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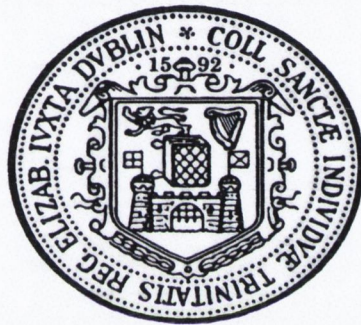
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# **Evaluation of polarising stimuli on bone marrow-derived macrophage phenotypes in two models of neuroinflammation**

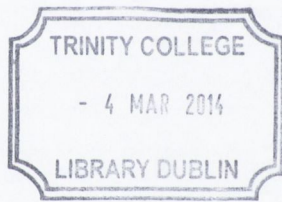
James Barrett



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

Department of Physiology  
Trinity College Institute of Neuroscience

2014



*Thesis 10226*

## **I: Declaration of authorship**

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work, with the exception of certain results kindly donated by Dr. Raasay Jones and Dr. Aedín Minogue and work carried out in conjunction with Dr. Thelma Cowley. I agree to deposit this thesis in the University's open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

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## II: Acknowledgements

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### III: Abstract

Inflammatory changes in the brain have been observed with aging and in neurodegenerative disorders, and these are believed to be associated with the accompanying decline in cognitive function. Recent evidence has suggested that peripheral immune cells may play a role in the modulation of inflammatory events within the central nervous system. The objective of this thesis was to investigate the responses of macrophages prepared from aged rats and mice which overexpress amyloid precursor protein and presenilin 1 (APP/PS1 mice) to stimuli which have been shown to polarise macrophages to different activation states. Therefore, the effects of lipopolysaccharide (LPS) and interferon- $\gamma$  (IFN $\gamma$ ), which induce classical activation of macrophages, which is also referred to as the M1 phenotype, and interleukin-4 (IL-4), which induces alternative activation, also referred to as the M2 state, were compared in bone marrow-derived macrophages (BMDMs) from young and aged rats and BMDMs from wildtype (WT) and APP/PS1 mice.

The data demonstrate that macrophages infiltrate the brains of aged rats and because of the age-related changes, it was important to investigate the responses of these macrophages to inflammatory stimuli. The data showed that macrophages from aged rats exhibited an enhanced response to LPS and IFN $\gamma$  compared with macrophages from young rats, however there was no age-related change observed in response to IL-4. It was also found that age was associated with increased hippocampal expression of the TLR4 agonist, high mobility group box 1 (HMGB1), suggesting that infiltrating macrophages responding to HMGB1 may exacerbate existing inflammation within the brain.

It has been consistently reported that microglial activation is observed in animal models of Alzheimer's disease and the present data shows that there is an increase in hippocampal concentration of IFN $\gamma$  in APP/PS1 mice which may trigger microglial activation. The present data also demonstrate that macrophage infiltration was increased in the brains of APP/PS1 mice and, significantly, BMDMs from APP/PS1 mice exhibit enhanced responsiveness to IFN $\gamma$ . Therefore infiltrating macrophages may be further stimulated by IFN $\gamma$  to exacerbate the inflammatory condition.

An additional aim of this study was to evaluate the signalling events triggered by IL-4 and IFN $\gamma$ . IL-4 increased activation of phosphorylated AKT (pAKT) in BMDMs from wildtype mice; in contrast, IFN $\gamma$  treatment decreased pAKT activation. The evidence indicates that activation of the PI3K/AKT pathway plays a role in the induction of the M2 markers Arginase-1, Chitinase-3-like-3 and found in inflammatory zone 1 and inhibition of this pathway was associated with a dramatic decrease in IL-4-induced expression of these markers. IFN $\gamma$ -induced tumour necrosis factor- $\alpha$ , major histocompatibility complex II and intercellular cell adhesion molecule-1 was exaggerated in the presence of the PI3K inhibitor Ly294002. Therefore it is concluded that manipulation of this pathway impacts on the polarisation of macrophages.

The most significant finding of this thesis is that macrophages in aged rats and APP/PS1 mice exhibit exaggerated responses to pro-inflammatory stimuli and that these macrophages gain entry into the CNS, where they potentially exacerbate the existing neuroinflammation. Therefore it is possible that therapies that aim to repair the integrity of the blood-brain barrier and restrict the entry of macrophages and other peripheral cells may be beneficial in disorders associated with increased inflammation within the CNS. The present data also demonstrate that the PI3K/AKT pathway is involved in the polarisation of macrophages, and future studies should investigate the ability of this pathway to modulate inflammatory conditions.

## IV: Table of contents

I: Declaration of Authorship	i
II: Acknowledgements	ii
III: Abstract	iii
IV: Table of contents	v
V: List of figures	xiv
VI: List of Tables	xvii
VII: Abbreviations	xviii

## Chapter 1

### Introduction

1.1. Macrophages	2
1.2. Macrophage Activation	3
1.3. M1 activation and markers of the M1 activation state	6
1.3.1. TNF $\alpha$ and NOS2 are the archetypal markers of the M1 activation state	7
1.3.1.1. TNF $\alpha$	7
1.3.1.2. NOS2	8
1.3.2. Inflammatory Cytokines as markers of M1 activation	9
1.3.2.1. IL-1 $\beta$	9



1.3.2.2. IL-6	9
1.3.3. Cellular proteins as markers of M1 activation	10
1.3.3.1 MHCII	10
1.3.3.2. CD11b	10
1.3.3.3. CD40	10
1.3.3.4. CD68	11
1.3.3.5. NOD2	11
1.3.3.6. ICAM-1	12
1.4. M2 activation	12
1.4.1. Arginase 1	13
1.4.2. Mannose Receptor	13
1.4.3. Chitinase-3-like-3	14
1.4.4. Fizz1	16
1.5. Macrophage signalling	16
1.5.1. TLR4 signalling	16
1.5.2. The IFN $\gamma$ signalling pathway	17
1.5.3. The IL-4 signalling Pathway	21
1.5.4. PI3K/AKT Pathway	21
1.6. The blood-brain barrier	23
1.7. Aging	23

<b>1.8 Alzheimer's Disease</b>	<b>25</b>
<b>1.9 Aims and objectives</b>	<b>29</b>

**Chapter 2**  
**Materials and methods**

<b>2.1 Animals</b>	<b>31</b>
<b>2.2. Cell Culture</b>	<b>32</b>
<b>2.2.1. Aseptic technique</b>	<b>32</b>
<b>2.2.2. Maintenance of L929 cell line</b>	<b>32</b>
<b>2.2.3. Cell treatments</b>	<b>33</b>
<b>2.2.4. Generation of bone marrow-derived macrophages (BMDMs)</b>	<b>33</b>
<b>2.2.5. Cell counting</b>	<b>34</b>
<b>2.3. Mononuclear cell isolation</b>	<b>35</b>
<b>2.3.1. Preparation of Percoll gradients</b>	<b>35</b>
<b>2.3.2. Cell isolation</b>	<b>35</b>
<b>2.4. Flow cytometry</b>	<b>37</b>

<b>2.4.1. Assessment of phagocytosis</b>	37
<b>2.4.2. Cell surface staining</b>	37
<b>2.5. Western Immunoblotting</b>	39
<b>2.5.1. Preparation of cell lysates</b>	39
<b>2.5.2. Preparation of tissue</b>	39
<b>2.5.3. Gel electrophoresis</b>	39
<b>2.6. Analysis of mRNA by reverse transcriptase polymerase chain reaction (RT-PCR)</b>	41
<b>2.6.1. Isolation of RNA</b>	41
<b>2.6.2. RNA quantification</b>	41
<b>2.6.3. cDNA synthesis</b>	42
<b>2.6.4. cDNA amplification by RT- PCR</b>	42
<b>2.6.5. RT-PCR quantification</b>	42
<b>2.7. Cytokine analysis</b>	45
<b>2.8. Mesoscale multiplex assays</b>	47
<b>2.8.1. Tissue preparation for Mesoscale</b>	47
<b>2.8.2. Mouse Pro-Inflammatory 7-Plex Assay</b>	47
<b>2.9. Statistical analysis</b>	47

## Chapter 3

### The Impact of aging on the activation state of bone-marrow derived macrophages

3.1. Introduction	50
3.2. Methods	52
3.3. Results	53
3.3.1. Age-related increase in the number of macrophages present in the brain	53
3.3.2. LPS induced CD11b and CD40 mRNA expression in BMDMs	53
3.3.3. The effect of LPS on cytokine mRNA and supernatant concentration in BMDMs from young and aged rats	53
3.3.4. The effect of IFN $\gamma$ on TNF $\alpha$ , and NOS2 mRNA expression in BMDMs from young and aged rats	54
3.3.5. The effect of IFN $\gamma$ on MHC II and CD40 mRNA expression in BMDMs from young and aged rats	55
3.3.6. The effect of IL-4 on MRC1 and ARG1 mRNA expression in BMDMs from young and aged rats	55
3.3.7. Phagocytic activity of BMDMs from young and aged rats.	55
3.3.8. HMGB1 protein expression in the hippocampus of young and aged rats	56
3.4 Discussion	67

## Chapter 4

### Analysis of the responsiveness of bone marrow-derived macrophages from WT and APP/PS1 mice to inflammatory stimuli

4.1. Introduction	73
4.2. Methods	76
4.3. Results	77
4.3.1. CD11b <sup>+</sup> /CD45 <sup>high</sup> cells are present in the brains of APP/PS1 animals.	77
4.3.2. The effect of IFN $\gamma$ on TNF $\alpha$ and NOS2 mRNA expression in BMDMs from WT and APP/PS1 mice	77
4.3.3. The effect of IFN $\gamma$ on MHC II and NOD2 mRNA expression in BMDMs from WT and APP/PS1 mice	77
4.3.4. The effect of LPS on CD11b and CD11b mRNA expression in BMDMs from WT and APP/PS1 mice	78
4.3.5. LPS increased cytokine concentration in BMDMs in BMDMs from WT and APP/PS1 mice	78
4.3.6. The effect of IL-4 on MRC1 and Arg1 mRNA expression in BMDMs from WT and APP/PS1 mice	78
4.3.7. IFN $\gamma$ R1 mRNA expression is increased in BMDMs from APP/PS1 mice	78

4.3.8. IFN $\gamma$ concentration is increased in the hippocampus of APP/PS1 mice	78
4.3.9. IFN $\gamma$ increases TNF $\alpha$ and NOS2 mRNA expression in BMDMs from WT and CD200 <sup>-/-</sup> mice	79
4.3.10. LPS increased TNF $\alpha$ and NOS2 mRNA expression in BMDMs from WT and CD200 <sup>-/-</sup> mice	79
4.3.11. IL-4 increased MRC1 and Arg1 mRNA expression in BMDMs from WT and CD200 <sup>-/-</sup> mice	80
4.3.12. IL-4 increased MRC1 and Arg1 mRNA expression in BMDMs from WT and CD200 <sup>-/-</sup> mice	80
4.4. Discussion	93

## Chapter 5

### Modulation of macrophage activation; a role for the PI3K/AKT pathway?

5.1. Introduction	99
5.2. Methods	101
5.3. Results	102
5.3.1. IL-4 induced the activation of pSTAT6 in BMDMs	102
5.3.2. The effect of IL-4 on PI3K/AKT pathway in BMDMs	102
5.3.3. IL-4 induced the MRC1, Arg1, Chi3li3 and FIZZ1 in BMDMs	102
5.3.4. The effect of Ly294002 on IL-4-induced markers of M2 activation in BMDMs	103
5.3.5. The effect of Ly294002 on IL-4-induced STAT6 and PI3K/AKT signalling in BMDMs	103
5.3.6. IFN $\gamma$ induced the activation of pSTAT1 in BMDMs	103
5.3.7. The effect of IFN $\gamma$ on PI3K/AKT activity in BMDMs	104
5.3.8. The effect of Ly294002 on IFN $\gamma$ -induced markers of M1 activation in BMDMs	104
5.3.9. The effect of Ly294002 on IFN $\gamma$ -induced STAT1 and PI3K/AKT signalling in BMDMs	105
5.4. Discussion	121

## Chapter 6

### General discussion

6.1. General discussion	127
6.2 Limitations and future studies	136
References	138
Appendix	156



## V: List of figures

<b>Figure 1.1</b> L-Arginine metabolism (Adapted from Varin & Gordon 2009)	15
<b>Figure 1.2</b> TLR4 signalling pathway	19
<b>Figure 1.3</b> IFN $\gamma$ induced classical activation	20
<b>Figure 1.4</b> The IL-4 signalling pathway	22
<b>Figure 2.1.</b> Image of Rat BMDMs	34
<b>Figure 2.2.</b> Percoll gradient, following centrifugation, for the isolation of glial cells from brain tissue	36
<b>Figure 3.1.</b> Age was associated with an increase in CD11b <sup>+</sup> /CD45 <sup>high</sup> cells in brain tissue	57
<b>Figure 3.2.</b> LPS induces CD11b and CD40 mRNA expression in BMDMs from young and aged rats	58
<b>Figure 3.3.</b> LPS increases IL-1 $\beta$ mRNA expression and supernatant concentration in BMDMs from young and aged rats	59
<b>Figure 3.4.</b> LPS increases TNF $\alpha$ mRNA expression and supernatant concentration in BMDMs from young and aged rats	60
<b>Figure 3.5.</b> LPS increases IL-6 mRNA expression and supernatant concentration in BMDMs from young and aged rats	61
<b>Figure 3.6.</b> The effect of IFN- $\gamma$ on TNF $\alpha$ , and NOS2 mRNA expression in BMDMs from young and aged rats	62
<b>Figure 3.7.</b> The effect of IFN- $\gamma$ on MHC II and CD40 mRNA expression in BMDMs from young and aged animals	63
<b>Figure 3.8.</b> The effect of IL-4 on MRC1 and ARG1 mRNA expression in BMDMs from young and aged rats	64
<b>Figure 3.9.</b> Phagocytic activity of BMDMs was increased with age	65
<b>Figure 3.10.</b> HMGB1 was increased in the hippocampus with age	66

<b>Figure 4.1.</b> Increased percentage of CD11b <sup>+</sup> /CD45 <sup>high</sup> cells present in the brains of APP/PS1 animals	81
<b>Figure 4.2.</b> The effect of IFN $\gamma$ on TNF $\alpha$ and NOS2 mRNA expression in BMDMs from WT and APP/PS1 mice	82
<b>Figure 4.3.</b> The effect of IFN $\gamma$ on MHC II and NOD2 mRNA in BMDMs from WT and APP/PS1 mice	83
<b>Figure 4.4.</b> The effect of LPS on CD40 and CD11b mRNA in BMDMs from WT and APP/PS1 mice	84
<b>Figure 4.5.</b> The effect of LPS on TNF $\alpha$ and NOS2 mRNA in BMDMs from WT and APP/PS1 mice	85
<b>Figure 4.6.</b> LPS increased TNF $\alpha$ and IL-6 in supernatant of BMDMs from WT and APP/PS1 mice	86
<b>Figure 4.7.</b> The effect of IL-4 on MRC1 and Arg1 mRNA expression in BMDMs from WT and APP/PS1 mice	87
<b>Figure 4.8.</b> IFNGR1 mRNA was increased in BMDMs from APP/PS1 mice	88
<b>Figure 4.9.</b> IFN $\gamma$ concentration was increased in hippocampus of APP/PS1, compared with WT mice	89
<b>Figure 4.10.</b> The effect of IFN $\gamma$ on TNF $\alpha$ , and NOS2 mRNA expression in BMDMs from WT and CD200 <sup>-/-</sup> mice	90
<b>Figure 4.11.</b> The effect of LPS on CD11b and CD40 mRNA in BMDMs from WT and CD200 <sup>-/-</sup> mice	91
<b>Figure 4.12.</b> The effect of IL-4 on MRC1 and Arg1 mRNA expression in BMDMs from WT and CD200 <sup>-/-</sup> mice	92
<b>Figure 5.1.</b> The effect of IL-4 on pSTAT6 expression in BMDMs	106

<b>Figure 5.2.</b> The effect of IL-4 on pPI3K and PI3K expression in BMDMs	107
<b>Figure 5.3.</b> The effect of IL-4 on pAKT and AKT expression in BMDMs	108
<b>Figure 5.4.</b> The effect of IL-4 on MRC1 and Arg1 mRNA expression in BMDMs	109
<b>Figure 5.5.</b> The effect of IL-4 on Chi3li3 and Fizz1 mRNA expression in BMDMs	110
<b>Figure 5.6.</b> The effect of Ly294002 on IL-4-induced MRC1 and Arg1 mRNA expression in BMDMs	111
<b>Figure 5.7.</b> The effect of Ly294002 on IL-4-induced Fizz1 and Chi3li3 mRNA expression in BMDMs	112
<b>Figure 5.8.</b> The effect of Ly294002 on IL-4 signalling in BMDMs	113
<b>Figure 5.9.</b> The effect of IFN $\gamma$ on pSTAT1 expression in BMDMs	114
<b>Figure 5.10.</b> The effect of IFN $\gamma$ on pPI3K and PI3K expression in BMDMs	115
<b>Figure 5.11.</b> The effect of IFN $\gamma$ on pAKT and AKT expression in BMDMs	116
<b>Figure 5.12.</b> The effect of Ly294002 on IFN $\gamma$ -induced TNF $\alpha$ and NOS2 mRNA in BMDMs	117
<b>Figure 5.13.</b> The effect of Ly294002 on IFN $\gamma$ -induced MHCII and ICAM1 mRNA in BMDMs	118
<b>Figure 5.14.</b> The effect of Ly294002 on IFN $\gamma$ -induced CD40 and NOD2 mRNA in BMDMs	119
<b>Figure 5.15.</b> The effect of Ly294002 on IFN $\gamma$ signalling in BMDMs	120
<b>Figure 6.1.</b> Proposed mechanism for AKT modulation on macrophage activation	133

## VI: List of tables

<b>Table 2.1.</b> Mouse and Rat antibodies used for flow cytometry	38
<b>Table 2.2.</b> Western immunoblot antibodies	40
<b>Table 2.3.</b> Rat TaqMan Gene Expression Assay numbers	43
<b>Table 2.4.</b> Mouse TaqMan Gene Expression Assay numbers	44
<b>Table 2.5.</b> Rat ELISA reagents	46
<b>Table 2.6.</b> Mouse ELISA reagents	46
<b>Table 6.1.</b> Summary of mRNA changes observed following LPS stimulation	134
<b>Table 6.2.</b> Summary of mRNA changes observed following IFN $\gamma$ stimulation	135

## VII: Abbreviations

ANOVA	Analysis of variance
APC	Antigen presenting cell
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complimentary deoxyribonucleic acid
CNS	Central nervous system
DAMP	Damage-associated molecular patterns
DMEM	Dulbecco's modified Eagle's medium
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
EAE	Experimental Allergic Encephalomyelitis
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FBS	foetal bovine serum
Fizz1	Found in inflammatory zone 1
HMGB1	High mobility group protein B1
ICAM-1	Intracellular adhesion molecule 1
IFN $\gamma$	Interferon- $\gamma$
IKK	I $\kappa$ B kinase
IL-10	Interleukin-10
IL-1 $\beta$	Interleukin-1 $\beta$
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-13	Interleukin-13
IRAK	Interleukin-1 receptor associated kinase
I $\kappa$ B	Inhibitor of $\kappa$ B
JAK	Janus tyrosine kinase
LAMP	Lysosomal-associated membrane protein

LBP	Lipopolysaccharide-binding protein
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LTP	Long-term potentiation
MAL	MyD88 adaptor-like protein
MAPK	Ras/Mitogen-activated protein kinase
MHCII	Major histocompatibility complex class II
mRNA	Messenger RNA
MS	Multiple sclerosis
MyD88	Myeloid differentiation primary response gene 88
NEMO	NF-kappa-B essential modulator
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural Killer cells
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NOS	Nitric oxide synthase
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase reaction chain
PI3K	phosphatidylinositol 3-kinases
PRR	Pattern recognition receptor
RasGAP	Ras GTPase activating protein
RNA	Ribonucleic acid
RNase	Ribonuclease
SEM	Standard error of mean
SHIP	Sh2-containing inositol phosphatase
SOCS	Suppressor of cytokine signaling
SphK	Spingosine phosphate kinase
STAT	Signal transducer and activator of transcription
Strep-HRP	Streptavidin-horseradish peroxidase linked
TGF-β	Transforming growth factor β
TLR	Toll-like receptor
Tyk2	Tyrosine kinase 2

# **Chapter 1**

## **Introduction**

## 1.1 Macrophages

Elie Metchnikoff first described macrophages in 1905 (Metchnikoff, 1905), recognising their ability to eliminate pathogens and function in homeostasis. Macrophages form the first line of defence and, as part of the innate immune response, help mediate the immune response to microorganisms (Varin & Gordon, 2009). A primary role of macrophages is the phagocytosis of pathogens and the removal of debris resulting from apoptotic cells (Town *et al.*, 2005).

Macrophage populations within tissue can be replenished from circulating blood monocytes; in fact as far back as 1939, Ebert and Florey observed that monocytes migrated from the blood and differentiated into macrophages in tissue (Gordon & Taylor, 2005). Other work has also described an increased number of monocytes recruited during an inflammatory event (Murray & Wynn, 2011). Blood monocytes originate in the bone marrow from hematopoietic stem cells, these cells then infiltrate tissue where they differentiate into mature macrophages and these tissue macrophages aid maintenance of homeostasis, with important roles in the clearance of cellular debris, tissue remodelling, repair and the initiation and resolution of inflammatory events. Macrophages exhibit great heterogeneity, as they adopt tissue specific functions which vary from osteoclasts that remodel bone and microglia that maintain homeostasis within the central nervous system (CNS) to liver macrophages, commonly referred to as Kupffer cells, which exhibit increased phagocytic activity as they remove foreign materials from the portal circulation. Alveolar macrophages are responsible for the removal of inhaled pathogens and, these macrophages can produce reactive oxygen species to aid this process. Perivascular macrophages are another subset of macrophages whose primary function is to remove debris from the perivascular space.

Microglia are the resident immune cells of the central nervous system (CNS), sometimes referred to as the tissue-based macrophages of the brain (Kofler & Wiley, 2011). The primary role of microglia is to maintain homeostasis and give support to neurons. However since they release an array of neurotrophic factors and anti-inflammatory cytokines it has also been suggested that they can enhance synaptic plasticity (Lull & Block, 2010). Genome wide microarray analysis has shown that microglia are most closely related to bone marrow-derived macrophages (BMDMs). There has been



much debate as to the origin of microglia, but recent findings have suggested that these cells are derived from primitive macrophages in the yolk sac that migrate to the CNS during development (Saijo & Glass, 2011). However, it has also been proposed that bone marrow-derived cells have the ability to cross the blood-brain barrier (BBB) and differentiate into microglia (Hess *et al.*, 2004; Malm *et al.*, 2005; Simard *et al.*, 2006). Simard and colleagues transplanted stem cells expressing green fluorescent protein (GFP) into lethally-irradiated mice and later observed that cells expressing GFP had infiltrated the CNS. This has led to the theory that there may be two distinct populations within the CNS - resident microglia and bone marrow-derived microglia. The merits of use of irradiation have been called into question as other groups have demonstrated that irradiation can lead to disruption of the blood-brain barrier (BBB) and this may account for the appearance of bone marrow cells within the CNS.

Bone marrow-derived macrophages (BMDMs) are primary macrophage cells derived from bone marrow stem cells that can differentiate into mature macrophages in the presence of growth factors *in vitro*. The major benefit of using these cells is that, unlike peritoneal macrophages or alveolar macrophages, a high number of cells can be generated from a single animal. They respond to a variety of stimuli including interleukin (IL)-4, interferon-(IFN) $\gamma$  and lipopolysaccharide (LPS) and, since they express the same genes as tissue macrophages, may prove to be a more relevant research tool than macrophage-like cell lines such as RAW 246.7 cells. It has been demonstrated that perivascular macrophages are bone marrow-derived, and that the turnover of these cells is approximately 30% over 2 months and, as mentioned above, it has been reported that bone marrow cells may migrate across the BBB and populate the CNS.

## 1.2 Macrophage Activation

Under normal conditions, macrophages are said to be in a "resting" condition, however this term is misleading as it is likely that these cells are constantly surveying the tissue environment. Once a threat is detected macrophages become "activated"; these activated macrophages release a variety of substances that mediate the immune response such as nitric oxide (NO), interleukin-(IL)1 $\beta$  and tumor necrosis factor (TNF)- $\alpha$  (Raes *et al.*, 2002a).

The role of macrophages is to maintain tissue homeostasis and, to achieve, this they express cell surface proteins that allow them to respond to a wide range of stimuli. To enable them to respond to invading pathogens, macrophages are activated by pattern associated molecular patterns (PAMPs), which are structures expressed by pathogens (Olson & Miller, 2004). These cells express pathogen recognition receptors (PRRs), an example of which is the toll-like receptor (TLRs) family. There are over 10 TLRs in humans which are capable of interacting with a diverse range of PAMPs (Takeda & Akira, 2005). TLR4 recognises LPS, a component of the cell wall of gram-negative bacteria (Buchanan *et al.*, 2010) and activation of TLR4 leads to the upregulation of pro-inflammatory mediators (Buchanan *et al.*, 2010). Macrophages also respond to endogenous molecules associated with 'danger' and stress (Plowden *et al.*, 2004). Release of damage-associated molecular patterns (DAMPs) into the microenvironment results in activation of nearby macrophages; adenosine triphosphate (ATP) is released from damaged or dying cells (Thomas & Salter, 2010) and induces the activation of the inflammasome both in macrophages (Mills, 2011) and microglia (Murphy & Lynch, 2012).

Soluble mediators such as cytokines and chemokines released from other immune cells can also lead to the activation of macrophages. The cytokine, IFN $\gamma$ , produced by Th1 cells, is a potent activator of macrophages (Nguyen & Benveniste, 2000) and it has been reported that IFN $\gamma$ -deficient mice are more susceptible to infection. While anti-inflammatory cytokines such as IL-10 restrict inflammation, mice lacking IL-10 exhibit an enhanced response to LPS (Berg *et al.*, 1995). Chemokines are released by various cells and their function is to recruit cells to the site of injury or infection. Macrophages express an array of chemokine receptors, including chemokine receptor 2 (CCR2) which is activated by the chemokine monocyte chemoattractant protein (MCP)-1. In a study carried out by Kurihara and colleagues it was demonstrated that deletion of the CCR2 gene led to decreased recruitment of macrophages to the site of inflammation and CCR2-deficient mice were unable to clear infection. (Kurihara *et al.*, 1997). Recent evidence from this laboratory has demonstrated an increased expression of chemokines within the CNS of CD200-deficient (CD200<sup>-/-</sup>) and APP/PS1 mice and this was associated with increased infiltration of peripheral immune cells including macrophages (Denieffe *et al.*, 2013; Minogue *et al.*, 2013 in review).

One of the main roles of macrophages is phagocytosis, which is defined as the uptake of particles greater than 0.5µm. The primary function of this process is the removal of dying or damaged cells and pathogens to maintain homeostasis, and for example, it has been suggested that macrophages remove almost  $2 \times 10^{11}$  erythrocytes each day (Mosser & Edwards, 2008). Phagocytosis is a crucial part of the immune response mediating the ingestion, destruction and processing of foreign materials and macrophages express receptors including TLRs and scavenger receptors (SRs) that mediate this process. Interaction of receptors on macrophages with ligands on the surface of the particle induces a signalling cascade that results in the plasma membrane surrounding the particle and internalising. The ingested material is then localised to the phagosome, which is a highly acidic environment equipped with peptides that degrade microbial components (Flannagan *et al.*, 2009). Macrophages also function as antigen presenting cells by displaying antigens from digested pathogens on their cell surface; this allows interaction with T cells and initiates the adaptive immune response.

Upon activation, macrophages release inflammatory mediators, such as pro-inflammatory cytokines, however they also express cell surface markers which enable macrophages to communicate with other cells of the immune system. Activated macrophages exhibit increased expression of MHCII which is involved in antigen presentation and allows communication between the innate and adaptive immune response. Increased expression of CD40 is a key feature of activated macrophages, CD40 receptor is expressed on macrophages while CD40 ligand is expressed on T cells, this allows further communication with the adaptive immune response.

While activation of macrophages is crucial in mediating the host immune response, if inflammation becomes dysregulated it can cause damage to nearby healthy tissue, this is referred to as the bystander effect. As a result it is of great importance that inflammatory responses are tightly regulated and several endogenous mechanisms exist to support this. These include expression of immune regulatory molecules like CD200. CD200 is an immunosuppressive molecule and the interaction of the ligand with its receptor, which is expressed on macrophages, maintains these cells in a quiescent state. Consistent with its modulatory role, it has been demonstrated that macrophages from CD200<sup>-/-</sup> mice exhibit an enhanced response to TLR stimulation (Snelgrove *et al.*, 2008).

In the last 100 years since the first description of macrophages, research has exposed the complexity of macrophage function. As stated by David Mosser 'macrophages may be more heterogeneous group of cells than originally appreciated, with different physiologies and performing immunological functions' (Mosser, 2003). These groups of macrophages are broadly divided into M1 and M2 activated cells, which in the simplest possible terming perform pro and anti inflammatory functions respectively.

### **1.3 M1 activation and markers of the M1 activation state**

As already described, macrophages become activated in response to changes within the tissue environment. M1 or classical activation of macrophages describes a shift in the cell phenotype from a quiescent state to a pro-inflammatory profile. The M1 state can be initiated by the Th1-derived cytokine IFN $\gamma$ , a PAMP such as LPS or DAMPs such as ATP or high mobility group box 1 (HMGB1). HMGB1 is primarily found within the nucleus and is released from necrotic cells but not apoptotic cells, which suggests that controlled cell death does not result in macrophage activation. HMGB1 has been shown to activate TLR4 signalling and results in the secretion of pro-inflammatory cytokines such as IL-1 $\beta$ , TNF $\alpha$  and IL-6. M1 macrophages are recruited to initiate the host response with the subsequent release of pro-inflammatory mediators.

IFN $\gamma$  was discovered almost 50 years ago in 1965 and has since been identified as a potent activator of macrophages/microglia (Brewington *et al.*, 2001). It is produced by a number of cells in the periphery including T cells and natural killer (NK) cells (Benveniste, 1998). IFN $\gamma$ -induced stimulation of macrophages induces a robust increase in the expression of TNF $\alpha$  and NOS2, the two archetypal markers of the M1 state. IFN $\gamma$  treated macrophages also exhibit an increased expression of other activation markers such as MHCII, CD40, ICAM-1 and NOD2. Macrophages exposed to IFN $\gamma$  exhibit increased antigen presentation capabilities and chemokine production which results in enhanced communication with other immune cells. Mice lacking IFN $\gamma$  or IFN $\gamma$ R1 exhibit a diminished ability to clear infection humans exhibiting a mutation in IFN $\gamma$ R are more susceptible to mycobacterial infection (Schroder *et al.*, 2004).

Activation of macrophages through PRR has been extensively studied; LPS, a component of gram negative bacteria, is frequently used to induce inflammation in experimental models (Lund *et al.*, 2006). LPS mediates its effects through TLR4, and induces a robust activation of macrophages, leading to the release of cytokines and expression of cell surface markers. The activation of TLRs is crucial to mount an immune response and macrophages from TLR4-deficient mice fail to mount a response to LPS treatment. While activation of TLRs is crucial in activating the immune system to mounting an effective immune response, it is also implicated in the pathogenesis of autoimmune, chronic inflammatory and infectious diseases

### **1.3.1. TNF $\alpha$ and NOS2 are the archetypal markers of the M1 activation state**

#### **1.3.1.1. TNF $\alpha$**

TNF $\alpha$  was first identified nearly 40 years ago as a product of macrophages (Carswell *et al.*, 1975). TNF $\alpha$  inherited its name from its ability to induce cell death especially in tumour cells, though it also has the ability to activate an effective immune response to combat infection when necessary (Walczak, 2011). TNF $\alpha$  can exist in two forms, as a membrane-associated protein and also in a soluble form (Locksley *et al.*, 2001). The membrane-bound TNF $\alpha$  forms heterodimers (Walczak, 2011) which the metalloprotease, TNF $\alpha$  converting enzyme (TACE), can cleave to release the soluble cytokine (Black *et al.*, 1997). Release of pro-inflammatory cytokines such as TNF $\alpha$  from microglia/macrophages is an indication of increased activation of these cells (Lynch, 2009).

In addition to its role in the periphery TNF $\alpha$  exerts many actions within the CNS. As is the case with many pro-inflammatory cytokines, prolonged exposure can become harmful and it has been reported that mice that over-express TNF $\alpha$  exhibit a learning impairment (Fiore *et al.*, 1996). Other groups have suggested a beneficial role for TNF $\alpha$  following brain injuries, since it was found that wildtype mice were more likely to suffer memory deficits following cortical brain injury than TNF $\alpha$ -deficient mice (Scherbel *et al.*, 1999). It has been recently shown in this laboratory that TNF $\alpha$  has the ability to increase the release of IL-6 and IL-1 $\beta$  in primary mixed glia (Minogue *et al.*, 2012). TNF $\alpha$  has also

been implicated in the chronic activation of microglia observed in neurodegenerative diseases possibly as a result of its ability to act as an autocrine/paracrine signal for microglia (Mir *et al.*, 2008).

#### **1.3.1.2. NOS2**

Like TNF $\alpha$  upregulation, an increase in the expression of the enzyme nitric oxide synthase (NOS) 2 is considered to be a marker of M1 activation. NOS can exist in 3 isoforms NOS1, NOS3 and NOS2 (iNOS). The NOS1 and NOS3 isoforms (also known as nNOS and eNOS) are constitutively expressed, while the expression of NOS2 is induced during an immune response (MacMicking *et al.*, 1997). NOS activity ultimately leads to the production of NO through an enzymatic pathway involving the substrate arginine from which NO and citrulline are produced (Colton, 2009).

Activation of microglia/macrophages is associated with an increase in the production of NO (Kim *et al.*, 2004; Mir *et al.*, 2008). The production of NO promotes the death of bacteria and it can mediate cell death in a number of ways. Firstly, it can induce the phosphorylation of p53 which is involved in triggering apoptosis (Messmer *et al.*, 1994). It can also inactivate cytochrome oxidase in the mitochondria and, as a result, disrupt cellular respiration (Colton, 2009). Thirdly, NO can mediate the oxidative and nitrosative damage to proteins, through the production of superoxides (MacMicking *et al.*, 1997). Excessive activity of NOS2 can have severe detrimental effects, and NO has been postulated to have a critical role in mediating neurotoxicity associated with neuroinflammation (Dawson & Dawson, 1998). NOS2 immunoreactivity has been reported to be increased in the spinal cord of ALS patients (Sasaki *et al.*, 2000), while LPS-induced production of NO by microglia has been associated with motor neuron injury (Zhao *et al.*, 2004).

Although activity of NOS2/NO is associated with neurotoxicity, beneficial effects have been described when its production is regulated correctly. In fact, following head injury, NOS2-deficient animals perform worse in behavioural tasks compared to their wildtype counterparts (Colton, 2009). The activity of NOS2 is tightly controlled in normal circumstances anti-inflammatory cytokines such as IL-4/IL-13 (Colton, 2009) and TGF- $\beta$  (Mills, 2011) downregulate its activity.

