. This was confirmed by treatment of mice with a neutralizing anti-IFN-γ Ab, which attenuated the effect of Th1 cells on Ab accumulation. In contrast with the effect of Th1 cells, transfer of Th17 cells, which have been associated with pathology in EAE and other autoimmune/inflammatory diseases, and Th2 cells, which have a more anti-inflammatory function in other diseases, did not enhance Ab accumulation in the brain. These findings are consistent with our earlier report that Ab-specific Th1 cells enhance proinflammatory cytokine production and MHC class II and costimulatory molecule expression by Ab-stimulated microglia, whereas Ab-specific Th2 cells suppress cytokine production by glial cells (32).

Under resting conditions, microglia are maintained in a quiescent state in the brain because of the presence of neuroimmune regulatory molecules that enable the interaction with other cells, low concentrations of stimulatory factors such as IFN-γ and other inflammatory cytokines, and the presence of minimal numbers of immune cells like T cells (13). However, microglial activation occurs following any insult, and an activated state is a characteristic of most, if not all, neurodegenerative diseases in which these cells can assume the role of APC. Modest microglial activation was observed in the hippocampus and cortex of 6- to 7-mo-old APP/PS1 mice but transfer of Th1 cells markedly increased activation. This is consistent with our previous findings that showed that Ab-specific Th1 cells increased microglial activation in vitro (32). In parallel with its effect on Ab accumulation, treatment of mice with anti-IFN-γ Ab attenuated the effect of Th1 cells on microglial activation. It is well established that IFN-γ is among the most potent activators of microglia (15, 48) and synergizes with Ab to increase expression of cell-surface markers of activation and production of inflammatory cytokines (15, 49). Chakrabarty et al. (50) reported that viral delivery of IFN-γ gene promotes microglial activation and clearance of Ab. We observed that Th1 cells also promoted microglial activation but that this was associated with an increase in Ab plaques. We do not have a definitive explanation for the discrepancy in these studies other than the differences in the experimental approaches: virally-delivered IFN-γ, which had effects, such as basal ganglia calcification, in
WT as well as Tg mice, compared with i.v. injected Aβ-specific Th1 cells, in which the effects were largely confined to Tg mice. One interpretation of the data, as suggested in this study, is that anti-IFN-γ prevents Th1 cell-induced activation of microglia, but it is possible that the Ab treatment affects infiltration of cells, perhaps by altering chemotaxis or exerting an effect on blood-brain barrier permeability.

Our studies with Th1 cells expressing GFP demonstrated that at least a proportion of the transferred Th1 cells did migrate from the periphery into the CNS. Interestingly, IFN-γ-secreting CD8 as well as CD4 T cells were detected in the brain following i.v. injection of Aβ-specific Th1 cells. This is consistent with studies in the EAE model that have demonstrated that Th1 cells preferentially infiltrate the CNS and facilitate recruitment of other inflammatory T cells (51). Interestingly, the migration of T cells into the brain and subsequent behavioral deficits was significantly more pronounced following transfer of Aβ-specific Th1 cells into APP/PS1 when compared with WT mice. This may reflect the higher Aβ burden in the APP/PS1 mice and might suggest local Ag stimulation of IFN-γ-secreting T cells, which were at a significantly higher frequency in the brains of APP/PS1 compared with WT mice.

Previous studies from this laboratory have shown that Aβ-specific Th1 cells enhanced Aβ-induced activation of microglia (32). Furthermore, the increase in microglial activation in APP/PS1 mice was accompanied by increased expression of inflammatory cytokines, including TNF-α and IL-1β (52). Interestingly, TNF-α and IL-1β have been shown to increase activity and/or expression of γ- and β-secretases (6, 7), which leads to Aβ deposition. Although activated microglia may phagocyte and remove Aβ aggregates (50), IL-1β-expressing microglia are associated with Aβ plaques and neurofibrillary tangles in the brain of AD patients, where they correlate with progressive neuronal damage (53). Furthermore, IL-1β can promote synthesis of APP in endothelial cells (54). It has also been reported that IFN-γ-induced activation of microglia enhanced processing of APP and suppressed Aβ clearance (55). We found that Th1 cells, which increase IL-1β expression by microglia (32), enhanced soluble and insoluble Aβ concentrations in the brains of APP/PS1 mice. However, it must be acknowledged that Aβ potently activates microglia in vitro and in vivo (56, 57); therefore, it is possible that there may be a feedback loop, leading to persistent microglial activation and Aβ accumulation with the subsequent pathogenic consequences.

Although there was significant Aβ accumulation in the brain of 6- to 7-month-old APP/PS1 mice, we found no evidence of genotyperelated changes during the training phase in the Morris water maze or during the probe test, contrasting with previous reports that indicated a deficit in slightly older (8-month-old) APP/PS1 mice (58, 59). It has been suggested that cognitive deficits correlate with insoluble Aβ in Tg2576 mice (60) and APP/PS1 mice (61), but this view is not supported by the present findings. However, we report that transfer of Th1 cells doubled the concentration of insoluble Aβ in brain tissue, and this was associated with deterioration in cognitive function in the probe test; this raises the possibility that a threshold concentration of Aβ must be reached before an impact on spatial learning is exerted. In contrast to the effect of Th1 cells on APP/PS1 mice, transfer of Th2 cells or Th17 cells exerted no effect in the spatial learning task or on either plaque number or Aβ accumulation. The present findings are at variance with another earlier report that indicated that adoptive transfer of a mixed T cell preparation improved performance of 10-month-old APP/PS1 mice in a radial arm maze task (62). Although no effect on insoluble Aβ or Aβ plaque numbers was observed, the authors suggested that microglia or monocytes were stimulated to clear Aβ because the distribution of Aβ-immunoreactive cells is hippocampus of mice that received T cells was similar to the distribution of MHC class II-positive cells. More recent data from this group suggested that the beneficial effects on behavior may be Th2 cell-mediated because the effect was evident when γ cells had been incubated in vitro in the presence of IL-2 and IL-13 (31). We have recently reported that the Aβ-induced microglia activation in vitro is attenuated by Th2 cells (32), and the current study found that Th2 cells, unlike Th1 cells, did not enhance plaque burden in vivo.

Although beneficial effects of T cells in the brain have also been observed (63), the evidence presented in this study indicates that Th1 cells, but not Th2 or Th17 cells, contribute to Aβ accumulation and development of a functional deficit in APP/PS1 mice during the early stages of development of pathology. This model, the effects appear to be mediated by IFN-γ and are associated with enhanced microglial activation, which may trigger inflammatory changes that propagate a damaging cascade of events and further development of pathology.

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