### **1.3.2 Inflammatory Cytokines as markers of M1 activation**

#### **1.3.2.1. IL-1 $\beta$**

The increased expression of inflammatory cytokines like IL-1 $\beta$  and IL-6 are also considered markers of the M1 activation state. IL-1 $\beta$  is considered to be the classic pro-inflammatory cytokine, and was first described in the 1980's. It is produced by activated immune cells including macrophages and microglia. Systemic administration of IL-1 $\beta$  has been shown to induce sickness behaviour, and increased expression of other pro-inflammatory cytokines in the CNS. It has been consistently shown that IL-1 $\beta$  impairs the induction of long-term potentiation (LTP) and its concentration in the hippocampus is increased in the brains of aged rats in which a deficit in LTP has been shown (Minogue *et al.*, 2007). In the brain, microglia are considered to be the primary source of IL-1 $\beta$ . Exposure of microglia to A $\beta$  has been shown to result in increased expression of IL-1 $\beta$ ; consequently it is not surprising that increased expression of IL-1 $\beta$  has also been observed in the brain of individuals with Alzheimer's disease (AD) (Griffin *et al.*, 1989).

#### **1.3.2.2. IL-6**

IL-6 is described as a pleiotropic cytokine, which can have both pro-inflammatory and anti-inflammatory effects. The effect of IL-6 on cells is mediated through the IL-6 receptor (IL-6R) and gp130 which is a signal transducer utilised by cytokines related to IL-6; this leads to the activation of the JAK/STAT and mitogen-activated protein kinase (MAPK) cascade. The increased expression of acute phase proteins during an inflammatory response is chiefly mediated by IL-6 activity and it is thought that IL-6 plays a role in the recruitment of leukocytes to the site of infection. The role of IL-6 in inflammation is complex as conflicting reports have suggested beneficial and detrimental effects of this cytokine.

IL-6 is produced by a number of cells in the brain including microglia, astrocytes, neurons and endothelial cells (Akiyama *et al.*, 2000). Under resting conditions IL-6 expression within the CNS is low; however it can be dramatically increased during inflammatory events. LPS-induced microgliosis was inhibited by injection of anti-IL-6 antibody suggesting that it induces inflammation which can have a detrimental effect in the

brain. However, it has also been shown that IL-6-deficient mice exhibit slower recovery following traumatic brain injury (Spooren *et al.*, 2011), identifying a reparative role. Work from this laboratory has suggested that in LPS-stimulated rat glia, IL-6 regulates IL-1 $\beta$  and TNF $\alpha$  production (Minogue *et al.*, 2012)

### **1.3.3. Cellular proteins as markers of M1 activation**

#### **1.3.3.1. MHCII**

MHCII is expressed on antigen presenting cells (APC) which includes macrophages and microglia, and its upregulation is a hallmark of activated cells. It has been proposed that expression of MHCII promotes phagocytosis of pathogens and cellular debris which contributes to increased release of pro-inflammatory mediators (Lynch, 2009). Its expression on macrophages/microglia, together with expression of co-stimulatory molecules CD80 and CD86, allows interaction with T cells (Frank *et al.*, 2006). It has been suggested that MHCII upregulation is an indicator of the M1 activation state in macrophages and microglia (Colton & Wilcock, 2010).

#### **1.3.3.2. CD11b**

CD11b is constitutively expressed on macrophages/microglia; and is used in many studies using flow cytometry to identify them within a mixed population of cells. It acts as a binding receptor for ICAM-1 and inactive complement 3b, and is believed to play a role in adhesion and migration. The expression of CD11b is increased following macrophage/microglial activation (Streit *et al.*, 1999) and is observed on microglia following treatment with LPS or amyloid- $\beta$ . CD11b upregulation is also reported with age (Sandhir *et al.*, 2008), in an animal model of AD and it is also increased following brain injury (Lynch, 2009). Indeed the degree of microglial activation appears to correlate closely with the increase in CD11b expression (Roy *et al.*, 2008).



#### 1.3.3.3. CD40

CD40 is a member of the TNF receptor superfamily and is expressed on many immune cells such as macrophages and dendritic cells (Nguyen & Benveniste, 2002). Microglia express CD40 within the CNS, although expression is low under resting conditions though it is increased with age and following inflammatory stimuli (Lynch 2009). LPS treatment of macrophages or co-treatment of macrophages with Th1 cells leads to an increase in CD40 expression *in vitro* (Aloisi *et al.*, 1998; Aloisi *et al.*, 2000; Nguyen & Benveniste, 2000; Lynch, 2009). CD40 ligand (CD40L), also known as CD154 is expressed on T cells (Henn *et al.*, 1998). CD40-CD40L interaction is involved in the development of many immune responses and this is a key mechanism in enabling the interactions between APC and T cells (Aloisi, 2001). Binding of CD40 on microglial cells with its ligand CD40L leads to an increased production of pro-inflammatory mediators such as TNF- $\alpha$ , IL-12 and NO (Chen *et al.*, 2006). Activated macrophages/microglia and CD40L-expressing T cells have been identified in MS and EAE (Gerritse *et al.*, 1996) and this may contribute to the inflammation observed in these conditions. Indeed upregulation of CD40 accompanies upregulation of other markers, including MHCII in M1 activated microglia (Colton & Wilcock, 2010). CD40 has also been implicated in a number of disorders that involve hyperactive immune response such as rheumatoid arthritis (RA) and MS (Aloisi, 2001). Blocking the CD40-CD40L interaction has been demonstrated to be beneficial in an animal model of MS (Gerritse *et al.*, 1996)

#### 1.3.3.4. CD68

CD68 is localised within the cell and it is a marker of lysosomal activity and phagocytosis (Lynch, 2009). It is a member of the lysosomal-associated membrane glycoprotein (LAMP) family (da Silva & Gordon, 1999), which play a role in maintaining the structure of the lysosomal membrane. The function of CD68 is still relatively unknown; but its location suggests a role in antigen processing or in the protection of lysosomal membranes against lysosomal hydrolases (Gottfried *et al.*, 2008).

### **1.3.3.5. NOD2**

NOD-like receptors (NLRs) are a family of PRR, which are localised intracellularly and involved in the recognition of peptidoglycan moieties from bacteria. NOD2 activation is induced by muranyl dipeptide (MDP) which is a peptidoglycan expressed by both gram-positive and gram-negative bacteria. It is suggested that MDP treatment in combination with TLR activation can induce the production of pro-inflammatory mediators (Moreira & Zamboni, 2012), though it has been argued by others that this is only. NOD2 mutations have been associated with susceptibility to infectious diseases such as leprosy and tuberculosis. Interestingly BMDMs from NOD2-deficient mice show no response to MDP stimulation (Pauleau & Murray, 2003), and a separate study reported that NOD2-deficient mice exhibit increased bacterial burden following oral administration of *Listeria* (Kobayashi *et al.*, 2005). NOD2 activation may also be involved in the recruitment of other immune cells to the site of infection, as NOD2 agonists have been shown to induce the production of RANTES (CCL5) by macrophages (Werts *et al.*, 2007).

### **1.3.3.6. ICAM-1**

Intracellular adhesion molecule 1(ICAM-1), also known as CD54, is an adhesion molecule that is important in the cell-cell interaction of endothelial cells and leukocytes. It is also expressed on other cells including macrophages and microglia. ICAM-1 interacts with LFA-1, a receptor found on leukocytes, and it is this interaction that allows binding of leukocytes to endothelial cells and migration into the tissue. Expression on macrophages and microglia is low under resting conditions, however LPS and IFN $\gamma$  stimulation have been shown to upregulate ICAM-1 expression. It is expressed on a number of cells and has been suggested that it mediates the infiltration of leukocytes.

## **1.4. M2 activation**

M2 or alternatively-activated macrophages, were first described by Siamon Gordon and colleagues in the early 1990's (Stein *et al.*, 1992) following their observation that treatment of macrophages with IL-4 induced expression of the mannose receptor (MRC1). The term alternative activation was adopted since these cells exhibited a distinct

morphology from classically-activated macrophages. In addition to MRC1 which is involved in endocytosis, M2 macrophages are characterised by increased expression of arginase which is involved in NO homeostasis, and the molecules chitinase-3-like-3 (Chi3li3) and found in inflammatory zone 1 (Fizz1), both of which are involved in the rebuilding of the extracellular matrix (Colton, 2009). The M2 state can also be induced by the anti-inflammatory cytokines IL-5 and IL-13 (Varin & Gordon, 2009).

M2 macrophages prevent excessive inflammation, mediating tissue repair and restoration of homeostasis (Gordon & Martinez, 2010) and this is thought to be initiated by the release of trophic factors. Although responses of M2 activated macrophages are beneficial in the resolution of the inflammatory response, it dampens the host immune response and it has been demonstrated that M2 macrophages promote *Leishmania* infection by inhibiting the Th1 response and macrophage-killing activity (Holscher *et al.*, 2006).

#### 1.4.1. Arginase 1

Arginase can exist in two isoforms, arginase 1 (Arg1) and arginase 2 (Arg2) that share approximately 60% amino acid sequence homology (Durante *et al.*, 2007). Arg1 is an inducible isoform that is upregulated in M2 macrophages, while Arg2 is a mitochondrial-associated protein constitutively expressed in many cells throughout the body (Colton, 2009). Arg1 is expressed at high levels within areas of the brain such as cerebellum, though expression of Arg2 appears to be low throughout the brain (Yu *et al.*, 2003). The role of Arg1 remains to be fully elucidated however a key function is considered to be in the regulation of NO production.

NO is produced by classically-activated macrophages/microglia via the processing of arginine by NOS2. Arginine is also a substrate for arginase, since the availability of arginine is the rate-limiting step in the production of NO by NOS2 (White *et al.*, 2006) arginase activity affects NO production by competitive inhibition (see figure 1.1). This increased expression of arginase observed in alternatively-activated macrophages enhances utilisation of arginine by arginase decreasing the amount of arginine available for NO production via NOS2 (Durante *et al.*, 2007). Evidence from studies using the BV2

microglial cell line showed that treatment with IL-4/IL-13 attenuated the IFN $\gamma$  increase in NOS2 and TNF $\alpha$  mRNA (Colton, 2009).

The role of arginase in alternative activation may not be limited to decreasing the levels of NO during inflammatory events as it has been suggested that arginase may also be involved in the production of ornithine-derived proline a precursor in the production of collagen (Munder *et al.*, 1999) which may be used in the rebuilding of the ECM.

#### **1.4.2. Mannose Receptor**

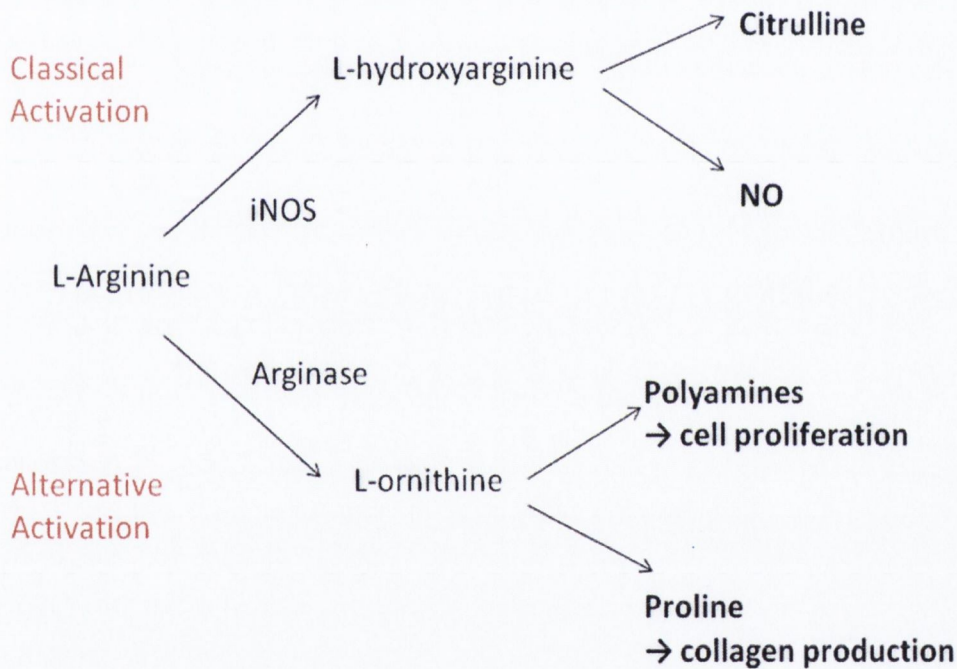
MRC1 is a member of the C-type lectins receptor (CLRs) family (Underhill & Ozinsky, 2002) and obtains its name from its ability to recognise sugars terminating in mannose, fucose or N-acetyl glucosamine (Taylor *et al.*, 2005). MRC1 mediates the phagocytosis of bacteria/yeasts (Burudi & Regnier-Vigouroux, 2001) and is highly expressed on alternatively-activated macrophages/microglia as well as dendritic cells.

MRC1 exists in a membrane-bound or soluble form (Burudi & Regnier-Vigouroux, 2001) and cleavage of the MRC1 from the cell membrane leads to release of the soluble form (Martinez-Pomares *et al.*, 1998). Engagement of the macrophage-bound MRC1 with its ligand initiates an anti-inflammatory signalling cascade which results in the production of IL-10 and IL-1Ra and a decrease in IL-12 and TNF $\alpha$  mRNA and protein (Colton, 2009).

MRC1 is a very reliable marker of alternative activation as, unlike other markers, it is not expressed in classically-activated macrophages/microglia and IFN $\gamma$  and LPS stimulation result in decreased expression of MRC1 (Colton, 2009). The important role of MRC1 is highlighted in MRC1 knock-out (MRC1<sup>-/-</sup>) mice. It was found that BMDMs cultured from MRC1<sup>-/-</sup> mice show an increased expression of CD40 in conjunction with increased IL-12 and IL-6 production (Paveley *et al.*, 2011). In the same study it was also found that CD45<sup>+</sup> macrophages from MRC1<sup>-/-</sup> mice exhibited decreased phagocytosis compared to wildtype mice.

### 1.4.3. Chitinase-3-like-3

Also known as YM1, chitinase-3-like-3 (Chi3li3) is another marker of alternative activation and is associated with rebuilding of the ECM. Chitin is a common structural element found in many organisms such as fungi and bacteria, however it is not found in mammalian tissue (Welch *et al.*, 2002) and this allows for the host defence to target chitin during an immune response. IL-4 causes a dramatic increase in the expression of Chi3li3 mRNA (Raes *et al.*, 2002b); this IL-4 induced increase is not observed in STAT6-deficient mice, suggesting that CHIL3 is induced after activation of the IL-4/STAT6 pathway (Welch *et al.*, 2002).



**Figure 1.1 L-Arginine metabolism.** L-arginine can be metabolised down two pathways, alternative and classical activation drive the activity of separate pathways (Adapted from Varin & Gordon 2009).

It has been suggested that Chi3li3 may have the ability to bind heparin sulphate (Raes *et al.*, 2002a), which increases the localisation of growth factors in the ECM. During

an inflammatory response the levels of heparin are decreased and binding of Chi3li3 to heparin is believed to decrease the loss of growth factors from the ECM which ultimately allows rebuilding of the ECM after an inflammatory event (Colton, 2009). Others have suggested that Chi3li3 may have a role in the recruitment of eosinophils (Owhashi *et al.*, 2000), suggesting a possible mechanism for macrophage and eosinophil communication. In further support of this function, it was demonstrated that blocking IL-4 and IL-13 activity or deletion of STAT6 prevents eosinophil recruitment in murine models of allergy (Welch *et al.*, 2002).

#### 1.4.4. Fizz1

Fizz1 also known as RELM- $\alpha$ , a member of the family of resistin-like molecules (RELM) is also upregulated in alternatively-activated macrophages/microglia. Fizz1 was first identified in the alveolar lavage of mice with experimentally-induced asthma (Nair *et al.*, 2003) and it has been suggested that Fizz1 may act in an anti-inflammatory manner during lung allergy and asthma (Nair *et al.*, 2009). Similar to YM1, Fizz1 has been implicated in the reconstruction of the extracellular matrix (ECM). Fizz1 upregulation increases the expression of collagen (Liu *et al.*, 2004b), which is integral to the integrity of the ECM.

IFN $\gamma$  decreases the IL-4-induced increase in the expression of Fizz1 in macrophages (Raes *et al.*, 2002a); in the same study the authors showed that both IL-4- and IL-4R-deficient mice showed no detectable levels of Fizz1. Although Fizz1 has been suggested to have roles in asthma and allergies in the periphery, its role within the CNS is still uncertain. A recent study published by Pesce and colleagues showed that following an immune challenge Fizz1-deficient mice show increased IL-4 and IL-13 levels compared with wildtype mice. The authors argued that the increase in the Th2-response suggests that Fizz1 is not merely a marker of alternative activation but may have a role in suppression of such responses (Pesce *et al.*, 2009).

## 1.5. Macrophage signalling

### 1.5.1. TLR4 signalling

Signalling through TLR4 is mediated through two different pathways, the MyD88-dependent and TRIF-associated (MyD88 independent) pathways (Figure 1.2). The MyD88-dependent pathway is conserved throughout most of the TLR family; only TLR4 and TLR3 employ an additional pathway (Akira, 2006). Signalling through the MyD88-dependent pathway is initiated through the interaction of the Toll/interleukin-1 receptor (TIR) domains of TLR4 and MyD88 (Horng *et al.*, 2002). MyD88 interacts with the interleukin-1 receptor-associated kinase (IRAK) family; the interaction of the death domains of MyD88 and IRAK4 allows the propagation of the signalling event (Kawai & Akira, 2007). Both pathways lead to the activation of a phosphorylation cascade which results in the activation of nuclear factor kappa-B (NF- $\kappa$ B) transcription factor. The TRIF-dependent pathway is referred to as the 'late phase' of NF- $\kappa$ B activation, as NF- $\kappa$ B activation occurs much earlier through the MyD88-dependent pathway which is aptly called 'early phase' (Buchanan *et al.*, 2010). NF- $\kappa$ B controls the expression of several genes that are involved in many processes within the CNS including synaptic plasticity and neurogenesis (Sarnico *et al.*, 2009a). Although NF- $\kappa$ B activation can ultimately be neuroprotective, it can also contribute to inflammatory processes and lead to apoptosis of cells after injury (Caso *et al.*, 2007; Sarnico *et al.*, 2009b).

### 1.5.2. The IFN $\gamma$ signalling pathway

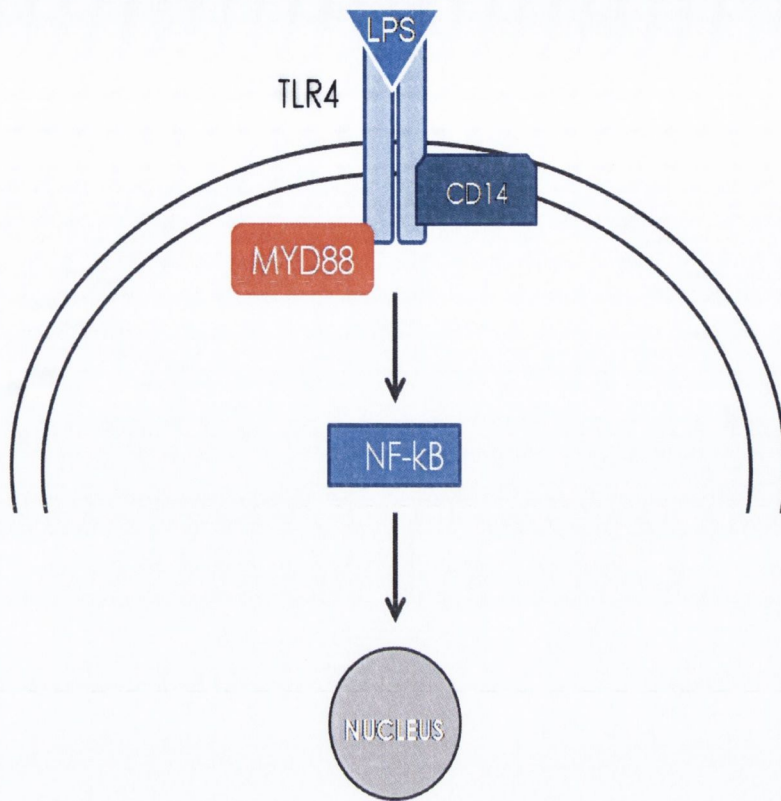
The IFN family comprises the, type I IFNs (such as IFN $\beta$ ) and type II IFN of which IFN $\gamma$  is the only member. While IFN $\gamma$  differs from the type I IFNs its inclusion within the IFN family occurred because of its ability to 'interfere' with microbial infections (Platanias, 2005). IFN $\gamma$  binding to its receptor induces a signalling cascade that utilises the Janus-activated kinases/signal transducer of transcription (JAK/STAT) pathway, ultimately inducing gene transcription (Saha *et al.*, 2010) (Figure 1.3).

The IFN $\gamma$  receptor comprises two subunits, IFN $\gamma$  receptor (IFN $\gamma$ R) 1 and 2. Both receptor subunits interact with a member of the JAK family; JAKs are non-receptor protein tyrosine kinases. IFN $\gamma$ R1 interacts with JAK1 while IFN $\gamma$ R2 interacts with JAK2. Binding of

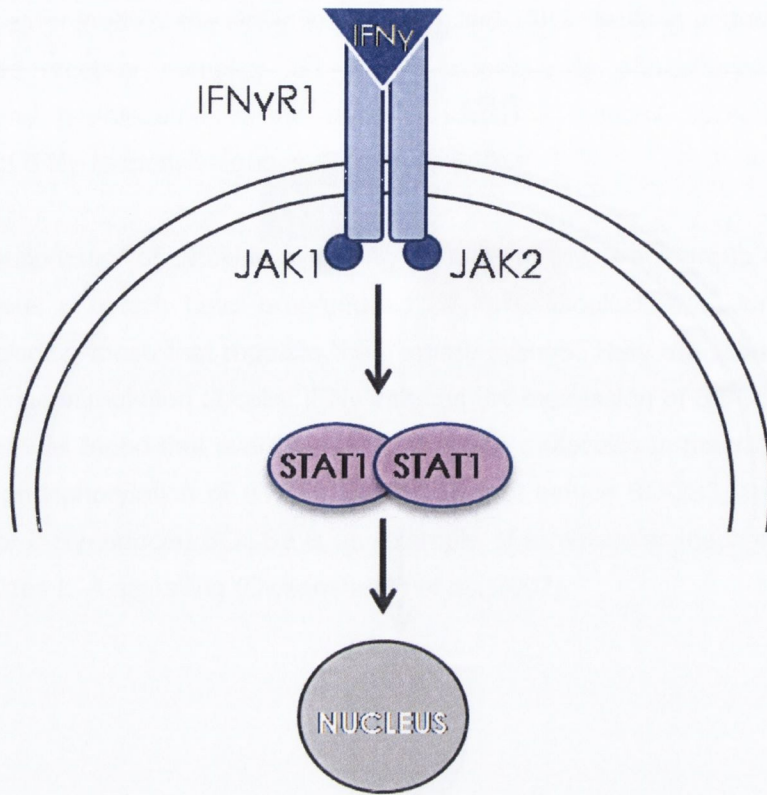
IFN $\gamma$  to its receptor leads to the activation of JAK1 and JAK2 resulting in the recruitment of STAT1 to the receptor complex. STAT1 is subsequently phosphorylated, forms a homodimer and translocates to the nucleus where it induces upregulation of the transcription of IFN $\gamma$ -responsive genes (Platanias, 2005).

The suppressor of cytokine signalling (SOCS) family, are a group of intracellular proteins, several of which have emerged as key physiological regulators of cytokine responses including those that regulate the immune system. They are robustly increased following cytokine stimulation of cells. IFN $\gamma$  induces the expression of SOCS 1 and 3 and, interestingly, it was found that overexpression of these molecules in macrophages blocks IL-4 induced phosphorylation of STAT6. IL-4 does not induce SOCS3, and it has been suggested that IFN $\gamma$ -induced SOCS3 is an example of a molecular mechanism by which IFN $\gamma$  antagonizes IL-4 signalling (Dickensheets *et al.*, 2007).





**Figure 1.2 TLR4 signalling pathway.** Binding of LPS to TLR4 initiates a signalling pathway lead to the activation of a phosphorylation cascade which results in the activation of nuclear factor kappa-B (NF- $\kappa$ B) transcription factor



**Figure 1.3 IFN $\gamma$  induced classical activation.** Interaction of IFN $\gamma$  with its receptor leads to the activation of JAK1 and JAK2, which induces the recruitment of STAT1 to the receptor complex. Following this STAT1 is subsequently phosphorylated, forms a homodimer and translocates to the nucleus where it induces gene transcription.

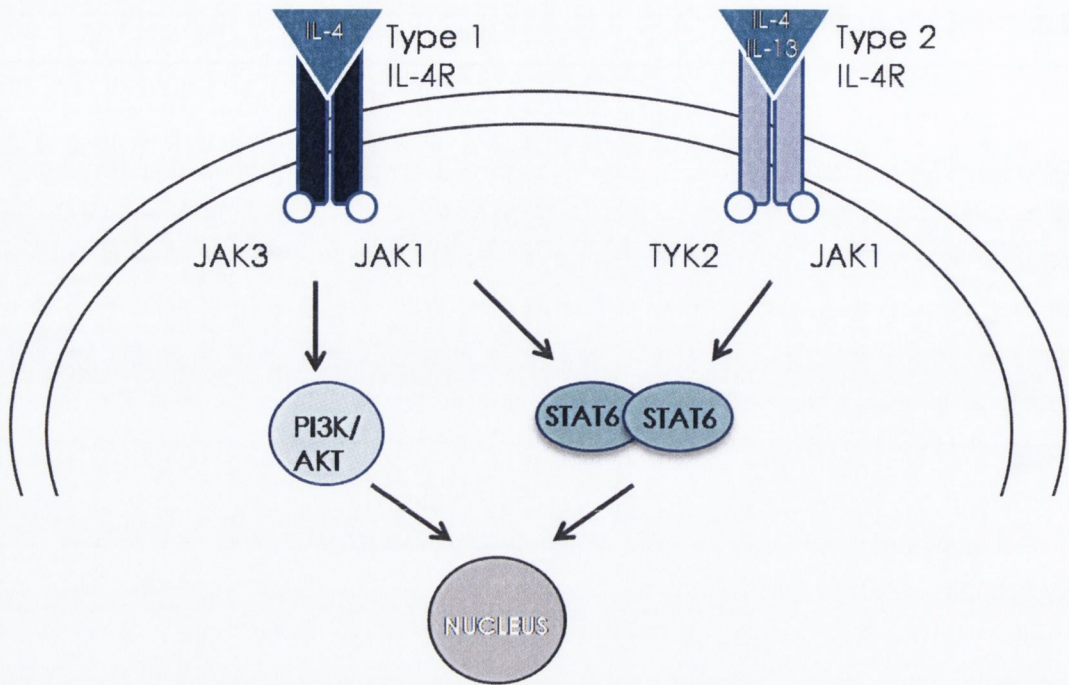
### 1.5.3. The IL-4 signalling Pathway

IL-4 and IL-13 signal transduction is mediated through the JAK/STAT pathway. IL-4 can signal through two receptors, type I IL-4 receptor (composed of two subunits, IL-4R $\alpha$  and IL-2R $\gamma$ ) and type II receptor (IL-4R $\alpha$  and IL-13R $\alpha$ 1). IL-13 can only signal through the type II receptor. IL-13 has a high affinity for the IL-13R $\alpha$ 2, though it has been suggested that this may act as a decoy receptor (Lupardus *et al.*, 2010). Type I IL-4 receptor is expressed predominantly on haemopoietic cells while type II IL-4R is found on non-haemopoietic cells, however some cells such as macrophages express both types (Gordon & Martinez, 2010).

Ligation of IL-4 to type I IL-4R induces the heterodimerisation of the IL-4R $\alpha$  and IL-2R $\gamma$  subunits leading to activation of JAK1 and JAK3. JAK1 phosphorylates the tail of the receptor which acts as a docking site for STAT6 where it is phosphorylated. STAT6 forms a homodimer with other phosphorylated STAT6 molecules and translocates to the nucleus where it causes the induction of IL-4-responsive genes (Varin & Gordon, 2009) (Figure 1.4). Activation of type II IL-4R also results in the homodimerisation of STAT6 though this is mediated through the activation of JAK1 and tyrosine kinase 2 (Tyk2). Unlike type I IL-4R, type II IL-4R can be activated by either IL-4 or IL-13. The type I IL-4R also activates IRS-1/2 which results in activation PI3K and RAS-MAPK and induces cell proliferation (Gordon & Martinez, 2010). IL-4 treatment also decreases the expression of Sh2-containing inositol phosphatase (SHIP), a negative regulator of the PI3K/AKT pathway, and this leads to increased activation of the PI3K/AKT pathway.

### 1.5.4. PI3K/AKT Pathway

The phosphatidylinositol 3-kinases (PI3K) /AKT pathway is involved in a number of cellular processes including differentiation, proliferation, survival, metabolism, autophagy, and motility. The PI3Ks are a family of lipid kinases that phosphorylate the 3-hydroxyl group of phosphoinositides. Although there are several different classes of PI3K, IL-4 stimulation activates the complex of two subunits, 85-kDa regulatory (p85) and 110kDa catalytic (p110). The central mediator of the PI3K pathway is AKT, a serine



**Figure 1.4. The IL-4 signalling pathway.** Activation of the Type 1 and Type 2 IL-4R leads to the downstream activation of STAT6. The Type 1 IL-4R mediates its effects through JAK1 and JAK2, while the Type 2 IL-4R associates with TYK2 and JAK1, ligation of these receptors leads to the recruitment of STAT6 to the receptor complex. Following this STAT6 is subsequently phosphorylated, forms a homodimer and translocates to the nucleus where it induces gene transcription. Interaction of IL-4 with its Type 1 receptor also induces the activation of the PI3K/AKT pathway, which is involved in the regulation of a number of cellular processes.

/threonine kinase and recent reports suggest that AKT act on as many as 40 substrates. Although this pathway is involved often crucial to the survival and proliferation of cells, hyperactivation of this pathway has been implicated in some human cancers.

The PI3K/AKT pathway is activated by molecules including growth factors and cytokines, leading to the activation of receptor tyrosine kinases (RTKs) on the cell surface. Activation of RTKs leads to interaction of the p85 catalytic subunit of PI3K with the tyrosine kinase domain; binding leads to the liberation of the p110 subunit and this leads to full activation of PI3K. IL-4 signalling triggers activation of IRS1/2, which contains phosphotyrosine residues that interact with the Src homology 2 (Sh2) domain of p85 subunit of PI3K which activates p110 activity. The p110 subunit converts phosphatidylinositol-4,5-biphosphate 2 (PIP<sub>2</sub>) to PIP<sub>3</sub>, which acts as a docking site which allows the interaction of PDK1 and AKT. This results in phosphorylation of AKT. AKT mediates its effects through which phosphorylation of several proteins (Minichiello, 2009).

In recent years, focus has turned to the possible role the PI3K/AKT pathway plays in modulating inflammation. Activation of the PI3K/AKT pathway by IL-4 has been shown to be important in the induction of the M2 state, and the evidence indicates that IL-4-induced Arg1, Chi3li3 and Fizz1 is significantly attenuated when this pathway is inhibited. Other groups have also demonstrated that TLR-induced cytokine production is negatively regulated by the PI3K/AKT pathway (Luyendyk *et al.*, 2008). Consequently, it has been demonstrated that LPS induced TNF $\alpha$  production is exaggerated in the THP-1 monocytic cell line following PI3K inhibition (Guha & Mackman, 2002).

## **1.6. The blood-brain barrier**

The blood-brain barrier (BBB) is a physical barrier composed of endothelial cells that line the cerebral vessels and act as a barrier separating the periphery and the CNS. The BBB has several roles in brain homeostasis; it allows the entry of many essential nutrients and metabolites as well as orchestrating the removal of waste products. The BBB is connected to neurons through astrocytes and maintains a stable environment for neurons, maintaining the appropriate ion concentration within the brain that is essential for synaptic function. The integrity of the BBB is determined by tight junctions, which are

domains of occluded intercellular clefts and the two tight junction proteins most extensively studied are occludin and claudins. Occludin is thought to be involved in the regulation of paracellular permeability and cell adhesion, while it has been suggested that claudins establish the barrier properties and may mediate paracellular ion permeability.

Recent evidence from this laboratory has demonstrated an increase in the permeability of the BBB in aged rats and mouse models in which neuroinflammation were marked (Blau *et al.*, 2012; Denieffe *et al.*, 2013; Kelly *et al.*, 2013) and this was associated with increased infiltration of peripheral immune cells including T cells. Although the concentration of IFN $\gamma$  in the brain is negligible under normal conditions, increased concentration of the Th1 cytokine IFN $\gamma$  has also been observed in the brains of these animals. Predictably increased IFN $\gamma$  was accompanied with evidence of increased microglial activation and this is consistent with the suggestion that the infiltration of peripheral cells into the CNS is the source of IFN $\gamma$  (Benveniste, 1998). The origin of IFN $\gamma$  within the CNS is still undetermined and a great deal of evidence suggests its expression in resident cells is very low. However studies have detected IFN $\gamma$  mRNA expression in neurons, astrocytes and microglia (Neumann *et al.*, 1997; De Simone *et al.*, 1998) and although De Simone and colleagues showed that isolated cultures of microglia and astrocytes expressed IFN $\gamma$  mRNA; no detectable levels of IFN $\gamma$  protein were demonstrated. Overall, it seems likely that whenever IFN $\gamma$  concentrations in the brain are detected, the sources is infiltrating cells.

### **1.7. Aging**

Many studies have demonstrated an age-related increase in inflammation in the brain (Maher *et al.*, 2005; Nolan *et al.*, 2005; Griffin *et al.*, 2006; Lyons *et al.*, 2009), and this is characterised by increased levels of pro-inflammatory cytokines such as IL-1 $\beta$ , IL-6 and TNF $\alpha$  (Godbout *et al.*, 2005; Maher *et al.*, 2005). These increases in are also seen in neurodegenerative disorders, especially Alzheimer's disease (Mrak & Griffin, 2005; Griffin, 2006) (Stout & Suttles, 2005)

Among possible explanations for the increase in microglial activation seen in the aged brain; is the loss of immunosuppressive molecules such as CD200 and fractalkine

(Lyons *et al.*, 2007a; Lyons *et al.*, 2009). Microglia express the receptors for both of these molecules and their activation helps to maintain microglia in a quiescent state. A decrease in CD200 with age was accompanied by an age-related increase in major histocompatibility complex II (MHCII), a marker of microglial activation (Lyons *et al.*, 2007a) and this increased in MHCII was reversed by injection of CD200 fusion protein. Similarly age-related increases in CD40, IL-1 $\beta$  and MHC II mRNA were associated with a decrease in fractalkine protein and mRNA (Lyons *et al.*, 2009). Evidence has also revealed that a decrease in the expression of anti-inflammatory cytokines, such as IL-4 and IL-10 occurs with age (Frank *et al.*, 2006) (Ye & Johnson, 2001; Maher *et al.*, 2005; Nolan *et al.*, 2005).

Another possible explanation for the inflammatory environment within the aged brain is the age-related changes observed in BBB permeability (Pakulski *et al.*, 2000; Pelegri *et al.*, 2007; Blau *et al.*, 2012) and this is often accompanied by infiltration of cells (Lynch, 2013). Entry of peripheral immune cells may exacerbate any inflammatory activity that already exists in the brain and Browne and colleagues showed that injection Th1 cells markedly increased inflammation (Browne *et al.*, 2013a). A detailed study carried out by Allan and colleagues demonstrated that macrophages induced cell death in organotypic hippocampal slices (Girard *et al.*, 2013). Whereas the infiltration of macrophages in CD200<sup>-/-</sup> mice increased inflammation (Denieffe *et al.*, 2013). Previous studies have reported an increase in the concentration of IFN $\gamma$  in the CNS of aged animals (Maher *et al.*, 2005; Clarke *et al.*, 2008), and this has been attributed to the infiltration of peripheral immune cells.

Although it is clear that age is accompanied by inflammatory changes in the brain, there are conflicting reports relating to the effect of age on macrophage function. Some studies have suggested that LPS-induced IL-1 $\beta$ , TNF $\alpha$  and IL-6 production is decreased in macrophages from aged rodents (Stout & Suttles, 2005) and this has been attributed to the reduction in TLR expression (Renshaw *et al.*, 2002). However, another study has suggested that TLR expression is unchanged and that intracellular signalling is compromised in macrophages from aged animals (Boehmer *et al.*, 2004). This decreased response to inflammatory activity has been proposed as an explanation for the observation that aged individuals are more susceptible to infection. An age-related decrease in the number of macrophages in tissue has also been proposed as a factor

which contributes to the susceptibility of aged mice to streptococcal infection (Goldmann *et al.*, 2010). It has also been reported that LPS-induced TNF $\alpha$  production was increased in macrophages from aged compared to young rats (Tang *et al.*, 2000) and several studies showed that LPS-induced IL-6 and IL-8 production was significantly increased in monocytes isolated from aged, compared with young, human subjects (Plowden *et al.*, 2004). Kohut and colleagues showed that age-related responses in macrophages were variable depending upon the nature of the stimulus and the origin of the macrophages (Kohut *et al.*, 2004). They demonstrated that splenic and alveolar macrophages from aged animals exhibited an enhanced response to a combined LPS and IFN $\gamma$  stimulation, although peritoneal macrophages from aged animals showed an impaired response.

### 1.8. Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common form of dementia affecting over 35.6 million people worldwide. Auguste D was the first documented case and was described in 1906 by Alois Alzheimer; she suffered from progressive cognitive decline and disorientation. Postmortem analysis of the AD brain reveals extensive neuronal loss, the presence of A $\beta$  plaques and neurofibrillary tangles. The accumulation of aberrant proteins is thought to contribute to the progressive neuronal loss that leads to subsequent symptoms.

Similar to other neurodegenerative disorders, evidence of increased inflammation is observed in the parenchyma of the AD brain. Post-mortem analysis of AD brains revealed enhanced expression of MHCII, a classic marker of microglial activation (Luber-Narod & Rogers, 1988). Increased expression of the pro-inflammatory cytokines IL-1 $\beta$  and IL-6 has also been observed in the AD brain (Wood *et al.*, 1993) and activated microglia have been shown to surround A $\beta$  plaques (Combs, 2009). In experimental systems it is well documented that A $\beta$  activates microglia *in vivo* and *in vitro* (Clarke *et al.*, 2007; Lyons *et al.*, 2007b; Lull & Block, 2010). Studies using imaging techniques have reported that there is a correlation between microglial activation and cognitive decline in patients with AD (Edison *et al.*, 2008) and mild cognitive impairment (Okello *et al.*, 2009).



The presence of A $\beta$  plaques is one of the hallmarks of AD pathology. A $\beta$  is produced by the enzymatic cleavage of the type 1 transmembrane glycoprotein, amyloid precursor protein (APP). The primary function of APP remains largely unknown although it has been suggested that it may regulate neurite outgrowth. It has also been demonstrated that APP deficient mice exhibit impaired LTP as well as impaired performance in spatial learning (Seabrook *et al.*, 1999; Ring *et al.*, 2007). APP cleavage can occur through two distinct pathways, the amyloidogenic and the non-amyloidogenic pathways, this proteolytic action is carried out by three enzymes,  $\alpha$ -, $\beta$ - and  $\gamma$ -secretases. The amyloidogenic pathway results in the production of A $\beta$ ,  $\beta$ -secretase cleaves APP resulting in the production of sAPP $\beta$  and a membrane bound C-terminal fragment which is further cleaved by  $\gamma$ -secretase resulting in the production of A $\beta$  peptide.  $\gamma$ -secretase may cleave this fragment at several sites, resulting in the production of a number of A $\beta$  species varying in size (39-43 amino acids). A $\beta$ <sub>1-40</sub> is the most common product compared to the less abundant A $\beta$ <sub>1-42</sub>. In the non-amyloidogenic pathway, APP is cleaved by  $\alpha$ -secretase which results in the production of sAPP $\alpha$  and a short membrane bound C-terminal fragment. However since  $\alpha$ -secretase cleaves APP within the A $\beta$  domain subsequent cleavage by  $\gamma$ -secretase does not result in A $\beta$  generation.

Age is the biggest risk factor for AD; however there are a number of genes that may act as risk factors. Familial AD (FAD) is associated with approximately 5-10% of cases of AD and most cases of FAD can be attributed to mutations in three genes APP, and presenilin (PS) 1 and 2 (Mattila *et al.*, 1998). A double mutation in APP found in a Swedish family (APP<sub>swe</sub>) has been found to increase the cleavage of APP by  $\beta$ -secretase, and this has been shown to favour the production of A $\beta$  (Haass *et al.*, 1995). PS1 and PS2 are catalytic subunits of  $\gamma$ -secretase, mutations in these genes have also been associated with increased A $\beta$ <sub>1-42</sub> production (Albani *et al.*, 2007). Identifying genes associated with AD has allowed the development of genetically-modified mouse models of AD. Although these animals do not exhibit enhanced neuronal loss they do exhibit other hallmarks of AD, such as A $\beta$  plaques, microglial and astrocyte activation and cognitive deficits (Eriksen & Janus, 2007). One of the most commonly used mouse models of AD is the APP<sub>swe</sub>/PS1dE9 (APP/PS1), both of these mutations are associated with increased A $\beta$  production. Accumulation of A $\beta$  within the brains of these mice has been observed as early as 4 months of age and plaque numbers increase with age (Garcia-Alloza *et al.*, 2006).

In the last decade evidence is mounting that peripheral immune cells may play a role in the pathology of AD. An increase in BBB permeability has been observed in AD patients (Desai *et al.*, 2007), and evidence from this laboratory has also demonstrated that integrity of the BBB is compromised in a mouse model of AD (Kelly *et al.*, 2013). Attention has now turned to the possible roles infiltrating peripheral immune cells may play in AD pathology and specifically the infiltration of IFN $\gamma$ -producing cells has been proposed to increase inflammation within the CNS. Recent evidence from this laboratory has shown that intrahippocampal injection of IFN $\gamma$  induced microglial activation in mice (Denieffe *et al.*, 2013).

There are several studies that report macrophages infiltrate the CNS in mouse models of AD (Simard *et al.*, 2006; El Khoury *et al.*, 2007; Kelly *et al.*, 2013). It has also been proposed that these infiltrating macrophages may play a role in the clearance of A $\beta$  (Simard *et al.*, 2006; El Khoury *et al.*, 2007) and work from this lab has shown that infiltrating macrophages exhibit an increased expression of CD68, which is thought to be indicative of phagocytic activity (Kelly *et al.*, 2013). Simard *et al* demonstrated that these macrophages associate with plaques and El Khoury and colleagues noted that inhibiting the entry of macrophages into the brains of a transgenic model led to an increase in A $\beta$  accumulation. Other studies have shown that perivascular macrophages play a role in A $\beta$  clearance; loss of perivascular macrophages is associated with an increase in A $\beta$  burden (Hawkes & McLaurin, 2009). Interestingly the same group also demonstrated that stimulating an increase in the turnover of perivascular macrophages results in enhanced A $\beta$  clearance. These groups concluded that infiltration of macrophages was beneficial in models of AD. Conversely, it has been shown that monocytes from AD patients exhibit a decreased ability to phagocytose A $\beta$  (Fiala *et al.*, 2005)

## 1.10 Aims

The present studies were prompted by considering how macrophages which infiltrate a brain in which inflammatory changes are ongoing, specifically the brains of aged animals and APP/PS1 mice, might respond to the inflammatory Stimuli. The specific aims of the study are:

1. To investigate whether macrophages infiltrate the brains of young or middle-aged rats. To examine the phenotype of BMDMs cultured from young and aged rats, and their responses to inflammatory stimuli.
2. To investigate the phenotype of BMDMs cultured from WT and APP/PS1 mice, and their responses to inflammatory stimuli and to assess the impact of CD200 expression on BMDM phenotype.
3. To investigate the differential effects of IL-4 and IFN $\gamma$  on PI3K/AKT activity in BMDMs and to determine the role of the PI3K/AKT pathway in the induction of M2 activation.

## Chapter 2

### Materials and Methods

## 2.1. Animals

All animals were maintained in a specific pathogen-free environment under constant veterinary supervision at the Bioresources Unit of Trinity College Dublin. Animals were housed in groups of 2 or 3 per cage, at 22-23°C with a 12-hour light-dark cycle. All animals used in these studies had free access to food and water and were fed a standard laboratory diet. Experiments were carried out under licence obtained from the Department of Health and Children (Ireland) under the Cruelty to Animals Act 1876, the European Community Directive, 86/609/EEC, and in agreement with experimental procedures laid down by the local ethics committee. During all studies, every effort was made to minimise stress to the animals. All animals were sacrificed by decapitation whilst under anaesthesia induced by urethane (1.5 g/kg; Sigma Aldrich; IRE).

Infiltration of monocytes into the brain was assessed in groups of young (4 months; n=4) and middle-aged (16 months; n=4) male rats. The bone marrow of femurs and tibias for preparation of Bone marrow-derived macrophages (BMDMs) were obtained from young (3-5 months) and aged (24-27 months) male Wistar rats. Hippocampal tissue was snap frozen for protein analysis.

APP/PS1 mice contain 2 transgenes inserted at a single locus; a chimeric APP and a deltaE9 mutation of human PS1. APP/PS1 and WT littermates (17-23 months; n=8-11) mice were used to obtain BMDMs. Mice were initially purchased from the Jackson Laboratory (USA) and bred with C57BL/6 animals. Hippocampal tissue was taken for cytokine and mRNA analysis, remaining tissue was analysed by flow cytometry to measure peripheral immune cell infiltration.

Male C57/BL6 mice were supplied by the Bioresources Unit of Trinity College Dublin. BMDMs were cultured from the bone marrow of tibia and femurs. Cells were harvested for protein and mRNA analysis.

## **2.2. Cell Culture**

### **2.2.1. Aseptic technique**

All cell culture-related experiments were carried out in a sterile laminar flow hood under aseptic conditions. The laminar flow hood was used to filter the air and eliminate any risk of contamination by airborne pathogens. To ensure that the flow hood remained sterile, it was sprayed with 70% ethanol (EtOH) in distilled water (dH<sub>2</sub>O) before and after use. Further to this, when all work was completed, the hood was exposed to UV light for 20 minutes. Any equipment used in the hood was sprayed down with 70% EtOH. Disposable latex gloves were also sprayed with 70% EtOH. Molecular grade pipette tips were used for all cell culture work and dH<sub>2</sub>O was autoclaved prior to use. All cells were maintained in an incubator at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.

### **2.2.2. Maintenance of L929 cell line**

L929 cells were kindly donated by Professor Luke o'Neill. L929 cells are a murine fibroblast cell line which is commonly used in experiments involving macrophages as a source of M-CSF. A bottle of Dulbecco's modified Eagle's medium (DMEM; 500ml; Invitrogen, UK) was supplemented with heat-inactivated foetal bovine serum (FBS; 50ml; 10%; Gibco, UK), penicillin-streptomycin (5ml; 1%; Gibco, UK) and fungizone (500µl; 0.25µg/ml Invitrogen, UK) were added into to yield complete DMEM (cDMEM) which was used in the preparation of cell cultures, including maintenance of L929 cells. cDMEM was stored at 4°C for up to 2 weeks. To differentiate bone marrow cells into BMDMs, L929 conditioned media was added to cDMEM (20%). Cells were grown in T175 cm<sup>2</sup> flasks until confluent and harvested using heat-activated trypsin. The cells were centrifuged at 850 x g for 4 minutes. The resulting pellet was resuspended in cDMEM and the cells were then counted as described in section 2.2.5. The cells were seeded in cDMEM (40ml) in T175cm<sup>2</sup> flasks (Sarstedt Ltd, UK) at a density of 0.5x10<sup>6</sup> cells/ml and maintained in culture for 1 week, at which time the L929 conditioned media was removed and the cells reseeded at 0.5x10<sup>6</sup> cells/ml in a fresh T175cm<sup>2</sup> flask. To ensure that there were no L929 cells present, the conditioned media was centrifuged (850 x g, 4 minutes), the upper 80% of supernatant collected and filter sterilised through a cellulose acetate membrane filter.

### **2.2.3. Cell treatments**

Reagents used for cell treatments were diluted to the appropriate concentration using pre-warmed culture media and all treatments were carried out for 24 hours unless stated otherwise.

LPS from *Escherichia coli* (Sigma Aldrich, UK) was diluted to a working concentration of 100ng/ml in cDMEM. A stock solution of recombinant IFN $\gamma$  (rat and mouse) (R&D Systems, UK) was prepared by reconstitution in sterile PBS containing BSA (0.1%) and a final concentration of 50ng/ml was used in these experiments. Recombinant IL-4 (rat and mouse) (R&D Systems, UK) was prepared as a stock solution in sterile PBS containing BSA (0.1%), all IL-4 treatments were carried out at a concentration of 200ng/ml. For experiments involving Ly290042 (10mM in DMSO; Merck Millipore, Germany), cells were pre-treated with Ly290042 (10 $\mu$ M) for 30 minutes prior to co-incubation with IL-4 or IFN $\gamma$  for 24 hours.

Following cell treatments, supernatants were removed using a sterile Pasteur pipette and transferred into sterile tubes and stored at -80°C for future analysis. Cells were harvested for PCR by washing once in PBS and adding RA1 buffer (350 $\mu$ l) and 2-mercaptoethanol (100:1); samples were stored in RNase free tubes at -80°C for RNA extraction. For protein analysis, cells were washed with PBS and lysis buffer (See Appendix) (70  $\mu$ l) was added; samples were stored at -80°C for later analysis.

### **2.2.4. Generation of bone marrow-derived macrophages (BMDMs)**

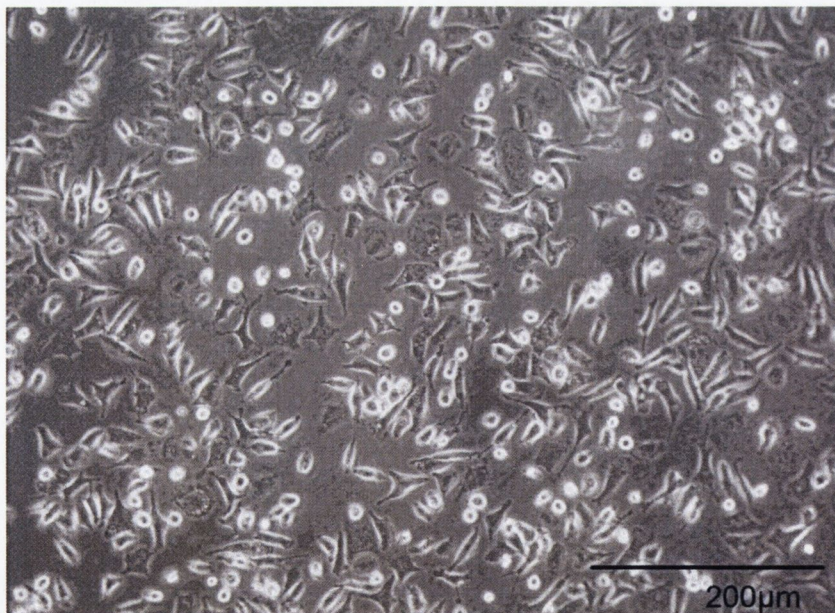
BMDMs were isolated from the marrow of the femurs and tibias. The legs of the animals were sprayed with 70% EtOH, the skin and muscle tissue then removed from the bones. The bones were sprayed with 70% EtOH, transferred to a sterile flow hood and cut at both ends. The marrow was then flushed out into a sterile falcon tube in cDMEM. The cell suspension was triturated using a sterile Pasteur pipette, filtered through a nylon mesh filter (40 $\mu$ m; BD Biosciences, US) into a sterile tube and centrifuged (400 x *g*, 5 minutes). The supernatant was removed and the pellet was resuspended in red blood cell lysis buffer (Sigma Aldrich, UK) to remove any red blood cells. The suspension was centrifuged

(400 x g, 5 minutes), supernatant was discarded, the cells were washed using cDMEM and centrifuged once more (400 x g, 5 minutes). The pellet was resuspended in 10 ml of cDMEM supplemented with L929 conditioned media (20%).

Cells were seeded in sterile cell culture flasks (rat: T175cm<sup>2</sup> flasks, mouse: T75cm<sup>2</sup> flasks). On day 2, non-adherent cells were removed from the flask, while fresh media was added to the original flask containing the adherent cells. On day 4, fresh L929 conditioned media (4ml) was added to the flask. The cells were cultured in the flasks for 6 days, after which time, they were transferred to 6-well plates ( $0.5 \times 10^6$  cells/well) and cultured for a further 2 days before treatment.

### 2.2.5. Cell counting

Cell counts were carried out using a haemocytometer. Cells were resuspended in a known volume of media and cell suspension (20µl) was loaded onto a glass haemocytometer. The cells were counted under a light microscope and the cell suspension was diluted to the desired density.



**Figure 2.1. Image of Rat BMDMs.**



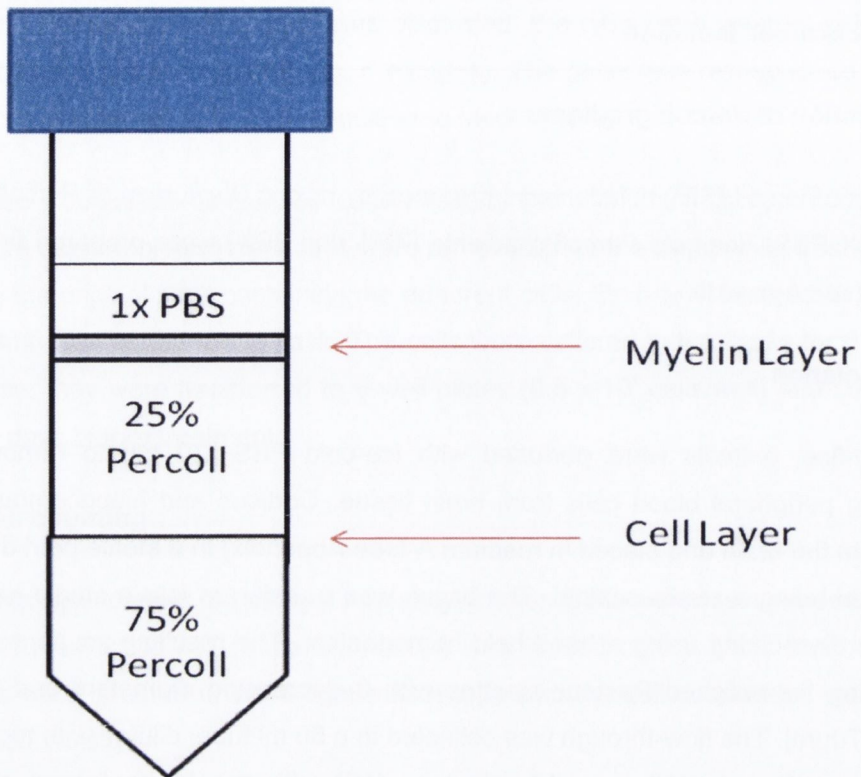
## **2.3. Mononuclear cell isolation**

### **2.3.1. Preparation of Percoll gradients**

A stock isotonic Percoll (SIP) solution was prepared by mixing 9 volumes of Percoll with 1 volume of 10X PBS. Ice-cold Percoll gradients (75% and 25%) were prepared from SIP using HBSS (see Appendix).

### **2.3.2. Cell isolation**

Prior to sacrifice, animals were perfused with ice-cold PBS (20 ml) to remove any contaminating peripheral blood cells from brain tissue. Cortices and hippocampus were dissected from the brain and placed in medium A (see Appendix) in a sterile petri dish and cross-chopped using a sterile scalpel. The tissue was transferred into a sterile tube and mechanically dissociated using a hand-held homogeniser. The resulting suspension was triturated using fire-polished Pasteur pipettes with 3 decreasing diameters and flushed onto a filter (70µm). The flow-through was collected in a 50 ml tube, diluted with medium A (25ml) and centrifuged (200 x *g*, 10 minutes, 4°C) with the brake turned off. The supernatant was discarded and the resultant pellet was re-suspended in 75% Percoll (10ml) and overlaid with 25% Percoll (10ml) and 1X PBS (6ml). This 3 layer gradient was centrifuged (800 x *g*, 30 minutes, 4°C) with the brake turned off, the tube was carefully removed from the centrifuge so as to not to disturb the gradient and the thick myelin-containing layer was collected at the 0-25% Percoll interface using a Pasteur pipette. The enriched microglia/monocyte population was collected at the 25-75% interface. Cells were gently removed using a Pasteur pipette and washed in 3 volumes FACS buffer (see Appendix) in preparation for analysis by flow cytometry (as described in section 2.4.2). Microglia were identified as CD11b<sup>+</sup>/CD45<sup>low</sup> cells, while infiltrating monocytes were identified as CD11b<sup>+</sup>/CD45<sup>high</sup> cells



**Figure 2.2. Percoll gradient, following centrifugation, for the isolation of glial cells from brain tissue.**

A 3-layer Percoll gradient was set up as illustrated above and centrifuged at  $800 \times g$  for 30 minutes. The 25-75% Percoll interface was collected for analysis by flow cytometry.

## 2.4. Flow cytometry

#### **2.4.1. Assessment of phagocytosis**

BMDMs from young and aged rats were seeded at a density of  $5 \times 10^5$  cells/ml in FACS tubes. Cells were incubated with fluorescently-labelled yellow/green latex beads (Absorbance/Emission=470/505nm; 1:200 dilution) for 2 hours. Media was replaced with trypan blue (0.2mg/ml) and samples were incubated for 2 minutes to quench extracellular fluorescence. Cells were washed 3 times with FACS buffer, centrifuged ( $350 \times g$ , 5 minutes) and resuspended in blocking solution (50% FBS in FACS buffer). Cells were centrifuged ( $350 \times g$ , 5 minutes), resuspended in FACS buffer with the appropriate antibodies for 30 minutes (see table 2.1.), and washed in FACS buffer 3 times. After the final wash, cells were re-suspended in FACS buffer (300 $\mu$ l) and immunofluorescence was immediately read on a DAKO CyAn-ADP 7 colour flow cytometer with Summit software v4.3 for acquisition. Further flow cytometric analysis was carried out using FlowJo v7.6.5.

Unstained cells and fluorescence minus one (FMO) tubes were used to gate the percentage of positive cells in any channel. Compensation beads were used to optimise fluorescence settings for multicolour flow cytometric analyses. These beads consist of two polystyrene microparticle populations, one which binds any  $\kappa$  light chain-bearing IgG and one lacking any binding capacity. When mixed together with a fluorochrome-conjugated antibody, the compensation beads provide distinct positive and negative populations used to manually set compensation levels.

Phagocytic BMDMs were represented as the percentage of CD11b<sup>+</sup> cells in the fluorescein isothiocyanate (FITC) channel.

#### **2.4.2. Cell surface staining**

For analysis of cell surface immunofluorescence by flow cytometry, cells were washed 3 times with FACS buffer, centrifuged ( $350 \times g$ , 5 minutes) and resuspended in blocking solution. Cells were centrifuged ( $350 \times g$ , 5 minutes), resuspended in FACS buffer with appropriate antibodies at room temperature for 30 minutes (see table 1 for mouse and rat antibodies) and washed in FACS buffer 3 times. After the final wash, cells were re-suspended in FACS buffer (300 $\mu$ l) and immunofluorescence analysis was carried out on a DAKO CyAn-ADP 7 colour flow cytometer with Summit software v4.3 for acquisition.

Further flow cytometric analysis was carried out using FlowJo v7.6.5. Unstained cells, FMOs and compensation tubes were set up as appropriate to gate the percentage of positive cells in any channel.

Antibody	Company	Dilution
<b>Anti-Rat CD11b Alexa Fluor 647</b>	Serotec Cat # MCA275A647	1:100
<b>Anti-Rat CD45 PE-Cy7</b>	BD Bioscience Cat # 561588	1:100
<b>Anti-mouse CD45 PE-Cy7</b>	BD Bioscience Cat # 552848	1:100
<b>Anti-mouse CD11b Alexa Fluor 647</b>	BD Bioscience Cat # 557686	1:100

**Table 2.1. Mouse and Rat antibodies used for flow cytometry.**

## 2.5. Western Immunoblotting

### **2.5.1. Preparation of cell lysates**

Following treatment, supernatant was removed and cells were gently washed with 1X PBS and incubated in lysis buffer (70µl) for 5 minutes on ice. Cells were removed using a sterile scraper and samples were centrifuged (14 000 x *g*, 10 minutes, 4°C). The resultant supernatant was collected and protein concentration assessed using the bicinchoninic acid (BCA) protein assay.

### **2.5.2. Preparation of tissue**

Hippocampal tissue was rapidly thawed and homogenised in RIPA solution (See Appendix). Protein concentration of samples was determined using a BCA assay kit. An 8 point standard curve with BSA concentrations ranging from 0 to 2µg/ml was prepared in RIPA. Samples and standards (25ul) were loaded in duplicate on a 96 well plate, BCA solution (200ul) was added to each well (50:1 Reagent 1 : Reagent 2). The plate was incubated (37°C, 30 minutes) and absorbance read at 562nm.

Samples were equalised for protein concentration and diluted with 4X Laemmli sample buffer (See Appendix) and heated to 80°C for 5 minutes.

### **2.5.3. Gel electrophoresis**

Proteins were separated by molecular weight on polyacrylamide gels (1mm thick) with a monomer concentration of 7.5% or 10%. Sample and protein ladder were loaded onto gels and run at 90V for approximately 1.5 hours.

Proteins were transferred to nitrocellulose membrane (0.45µm) which was blocked at room temperature for 1 hour using 5% non-fat dried milk in 1X Tris buffered-saline containing 0.05% Tween (TBS-T; See Appendix). Primary and peroxidase-conjugated secondary antibodies were prepared in 5% non-fat dried milk in TBS-T (10ml).

Membranes were incubated overnight at 4°C with primary antibody, washed for 1 hour with TBS-T and incubated with the appropriate secondary antibody for 2 hours at room temperature. Immunoreactive bands were visualised by chemiluminescence on a Fujifilm LAS-4000 system. Blots were stripped using Re-Blot Plus and re-probed for β-actin and immunoreactive bands were visualised as above.

Western Immunoblot Antibodies

Target	Company	MW	Dilution
AKT	Cell Signalling #9272	60 kDa	1:1000
pAKT	Cell Signalling #9721	60 kDa	1:1000
STAT1	Cell Signalling #9172	84,91 kDa	1:1000
pSTAT1	Cell Signalling #9171	84,91 kDa	1:1000
STAT6	Cell Signalling #5397	110 kDa	1:1000
pSTAT6	Cell Signalling #9361	110 kDa	1:1000
PI3K	Cell Signalling #42575	85 kDa	1:1000
pPI3K	Cell Signalling #42285	85 kDa	1:1000
HMGB1	Abcam #Ab18256	29 kDa	1:2000
Actin	Sigma Aldrich #A5316	42 kDa	1:5000

Table 2.2. Western immunoblot antibodies

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

### **2.6.1. Isolation of RNA**

RNA was extracted from lysates of BMDMs using a Nucleospin® RNAII KIT (Macherey-Nagel, Duren, Germany). Cells were scraped from 6-well plates in a cell lysis buffer containing RA1 buffer and 2-mercaptoethanol (100:1). Cell lysates were filtered using NucleoSpin Filters, collected in eppendorf tubes and centrifuged (11,000 x *g*, 1 minute). EtOH (70%, 350µl) was added to the filtrate, mixed and loaded onto NucleoSpin RNA II columns. Samples were centrifuged (11,000 x *g*, 30 seconds) to bind the RNA to the column. Membrane desalting buffer was added to desalt the silica membrane, which was dried by centrifugation (11 000 x *g*, 1 minute). To digest any DNA present, a DNase reaction mixture (95µl; 10µl DNase and 90µl reaction buffer per sample) was added to the column and incubated at room temperature for 15 minutes. RA2 buffer (200µl), which inactivates DNase, was added to the columns and centrifuged (11 000 x *g*, 30 seconds). The column was then placed in a new collecting tube, washed with 600µl of RA3 buffer and centrifuged (11 000 x *g*, 30 seconds). The flow through was discarded and the column was placed into a fresh collecting tube. For the final wash, RA3 buffer (250µl) was added to the column and centrifuged (11 000 x *g*, 2 minutes). The column was placed in a 1.5 ml RNase-free tube, RNA was eluted by addition of nuclease-free H<sub>2</sub>O (40µl) and centrifuged (11 000 x *g*, 1 minute).

### **2.6.2. RNA quantification**

RNA concentration was quantified using a NanoDrop-spectrophotometer (ND-1000 V3.5, Nanodrop Technologies Inc., USA). RNA concentration was measured based on absorbance at 260 nm and values were expressed as ng/µl. The ratio of absorbance at 260 and 280 nm (A<sub>260</sub>:A<sub>280</sub>) is indicative of RNA purity. A ratio of ~2.0 suggests that the RNA is of high quality and purity. All RNA samples used in these studies had an A<sub>260</sub>:A<sub>280</sub> ratio >1.7.

### **2.6.3. cDNA synthesis**

Equalised RNA was reverse transcribed into cDNA using a High-Capacity cDNA Archive Kit (Applied Biosystems, UK) according to the protocol supplied by the manufacturer. Briefly, master mix was prepared with 10X Reverse Transcription Buffer (1:5), 25X dNTPs (1:12.5), 10X Random Primers (1:5), MultiScribe Reverse Transcriptase (1:10) and nuclease-free H<sub>2</sub>O (1:2.4). For each sample, RNA (20µl) was added to 2X master mix (20µl) in a mini-centrifuge tube. Samples were incubated for 10 minutes at 25°C followed by 2 hours at 37°C on a thermocycler (PTC-200, Peltier Thermal Cycler, MJ Research, Biosciences Ireland). The cDNA was stored at -20°C until required.

#### **2.6.4. cDNA amplification by RT- PCR**

Real-time PCR primers and probes (see tables 2.3 & 2.4) were delivered as “TaqMan® Gene Expression Assays” (Applied Biosystems, Darmstadt, Germany).

cDNA (5µl) was added to a well of a PCR plate, along with nuclease-free water (15µl), target primer (2.5µl), β-actin primer (2.5µl) and TaqMan® Universal PCR Master Mix (25µl). Real-time PCR was performed on an Applied Biosystems ABI Prism 7300 Sequence Detection System v1.3.1 in 96-well format. Samples were measured in a single RT-PCR run. Each run comprised of 3 stages: 50°C for 2 minutes, 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. In all of the studies the target gene expression was measured relative to the endogenous control, β-actin.

#### **2.6.5. RT-PCR quantification**

All RT-PCR analysis was carried out using the delta CT method to determine gene expression. This method allows the user to determine the change in gene expression of treated samples compared to their endogenous controls. Fold changes in gene expression can then be assessed between groups.



List of Rat primers (Applied Biosystems, Darmstadt, Germany)		
Gene name	Gene description	Assay I.D.
CD40	Cluster of differentiation 40	Rn01423583-m1
CD11b	Cluster of differentiation 11b	Rn00709342-m1
CD68	Cluster of Differentiation 68	Rn01495634_g1
MHCII	Major Histocompatibility Complex II	Rn00585451_m1
TNF- $\alpha$	Tumour necrosis factor- $\alpha$	Rn99999017_m1
NOS2	Nitric Oxide Synthase 2	Rn00561646_m1
ARG1	Arginase 1	Rn00567522_m1
MRC1	Mannose Receptor	Rn01487342_m1
IL-1 $\beta$	Interleukin-1 beta	Rn00580432_m1
IL-6	Interleukin-6	Rn00561420_m1

**Table 2.3. Rat TaqMan Gene Expression Assay numbers.**

List of mouse primers (Applied Biosystems, Darmstadt, Germany)

Gene name	Gene description	Assay I.D.
CD40	Cluster of differentiation 40	Mm00441891_m1
CD11b	Cluster of differentiation 11b	Mm00434455_m1
MHCII (CITA)	Major Histocompatibility Complex II	Mm00482914_m1
TNF- $\alpha$	Tumour necrosis factor- $\alpha$	Mm00443258_m1
NOD2	Nucleotide-binding oligomerization domain-containing protein 2	Mm00467543_m1
ICAM-1	Intracellular Adhesion Molecule 1	Mm00516027_g1
NOS2	Nitric Oxide Synthase 2	Mm00440502_m1
Arg1	Arginase 1	Mm00475988_m1
MRC1	Mannose Receptor	Mm00485148_m1
Chi3li3	Chitinase-3-like-3	Mm00657889_mH
Fizz1	Found in inflammatory zone 1	Mm00445109_m1
IFN $\gamma$ R	Interferon gamma receptor	Mm00599890_m1

**Table 2.4. Mouse TaqMan Gene Expression Assay numbers.**

## 2.7. Cytokine analysis

Enzyme-linked immunosorbent assay (ELISA) was used to determine the cytokine concentrations in supernatant from BMDMs prepared from rats and mice. ELISA plates were coated with capture antibody and incubated overnight at 4°C (100µl; see Tables 4 and 5 for specific details). Plates were washed 3 times with wash buffer (200µl; PBS containing 0.05% Tween-20) and incubated at room temperature for a minimum of 1 hour in blocking buffer (200µl; Assay Diluent). Plates were washed 4 times with wash buffer (200µl), samples and standards were loaded (100µl in duplicate) and incubated at room temperature for 2 hours. Plates were washed 4 times with wash buffer (200µl) and incubated at room temperature with detection antibody (100µl; see Tables 4 and 5 for specific details). Plates were washed 4 times with wash buffer (200µl) and incubated in the dark at room temperature for 30 minutes with horseradish peroxidase-conjugated streptavidin (strep-HRP; 100µl; 1:200 dilution in Assay Diluent). Plates were washed 4 times with wash buffer (200µl) and incubated with substrate solution (100µl; 1:1 H<sub>2</sub>O<sub>2</sub>:tetramethylbenzidine) in the dark at room temperature until colour developed (approximately 20 minutes). The reaction was stopped using stop solution (50µl; 1M H<sub>2</sub>SO<sub>4</sub>) and absorbance was read at 450nm using a BioTek ELx800 Microtitre Plate Reader. Sample concentrations were determined by interpolating values from a standard curve, and expressed as pg/ml of supernatant.

Cytokine	Supplier	Block	Capture Antibody	Standard	Detection Antibody
IL-6	BD Biosciences, USA	1% BSA in PBS	Monoclonal anti-rat IL-6	Rat recombinant IL-6 standards	Biotinylated anti-rat IL-6
TNF- $\alpha$	BD Biosciences, USA	1% BSA in PBS	Monoclonal anti-rat TNF- $\alpha$	Rat recombinant TNF- $\alpha$ standards	Biotinylated anti-rat TNF- $\alpha$
IL-1 $\beta$	R & D Systems	1% BSA in PBS	Monoclonal anti-rat IL-1 $\beta$	Rat recombinant IL-1 $\beta$ standards	Biotinylated anti-rat IL-1 $\beta$

**Table 2.5. Rat ELISA reagents.**

Cytokine	Supplier	Block	Capture Antibody	Standard	Detection Antibody
IL-6	BD Biosciences, USA	1% BSA in PBS	Monoclonal rat Anti-mouse IL-6	Mouse recombinant IL-6 standards	Biotinylated anti-mouse IL-6
TNF- $\alpha$	BD Biosciences, USA	1% BSA in PBS	Monoclonal anti-mouse TNF $\alpha$	Mouse recombinant TNF $\alpha$ standards	Biotinylated anti-mouse TNF $\alpha$

**Table 2.6. Mouse ELISA reagents.**

## **2.8. Mesoscale multiplex assays**

### **2.8.1. Tissue preparation for Mesoscale**

Mesoscale analysis of hippocampal cytokine concentration was carried out by Dr. Raasay Jones and Dr. Aedin Minogue. Hippocampal tissue was harvested from WT and APP/PS1 mice and prepared for a Pro-Inflammatory 7-Plex Assay. Briefly, hippocampal tissue (~20mg) was homogenised in 1% Triton-X in 1X PBS (100µl) using a hand-held homogeniser. Samples were centrifuged (21 500 x *g*, 20 minutes, 4°C) and the supernatant collected. A BCA protein assay was carried out on the supernatant and samples were equalised to 2mg/ml in 1% Triton-X in 1X PBS and stored at -80°C.

### **2.8.2. Mouse Pro-Inflammatory 7-Plex Assay**

Briefly, blocker A solution (1%; 150µl) was added to each well of the 96-well plate and incubated at room temperature for 1 hour. The plate was washed 3 times with 1X Tris-wash buffer. Standards were prepared as per manufacturer's instructions and sample or standard (25µl) was dispensed into separate wells of the MSD plate and incubated overnight at 4°C with vigorous shaking. Following overnight incubation, the plate was washed 3 times with 1X Tris-wash buffer and detection antibody solution (25µl) was added to each well. The plate was sealed and incubated with vigorous shaking for 2 hours at room temperature. The plate was washed 3 times with 1X Tris-wash buffer and 2X MSD read buffer T (150µl) was added to each well. The plate was read using a Mesoscale Sector Imager and cytokine concentrations were calculated relative to the standard curve (expressed as pg/mg of protein).

## **2.9. Statistical analysis**

All data were expressed as mean ± standard error of the mean (SEM). Data were analysed using a Student's *t*-test, 1- or 2-way analysis of variance (ANOVA) where indicated, significance denoted as  $p < 0.05$ . If significance was found following the use of

ANOVA, the data were further analysed using Bonferroni *post hoc* test. Analysis was carried out using Prism software (Graphpad, US)

## Chapter 3

### The Impact of aging on the activation state of bone-marrow derived macrophages

### 3.1. Introduction

Normal healthy aging has been associated with increased inflammation in the brain, characterised by an increased concentration of pro-inflammatory cytokines like IL-1 $\beta$  and TNF $\alpha$  particularly in the hippocampus. This increase in pro-inflammatory cytokines is accompanied by a decreased concentration of anti-inflammatory cytokines such as IL-4 and IL-10 (Ye & Johnson, 2001; Maher *et al.*, 2005; Nolan *et al.*, 2005; Clarke *et al.*, 2008). It has been well documented that LTP is impaired in the hippocampus of aged animals (Nolan *et al.*, 2005). Previous studies have shown that pro-inflammatory mediators inhibit hippocampal-dependent learning and memory.

Microglia are the resident immune cells of the brain, and often referred to as the macrophages of the CNS. Under normal conditions microglia are said to be 'resting', however they are constantly surveying their microenvironment. Similar to macrophages in the periphery, microglia can become activated in response to immunological stimuli, infection or injury. Activated microglia/macrophages undergo a conformational change and express a number of cell surface markers such as, MHCII, CD40 and CD11b, and release pro-inflammatory cytokines (Lull & Block, 2010). The principal function of microglia within the healthy brain is protection, but it has been suggested that in disease and normal aging that microglial activation becomes dysregulated and consequently detrimental to neuronal health, this often referred to as the 'bystander effect' (Colton, 2009). Several studies have previously reported evidence of increased microglial activation in the aged brain (Griffin *et al.*, 2006; Lyons *et al.*, 2009). One possible reason for an increase in microglial activation may be the age-related loss of immunosuppressive molecules such as CD200 (Lyons *et al.*, 2007a) and fractalkine (Lyons *et al.*, 2009).

Age-related changes in the permeability of the BBB have been reported in rats (Blau *et al.*, 2012), mice (Pelegri *et al.*, 2007) and humans (Pakulski *et al.*, 2000). Increased permeability of the BBB has been associated with an increase in the number of peripheral immune cells, such as macrophages, infiltrating the brain (Blau *et al.*, 2012). Entry of these cells may exacerbate any inflammatory imbalance that may already exist. IFN $\gamma$ , one of the most potent endogenous activators of microglia/macrophages (Nguyen & Benveniste, 2000), is produced by a number of peripheral immune cells such as T cells and NK cells. Previous studies have reported an increase in the concentration of IFN $\gamma$  in



the CNS of aged animals (Maher *et al.*, 2005; Clarke *et al.*, 2008), and, since resident cells of brain do not produce significant amounts of IFN $\gamma$ , the possibility that infiltrating cells are responsible has to be considered.

It is now widely accepted that macrophages can adopt several activation states depending on the stimuli present within their environment (Colton, 2009). Pro-inflammatory stimuli such as LPS and IFN $\gamma$  are said to induce an activated state described as M1, which is also referred to as classical activation. M1 activated macrophages are associated with increased release of pro-inflammatory mediators; M1 macrophages are involved with the induction of an immune response and is often referred to the 'killing phase' of macrophage activation. Gordon and colleagues showed that upon treatment with IL-4, macrophages exhibited a distinct phenotype (Stein *et al.*, 1992). IL-4 induces an anti-inflammatory phenotype often referred to as alternative or M2 activation. M2 macrophages are thought to be involved with the repair and resolution of inflammatory processes.

### **Aims of Study**

1. To investigate whether macrophages infiltrate in the brains of young or middle-aged rats.
2. To investigate the phenotype of BMDMs cultured from young and aged rats, and their responses to inflammatory stimuli.

### 3.2. Methods

In the monocyte cell infiltration study a group of young rats (4 months n=4) and middle-aged rats (16 months n=4) were used. A separate group of young rats (4 months n=10) and aged rats (23-27 months n=11) were used in a study to characterise the response of BMDMs to different inflammatory stimuli.

In the monocyte cell infiltration study, animals were perfused with ice-cold PBS prior to sacrifice to ensure removal of all contaminating cells from peripheral blood. Brain tissue was taken for isolation of mononuclear cells as described in section 2.3. by using flow cytometry microglia were identified as CD11b<sup>+</sup>/CD45<sup>low</sup> cells, while infiltrating monocytes were identified as CD11b<sup>+</sup>/CD45<sup>high</sup> cells. For the second study, animals were sacrificed by decapitation whilst under anaesthesia induced by urethane. Hippocampal tissue was taken for protein and RNA analysis. BMDMs were cultured from young and aged rats as described in section 2.2.4. BMDMs were treated with LPS (100ng/ml), IFN $\gamma$  (50ng/ml) and IL-4 (200ng/ml) for 24 hour. Cells were harvested for RNA and cytokine analysis.

All data are expressed as mean  $\pm$  standard error of the mean (SEM). Data were analysed using a student t-test or 2-way analysis of variance (ANOVA) where indicated, significance denoted as  $p < 0.05$ . If significance was found following the use of ANOVA, the data were further analysed with the use of a Bonferroni *post hoc* tests. Analysis was carried out using Prism software (Graphpad, US).

### **3.3. Results**

#### **3.3.1. Age-related increase in the number of macrophages present in the brain.**

Under normal conditions peripheral immune cells cannot infiltrate the CNS, however it has been suggested that in conditions such as aging, the BBB becomes leaky and this allows the increased entry of cells such as macrophages. Flow cytometric analysis of CD11b<sup>+</sup> cells in the brain revealed there was a significant increase in CD11b<sup>+</sup>/CD45<sup>high</sup> macrophages in the brains of middle-aged, compared with young rats when expressed as a % of the total number of CD11b<sup>+</sup> cells ( $\hat{p}$ <0.05; Student's *t*-test; Figure 3.1).

#### **3.3.2. LPS induced CD11b and CD40 mRNA expression in BMDMs.**

In an effort to characterise the macrophages in aged, compared with young rats, BMDMs were incubated in the presence or absence of LPS (100ng/ml, 24 hours). The data show that LPS significantly increased CD11b mRNA expression in BMDMs from young and aged rats ( $^{***}p$ <0.001; ANOVA; Figure 3.2. A). The LPS-induced increase in CD11b mRNA was significantly greater in BMDMs from aged rats compared with BMDMs cultured from young rats ( $^{**}p$ <0.01; ANOVA; Figure 3.2.A). LPS significantly increased CD40 mRNA expression in BMDMs from young and aged rats ( $^{***}p$ <0.001; ANOVA; Figure 3.2.B). LPS-induced CD40 mRNA expression was significantly greater in BMDMs from aged rats compared with BMDMs cultured from young rats ( $^{+++}p$ <0.001; ANOVA; Figure 3.2.B).

#### **3.3.3. The effect of LPS on cytokine mRNA and supernatant concentration in BMDMs from young and aged rats.**

In the presence or absence of LPS, expression of IL-1 $\beta$ , IL-6 and TNF $\alpha$  mRNA was assessed by PCR in macrophages from young and aged rats. LPS significantly increased IL-1 $\beta$  mRNA expression in BMDMs from young and aged rats ( $^{***}p$ <0.001; ANOVA; Figure3.3.A) and this LPS-induced increase in IL-1 $\beta$  mRNA was significantly greater in BMDMs from aged rats ( $^{**}p$ <0.01; ANOVA; Figure3.3.A). LPS also significantly increased TNF $\alpha$  mRNA expression in BMDMs from rats ( $^{***}p$ <0.001; ANOVA; Figure 3.4.A) and the

LPS effect was significantly greater in BMDMs from aged rats ( $^{**}p < 0.01$ ; ANOVA; Figure 3.4.A). IL-6 mRNA expression in BMDMs from rats was significantly increased following LPS stimulation ( $^{***}p < 0.001$ ; ANOVA; Figure 3.5A). However in contrast to the data for IL-1 $\beta$  and TNF $\alpha$  the LPS-induced increase in IL-6 mRNA was significantly less in BMDMs from aged rats compared with BMDMs cultured from young rats ( $^{+}p < 0.05$ ; ANOVA; Figure 3.5A).

IL-1 $\beta$  concentration in supernatant samples from BMDMs was measured by ELISA. LPS significantly increased IL-1 $\beta$  supernatant concentration from BMDMs from both age groups ( $^{***}p < 0.001$ ; ANOVA; Figure 3.3B), there was no age-related difference observed. In parallel LPS increased the concentration of TNF $\alpha$  in supernatant samples from BMDMs prepared from young and aged animals ( $^{***}p < 0.001$ ; ANOVA; Figure 3.4B), and as shown for IL-1 $\beta$ , there was no age-related difference in release of TNF $\alpha$ . LPS also significantly increased IL-6 concentration in supernatant from BMDMs prepared from young and aged rats ( $^{***}p < 0.001$ ; ANOVA; Figure 3.5B), similar to IL-1 $\beta$  and TNF $\alpha$  no age-related changes were observed.

#### **3.3.4. The effect of IFN $\gamma$ on TNF $\alpha$ , and NOS2 mRNA expression in BMDMs from young and aged rats.**

Similar to LPS, IFN $\gamma$  triggers macrophages to adopt the M1 state and here the effect of IFN $\gamma$  on BMDMs was also examined. Cells were treated with IFN $\gamma$  (50ng/ml, 24 hours) and assessed for changes in the archetypal markers of classical activation, TNF $\alpha$  and NOS2. The data show that IFN $\gamma$  significantly increased TNF $\alpha$  mRNA expression in BMDMs from young and aged rats ( $^{***}p < 0.001$ ; ANOVA; Figure 3.6A) but was significantly greater in BMDMs from aged rats compared with BMDMs cultured from young rats ( $^{+++}p < 0.001$ ; ANOVA; Figure 3.6A). IFN $\gamma$  significantly increased NOS2 mRNA expression in BMDMs from young and aged rats ( $^{***}p < 0.001$ ; ANOVA; Figure 3.6B), but there were no significant age-related differences.

### **3.3.5. The effect of IFN $\gamma$ on MHCII and CD40 mRNA expression in BMDMs from young and aged rats.**

MHCII and CD40 are considered to be markers of microglial and macrophage activation and age-related changes in both of these markers have been reported in the brain. Here the data demonstrate an age-related increase in MHCII mRNA expression ( $^+p<0.05$ ; ANOVA). IFN $\gamma$  significantly increased MHCII mRNA expression in BMDMs from rats ( $^{***}p<0.001$ ; ANOVA; Figure 3.7A) and the IFN $\gamma$ -induced effect was significantly greater in BMDMs from aged rats ( $^{+++}p<0.001$ ; ANOVA; Figure 3.7A).

IFN $\gamma$  treatment also significantly increased CD40 mRNA expression in BMDMs from rats ( $^{**}p<0.01$ ; ANOVA; Figure 3.7B) and the IFN $\gamma$ -induced effect was significantly greater in BMDMs from aged rats compared ( $^+p<0.05$ ; ANOVA; Figure 3.7B).

### **3.3.6. The effect of IL-4 on MRC1 and ARG1 mRNA expression in BMDMs from young and aged rats.**

LPS and IFN $\gamma$  induce the M1 activation state, while the anti-inflammatory cytokine IL-4 induces the M2 or alternative activation state and M2 macrophages are believed to be associated with the resolution of inflammation. IL-4 treated macrophages are associated with a decrease in pro-inflammatory mediators such as NOS2 (Colton, 2009) and M2 macrophages exhibit a high expression of MRC1 and Arg1. BMDMs from rats were treated with IL-4 (200ng/ml, 24 hours). It was found that IL-4 treatment significantly increased MRC1 mRNA expression in BMDMs from young animals ( $^{***}p<0.001$ ; ANOVA; Figure 3.8A), and this change was significantly less BMDMs in aged animals ( $^+p<0.05$ ; ANOVA; Figure3.8.A). IL-4 significantly increased ARG1 mRNA expression in BMDMs from young and aged rats ( $^{***}p<0.001$ ; ANOVA; Figure 3.8B), but in contrast to the effects on age-related changes were observed.

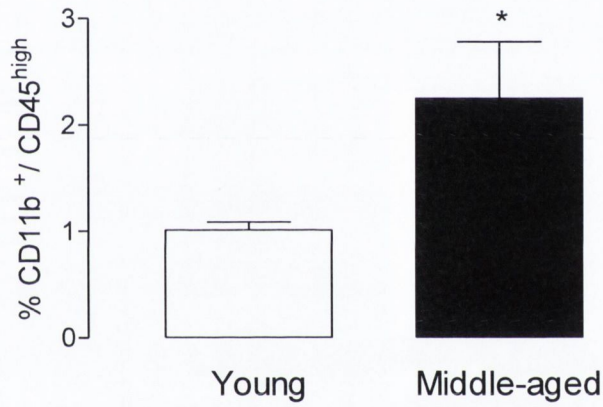
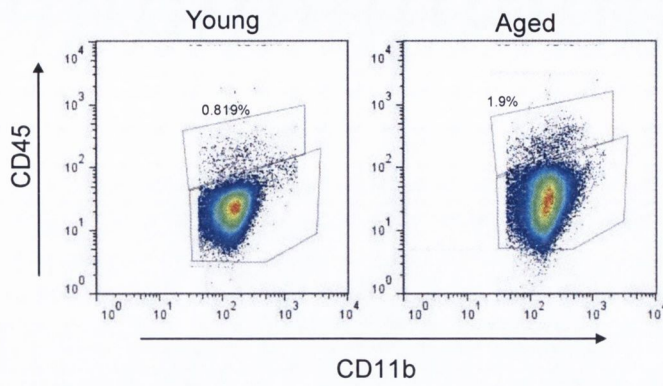
### **3.3.7. Phagocytic activity of BMDMs from young and aged rats.**

Phagocytic activity of BMDMs from young and aged rats was measured by assessing the uptake of fluorescently-labelled yellow/green latex bead (Abs/Em=470/505nm; 1:200 dilution). Using Flow Cytometry the data show that latex bead uptake was significantly greater in BMDMs cultured from aged rats compared to BMDMs derived from young rats (\*\* $p < 0.01$ ; Student's *t*-test; Figure 3.9A).

CD68 is a macrophage/microglia-specific protein; it is a marker of lysosomal activity and is considered to be a marker of phagocytosis. Consistent with the data showing an age-related increase in phagocytosis, an increase in CD68 mRNA expression was also observed in BMDMs from aged animals compared to young controls ( $p < 0.05$ ; Student's *t*-test; Figure 3.9B)

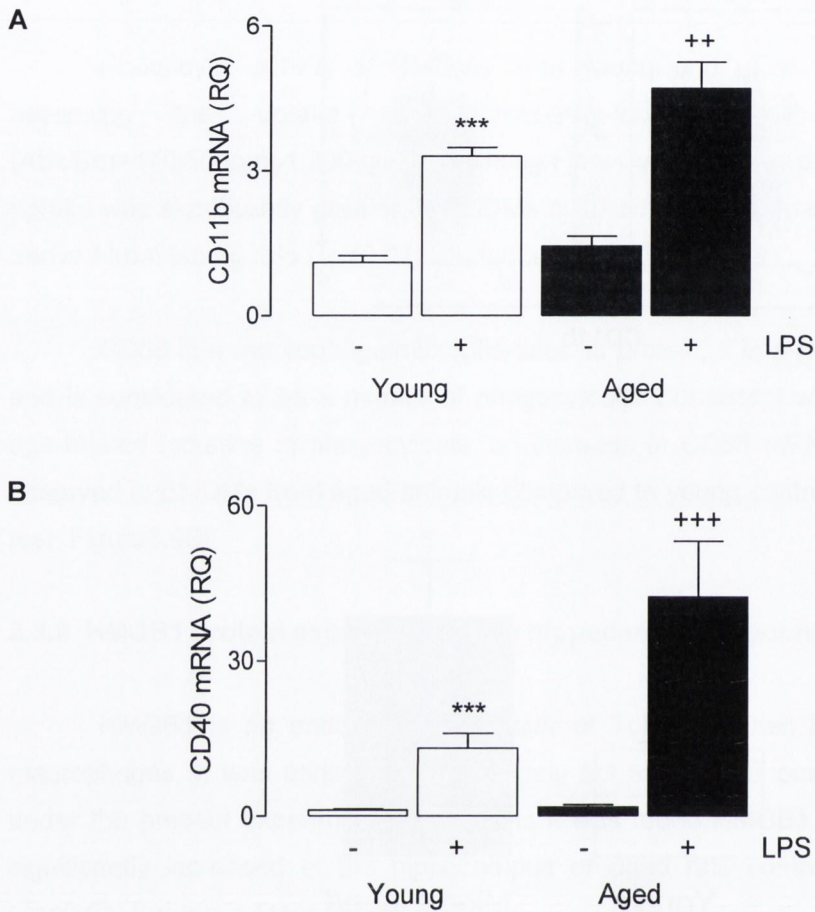
### **3.3.8. HMGB1 protein expression in the hippocampus of young and aged rats.**

HMGB1 is an endogenous activator of TLR4 and has been shown to activate macrophages. It was considered that it may act as endogenous activator of TLR4 and under the present experimental conditions it was found HMGB1 protein expression was significantly increased in the hippocampus of aged rats compared to young controls (\*\* $p < 0.01$ ; Student's *t*-test; Figure 3.10)



**Figure 3.1. Age was associated with an increase in CD11b<sup>+</sup>/CD45<sup>high</sup> cells in brain tissue.**

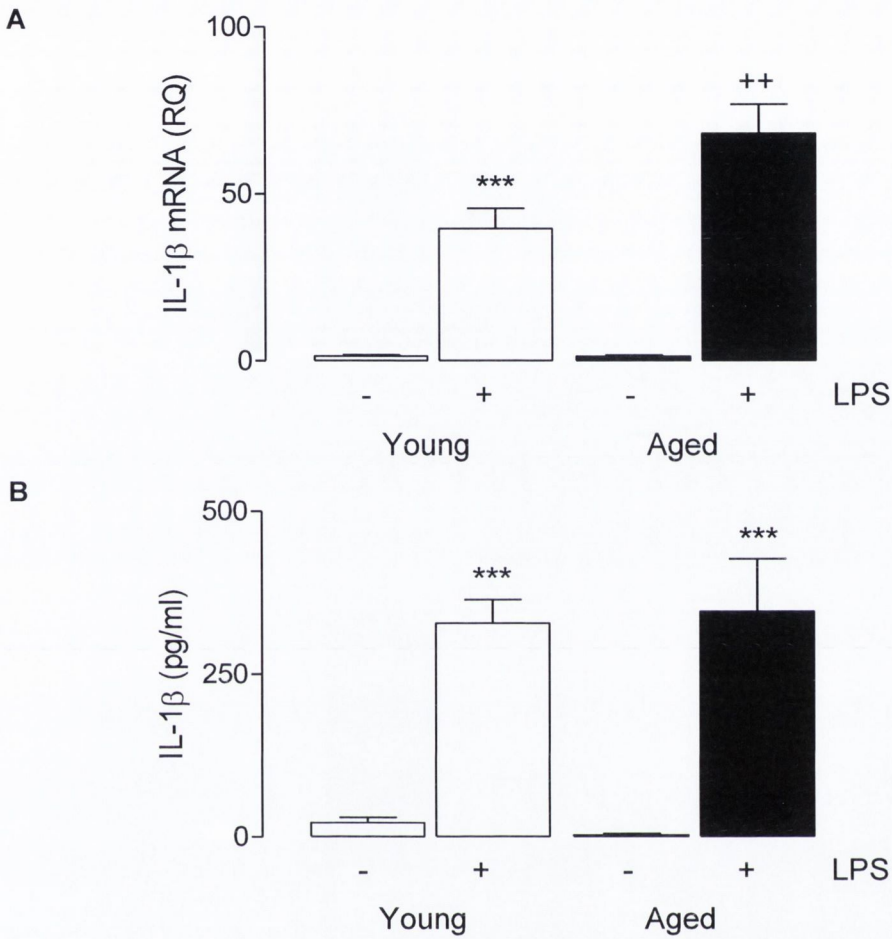
There was a significant increase in the number of macrophages in the brains of older rats (\* $p < 0.05$ , Student's *t*-test). Values are presented as means ( $\pm$ SEM;  $n=4$ ) and expressed as a percentage of all CD11b<sup>+</sup> cells.



**Figure 3.2. LPS induces CD11b and CD40 mRNA expression in BMDMs from young and aged rats.**

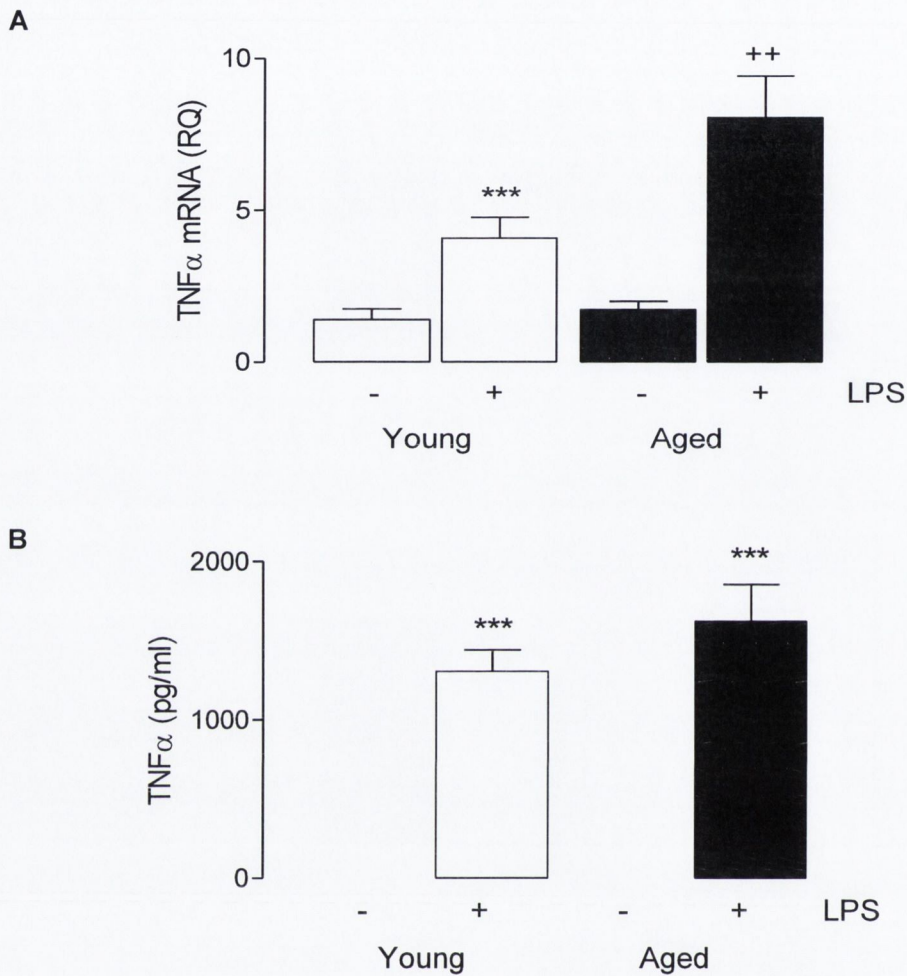
BMDMs were treated with LPS (100ng/ml; 24 hours). LPS significantly increased CD11b and CD40 mRNA in BMDMs from young and aged rats (\*\* $p < 0.001$ , ANOVA; A and B). The LPS-induced increase in CD11b and CD40 mRNA expression was significantly greater in BMDMs cultured from aged, compared with young rats (\*\* $p < 0.01$ ; +++ $p < 0.001$ ; ANOVA; A and B respectively). Values are presented as means ( $\pm$ SEM;  $n = 8-10$ ) expressed as a ratio to  $\beta$ -actin mRNA.





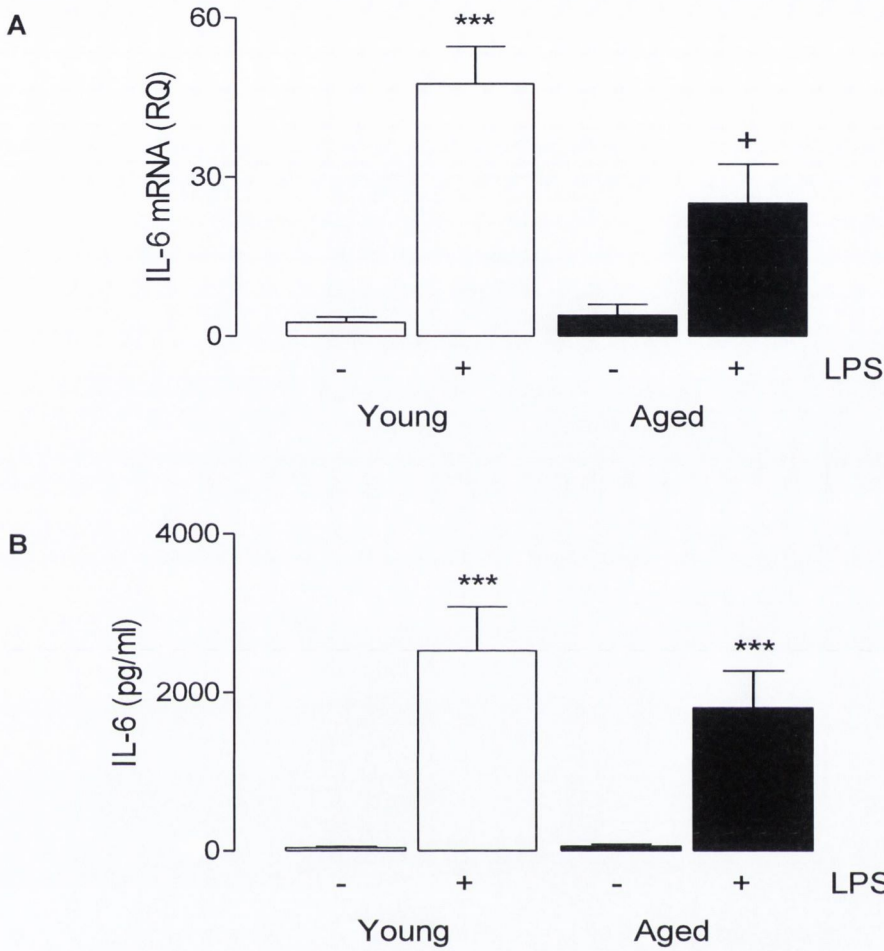
**Figure 3.3. LPS increases IL-1 $\beta$  mRNA expression and supernatant concentration in BMDMs from young and aged rats.**

BMDMs were treated with LPS (100ng/ml; 24 hours). LPS significantly increased IL-1 $\beta$  mRNA expression in BMDMs from young and aged rats (\*\*\* $p$ <0.001, ANOVA; A). The LPS-induced IL-1 $\beta$  mRNA expression was significantly greater in BMDMs cultured from aged rats (\*\* $p$ <0.01; ANOVA; A). LPS significantly increased IL-1 $\beta$  release from BMDMs from young and aged animals (\*\*\* $p$ <0.001, ANOVA; B). There was no age-related change in IL-1 $\beta$  release. Values are presented as means ( $\pm$ SEM;  $n$ =8-10) expressed as a ratio to  $\beta$ -actin mRNA or as pg/ml.



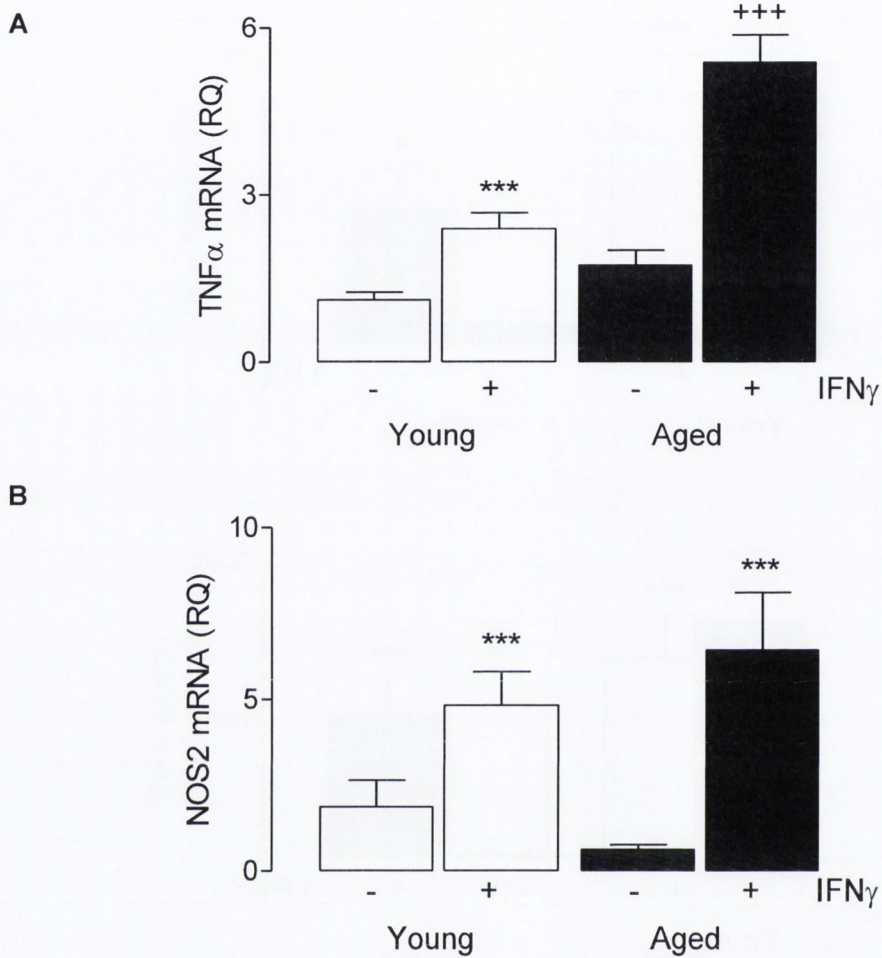
**Figure 3.4. LPS increases TNF $\alpha$  mRNA expression and supernatant concentration in BMDMs from young and aged rats.**

BMDMs were treated with LPS (100ng/ml; 24 hours). LPS significantly increased TNF $\alpha$  mRNA expression in BMDMs from young and aged rats (\*\* $p < 0.001$ , ANOVA; A). The LPS-induced increase in TNF $\alpha$  mRNA expression was significantly greater in BMDMs cultured from aged rats (\*\* $p < 0.01$ ; ANOVA). LPS significantly increased TNF $\alpha$  release from BMDMs from young and aged animals (\*\* $p < 0.001$ , ANOVA; B). There was no age-related change in TNF $\alpha$  release. Values are presented as means ( $\pm$ SEM;  $n=8-10$ ) expressed as a ratio to  $\beta$ -actin mRNA or as pg/ml.



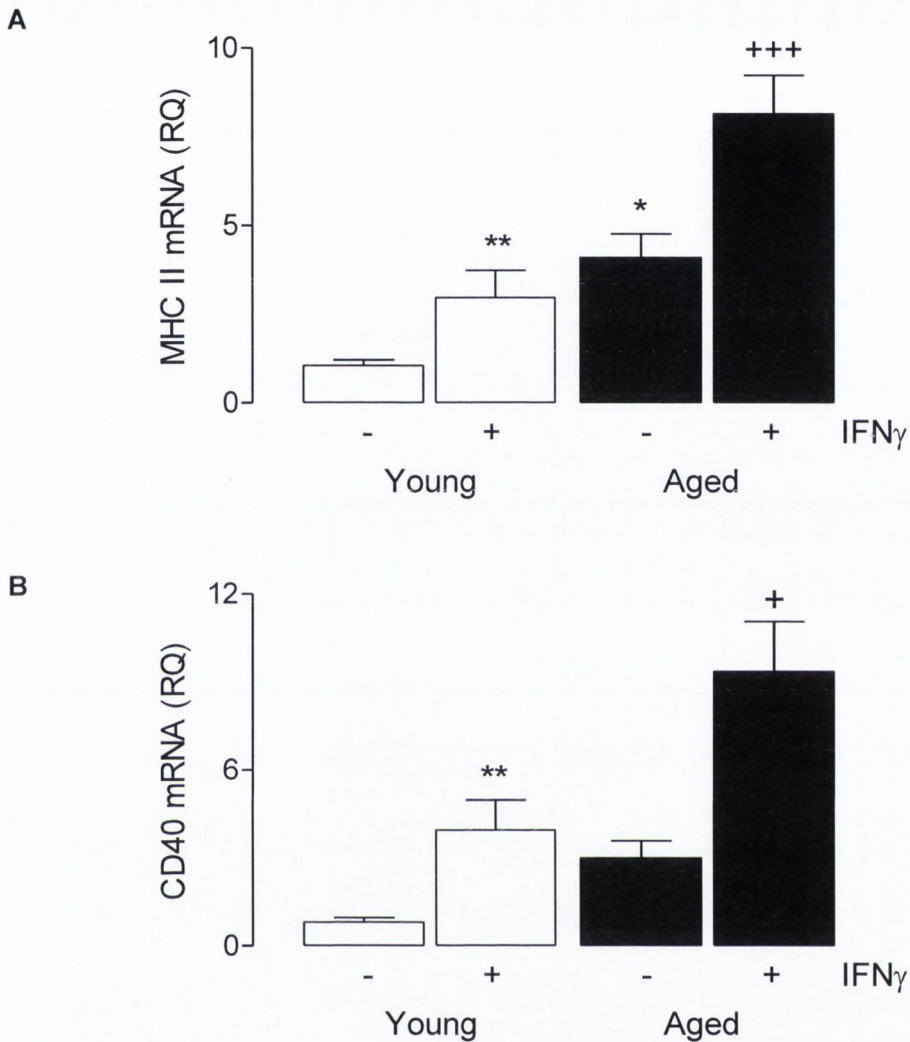
**Figure 3.5. LPS increases IL-6 mRNA expression and supernatant concentration in BMDMs from young and aged rats.**

BMDMs were treated with LPS (100ng/ml; 24 hours). LPS significantly increased IL-6 mRNA expression in BMDMs from young and aged rats ( $***p < 0.001$ , ANOVA; A). The LPS-induced increase in IL-6 mRNA expression was significantly greater in BMDMs cultured from young rats ( $^+p < 0.05$ ; ANOVA; A). LPS significantly increased IL-6 release from BMDMs from young and aged animals ( $***p < 0.001$ , ANOVA; B). There was no age-related change in IL-6 supernatant concentration. Values are presented as means ( $\pm$ SEM;  $n=$ ) expressed as a ratio to  $\beta$ -actin mRNA or as pg/ml.



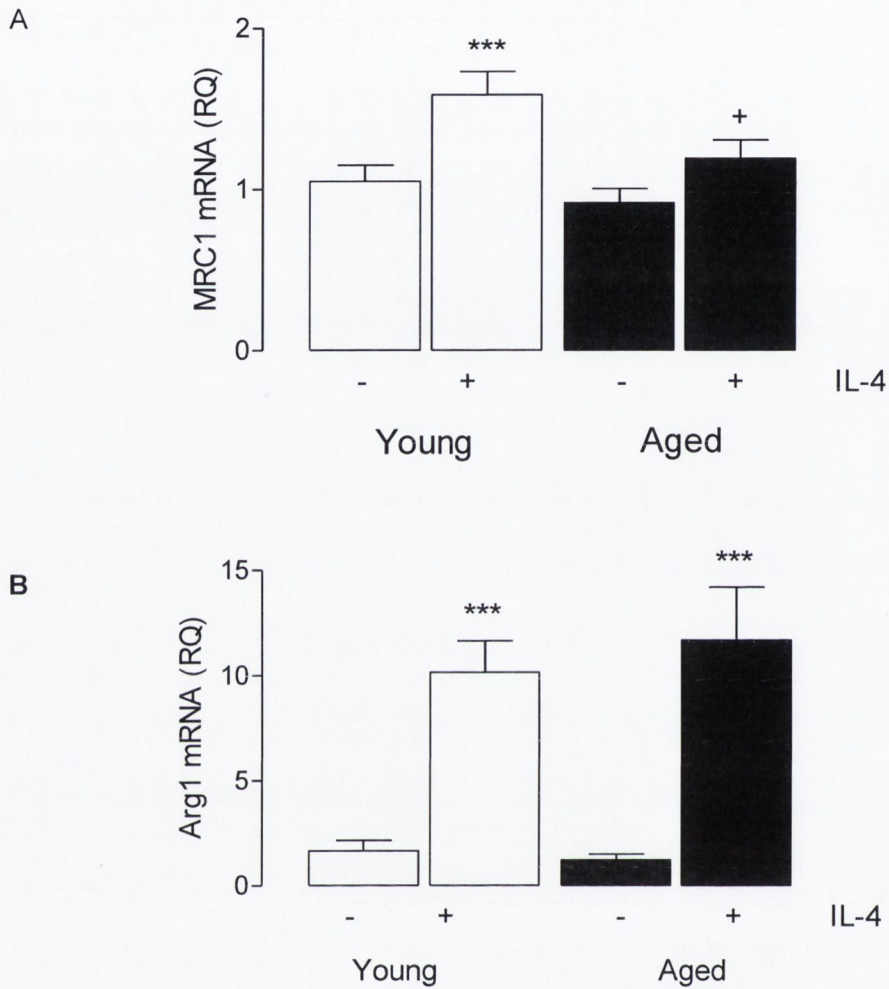
**Figure 3.6. The effect of IFN $\gamma$  on TNF $\alpha$ , and NOS2 mRNA expression in BMDMs from young and aged rats.**

BMDMs were treated with IFN $\gamma$  (50ng/ml; 24 hours). IFN $\gamma$  significantly increased TNF $\alpha$  mRNA in BMDMs from young and aged rats (\*\* $p$ <0.01; ANOVA; A) and BMDMs cultured from aged rats exhibited an exaggerated response compared to cells derived from young rats (\*\*\* $p$ <0.001; ANOVA; A). IFN $\gamma$  significantly increased NOS2 mRNA in BMDMs from young and aged rats (\*\* $p$ <0.01; ANOVA; B), however no age-related difference was observed. Values are presented as means ( $\pm$ SEM;  $n$ =8) expressed as a ratio to  $\beta$ -actin mRNA.



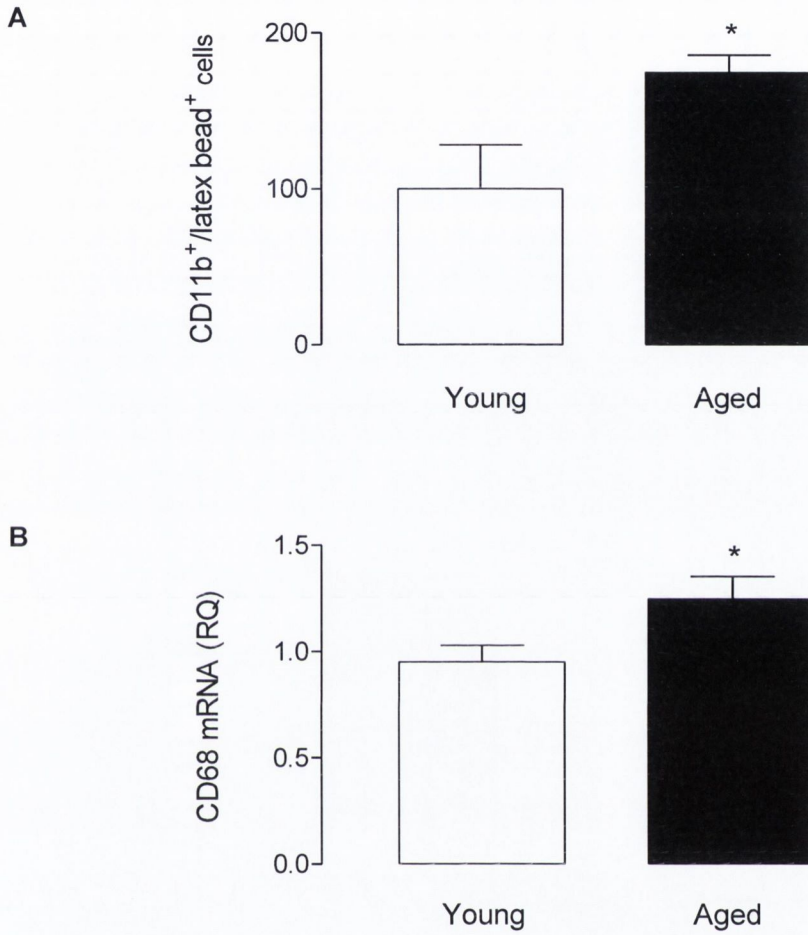
**Figure 3.7. The effect of IFN $\gamma$  on MHCII and CD40 mRNA expression in BMDMs from young and aged animals.**

An age-related increase in the expression of MHCII mRNA was observed ( $\dot{p}<0.05$ , ANOVA; A). IFN $\gamma$  significantly increased MHCII mRNA expression in BMDMs from young and aged rats ( $\dot{\dot{p}}<0.01$ , ANOVA; A) and this IFN $\gamma$ -induced change was significantly greater in BMDMs from aged animals ( $\dot{\dot{\dot{p}}}<0.001$ , ANOVA; A). IFN $\gamma$  significantly increased CD40 mRNA expression in BMDMs from young and aged rats ( $\dot{\dot{p}}<0.01$ , ANOVA; B) and the IFN $\gamma$ -induced change was significantly greater in BMDMs from aged rats ( $\dot{p}<0.05$ , ANOVA; B). Values are presented as means ( $\pm$ SEM; n=8-10) expressed as a ratio to  $\beta$ -actin mRNA.



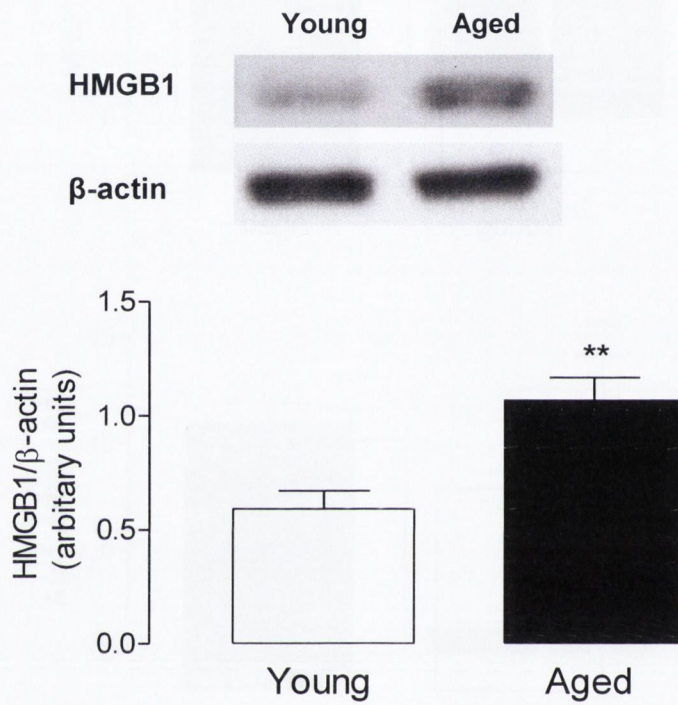
**Figure 3.8. The effect of IL-4 on MRC1 and Arg1 mRNA expression in BMDMs from young and aged rats.**

BMDMs were treated with IL-4 (200ng/ml; 24 hours). IL-4 increased MRC1 mRNA expression in BMDMs (\*\* $p < 0.001$ , ANOVA). IL-4-induced increase in MRC1 mRNA was found to be less profound in BMDMs from aged rats ( $^+p < 0.05$ , ANOVA; A). IL-4 significantly increased Arg1 mRNA expression in BMDMs cultured from young and aged animals (\*\* $p < 0.001$ , ANOVA; B), however no age-related change was observed. Values are presented as means ( $\pm$ SEM;  $n = 8-10$ ) expressed as a ratio to  $\beta$ -actin mRNA.



**Figure 3.9. Phagocytic activity of BMDMs was increased with age.**

Uptake of fluorescently-labelled yellow/green latex bead (Abs/Em=470/505nm) was significantly greater in BMDMs cultured from aged rats compared to BMDMs derived from young rats ( $p < 0.05$ ; Student's *t*-test; A). CD68 mRNA expression was significantly greater in BMDMs cultured from aged rats compared to young rats ( $p < 0.05$ ; Student's *t*-test; B). Values are presented as means ( $\pm$ SEM;  $n=6$ ) and expressed as a ratio to  $\beta$ -actin mRNA



**Figure 3.10. HMGB1 was increased in the hippocampus with age.**

HMGB1 protein expression was significantly greater in hippocampal tissue prepared from aged compared to young rats (\*\* $p < 0.01$ ; Student's *t*-test). A representative blot is shown. Data are presented as means ( $\pm$ SEM;  $n=5-7$ ) and expressed as HMGB1/ $\beta$ -actin (arbitrary units).



### 3.4 Discussion

One of the central aims of the present study was to establish whether there was an age-related variance in response of BMDMs to different stimuli. As aging is associated with an increased inflammatory profile, the hypothesis was that BMDMs from aged rats would exhibit an exaggerated response to pro-inflammatory stimuli such as LPS and IFN $\gamma$ , with the possibility that the response of these macrophages to IL-4 would also be altered. The significance of this study lies in the fact that an age-related permeability of the BBB has been reported in several studies, which can result in increased infiltration of peripheral immune cells. In this context an additional aim of this study was to investigate macrophage infiltration into the brains of young and middle-aged rats. The hypothesis to be tested was that there is an increase in the number of macrophages within the brains of older rats and that these cells exhibit an exaggerated response to pro-inflammatory stimuli. The increased susceptibility of these cells to pro-inflammatory stimuli may exacerbate any inflammatory imbalances that exist within the CNS.

The first significant result is that there was a significant increase in the number of macrophages within the brain of middle-aged, compared with young, rats. This is in agreement with previous evidence from this laboratory which reported an age-related increase in infiltrating macrophages (Blau *et al.*, 2012). Previous studies have provided evidence of increased microglial activation and inflammation within the brains of aged rats (Griffin *et al.*, 2006). It still remains unclear whether these cells play a beneficial or detrimental role within the CNS, with conflicting reports in the literature. Previous work in an animal model of AD reported that peripheral immune cells may be more efficient phagocytes of A $\beta$  than resident microglia (Simard *et al.*, 2006). El Khoury and colleagues demonstrated that in a mouse model of AD lacking CCR2, peripheral monocyte trafficking to the CNS was decreased and this was associated with increased A $\beta$  plaque burden and mortality (El Khoury *et al.*, 2007). It has also been reported that monocyte infiltration correlates with the progression to the paralytic stage of EAE and inhibiting monocyte recruitment to the CNS blocked EAE progression (Ajami *et al.*, 2011). A detrimental effect of macrophages on CNS cells has been reported by Allan and colleagues; they showed macrophages to be cytotoxic to organotypic hippocampal slice cultures independent of M1 or M2 activation (Girard *et al.*, 2013). As a first step in addressing this, it was important to determine the phenotype of these cells and their response to inflammatory stimuli.

Macrophages respond to a variety of stimuli (Gordon, 2003; Mosser, 2003) and in this study, LPS induced the upregulation of several markers associated with macrophage activation. Macrophages/microglia exhibit an upregulation of CD11b and CD40 expression during inflammatory conditions (Lynch, 2009) and CD11b expression is reported to correlate with the degree of activation (Roy *et al.*, 2008). The present data show that LPS enhanced the expression of CD11b and CD40 mRNA in BMDMs from young and aged rats. However the LPS-induced increase in CD11b and CD40 mRNA was exaggerated in BMDMs from aged animals. CD40-CD40L engagement is a key mechanism in enabling the interactions between APC and T-cells (Aloisi, 2001). CD40 has been implicated in disorders that involve hyperactive immune response such as rheumatoid arthritis and MS (Aloisi, 2001), while depletion of CD40 or CD40L decreases the progression of AD (Chen *et al.*, 2006). The enhanced LPS-induced response of BMDMs prepared from older rats described here corresponds with similar changes observed in microglia (Nolan *et al.*, 2005; Griffin *et al.*, 2006; Cox *et al.*, 2012) and is indicative of an age-related inflammatory profile.

The production of pro-inflammatory cytokines is a hallmark of macrophage/microglial activation. In this study, LPS induced production and release of IL-1 $\beta$ , TNF $\alpha$  and IL-6 and in the case of IL-1 $\beta$  and TNF $\alpha$ ; LPS-induced changes were significantly greater in BMDMs derived from aged rats, which correlates with the change in CD11b and CD40 mRNA expression. Conversely LPS-induced IL-6 mRNA expression was significantly reduced in BMDMs from aged animals compared to young controls. In contrast to the present findings where LPS-induced release of IL-1 $\beta$  and TNF $\alpha$  was not age dependent, it has been reported LPS-induced release of IL-1 $\beta$  and TNF $\alpha$  from alveolar macrophages is greater in cells from aged animals compared to young controls (Kohut *et al.*, 2004). The variability in findings may be due to interspecies differences however it is also possible that macrophages from different tissues respond differently to a given stimulus and this is supported by the additional finding that an age-related decrease in IL-1 $\beta$  and TNF $\alpha$  release from peritoneal macrophages was observed in response to LPS (Boehmer *et al.*, 2004) In contrast to the changes in IL-1 $\beta$  and TNF $\alpha$  the LPS-induced change in IL-6 mRNA was decreased with age, even though IL-6 has been implicated in a number of pro-inflammatory processes, it is well documented that IL-6 is a pleiotropic cytokine and it has been recently shown that LPS-induced IL-6 modulates the release of

IL-1 $\beta$  from cultured rat glia (Minogue *et al.*, 2012). A study carried out by Steensberg and colleagues showed that IL-6 treatment increased IL-1ra and IL-10 plasma concentration in humans (Steensberg *et al.*, 2003). In the present study, a decrease in LPS-induced IL-6 mRNA in BMDMs from aged animals may represent a decrease in the capacity to control inflammatory events.

IFN $\gamma$  is a potent endogenous activator of macrophages and microglia and can induce the expression of pro-inflammatory molecules. It is produced by a number of cells including Th-1 and NK cells. The present data show that IFN $\gamma$  induced expression of TNF $\alpha$  and NOS2, the 2 archetypal markers of the M1 activation state. Significantly, the effect of IFN $\gamma$  was increased in BMDMs from aged compared with young rats indicating an age-related susceptibility to inflammatory stimulus. The increase in MHCII and CD40 mRNA expression following IFN $\gamma$  treatment was also greater in BMDMs derived from aged rats, this is in agreement with previous findings that have highlighted an age-related increase in these markers in the brains of aged animals (Griffin *et al.*, 2006; Lyons *et al.*, 2009; Cox *et al.*, 2012). It also emphasises the heightened responsiveness of BMDMs with age to pro-inflammatory stimuli such as LPS and IFN $\gamma$ . In a comprehensive review Stout and Suttles discuss the changes associated with macrophage function and suggested that macrophages from aged animals exhibit a diminished response to inflammatory stimuli such as LPS (Stout & Suttles, 2005). Which is at variance with present findings, however an elegant study by Kohut and colleagues showed that age related responses in macrophages were variable depending upon the nature of the stimulus and the origin of the macrophages (Kohut *et al.*, 2004). Whereas splenic and alveolar macrophages from aged rodents exhibited an increased response to LPS+IFN $\gamma$ , peritoneal macrophages from aged animals showed an impaired response. This suggests that the discrepancy in findings reported in the literature may be explained by the origin of the macrophages.

Alternative or M2 activation of BMDMs is associated with the resolution of inflammation and wound repair (Gordon & Martinez, 2010) and hallmarks of alternative activation include increased expression of MRC1, factors that limit inflammatory activity like Arg1 and that are associated with repair of the extracellular matrix like Chi3li3 and Fizz1. IL-4 increased expression of MRC1 and Arg1 mRNA in BMDMs from young and aged rats and whereas a similar IL-4-induced effect was observed for Arg1 the change in MRC1 was decreased in cells prepared from old animals. A previous study reported that

BMDMs cultured from MRC1<sup>-/-</sup> mice show an increased pro-inflammatory phenotype (Paveley *et al.*, 2011). Similar to BMDMs cultured from MRC1<sup>-/-</sup> mice, BMDMs from aged rats exhibit an enhanced pro-inflammatory phenotype and this corresponds with age-related decrease in MRC1 expression. Interestingly, it has been reported that treatment of macrophages with IFN $\gamma$  or LPS leads to a decrease in MRC1 expression (Colton, 2009). It is possible that the increased inflammatory profile of BMDMs cultured from aged animals described here may be linked to the decreased expression of MRC1.

The main role of Arg1 is regulation of NO production and consistent with this it has been shown that IL-4 inhibited NO production in murine macrophages (Nemoto *et al.*, 1999). The finding that there was no age-related alteration in Arg1 following IL-4 treatment in macrophages is in agreement with previous evidence which reported Arg1 expression and activity was similar in brain tissue of young and aged animals (Liu *et al.*, 2004a). It should be acknowledged that although the findings are consistent, the preparations are different with evidence obtained from BMDMs and prefrontal cortical tissue.

Phagocytosis is a fundamental function undertaken by macrophages in the resolution of an inflammatory event (Mantovani *et al.*, 2013). Here phagocytic activity was assessed by flow cytometry measuring the uptake of fluorescently-tagged latex beads. A significant increase in the uptake of latex beads was observed in BMDMs from aged rats compared with young rats. This was accompanied by an age-related increase in CD68 mRNA expression. CD68 is a macrophage/microglia-specific protein; it is a marker of lysosomal activity and is considered to be a marker of phagocytosis (Lynch, 2009) therefore these results both suggest increased cell activation and phagocytic activity in BMDMs cultured from aged rats.

Because of the infiltration of macrophages into the brain with age and the increased responsiveness to LPS-induced TLR4 activation, it was of interest to establish whether brain tissue from aged rats might express increased endogenous TLR4 agonist. HMGB1 is an endogenous activator of TLR4 and has been shown to activate macrophages (Yang *et al.*, 2010). HMGB1 is considered to be a DAMP as it can be released by damaged or necrotic cells, it modulates pro-inflammatory cytokine production but suppresses the production of anti-inflammatory cytokines in the murine macrophage cell line RAW 264.7 (El Gazzar, 2007). In this study HMGB1 expression was significantly

increased in the hippocampus of aged animals compared with young controls and as BMDMs from aged rats exhibit an enhanced response to TLR4 stimulation, the implication of these findings is that macrophages that gain entry into the brain of aged animals have a greater probability of becoming activated by HMGB1 which leads to increased inflammation.

The main finding of this study is that there was an age-related increase in macrophages found in the brain accompanying an age-related increase in the response to LPS and IFN $\gamma$ . Therefore infiltrating macrophages responding to HMGB1 may exacerbate any existing inflammation. It is important to note that peripheral cells such as T cells and NK cells which also infiltrate the brain with age, produce IFN $\gamma$ , providing another potential mechanism by which macrophages may become activated. Therapies that aim to repair the integrity of the BBB and restrict the entry of these peripheral cells including macrophages may be beneficial in disorders associated with increased inflammation within the CNS.

## Chapter 4

Analysis of the responsiveness of bone marrow-derived macrophages from WT and APP/PS1 mice to inflammatory stimuli

## 4.1. Introduction

The evidence presented in Chapter 3 suggests that macrophages from aged animals exhibit an increased susceptibility to pro-inflammatory stimuli such as LPS and IFN $\gamma$ . These findings gave rise to the hypothesis that macrophages from other animal models which exhibit enhanced inflammatory activity would also show altered responses to inflammatory stimuli. The APP/PS1 mouse is a commonly-used model of AD and these animals exhibit an increased inflammatory profile. CD200 is an immunosuppressive molecule that maintains macrophages/microglia in a quiescent state, CD200-deficient (CD200<sup>-/-</sup>) mice lack CD200 ligand and this is associated with an increase in macrophage/microglial activation. The aim of the current study was to investigate the phenotype of BMDMs from WT, APP/PS1 and CD200<sup>-/-</sup> mice and their responses to inflammatory stimuli.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and is characterised by neuronal loss, the presence of amyloid- $\beta$  (A $\beta$ ) plaques and neurofibrillary tangles. AD is the most common cause of dementia and, similar to other neurodegenerative disorders, evidence of increased inflammation is observed in AD brain parenchyma. Post-mortem analysis of AD brains revealed enhanced expression of MHCII, a classic marker of microglial activation (Luber-Narod & Rogers, 1988). Increased expression of the pro-inflammatory cytokines IL-1 $\beta$  and IL-6 has also been observed in the AD brain (Wood *et al.*, 1993) and activated microglia have been shown to surround A $\beta$  plaques (Combs, 2009). It has been well documented that A $\beta$  treatment has the ability to activate microglia *in vivo* and *in vitro* (Clarke *et al.*, 2007; Lyons *et al.*, 2007b; Lull & Block, 2010). Studies using imaging techniques have reported that there is a correlation between microglial activation and cognitive decline in AD (Edison *et al.*, 2008) and mild cognitive impairment patients (Okello *et al.*, 2009).

Identifying genes associated with AD has allowed the development of genetically-modified animal models of AD. These animal models exhibit hallmarks of AD, such as A $\beta$  plaques, microglial and astrocyte activation and some cognitive deficits (Eriksen & Janus, 2007). The APP/PS1 double transgenic mouse is one of the most commonly-used animal models of AD. Familial AD has been attributed to mutations in 3 genes APP, PS1 and PS2, the APP/PS1 mice overexpress both APP and PS1. The presence of A $\beta$  plaques in

the brains of these animals has been observed as early as 4 months of age and plaque numbers increase in numbers with age (Garcia-Alloza *et al.*, 2006). These mice exhibit memory deficits (Jankowsky *et al.*, 2005; Cao *et al.*, 2007), as well as evidence of enhanced microglial activation (Gallagher *et al.*, 2013). Recent evidence has also shown that the integrity of the BBB is impaired in these animals and this is accompanied by the presence of IFN $\gamma$  producing peripheral immune cells in the brain (Kelly *et al.*, 2013).

Increased numbers of macrophages have been found in the brains of APP/PS1 mice (Kelly *et al.*, 2013). It has been suggested that these infiltrating macrophages have an important role in the clearance of A $\beta$  plaques (Simard *et al.*, 2006; El Khoury *et al.*, 2007), El Khoury and colleagues reported that decreased recruitment of mononuclear cells into the CNS coincides with an increased plaque burden. Evidence from this laboratory has shown that infiltrating macrophages exhibit increased expression of the phagocytic marker CD68 (Kelly *et al.*, 2013) and this is accompanied by increased expression of M1 activation state markers in the brains of APP/PS1 mice (Minogue *et al.*, 2013 in review). The literature regarding macrophages in AD and models of AD has focused on the role of these cells in the phagocytosis of A $\beta$  while the phenotype or responsiveness of these cells to inflammatory stimuli has not yet been characterised.

In addition to assessing the phenotype of macrophages in APP/PS1 mice, the present study also aimed to investigate the phenotype of BMDMs in additional animal model exhibiting increased inflammatory activity, CD200<sup>-/-</sup> mice. Evidence of enhanced microglial activation has been observed in the brains of CD200<sup>-/-</sup> mice, accompanied by a deficit in LTP (Lyons *et al.*, 2007a; Costello *et al.*, 2011). Microglia from CD200<sup>-/-</sup> mice exhibit an enhanced responsiveness to IFN $\gamma$ , suggesting loss of CD200 confers a susceptibility to inflammatory stimulation (Denieffe *et al.*, 2013). Increased infiltration of macrophages is also seen in the brains of CD200<sup>-/-</sup> mice, these macrophages also exhibit an increase in MHCII expression, a classic marker of microglial/macrophage activation (Denieffe *et al.*, 2013). The hypothesis to be addressed is that loss of CD200 may result in BMDMs exhibiting an enhanced response to pro-inflammatory stimuli.



Aims of Study:

1. To investigate the phenotype of BMDMs cultured from WT and APP/PS1 mice, and their responses to inflammatory stimuli.
2. To assess the impact of CD200 expression on BMDM phenotype.

## 4.2. Methods

APP/PS1 and WT mice (17-24 months n=11-13) were used in this study. Animals were anaesthetised and perfused with ice-cold PBS to ensure removal of all contaminating cells from peripheral blood. Brain tissue was taken for isolation of mononuclear cells as described in section 2.3. by using flow cytometry microglia were identified as CD11b<sup>+</sup>/CD45<sup>low</sup> cells, while infiltrating monocytes were identified as CD11b<sup>+</sup>/CD45<sup>high</sup> cells. Hippocampal tissue was taken for protein analysis. BMDMs were cultured from WT and APP/PS1 mice (17-24 months n=11-13) and WT and CD200<sup>-/-</sup> mice (3 months n=4) as described in section 2.2.4. BMDMs were treated with LPS (100ng/ml); IFN $\gamma$  (50ng/ml) and IL-4 (200ng/ml) for 24 hours. Cells were harvested for RNA and cytokine analysis.

All data are expressed as mean  $\pm$  standard error of the mean (SEM). Data were analysed using a Student's *t*-test or 2-way analysis of variance (ANOVA) where indicated, with significance denoted as  $p < 0.05$ . If significance was found following the use of ANOVA, the data were further analysed with the use of a Bonferroni *post hoc* tests. Analysis was carried out using Prism software (Graphpad, US).

## 4.3 Results

### 4.3.1. CD11b<sup>+</sup>/CD45<sup>high</sup> cells are present in the brains of APP/PS1 animals.

Flow cytometry analysis revealed that there was a significant increase in the number of CD11b<sup>+</sup>/CD45<sup>high</sup> macrophages in the brains of APP/PS1 mice compared with WT mice (\*\*p<0.01; Student's *t*-test; Figure 4.1).

### 4.3.2. The effect of IFN $\gamma$ on TNF $\alpha$ and NOS2 mRNA expression in BMDMs from WT and APP/PS1 mice.

IFN $\gamma$  induces the M1 or classical activation state, which play a role in the induction of an immune response and is often referred to the 'killing phase' of macrophage activation. BMDMs were treated with IFN $\gamma$  (50ng/ml; 24 hours). IFN $\gamma$  significantly increased TNF $\alpha$  mRNA in BMDMs from APP/PS1 mice (\*\*p<0.01; ANOVA; Figure 4.2A) and this IFN $\gamma$ -induced change was significantly greater in BMDMs from APP/PS1 mice (\*\*\*p<0.01, ANOVA; Figure 4.2A). IFN $\gamma$  also significantly increased NOS2 mRNA (\*\*p<0.01; ANOVA; Figure 4.2B) but, in contrast to the effects seen on TNF $\alpha$ , no genotype-related changes were observed.

### 4.3.3. The effect of IFN $\gamma$ on MHCII and NOD2 mRNA expression in BMDMs from WT and APP/PS1 mice.

MHCII and NOD2 are two inflammatory molecules that are also modulated by IFN $\gamma$ . MHCII is involved in antigen presentation while NOD2 is an intracellular pathogen recognition receptor. IFN $\gamma$  significantly increased MHCII mRNA in BMDMs from WT and APP/PS1 mice (\*\*p<0.01, ANOVA; Figure 4.3A) and this IFN $\gamma$ -induced change was significantly greater in BMDMs from APP/PS1 mice (\*\*\*p<0.01, ANOVA; Figure 4.3A). IFN $\gamma$  significantly increased NOD2 mRNA in BMDMs from WT and APP/PS1 mice (\*\*p<0.01, ANOVA; Figure 4.3B), and the IFN $\gamma$ -induced effect was significantly greater in BMDMs from APP/PS1 mice (\*\*\*p<0.01, ANOVA; Figure 4.3B).

#### **4.3.4. The effect of LPS on CD40 and CD11b mRNA expression in BMDMs from WT and APP/PS1 mice.**

The effect of LPS (100ng/ml; 24 hours) was also assessed and the data show that LPS significantly increased CD40 mRNA expression in BMDMs from WT and APP/PS1 mice ( $^{***}p<0.001$ ; ANOVA; Figure 4.4A), but there were no genotype-related change. LPS treatment increased CD11b mRNA expression in BMDMs from WT and APP/PS1 mice ( $^{\dagger}p<0.05$ ; ANOVA; Figure 4.4B), but in this case there was a genotype-related increase in CD11b mRNA expression ( $^{\dagger}p<0.05$ ; ANOVA; Figure 4.4B).

#### **4.3.5. LPS increased TNF $\alpha$ and NOS2 mRNA in BMDMs from WT and APP/PS1 mice.**

LPS significantly increased TNF $\alpha$  mRNA expression in BMDMs from WT and APP/PS1 mice ( $^{***}p<0.001$ , ANOVA; Figure 4.5A), and the LPS-induced effect was significantly greater in BMDMs from APP/PS1 mice ( $^{\dagger}p<0.05$ , ANOVA; Figure 4.5A). LPS stimulation significantly increased NOS2 mRNA expression in a similar fashion in BMDMs from WT and APP/PS1 mice ( $^{***}p<0.001$ , ANOVA; Figure 4.5B).

#### **4.3.6. LPS increased cytokine concentration in BMDMs in BMDMs from WT and APP/PS1 mice.**

It is well documented that in the presence of a pro-inflammatory stimulus like LPS that macrophages release pro-inflammatory mediators such as cytokines. The current data show that in the presence of LPS there was a significantly increased supernatant concentration in TNF $\alpha$  and IL-6 ( $^{***}p<0.001$ ; ANOVA; Figure 4.6A and Figure 4.6B respectively), but no genotype-related changes were observed.

#### **4.3.7. The effect of IL-4 on MRC1 and Arg1 mRNA expression in BMDMs from WT and APP/PS1 mice.**

BMDMs from WT and APP/PS1 mice were treated with IL-4 (200ng/ml; 24 hours), to evaluate the effect of genotype on the ability of BMDMs to adopt the M2 activation state which is characterised by the expression of MRC1 and Arg1 mRNA. IL-4 significantly increased MRC1 mRNA expression in BMDMs from WT and APP/PS1 mice ( $^{***}p<0.001$ ;

ANOVA; Figure 4.7A). IL-4 also significantly increased Arg1 mRNA expression in BMDMs from WT and APP/PS1 mice ( $***p < 0.001$ ; ANOVA; Figure 4.7B), but no genotype-related changes were observed.

#### **4.3.8. IFN $\gamma$ R1 mRNA expression is increased in BMDMs from APP/PS1 mice.**

It was considered that the increased responsiveness of BMDMs from APP/PS1 mice might be a consequence of increased IFN $\gamma$  and, mRNA expression of the receptor was assessed. The data shows that there was a significant increase in the expression of IFN $\gamma$ R1 mRNA in BMDMs from APP/PS1 mice compared to WT mice ( $*p < 0.05$ , student *t* test; Figure 4.8).

#### **4.3.9. IFN $\gamma$ concentration is increased in the hippocampus of APP/PS1 mice.**

BMDMs from APP/PS1 mice exhibit an enhanced response to IFN $\gamma$  and the evidence shows an increase in the number of macrophages in the brains of these mice. Therefore if infiltrating cells are exposed to increased IFN $\gamma$  *in vivo*, they will adopt an M1 state. The data show that IFN $\gamma$  concentration was significantly greater in hippocampus of APP/PS1 compared with WT mice ( $*p < 0.05$ ; Student's *t*-test; Figure 4.9).

#### **4.3.10. IFN $\gamma$ increases TNF $\alpha$ and NOS2 mRNA expression in BMDMs from WT and CD200 $^{-/-}$ mice.**

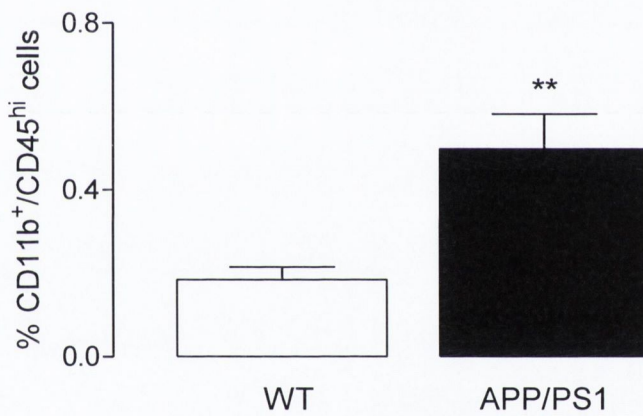
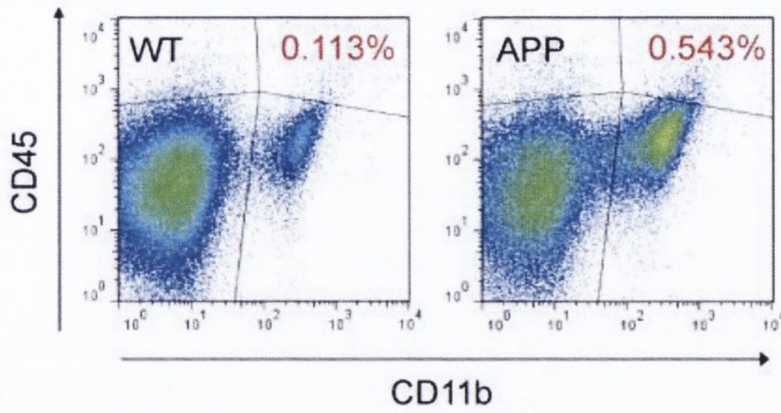
Neuroinflammatory changes have been observed in CD200 $^{-/-}$  mice, and it was hypothesised that BMDMs from CD200 $^{-/-}$  mice might act in a similar to microglia from CD200 $^{-/-}$  mice exhibiting an enhanced responsiveness to IFN $\gamma$ . IFN $\gamma$  significantly increased TNF $\alpha$  mRNA in BMDMs from WT and CD200 $^{-/-}$  mice, but no genotype-related change was observed ( $**p < 0.01$ ; ANOVA; Figure 4.10A). Similarly IFN $\gamma$  significantly increased NOS2 mRNA ( $**p < 0.01$ ; ANOVA; Figure 4.10B); in this case the IFN $\gamma$ -induced change was significantly reduced in BMDMs from CD200 $^{-/-}$  compared with WT mice ( $+p < 0.01$ ; ANOVA; Figure 4.10B).

#### **4.3.11. LPS increased CD11b and CD40 mRNA expression in BMDMs from WT and CD200<sup>-/-</sup> mice.**

Previous evidence has shown that glia and macrophages from CD200<sup>-/-</sup> mice show an exaggerated response to TLR stimulation (Snelgrove *et al.*, 2008; Costello *et al.*, 2011). LPS significantly increased CD11b (\*p<0.05; ANOVA; Figure 4.11A) and CD40 (\*\*p<0.01; ANOVA; Figure 4.11B) mRNA in BMDMs from WT and CD200<sup>-/-</sup> mice, no genotype-related change was observed.

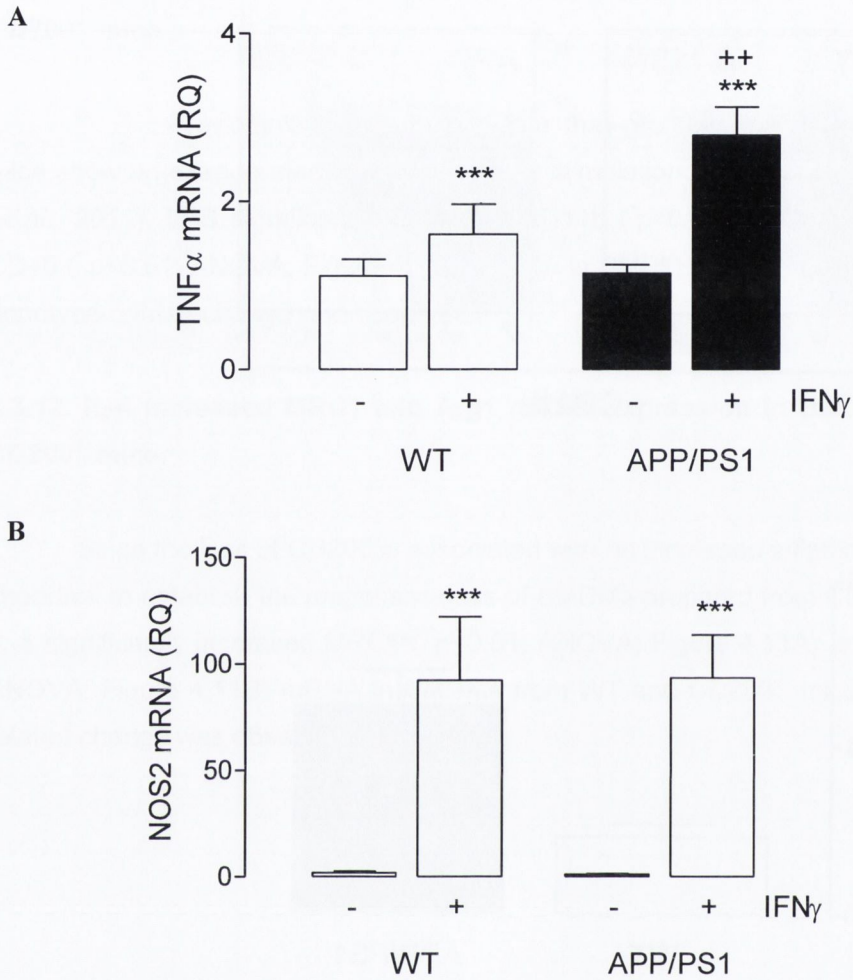
#### **4.3.12. IL-4 increased MRC1 and Arg1 mRNA expression in BMDMs from WT and CD200<sup>-/-</sup> mice.**

Since the loss of CD200 is associated with an increased inflammatory profile it was important to establish the responsiveness of BMDMs prepared from CD200<sup>-/-</sup> mice to IL-4. IL-4 significantly increased MRC1 (\*\*p<0.01; ANOVA; Figure 4.11A) and Arg1 (\*\*p<0.001; ANOVA; Figure 4.11B) mRNA in BMDMs from WT and CD200<sup>-/-</sup> mice, but no genotype-related change was observed.



**Figure 4.1. Increased percentage of CD11b<sup>+</sup>/CD45<sup>high</sup> cells present in the brains of APP/PS1 animals.**

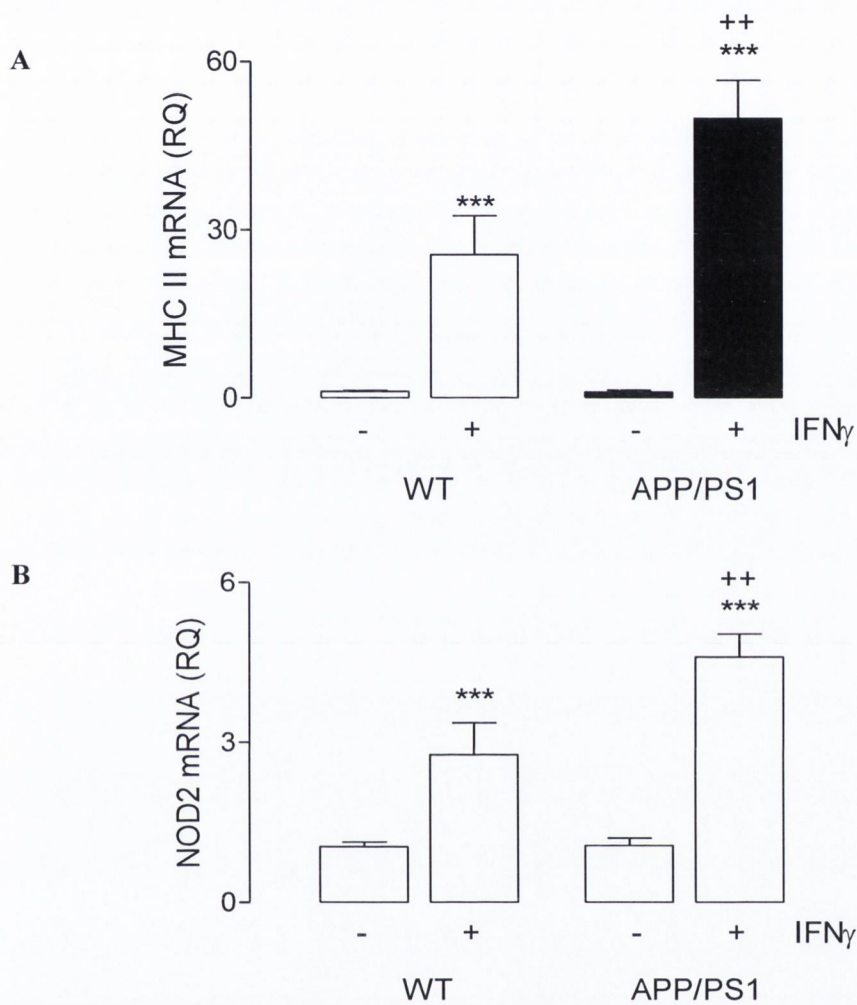
There was a significant genotype-related increase in the percentage of macrophages in the brains of APP/PS1 mice (\*\* $p < 0.01$ ; Student's *t*-test). Values are presented as means ( $\pm$ SEM;  $n=8$ ) and expressed as a percentage of CD11b<sup>+</sup> cells isolated from the brains. (Data generated by Dr Raasay Jones and Dr Aedin Minogue)



**Figure 4.2. The effect of IFN $\gamma$  on TNF $\alpha$  and NOS2 mRNA expression in BMDMs from WT and APP/PS1 mice.**

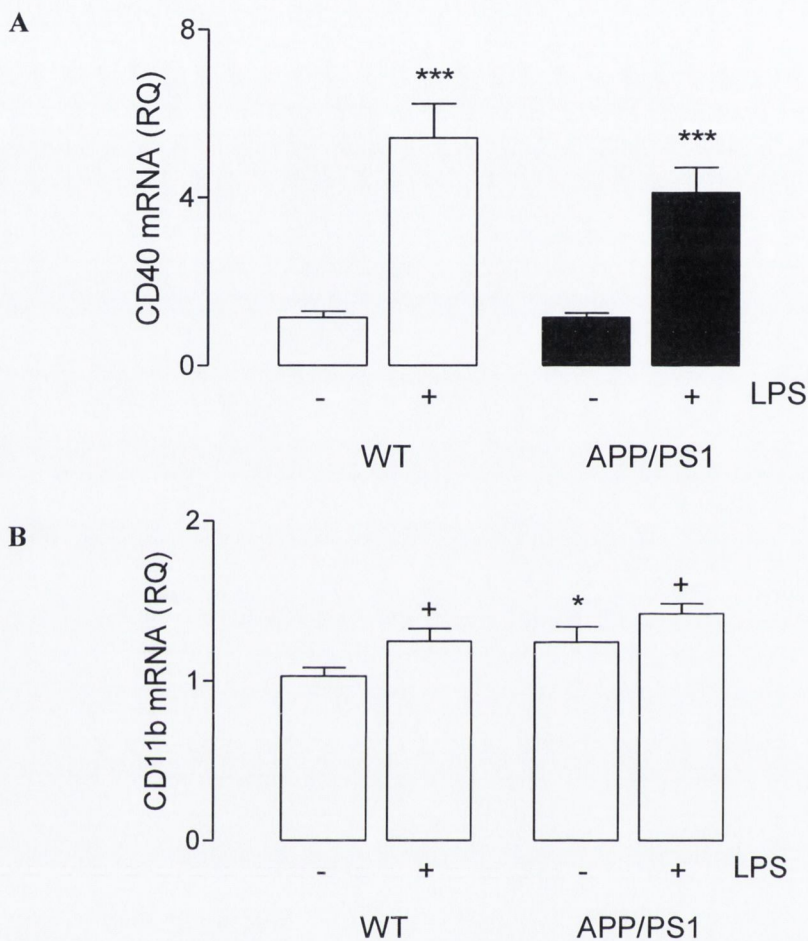
BMDMs were treated with IFN $\gamma$  (50ng/ml; 24 hours). IFN $\gamma$  increased TNF $\alpha$  mRNA expression in BMDMs from WT and APP/PS1 mice (\*\* $p$ <0.001; ANOVA; A and B respectively), the IFN $\gamma$  effect on TNF $\alpha$  mRNA was significantly greater in BMDMs from APP/PS1 mice (\*\* $p$ <0.01; ANOVA; A). Values are presented as means ( $\pm$ SEM;  $n$ =10-12) and expressed as a ratio to  $\beta$ -actin mRNA.





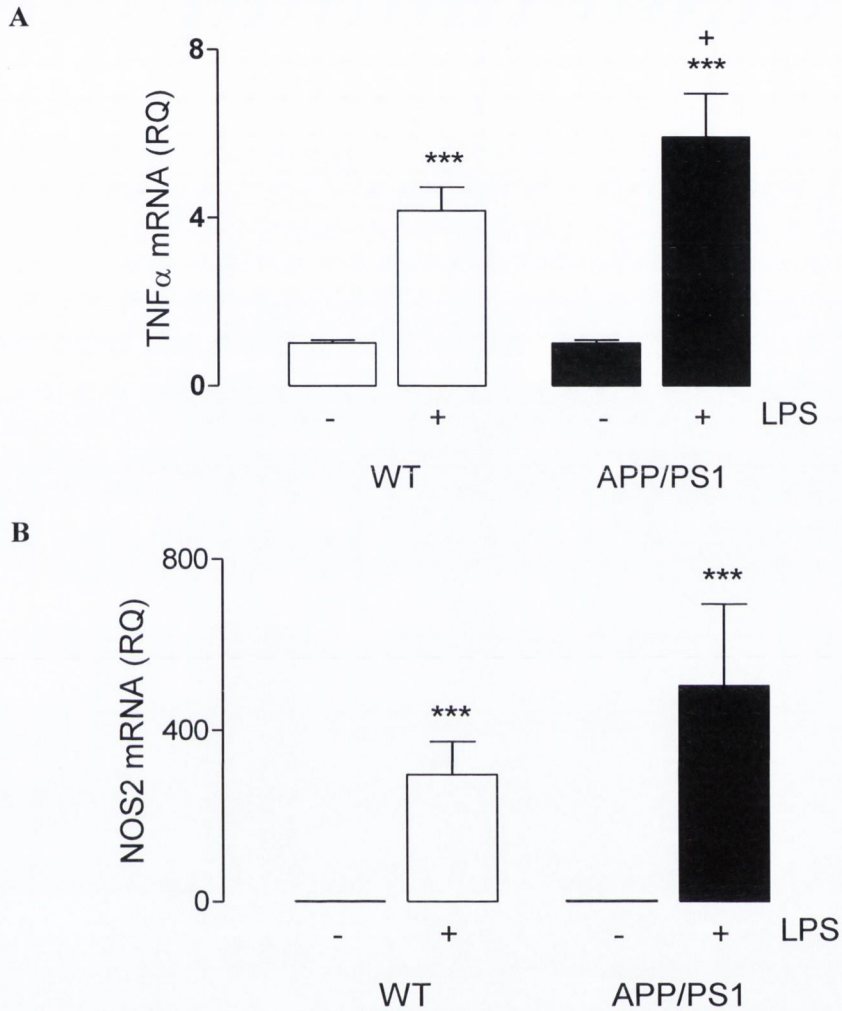
**Figure 4.3. The effect of IFN $\gamma$  on MHCII and NOD2 mRNA in BMDMs from WT and APP/PS1 mice.**

IFN $\gamma$  (50ng/ml; 24 hours) significantly increased MHCII and NOD2 mRNA expression in BMDMs from WT and APP/PS1 mice (\*\*\*) $p$ <0.01; ANOVA; A and B respectively) and the IFN $\gamma$ -induced change was significantly greater in BMDMs from APP/PS1 mice (\*\*) $p$ <0.01, ANOVA; A and B respectively). Values are presented as means ( $\pm$ SEM;  $n$ =10-12) and expressed as a ratio to  $\beta$ -actin mRNA.



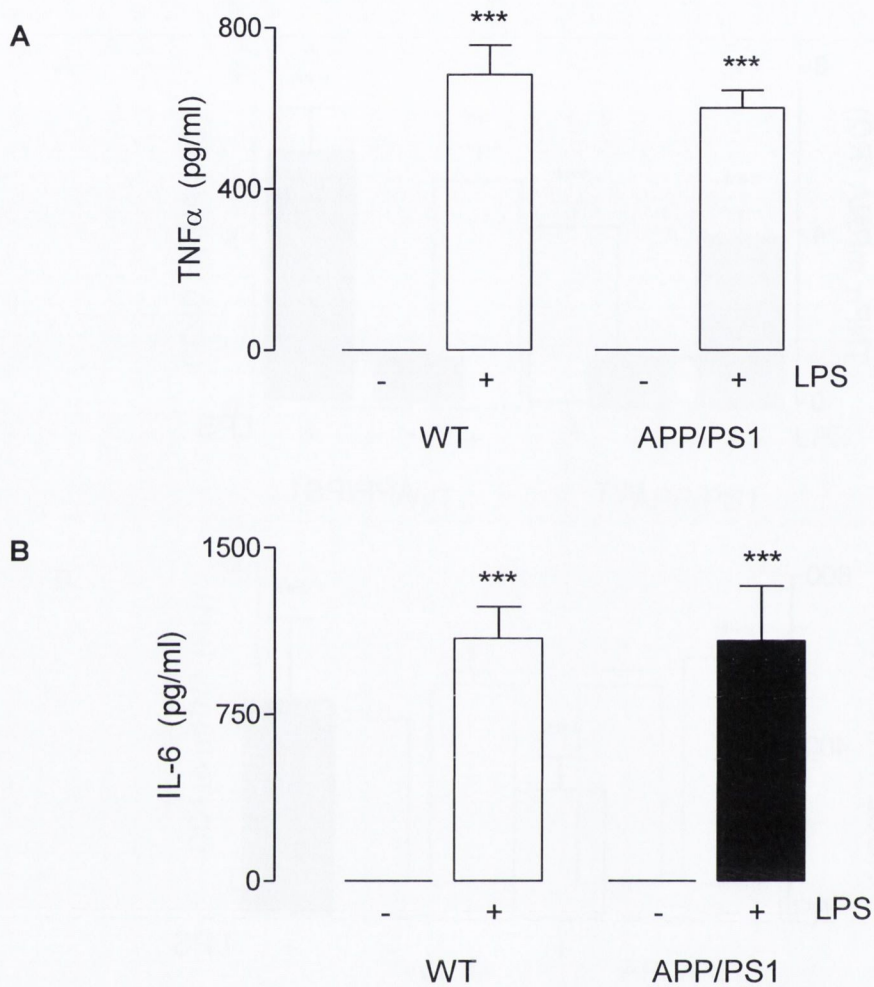
**Figure 4.4. The effect of LPS on CD40 and CD11b mRNA in BMDMs from WT and APP/PS1 mice.**

BMDMs from WT and APP/PS1 mice were treated with LPS (100ng/ml; 24 hours). LPS significantly increased CD40 in BMDMs from WT and APP/PS1 mice (\*\* $p < 0.001$ ; ANOVA; A) but no genotype-related changes were observed. There was a genotype-related increase in CD11b mRNA expression (\* $p < 0.05$ ; ANOVA; B), LPS increased CD11b mRNA ( $^+p < 0.05$ , ANOVA; B). Values are presented as means ( $\pm$ SEM;  $n=10-12$ ) and expressed as a ratio to  $\beta$ -actin mRNA.



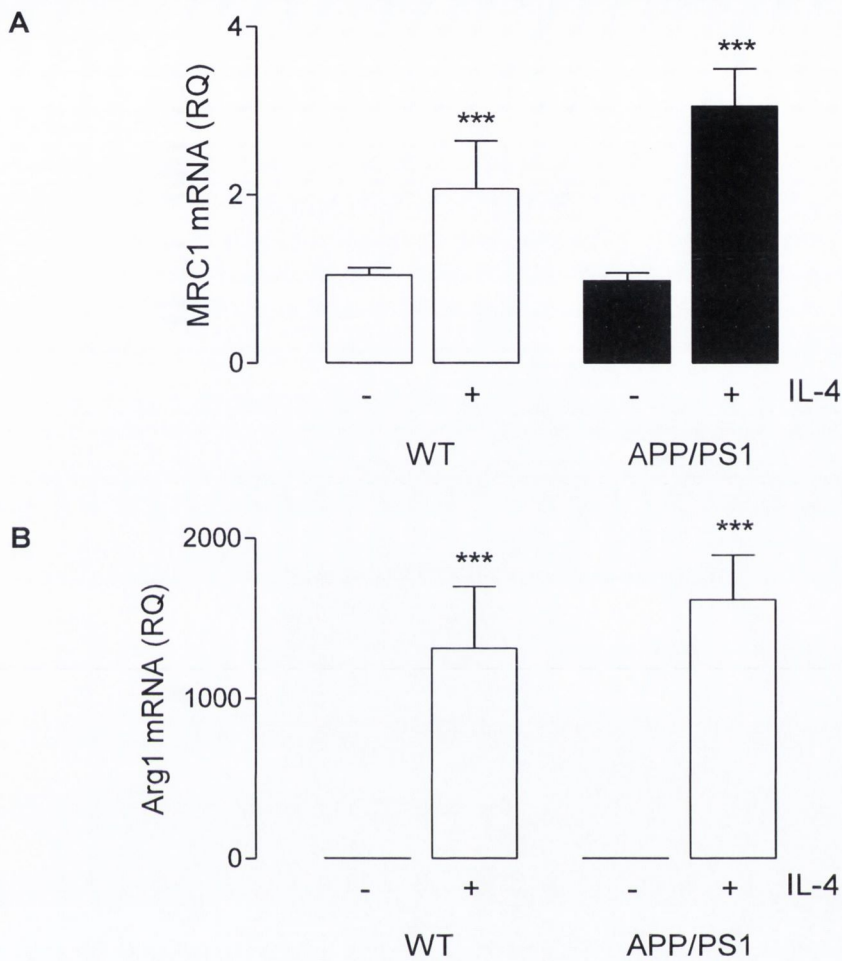
**Figure 4.5. The effect of LPS on TNF $\alpha$  and NOS2 mRNA in BMDMs from WT and APP/PS1 mice.**

BMDMs from WT and APP/PS1 mice were treated with LPS (100ng/ml; 24 hours). LPS significantly increased TNF $\alpha$  and NOS2 mRNA expression in BMDMs from WT and APP/PS1 mice (\*\* $p < 0.001$ , ANOVA; A and B respectively), and the LPS-induced increase in TNF $\alpha$  mRNA expression was significantly greater in BMDMs from APP/PS1 mice ( $^+p < 0.05$ ; ANOVA; A). Values are presented as means ( $\pm$ SEM;  $n = 10-12$ ) and expressed as a ratio to  $\beta$ -actin mRNA.



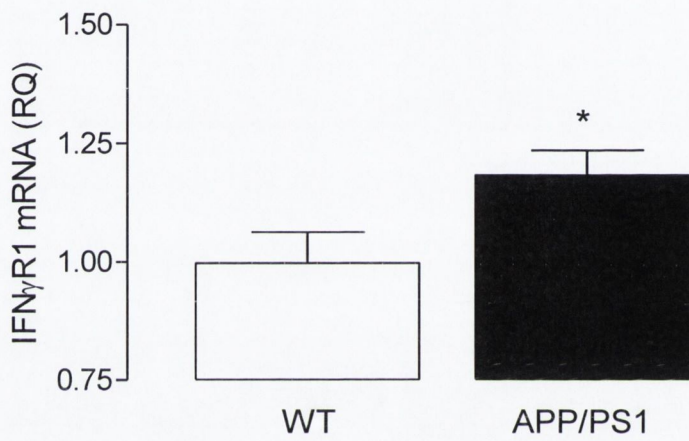
**Figure 4.6. LPS increased TNF $\alpha$  and IL-6 in supernatant of BMDMs from WT and APP/PS1 mice.**

LPS (100ng/ml; 24 hours) significantly increased supernatant concentration of IL-6 and TNF $\alpha$  in BMDMs from WT and APP/PS1 mice (\*\* $p < 0.001$ ; ANOVA; A and B respectively), no genotype related changes were observed. Values are presented as means ( $\pm$ SEM;  $n=10-12$ ) and expressed as pg/ml.



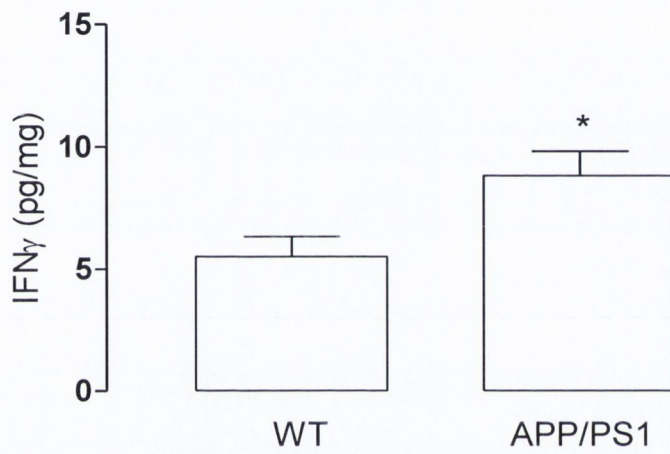
**Figure 4.7. The effect of IL-4 on MRC1 and Arg1 mRNA expression in BMDMs from WT and APP/PS1 mice.**

IL-4 (200ng/ml; 24 hours) increased MRC1 and Arg1 mRNA expression in BMDMs from WT and APP/PS1 mice (\*\**p*<0.001; ANOVA; A and B respectively), however no genotype-related changes observed. Values are presented as means ( $\pm$ SEM; n=10-12) and expressed as a ratio to  $\beta$ -actin mRNA.



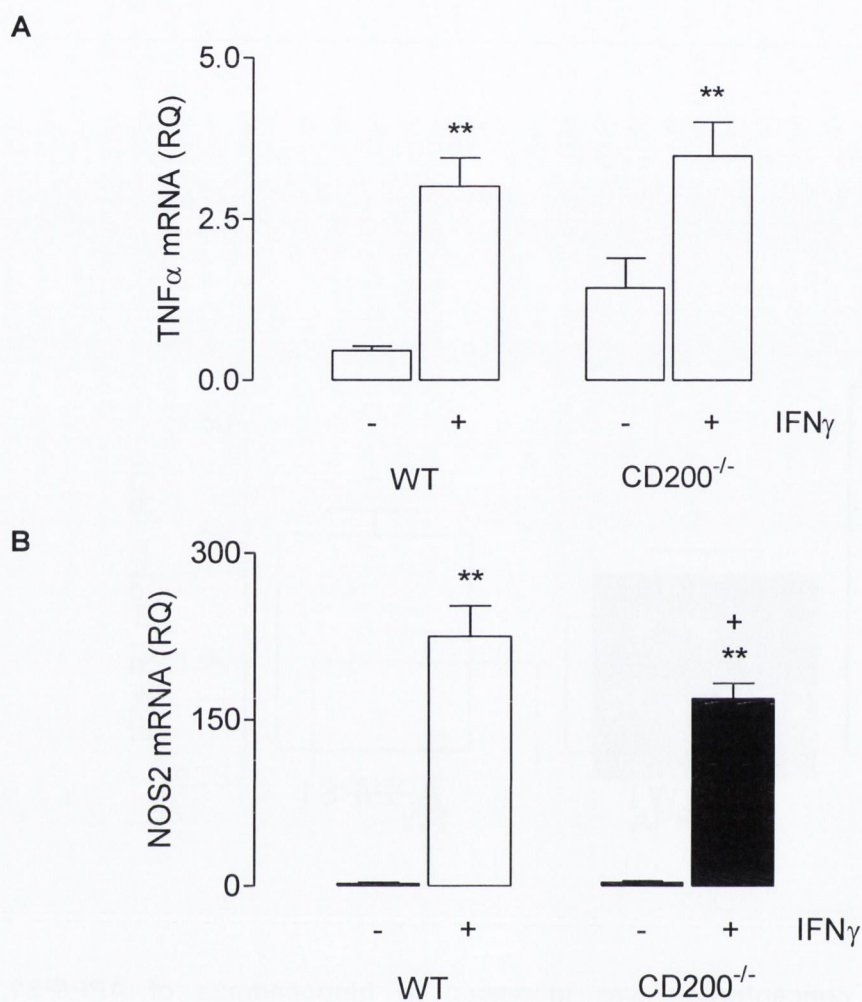
**Figure 4.8. IFN $\gamma$ R1 mRNA was increased in BMDMs from APP/PS1 mice.**

There was a significant increase in IFN $\gamma$ R1 mRNA in BMDMs prepared from APP/PS1 mice compared with WT mice (\* $p < 0.05$ ; Student's *t*-test). Values are presented as means ( $\pm$ SEM;  $n = 10-12$ ) and expressed as a ratio to  $\beta$ -actin mRNA.



**Figure 4.9. IFN $\gamma$  concentration was increased in hippocampus of APP/PS1, compared with WT mice**

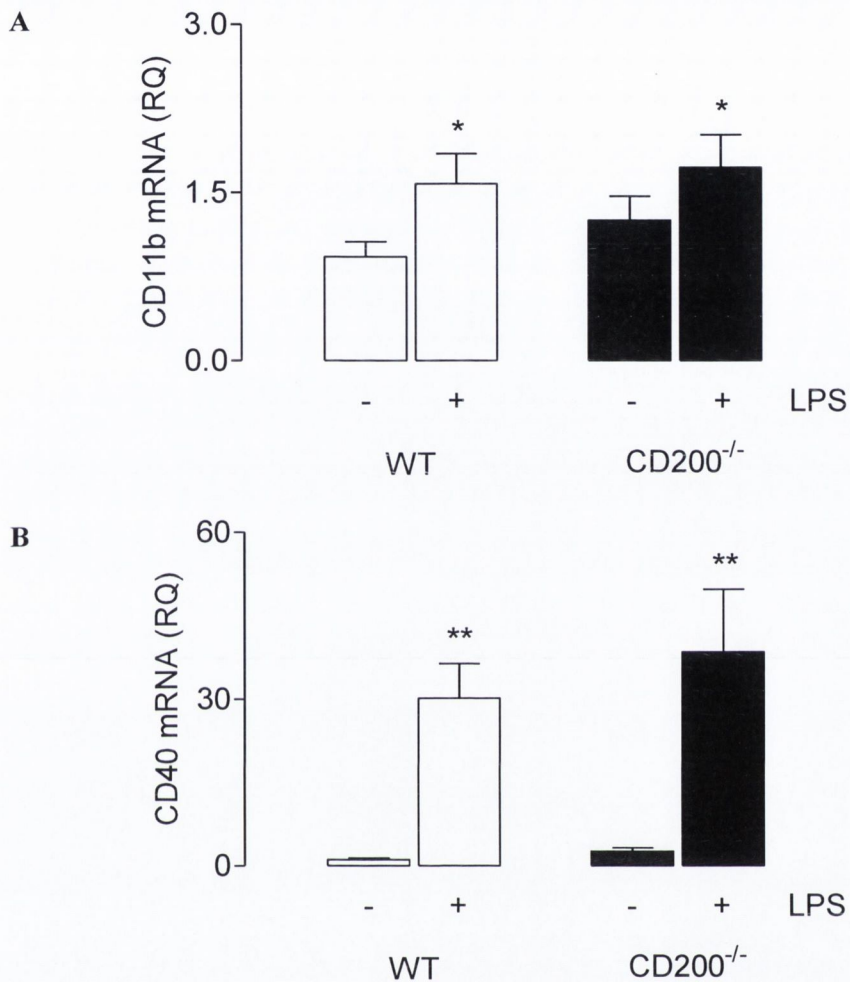
IFN $\gamma$  protein concentration was significantly greater in hippocampus of APP/PS1 mice (\* $p < 0.05$ ; Student's *t*-test). Data are presented as means ( $\pm$ SEM;  $n = 5-6$ ) and expressed as pg/mg. (Data generated by Dr Raasay Jones and Dr Aedin Minogue)



**Figure 4.10. The effect of IFN $\gamma$  on TNF $\alpha$ , and NOS2 mRNA expression in BMDMs from WT and CD200<sup>-/-</sup> mice.**

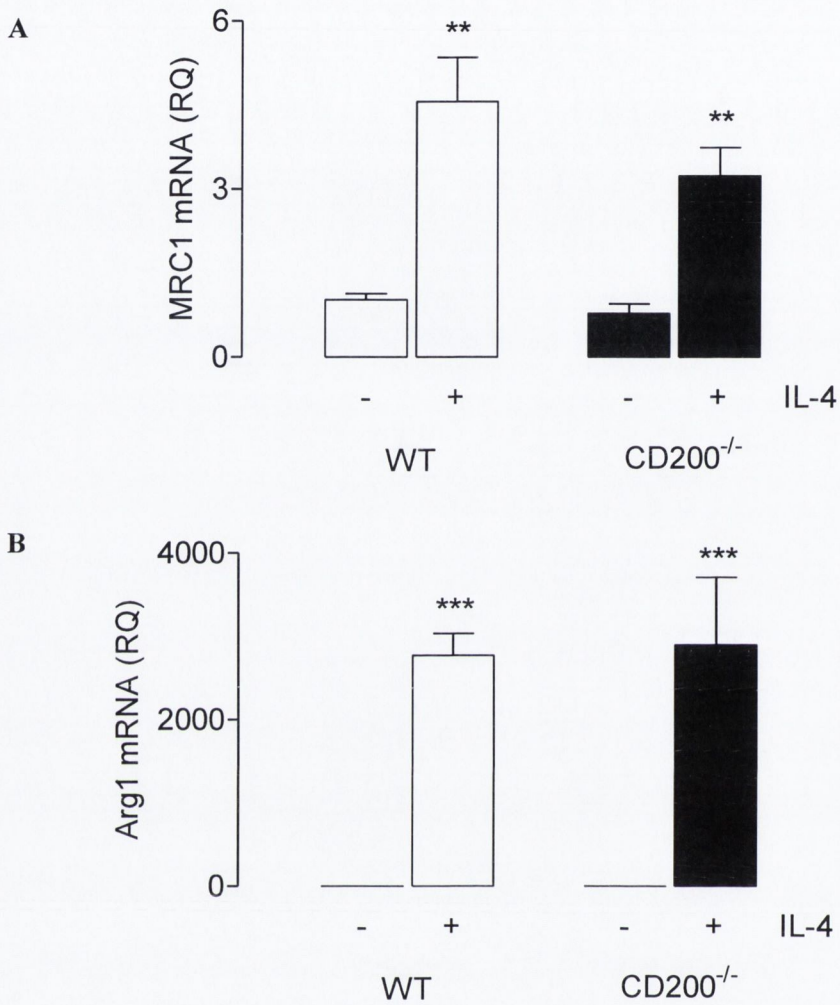
IFN $\gamma$  (50ng/ml; 24 hours) increased TNF $\alpha$  and NOS2 mRNA in BMDMs from WT and CD200<sup>-/-</sup> mice (\*\*p<0.01; ANOVA; A and B respectively) and the IFN $\gamma$ -induced NOS2 change was significantly reduced in BMDMs from CD200<sup>-/-</sup> mice. (\*p<0.05; ANOVA; B), Values are presented as means ( $\pm$ SEM; n=4) and expressed as a ratio to  $\beta$ -actin mRNA.





**Figure 4.11. The effect of LPS on CD11b and CD40 mRNA in BMDMs from WT and CD200<sup>-/-</sup> mice.**

LPS (100ng/ml; 24 hours) significantly increased CD11b mRNA in BMDMs from WT and CD200<sup>-/-</sup> mice (\*p<0.05, ANOVA; A). LPS also increased CD40 mRNA in BMDMs from WT and CD200<sup>-/-</sup> mice (\*\*p<0.01, ANOVA; B). No genotype-related genotype related changes were observed. Values are presented as means (±SEM; n=4) expressed as a ratio to β-actin mRNA.



**Figure 4.12. The effect of IL-4 on MRC1 and Arg1 mRNA expression in BMDMs from WT and CD200<sup>-/-</sup> mice.**

IL-4 (200ng/ml; 24 hours) significantly increased MRC1 (\*\* $p < 0.01$ , ANOVA; A) and Arg1 mRNA (\*\*\*) $p < 0.001$ , ANOVA; B) expression in BMDMs from WT and CD200<sup>-/-</sup> mice. No genotype-related change observed. Values are presented as means ( $\pm$ SEM;  $n=4$ ) and expressed as a ratio to  $\beta$ -actin mRNA.

#### 4.4. Discussion

The significant finding in this study is that BMDMs prepared from APP/PS1 mice respond more profoundly to LPS and IFN $\gamma$  than BMDMs from WT mice. In contrast, there was no change in responsiveness to IL-4. The importance of this finding lies in the fact that the inflammatory environment in the brain which is a characteristic of age, and also occurs in the brain of APP/PS1 mice, will stimulate infiltrating macrophages and further enhance the already-existing neuroinflammatory changes.

Flow cytometric analysis of a cell suspension prepared from perfused brain tissue revealed that there was a significant increase in the proportion of CD11b<sup>+</sup> CD45<sup>high</sup> macrophages in samples from APP/PS1 mice compared with WT mice. There is no definitive marker which distinguishes macrophages from microglia but it is agreed that the expression of CD45 is a useful indicator since it is expressed at very low levels in resident microglia although expression increases when cells are activated (Sedgwick *et al.*, 1991). Recent data from this laboratory has confirmed this genotype-related increase in infiltration of macrophages (Kelly *et al.*, 2013) which was also associated with infiltration of T lymphocytes. The primary role of these infiltrating macrophages has yet to be identified but use of animal models has yielded evidence suggesting that these infiltrating cells have an important role in the clearance of A $\beta$  plaques (Simard *et al.*, 2006; El Khoury *et al.*, 2007); however other groups have suggested that phagocytic activity of macrophages from AD patients is defective (Fiala *et al.*, 2005). Although the mechanism by which cell infiltration occurs remains to be clarified, Kelly and colleagues demonstrated that it was associated with increased blood brain barrier permeability as assessed by MRI using the signal generated by extravasation of gadolinium. Interestingly a similar increase in macrophage infiltration coupled with BBB permeability was observed in CD200<sup>-/-</sup> mice (Denieffe *et al.*, 2013). In both studies, and others (O'Reilly & Lynch, 2012; Gallagher *et al.*, 2013; Kelly *et al.*, 2013), there was evidence of increased expression of several markers of microglial activation including an increase in IL-12 concentration which may trigger macrophages to release IFN $\gamma$  (Darwich *et al.*, 2009). Indeed it has been suggested that the increase in microglial activation, which have the characteristics of the classical activation state, may be driven by IFN $\gamma$  released from Th1 cells or perhaps macrophages (Denieffe *et al.*, 2013) (Minogue *et al.* 2013 in review).

To characterize the BMDMs, cells prepared from WT and APP/PS1 mice were incubated in the presence of IFN $\gamma$  and LPS to induce macrophages to adopt the M1 pro-inflammatory activation state. The archetypal markers of this state are TNF $\alpha$  mRNA and NOS2 mRNA (Gordon, 2003) although recent data from this laboratory have indicated that increased expression of NOD2 may also be a reliable indicator of this state. In the present study, IFN $\gamma$  treatment caused an increase in TNF $\alpha$  and NOS2 mRNA, and the IFN $\gamma$ -induced change in TNF $\alpha$  mRNA was greater in BMDMs from APP/PS1 mice. This mirrors recent work carried out in this group which has reported a genotype-related increase in hippocampal TNF $\alpha$  protein and mRNA expression (Minogue in review) (Kelly *et al.*, 2013). IFN $\gamma$  treatment increased NOD2 mRNA expression in BMDMs from WT and APP/PS1 mice and this IFN $\gamma$ -induced change was significantly greater in BMDMs from APP/PS1 mice. NOD2 has been implicated in inflammatory conditions such as Crohn's disease and stimulation of NOD2 in conjunction with TLR activation has been associated with an increase in the production of a number of cytokines (Le Bourhis *et al.*, 2007; Moreira & Zamboni, 2012). IFN $\gamma$  treatment increased MHCII mRNA expression in BMDMs from both groups and this is consistent with similar findings in microglia (Lynch, 2009). Like TNF $\alpha$  and NOD2 mRNA the IFN $\gamma$ -induced change in MHCII was greater in BMDMs from APP/PS1 mice. Analysis of IFN $\gamma$ R mRNA revealed a genotype-related increase, which may explain the increased responsiveness to IFN $\gamma$  observed in BMDMs prepared from APP/PS1 mice. An increased expression of IFN $\gamma$ R has also been reported in the brains of APP/PS1 animals; in this case flow cytometric analysis of a single cell suspension prepared from the perfused brain identified a genotype-related increase in the percentage of CD11b<sup>+</sup>/IFN $\gamma$ R<sup>+</sup> cells (Minogue *et al.*, 2012).

LPS predictability induced the expression of pro-inflammatory markers associated with M1 activation. It increased the expression of CD11b and CD40 mRNA in BMDMs prepared from WT and APP/PS1 mice; but, interestingly, there was a genotype-related increase in the expression of CD11b mRNA which compliments previous work from this group that shows increased CD11b mRNA expression in the hippocampus of APP/PS1 animals (Kelly *et al.*, 2013). LPS treatment also increases TNF $\alpha$  and NOS2 mRNA in BMDMs, however only the LPS-induced change in TNF $\alpha$  mRNA was significantly greater in cells prepared from APP/PS1 mice. Activated macrophages release pro-inflammatory mediators including cytokines and here LPS increased the concentration of TNF $\alpha$  and IL-6 in supernatant samples from BMDMs prepared from WT and APP/PS1 mice, however no

genotype-related changes were observed. Previous work has reported that LPS-induced production of TNF $\alpha$  and IL-6 was enhanced in monocytes prepared from AD patients in comparison with controls (Guerreiro *et al.*, 2007). This group isolated monocytes from blood samples of AD patients. Macrophages were derived from bone marrow cells in the present study, it may also be that exposure to circulating factors may confer differences in the response. This difference may also indicate that it is inappropriate to extrapolate findings from a mouse model of AD to the human condition.

In contrast to the increased responsiveness of BMDMs from APP/PS1 mice to LPS and IFN $\gamma$ , no genotype-related changes in IL-4 induced increases in MRC1 and Arg1 mRNA expression were observed. These results compliment data from a recent study which reported no genotype-related differences in hippocampal expression of MRC1 or Arg1 mRNA in WT and APP/PS1 mice (Minogue *et al.*, 2012). Taken together, whereas clear differences in markers of M1 activation were observed these results suggest that microglia and macrophages respond to IL-4 in a similar fashion in WT and APP/PS1 mice. Although work from this lab reports no changes in the expression of markers of M2 activation, conflicting reports have suggested that the hippocampal expression of M2 markers are increased (Wilcock *et al.*, 2011) and decreased (Jimenez *et al.*, 2008) in mouse models of AD. These contrasting findings may be explained by the different transgenic models employed in the respective studies. One of the limitations of these results is that the expression of these markers was assessed in hippocampal tissue and so is not reflective of microglial expression.

Since it was found that there was a genotype-related increase in the infiltration of macrophages and that BMDMs from APP/PS1 mice exhibited an enhanced response to IFN $\gamma$ , it was of importance to assess the expression of IFN $\gamma$  in the brain of these animals. An increase in IFN $\gamma$  in the brain of APP/PS1 would provide a stimulus which would trigger the infiltrating cells to adopt an M1 phenotype and potentially exacerbate any existing inflammatory changes. IFN $\gamma$  concentration was found to be significantly greater in the hippocampus of APP/PS1 mice compared to their WT counterparts. This replicates previous work from this group that reported a genotype-related increase in hippocampal IFN $\gamma$  concentration (Kelly *et al.*, 2013). Increased cortical concentration of IFN $\gamma$  has been reported in another transgenic model and this was accompanied by a decrease in IL-4 (Abbas *et al.*, 2002). The cell source of IFN $\gamma$  in the brain remains to be identified since

resident cells produce little, if any, IFN $\gamma$ , although sporadic reports have suggested that microglia may release IFN $\gamma$  under specific circumstances. Research from this group suggested that the increased IFN $\gamma$  concentration may be attributed to increased infiltration of peripheral immune cells such as T cells (Browne *et al.*, 2013b) and NK cells (Kelly *et al.*, 2013). Brains of APP/PS1 mice exhibit evidence of increased inflammatory activity and the current data suggests that macrophages that gain entry into the brain will be activated by IFN $\gamma$  and exacerbate the inflammation. The consequences of increased IFN $\gamma$  in the brain on neuronal function may be significant. It has been shown that when IFN $\gamma$  is increased for example in aged rats, APP/PS1 or CD200<sup>-/-</sup> mice LTP is decreased. Consistent with this, recent evidence has shown that intrahippocampal injection of IFN $\gamma$  leads to increased microglial activation accompanied by a deficit in LTP (Kelly *et al.*, 2013).

CD200 is expressed on numerous cells in the CNS including endothelial cells, astrocytes and neurons, while CD200R is expressed on microglia, and the CD200/CD200R interaction maintains microglia/macrophages in a quiescent state. Loss of CD200 is associated with an increased inflammatory profile. In addition CD200 and CD200R expression is reduced in the brains of AD patients compared to aged-matched controls and this was associated with inflammatory changes (Walker *et al.*, 2009). Here the hypothesis was that macrophages from CD200<sup>-/-</sup> mice would exhibit an enhanced response to pro-inflammatory stimuli and although IFN $\gamma$  treatment increased TNF $\alpha$  mRNA expression in BMDMs prepared from WT and CD200<sup>-/-</sup> mice, there were no genotype-related enhancement. The findings in macrophages are at variance with findings in microglia, recent work from this laboratory has proposed that glia from CD200<sup>-/-</sup> mice exhibit enhanced responsiveness to IFN $\gamma$  (Denieffe *et al.*, 2013). These results indicate that BMDMs and microglia from CD200<sup>-/-</sup> mice show different responses to inflammatory stimuli.

Like IFN $\gamma$ -induced changes, the LPS-induced increases in CD11b and CD40 mRNA expression were similar in BMDMs prepared from WT and CD200<sup>-/-</sup> mice. Previous work has shown that glia and alveolar macrophages prepared from CD200<sup>-/-</sup> mice show an enhanced susceptibility to TLR activation (Snelgrove *et al.*, 2008; Costello *et al.*, 2011). The difference in findings may reflect variation in the responses of macrophages from different regions of the body, as previously demonstrated by (Kohut *et al.*, 2004). Another

possible reason for the differences in responses between the present study and that of Snelgrove and colleagues is the age of the animals; in the present study all mice were 12 weeks old, while alveolar macrophages from 24 week old animals.

Previous unpublished work from this group has reported that glia from CD200<sup>-/-</sup> mice respond to IL-4 in a similar fashion to their WT counterparts and the data here suggests that BMDMs prepared from WT and CD200<sup>-/-</sup> mice also respond to IL-4 in a similar fashion.

Therefore the principal finding of this study is that BMDMs from APP/PS1 mice respond more profoundly to inflammatory stimuli but not to anti-inflammatory stimuli, whereas CD200 deficiency had no effect on responsiveness of BMDMs to any stimuli. There was also an increase in macrophage infiltration into the brain of APP/PS1 mice and this was accompanied by an increase in hippocampal concentration of IFN $\gamma$ . Previous work has suggested that these cells play a beneficial role in A $\beta$  clearance, but the evidence from the current study suggests that they also have the potential to exacerbate inflammatory activity within the CNS. Therapies that restrict the entry of IFN $\gamma$  producing cells into the CNS may prove to be beneficial.

## Chapter 5

### Modulation of macrophage activation; a role for the PI3K/AKT pathway?



## 5.1. Introduction

Alternative or M2 activation is induced by the Th2 cytokines IL-4 and IL-13 and similar to many cytokines, IL-4 and IL-13 signal transduction is mediated through the JAK/STAT pathway. Activation of the specific cytokine receptor leads to autophosphorylation of JAKs which leads to the recruitment and activation of STATs which translocates to the nucleus to induce gene transcription. IL-4 and IL-13 signalling is primarily through activation of STAT6, while IFN $\gamma$  signalling is mediated through the activation of STAT1. M1 activation is associated with an increased expression of pro-inflammatory mediators; it can be induced by PAMPs such as LPS or endogenous activators such as IFN $\gamma$ .

IL-4 can signal through two receptors, the type I IL-4 receptor (composed of two subunits, IL-4R $\alpha$  and IL-2R $\gamma$ ) and type II receptor (IL-4R $\alpha$  and IL-13R $\alpha$ 1), however IL-13 signalling is restricted to the type II receptor. Type I IL-4 receptor is expressed predominantly on hematopoietic cells, while type II IL-4R is found on non-hematopoietic cells, although some cells such as macrophages express both types (Gordon & Martinez, 2010). Ligation of IL-4 to type I IL-4R leads to the activation of JAK1 and JAK3, JAK1 phosphorylates the tail of the receptor which acts as a docking site for STAT6 where it becomes phosphorylated. Activation of type II IL-4R also results in the phosphorylation of STAT6 and this is mediated through the activation of JAK1 and tyrosine kinase 2 (Tyk2). Phosphorylated STAT6 forms a homodimer with other phosphorylated STAT6 molecules and translocates to the nucleus where it causes the induction of IL-4 responsive genes (Varin & Gordon, 2009). Unlike type I IL-4R, type II IL-4R can be activated by either of IL-4 or IL-13. Activation of the type I IL-4R also activates IRS-1/2 which results in the activation of PI3K and RAS-MAPK which induce cell proliferation (Gordon & Martinez, 2010).

The IFN $\gamma$  signalling cascade also utilises the JAK/STAT pathway, (Saha *et al.*, 2010); the IFN $\gamma$  receptor comprises of two subunits, IFN $\gamma$  receptor (IFN $\gamma$ R) 1 and IFN $\gamma$ R2 and both receptor subunits interact with a member of the JAK family. IFN $\gamma$ R1 interacts with JAK1 while IFN $\gamma$ R2 interacts with JAK2 and binding of IFN $\gamma$  to its receptor activates JAK1 and JAK2 which leads to the recruitment of STAT1 to the receptor. When STAT1 is phosphorylated it forms a homodimer and translocates to the nucleus upregulating the transcription of IFN $\gamma$  responsive genes (Platanias, 2005). Changes in signalling can lead

to some detrimental effects, CD200<sup>-/-</sup> mice exhibit evidence of increased inflammation and it has been recently proposed that this is caused by an increase in STAT1 activation following IFN $\gamma$  stimulation leads to exaggerated M1 responses in glia prepared from CD200<sup>-/-</sup> (Denieffe *et al.*, 2013).

The PI3K/AKT pathway is a central signalling pathway that can be stimulated by growth factors, cytokines and TLR stimulation (Ruckerl, Jenkins *et al.* 2012). Activation of this pathway leads to the modulation of several cellular processes such as cell survival cell cycle progression and cellular growth (Fresno Vara, Casado *et al.* 2004). Activation of the PI3K/AKT pathway has also been associated with anti-inflammatory properties, for example evidence has suggested that the PI3K/AKT pathway is a negative regulator of TLR induced cytokine release (Luyendyk, Schabbauer *et al.* 2008; Medina, Morris *et al.* 2010) and mediates the anti-inflammatory effects of fractalkine in mixed glial cells (Lyons, Lynch *et al.* 2009). Since it has been reported that the PI3K/AKT pathway plays a role in the induction of M2 activation (Ruckerl, Jenkins *et al.* 2012) and IFN $\gamma$  has been shown to increase the expression of PTEN, a negative regulator of PI3K activity (Zhang, Banik *et al.* 2007), the aim of the present study was to investigate the differential effects of IL-4 and IFN $\gamma$  on the PI3K/AKT pathway.

Aim of Study:

1. To investigate the differential effects of IL-4 and IFN $\gamma$  on PI3K/AKT activity in BMDMs.
2. To determine the role of the PI3K/AKT pathway in the induction of M2 activation.

## 5.2. Methods

BMDMs were cultured from C57/BL6 mice (3 months n=5) as described in section 2.2.4. BMDMs were treated with IL-4 (200ng/ml) or IFN $\gamma$  and cells were harvested for RNA and protein analysis at 0, 0.5, 1, 2, 4 and 24 hours. In a separate experiment, BMDMs were treated in the presence or absence of IL-4 or IFN $\gamma$  and the PI3K inhibitor Ly294002 (10 $\mu$ M). BMDMs were pre-treated with Ly294002 for 30 minutes prior to the addition of IL-4 or IFN $\gamma$  for 24 hours; cells were harvested for RNA and protein analysis.

All data are expressed as mean  $\pm$  standard error of the mean (SEM). Data were analysed using a student t-test or 2-way analysis of variance (ANOVA) where indicated, with significance denoted as  $p < 0.05$ . If significance was found following the use of ANOVA, the data were further analysed with the use of a Bonferroni *post hoc* tests. Analysis was carried out using Prism software (GraphPad, US).

### 5.3. Results

#### 5.3.1. IL-4 induced the activation of pSTAT6 in BMDMs.

It is well documented that IL-4 mediates its effects through the activation of STAT6 (Gordon & Martinez, 2010). Here BMDMs were treated with IL-4 (200ng/ml) for 0, 0.5, 1, 2, 4 and 24 hours respectively. It was found that pSTAT6 was significantly increased by IL-4 treatment as early as 0.5 hour ( $***p<0.001$ ; ANOVA; Figure 5.1) and remained significantly increased up to 24 hours.

#### 5.3.2. The effect of IL-4 on PI3K/AKT pathway in BMDMs.

IL-4 stimulation activates the PI3K/AKT pathway in macrophages and it has been reported that activation of this pathway was crucial in the induction of M2 activation (Heller, Qi et al. 2008; Ruckerl, Jenkins et al. 2012). Here analysis was undertaken to evaluate the effect of IL-4 treatment on pPI3K and PI3K expression in BMDMs. BMDMs were treated with IL-4 for 0, 0.5, 1, 2, 4 and 24 hours, analysis concluded that IL-4 did not alter pPI3K (Figure 5.2A) or total PI3K (Figure 5.2B) expression at any timepoint. pAKT expression was significantly increased 24 hours following stimulation with IL-4 ( $**p<0.01$ ; Student's *t*-test; Figure 5.3A). IL-4 treatment had no effect on total AKT (Figure 5.3B) expression at any timepoint.

#### 5.3.3. IL-4 induced the MRC1, Arg1, Chi3li3 and FIZZ1 in BMDMs.

Increased expression of MRC1, Arg1, Chi3li3 and FIZZ1 are hallmarks of M2 activated macrophages (Colton, 2009). The aim of the experiment was to investigate the time course of IL-4 induced changes in these markers. IL-4 increased MRC1 mRNA as early as 1 hour ( $***p<0.001$ ; ANOVA; Figure 5.4A), however Arg1 mRNA was not significantly increased until 6 hours following IL-4 treatment ( $***p<0.001$ ; ANOVA; Figure 5.4B). IL-4 significantly increased Chi3li3 and FIZZ1 mRNA expression at 24 hours only ( $***p<0.001$ ; ANOVA; Figure 5.5A and B respectively).

#### **5.3.4. The effect of Ly294002 on IL-4-induced markers of M2 activation in BMDMs.**

Since IL-4 treatment increased pAKT expression in BMDMs at 24 hours, analysis was carried out to identify the role of pAKT in the induction of markers of alternative activation. This was assessed using the PI3K inhibitor Ly294002. IL-4 significantly increased MRC1 mRNA expression in BMDMs ( $^{***}p < 0.001$ ; ANOVA; Figure 5.6A) and, in the presence of Ly294002, this IL-4-induced increase was significantly greater ( $^{+++}p < 0.001$ ; ANOVA; Figure 5.6A). IL-4 also increased Arg1 mRNA expression in BMDMs ( $^{***}p < 0.001$ ; ANOVA; Figure 5.6B) and this IL-4-induced change was significantly attenuated in the presence of Ly294002 ( $^{+}p < 0.05$ ; ANOVA; Figure 5.6B). Similarly IL-4 increased Chi3li3 and FIZZ1 mRNA expression in BMDMs ( $^{***}p < 0.001$ ; ANOVA; Figure 5.7A and Figure 5.7B respectively) and Ly294002 significantly attenuated these IL-4-induced changes ( $^{+++}p < 0.001$ ; ANOVA; Figure 5.7A and Figure 5.7B respectively).

#### **5.3.5. The effect of Ly294002 on IL-4-induced STAT6 and PI3K/AKT signalling in BMDMs.**

Western immunoblot analysis was carried out to confirm that Ly294002 inhibited AKT phosphorylation and to examine its effects on IL-4-induced changes in BMDMs. IL-4 increased pAKT expression in BMDMs confirming the data in Figure 5.3 ( $^{+}p < 0.05$ ; ANOVA; Figure 5.8A) and this increase was significantly attenuated by Ly294002 ( $^{$$}p < 0.01$ ; ANOVA; Figure 5.8A). BMDMs incubated in the presence of Ly294002 alone also showed a significant decrease in pAKT expression ( $^{+++}p < 0.001$ ; ANOVA; Figure 5.8A). The specificity of the effect of Ly294002 was checked by investigating any potential effect on STAT6 activation, and the data show that while IL-4 significantly increased pSTAT6 expression ( $^{***}p < 0.001$ ; ANOVA; Figure 5.8B), Ly294002 had no effect on the IL-4-induced pSTAT6.

#### **5.3.6. IFN $\gamma$ induced the activation of pSTAT1 in BMDMs.**

IFN $\gamma$  signals through the JAK/STAT pathway and STAT1 is activated by incubation of macrophages and microglia with IFN $\gamma$ . BMDMs were incubated in the presence of IFN $\gamma$  (50ng/ml) for 0, 0.5, 1, 2, 4 and 24 hours to establish the time at which STAT1 was

activated and the data show that pSTAT1 expression was significantly increased as early as 0.5 hours ( $^{***}p < 0.001$ ; ANOVA; Figure 5.9), and remained elevated for 24 hours.

### **5.3.7. The effect of IFN $\gamma$ on PI3K/AKT activity in BMDMs.**

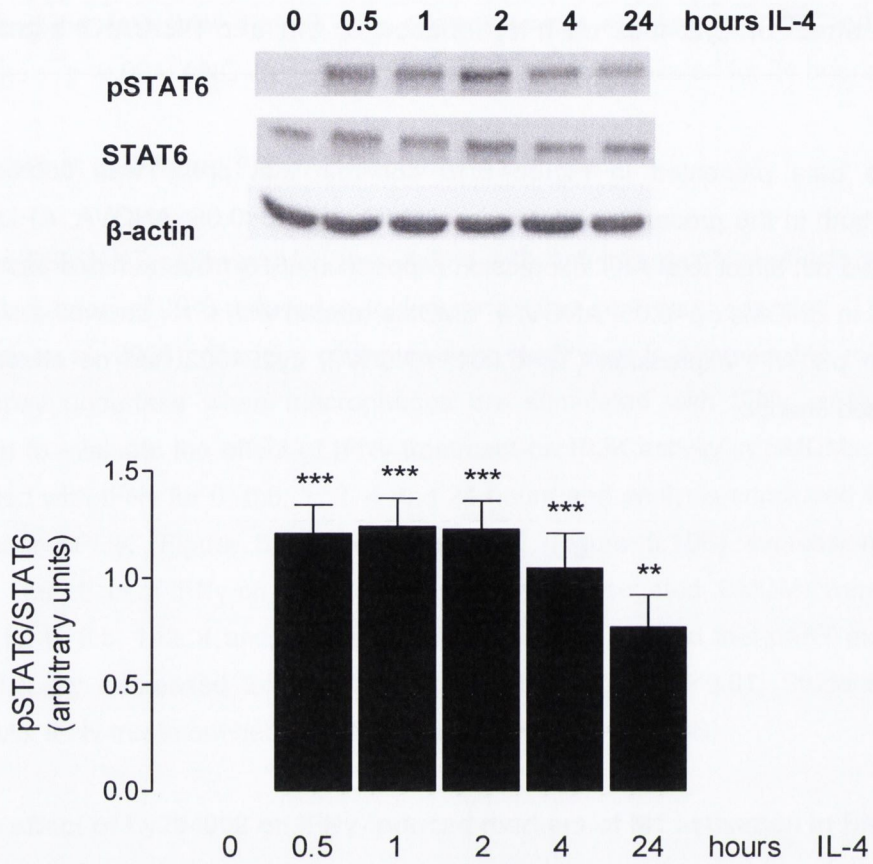
The PI3K/AKT pathway has been linked with anti-inflammatory effects and such negative regulation of LPS-induced cytokine production has been reported (Luyendyk, Schabbauer et al. 2008). The aim of the present study was to examine the role of this pathway may undertake when macrophages are stimulated with IFN $\gamma$ , analysis was undertaken to evaluate the effect of IFN $\gamma$  treatment on PI3K activity in BMDMs. BMDMs were treated with IFN $\gamma$  for 0, 0.5, 1, 2, 4 and 24 hours and analysis concluded that IFN $\gamma$  did not alter pPI3K (Figure 5.10A) or total PI3K (Figure 5.10B) expression at any timepoint. The effect of IFN $\gamma$  on pAKT expression was investigated, BMDMs were treated with IFN $\gamma$  for 0, 0.5, 1, 2, 4 and 24 hours and the analysis showed that pAKT expression was significantly decreased 24 hours after IFN $\gamma$  treatment ( $^{**}p < 0.01$ ; Student's *t*-test; Figure 6.3A). IFN $\gamma$  treatment had no effect on total AKT (Figure 6.3B).

### **5.3.8. The effect of Ly294002 on IFN $\gamma$ -induced markers of M1 activation in BMDMs.**

Because IFN $\gamma$  decreased pAKT, it was considered that inhibition of pAKT would result in an exaggerated response to IFN $\gamma$ . To assess this, BMDMs were treated with IFN $\gamma$  in the presence or absence of Ly294002 for 24 hours. IFN $\gamma$  significantly increased TNF $\alpha$  and NOS2 mRNA ( $^{***}p < 0.001$ ; ANOVA; Figures 5.12A and Figures 5.12B respectively), and the IFN $\gamma$  effect on TNF $\alpha$  mRNA expression was enhanced in the presence of Ly294002 ( $^{+}p < 0.05$ ; ANOVA; Figure 5.12A). IFN $\gamma$  also significantly increased MHCII and ICAM1 mRNA in macrophages ( $^{***}p < 0.001$ ; ANOVA; Figure 6.8A and Figure 6.8B respectively) and the IFN $\gamma$ -induced changes were significantly greater in the presence of Ly294002 ( $^{+++}p < 0.001$ ; ANOVA; Figure 5.13A and Figure 5.13B respectively). Although IFN $\gamma$  significantly increased CD40 and NOD2 mRNA ( $^{***}p < 0.001$ ; ANOVA; Figures 5.14A and Figure 5.14B respectively), there was no Ly294002-induced effect.

### **5.3.9. The effect of Ly294002 on IFN $\gamma$ -induced STAT1 and PI3K/AKT signalling in BMDMs.**

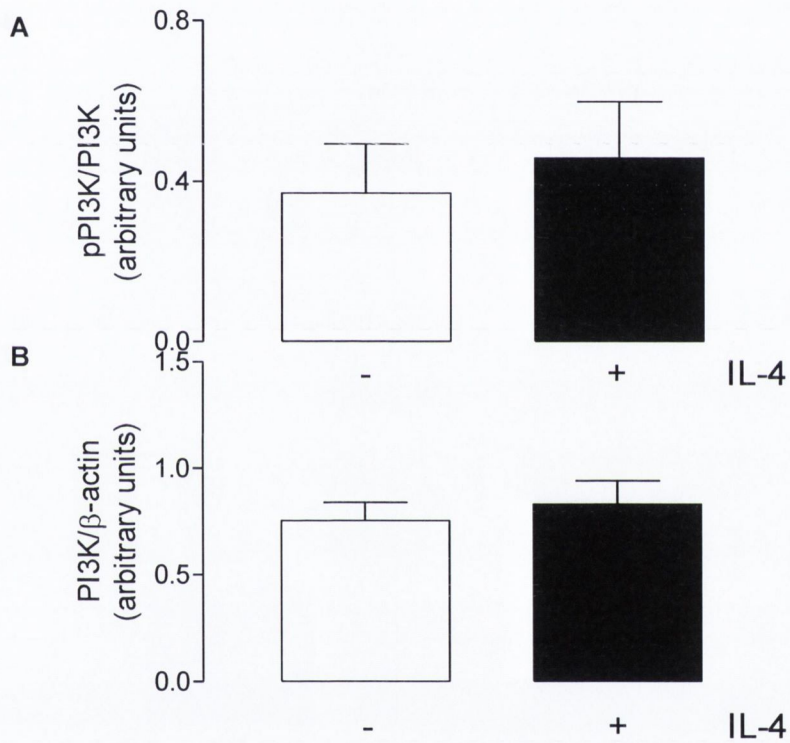
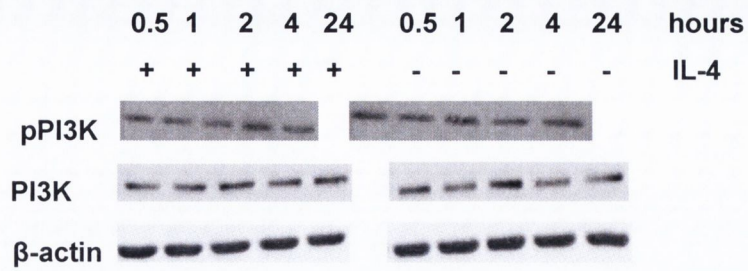
The data presented in Figure 5.15 confirms that pAKT was decreased by Ly294002 both in the presence and absence of IFN $\gamma$  (\*,  $^{\dagger}p < 0.05$ ; ANOVA; A) Ly294002 treatment did not affect total AKT expression (Appendix). IFN $\gamma$  treatment decreased pAKT expression in BMDMs ( $^{\dagger}p < 0.05$ ; ANOVA). BMDMs treated with IFN $\gamma$  showed a significant increase in pSTAT1 expression ( $^{***}p < 0.001$ ; ANOVA), Ly294002 had no effect on this IFN $\gamma$ -induced change.



**Figure 5.1. The effect of IL-4 on pSTAT6 expression in BMDMs**

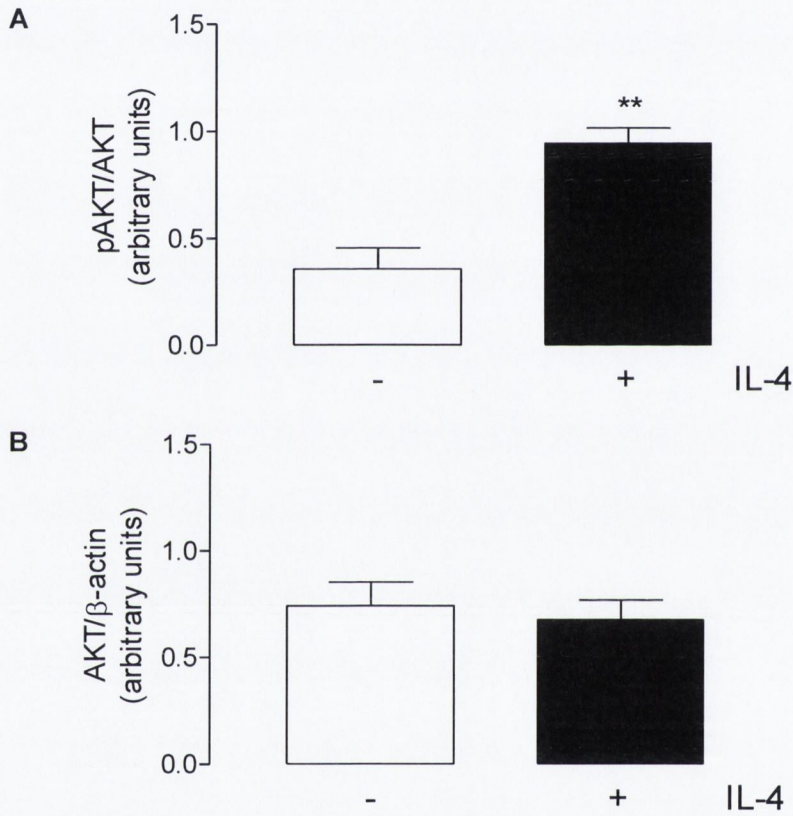
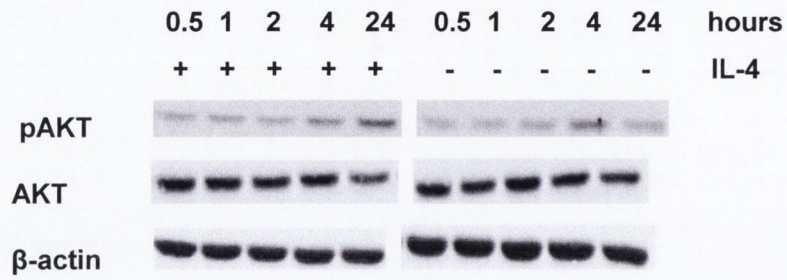
BMDMs were treated with IL-4 (200ng/ml) for 0, 0.5, 1, 2, 4 and 24 hours. IL-4 treatment significantly increased pSTAT6 expression as early as 0.5 hours (\*\*p<0.001; ANOVA) and remained elevated at 24 hours (\*\*p<0.01; ANOVA). Values are presented as means ( $\pm$ SEM; n=5) and expressed as pSTAT6/STAT6 (arbitrary units).





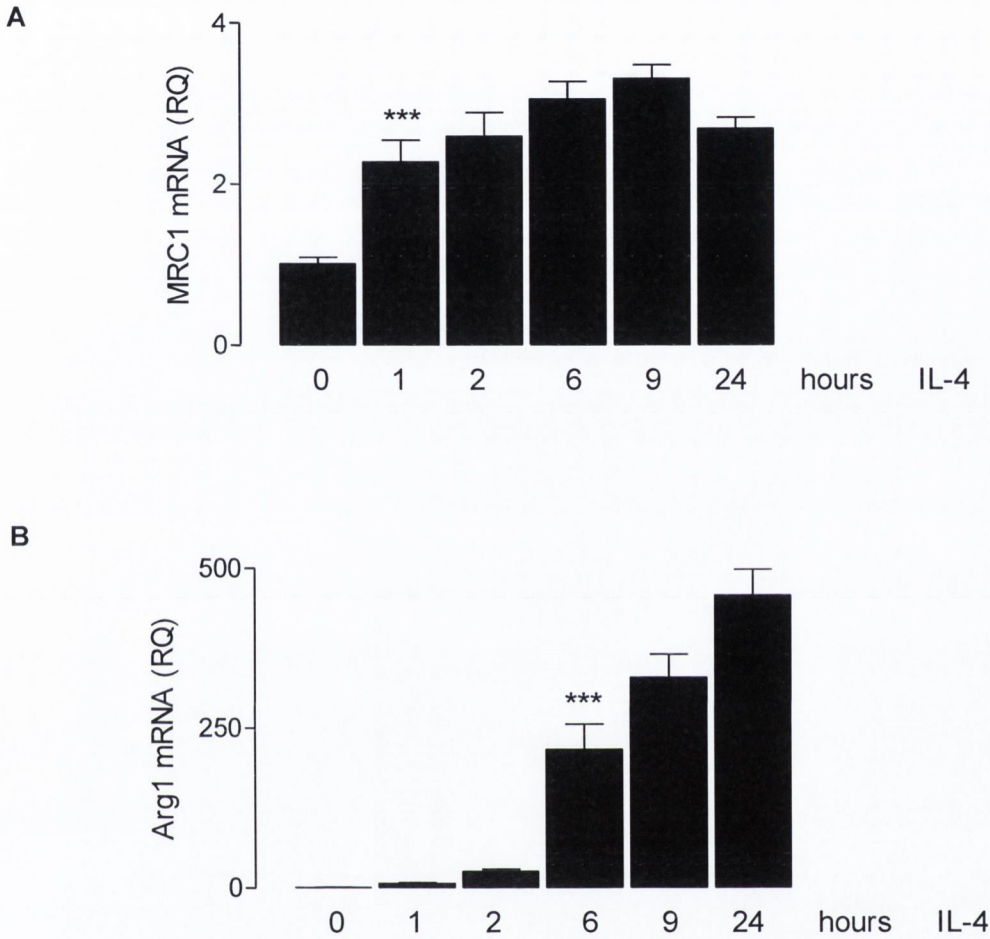
**Figure 5.2. The effect of IL-4 on pPI3K and PI3K expression in BMDMs**

BMDMs were treated with IL-4 (200ng/ml) for 0, 0.5, 1, 2, 4 and 24 hours. IL-4 treatment did not alter total pPI3K or total PI3K expression at any timepoint. The graphs illustrate the data at 24 hours. Values are presented as means ( $\pm$ SEM;  $n=5$ ) and expressed as pPI3K/PI3K or PI3K/ $\beta$ -actin (arbitrary units).



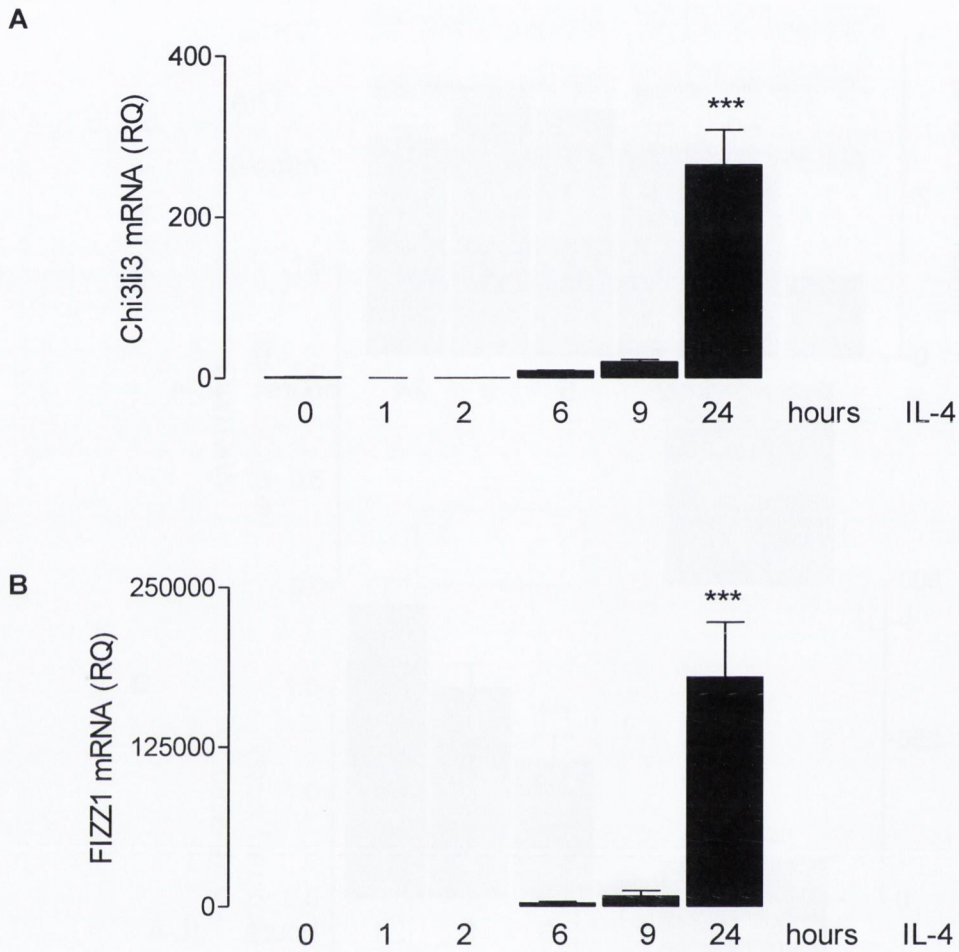
**Figure 5.3. The effect of IL-4 on pAKT and AKT expression in BMDMs**

BMDMs were treated with IL-4 (200ng/ml) for 0, 0.5, 1, 2, 4 and 24 hours. The graphs illustrate the data at 24 hours. IL-4 treatment significantly increased pAKT expression at 24 hours (\*\* $p < 0.001$ ; ANOVA; A). IL-4 treatment did not alter total AKT expression at any timepoint (B). Values are presented as means ( $\pm$ SEM;  $n=5$ ) and expressed as pAKT/AKT or AKT/ $\beta$ -actin (arbitrary units).



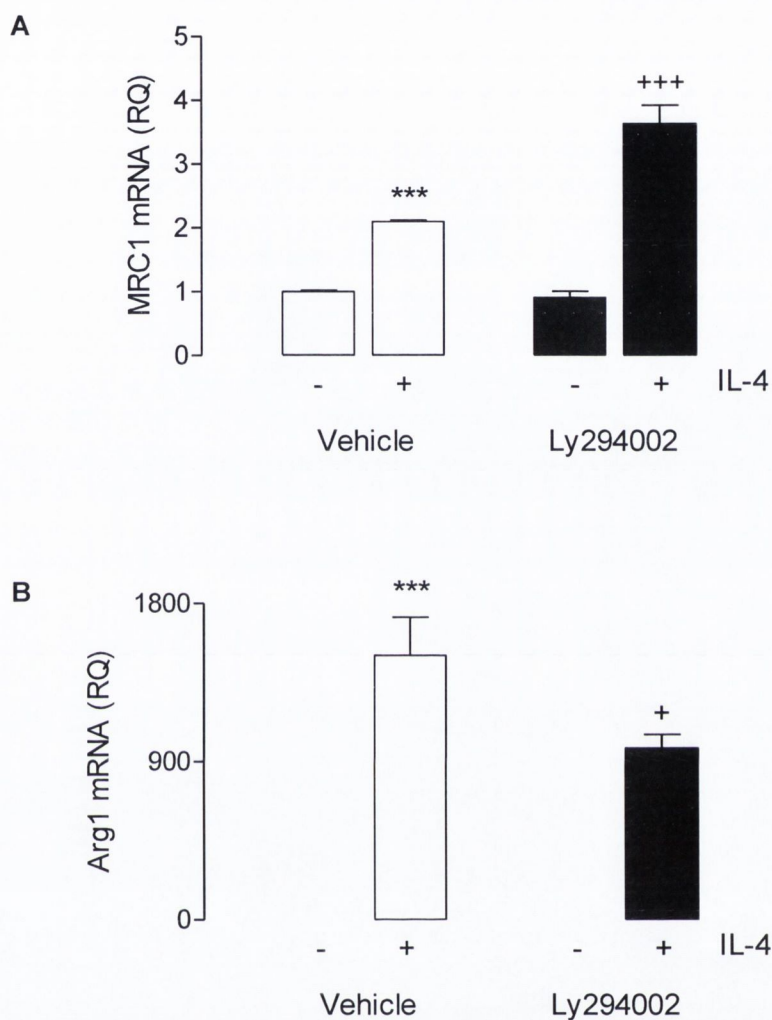
**Figure 5.4. The effect of IL-4 on MRC1 and Arg1 mRNA expression in BMDMs**

BMDMs were treated with IL-4 (200ng/ml) for 0, 1, 2, 6, 9 and 24 hours. IL-4 treatment significantly increased MRC1 mRNA at 1 hour (\*\* $p < 0.001$ ; ANOVA; A) and this remained elevated until 24 hours. Arg1 mRNA was significantly increased at 6 hours (\*\* $p < 0.001$ ; ANOVA; B), this IL-4-induced increase was further increased at 9 and 24 hours (\*\* $p < 0.001$ ; ANOVA; B). Values are presented as means ( $\pm$ SEM;  $n=5$ ) and expressed as a ratio to  $\beta$ -actin mRNA.



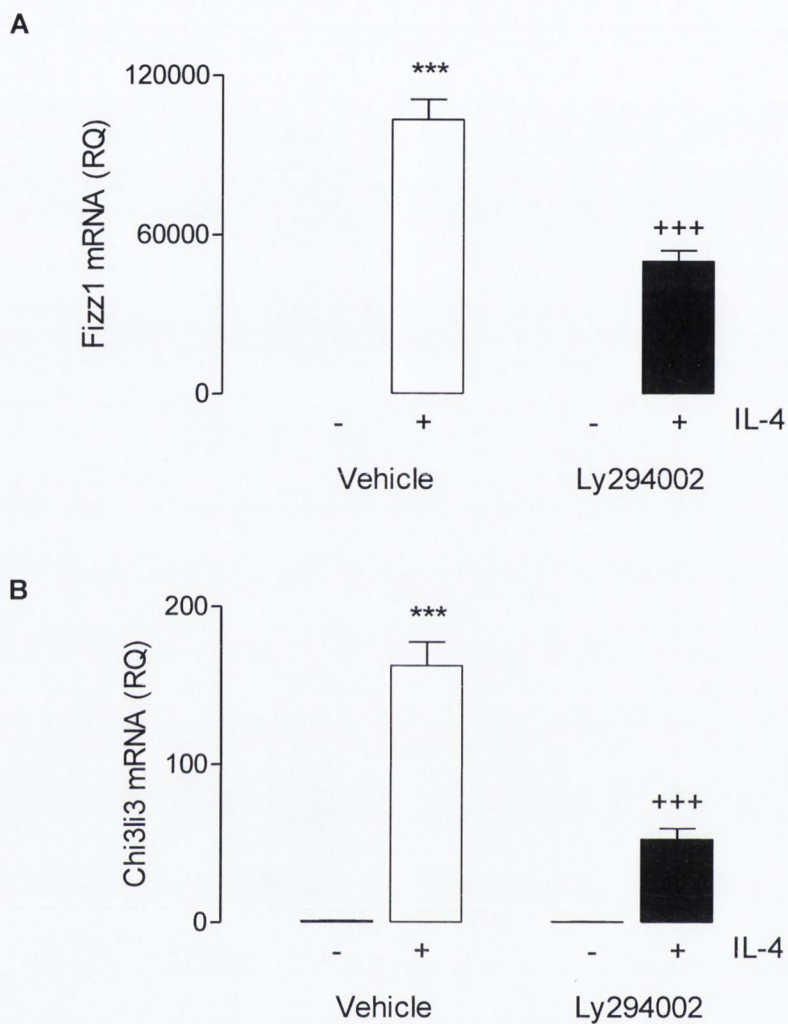
**Figure 5.5. The effect of IL-4 on Chi3li3 and Fizz1 mRNA expression in BMDMs**

BMDMs were treated with IL-4 (200ng/ml) for 0, 1, 2, 6, 9 and 24 hours. IL-4 treatment significantly increased Chi3li3 mRNA at 24 hours (\*\* $p < 0.001$ ; ANOVA; A). Fizz1 mRNA was significantly increased at 24 hours following IL-4 treatment (\*\* $p < 0.001$ ; ANOVA; B). Values are presented as means ( $\pm$ SEM;  $n=5$ ) and expressed as a ratio to  $\beta$ -actin mRNA.



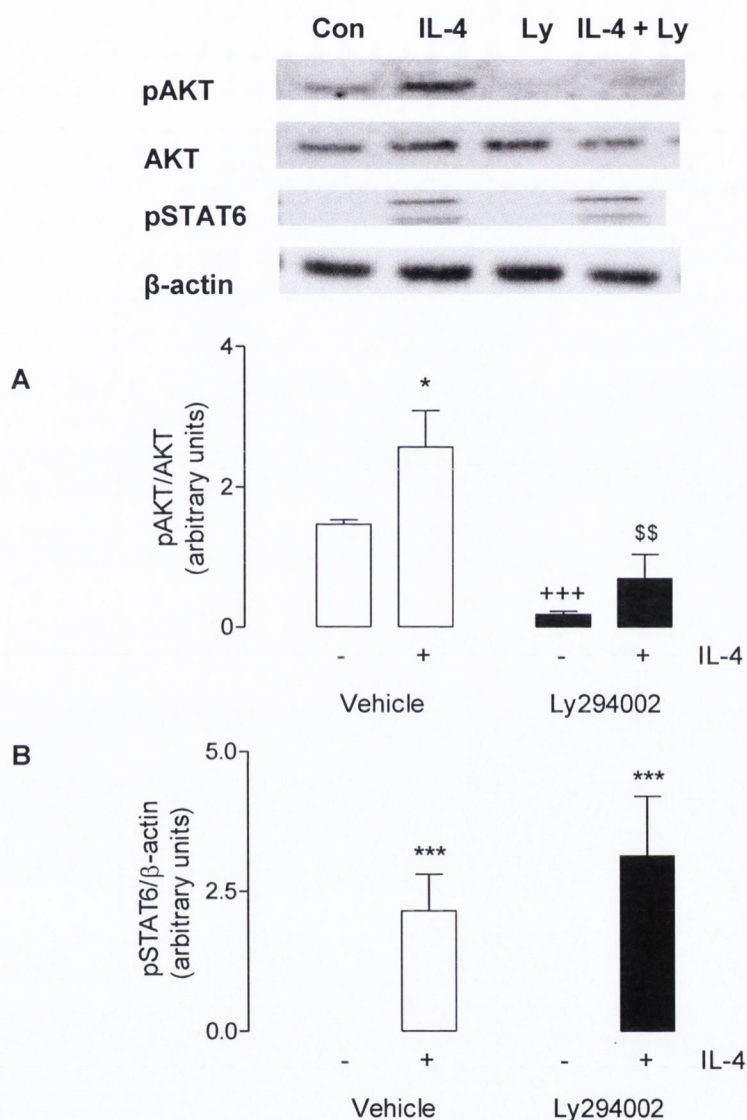
**Figure 5.6. The effect of Ly294002 on IL-4-induced MRC1 and Arg1 mRNA expression in BMDMS**

BMDMs were treated with IL-4 (200ng/ml; 24 hours) alone or in the presence of Ly294002 (10 $\mu$ m). IL-4 increased MRC1 and Arg1 mRNA (\*\* $p$ <0.001; ANOVA). In the presence of Ly294002 the IL-4-induced increase in MRC1 was significantly increased (\*\* $p$ <0.001; ANOVA; B). In contrast Ly294002 treatment significantly attenuated the IL-4-induced increase in Arg1 ( $^+p$ <0.05; ANOVA). Values are presented as means ( $\pm$ SEM;  $n$ =5) and expressed as a ratio to  $\beta$ -actin mRNA.



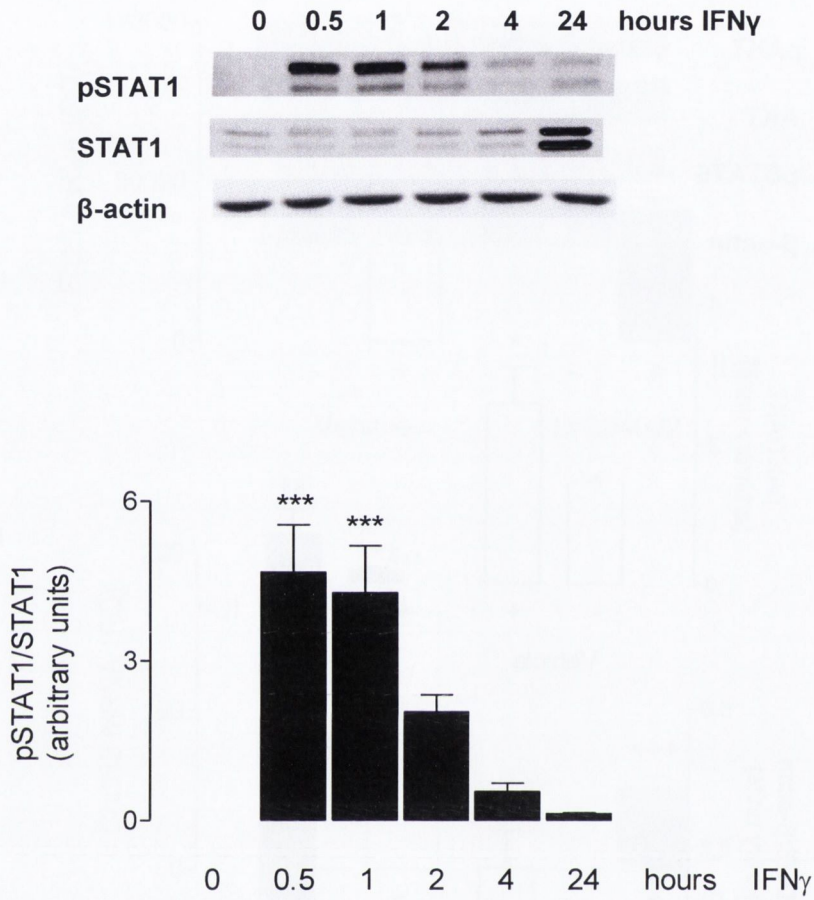
**Figure 5.7. The effect of Ly294002 on IL-4-induced Fizz1 and Chi3li3 mRNA expression in BMDMs**

BMDMs were treated with IL-4 (200ng/ml; 24 hours) alone or in the presence of Ly294002 (10 $\mu$ m). IL-4 increased Fizz1 and Chi3li3 mRNA (\*\* $p$ <0.001; ANOVA; A and B). Ly294002 treatment significantly attenuated the IL-4-induced increase in Fizz1 and Chi3li3 (\*\* $p$ <0.05; ANOVA; A and B). Values are presented as means ( $\pm$ SEM;  $n$ =5) and expressed as a ratio to  $\beta$ -actin mRNA.



**Figure 5.8. The effect of Ly294002 on IL-4 signalling in BMDMs**

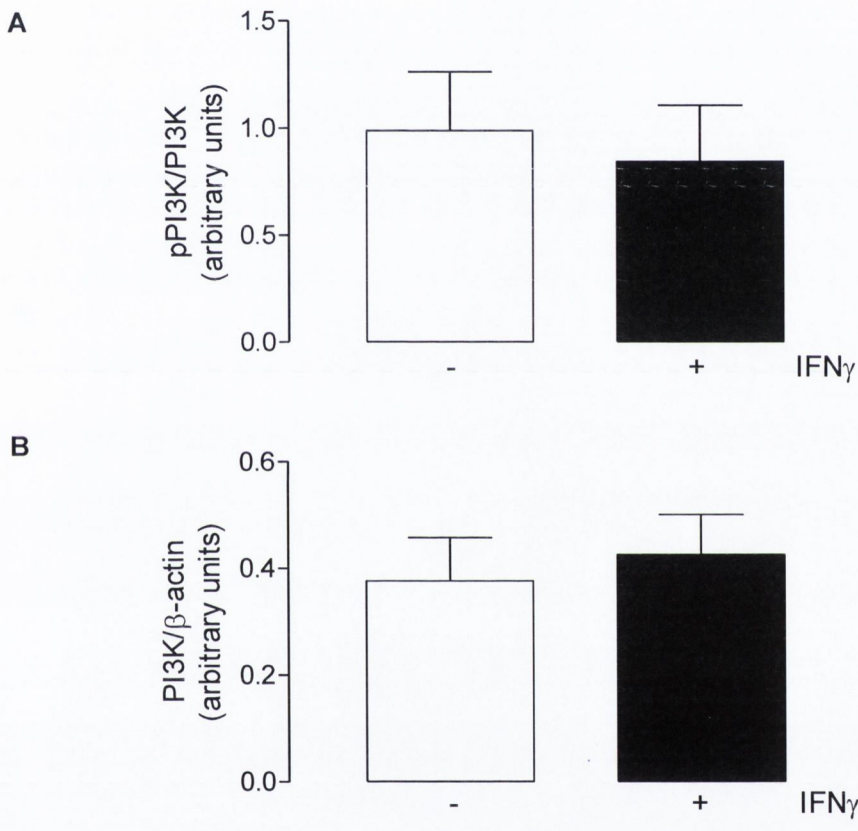
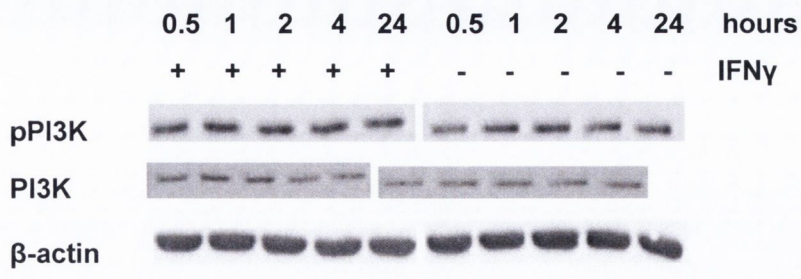
BMDMs were treated with IL-4 (200ng/ml; 24 hours) alone or in the presence of Ly294002 (10 $\mu$ m). IL-4 treatment increased pAKT expression in BMDMs ( $^*p < 0.05$ ; ANOVA; A) and this increase was significantly attenuated in the presence of Ly294002 ( $^{$$}p < 0.01$ ; ANOVA; A). Ly294002 treatment significantly decreased in pAKT expression ( $^{+++}p < 0.001$ ; ANOVA; A). BMDMs stimulated with IL-4 exhibited a significant increase in pSTAT6 expression ( $^{***}p < 0.001$ ; ANOVA; B), Ly294002 had no effect on IL-4-induced pSTAT6. Values are presented as means ( $\pm$ SEM; n=5) and expressed as pAKT/AKT or pSTAT6/  $\beta$ -actin.



**Figure 5.9. The effect of IFN $\gamma$  on pSTAT1 expression in BMDMs**

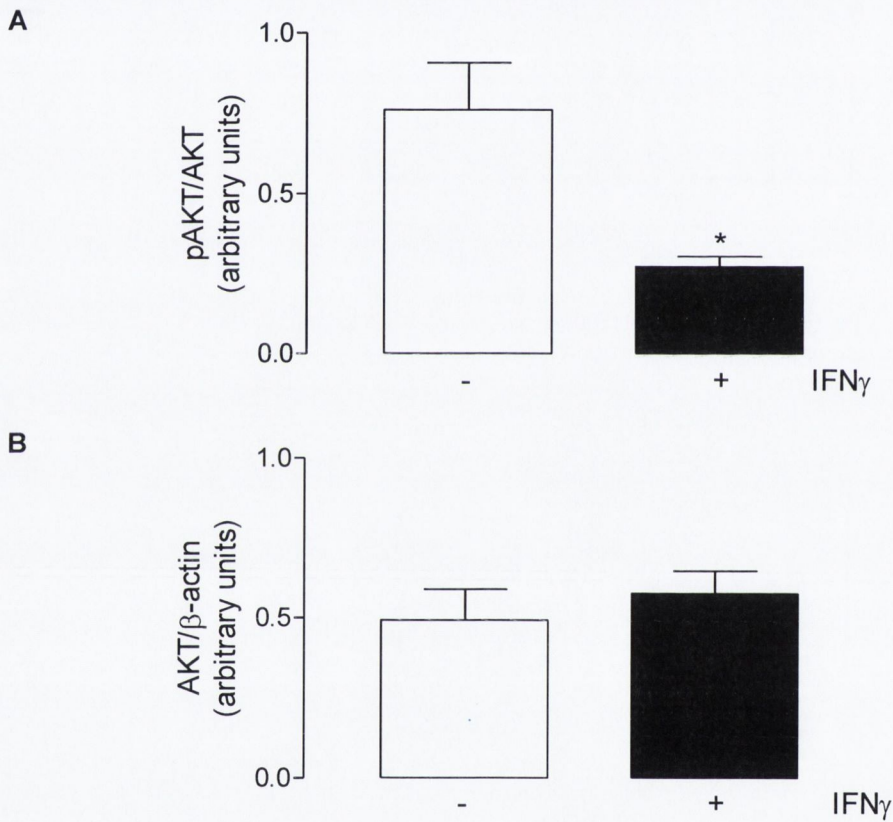
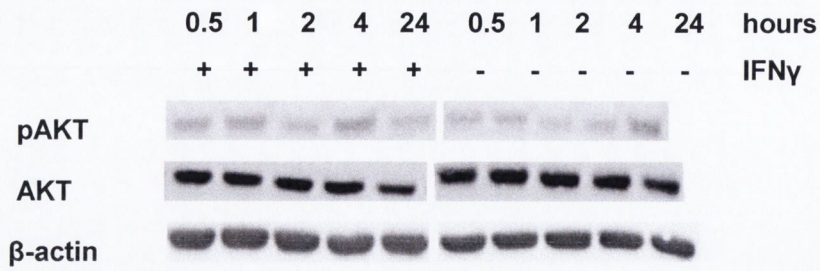
BMDMs were treated with IL-4 (200ng/ml) for 0, 0.5, 1, 2, 4 and 24 hours. IFN $\gamma$  treatment significantly increased pSTAT1 expression at 0.5 and 1 hour (\*\* $p < 0.001$ ; ANOVA), expression remained elevated later timepoints this was not statistically significant. Values are presented as means ( $\pm$ SEM;  $n=5$ ) and expressed as pSTAT1/STAT1 (arbitrary units).





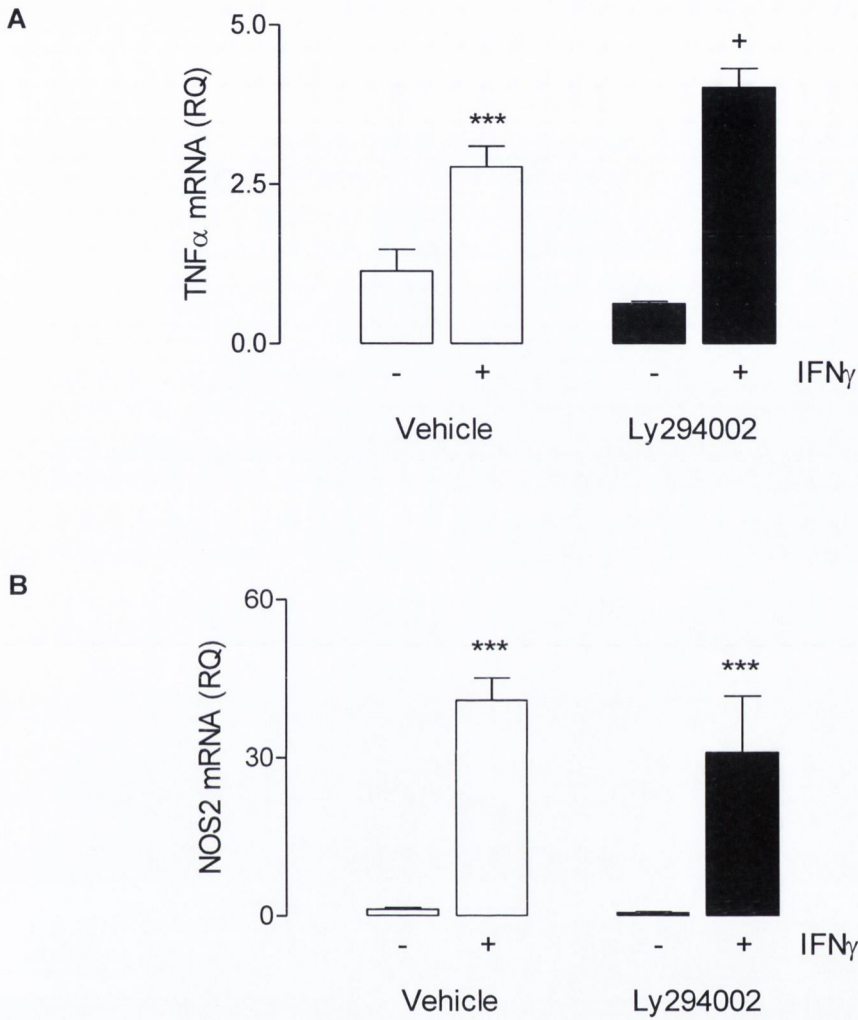
**Figure 5.10. The effect of IFN $\gamma$  on pPI3K and PI3K expression in BMDMs**

BMDMs were treated with IFN $\gamma$  (50ng/ml) for 0, 0.5, 1, 2, 4 and 24 hours. The graphs illustrate the data at 24 hours. IFN $\gamma$  treatment did not alter total pPI3K (A) or total PI3K (B) expression at any timepoint. Values are presented as means ( $\pm$ SEM; n=5) and expressed as pPI3K/PI3K or PI3K/ $\beta$ -actin (arbitrary units).



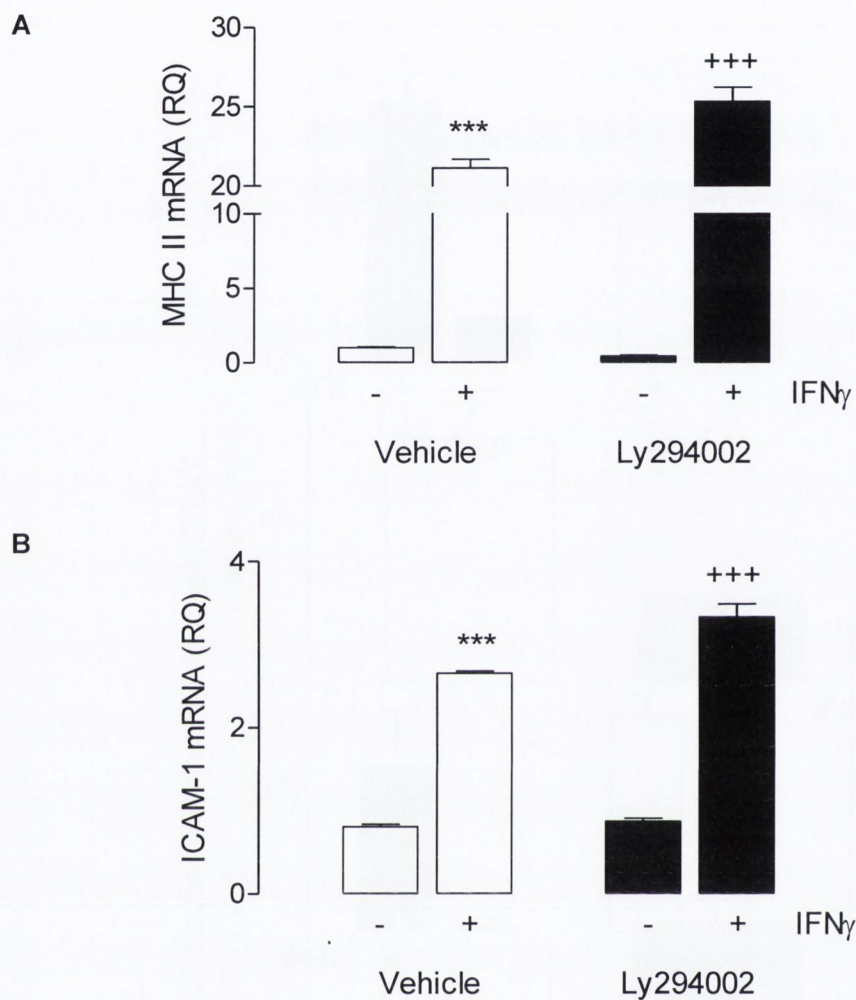
**Figure 5.11. The effect of IFN $\gamma$  on pAKT and AKT expression in BMDMs**

BMDMs were treated with IFN $\gamma$  (50ng/ml) for 0, 0.5, 1, 2, 4 and 24 hours. The graphs illustrate the data at 24 hours. IFN $\gamma$  treatment significantly decreased pAKT expression at 24 hours (\*\* $p < 0.01$ ; Student's  $t$ -test; B). IFN $\gamma$  treatment did not alter total AKT expression at any timepoint (B). Values are presented as means ( $\pm$ SEM;  $n=5$ ) and expressed as pAKT/AKT or AKT/ $\beta$ -actin (arbitrary units).



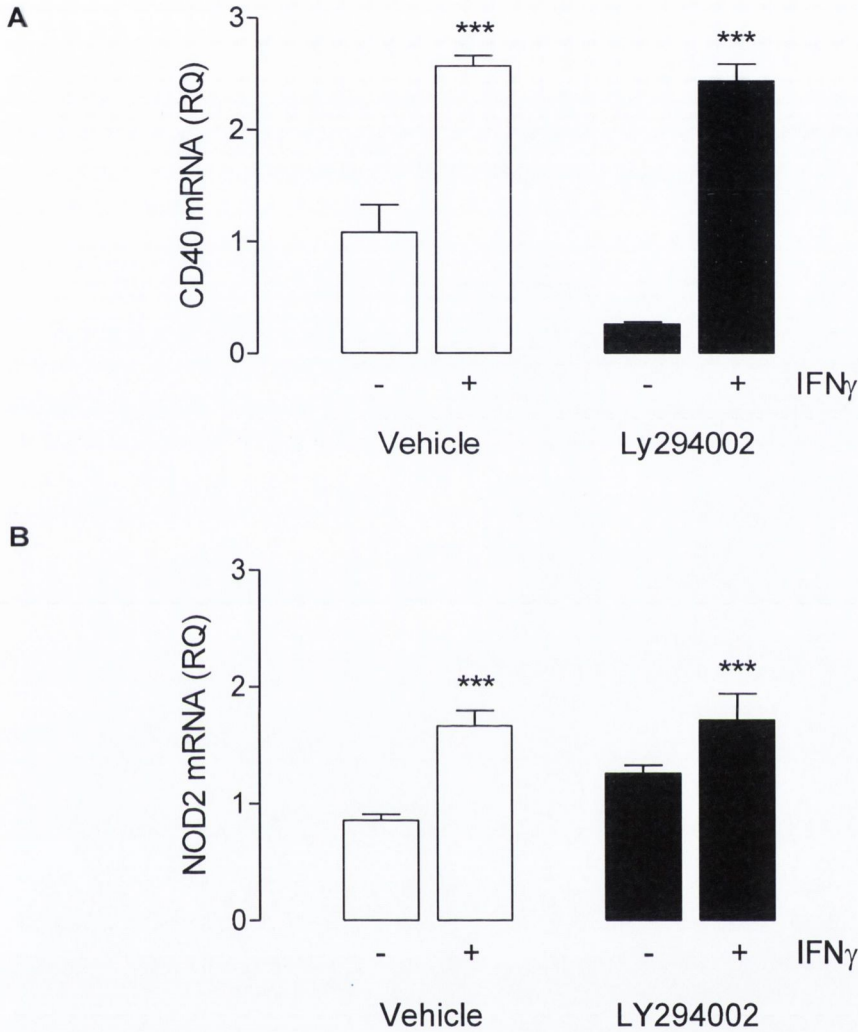
**Figure 5.12. The effect of Ly294002 on IFN $\gamma$ -induced TNF $\alpha$  and NOS2 mRNA in BMDMS**

BMDMs were treated with IFN $\gamma$  (50ng/ml; 24 hours) alone or in the presence of Ly294002 (10 $\mu$ m). IFN $\gamma$  increased TNF $\alpha$  and NOS2 mRNA (\*\*\*) $p$ <0.001; ANOVA; A and B respectively). In the presence of Ly294002, IFN $\gamma$ -induced TNF $\alpha$  mRNA was significantly increased (+ $p$ <0.05; ANOVA; A). Ly294002 treatment had no effect on IFN $\gamma$ -induced NOS2 mRNA. Values are presented as means ( $\pm$ SEM; n=5) and expressed as a ratio to  $\beta$ -actin mRNA.



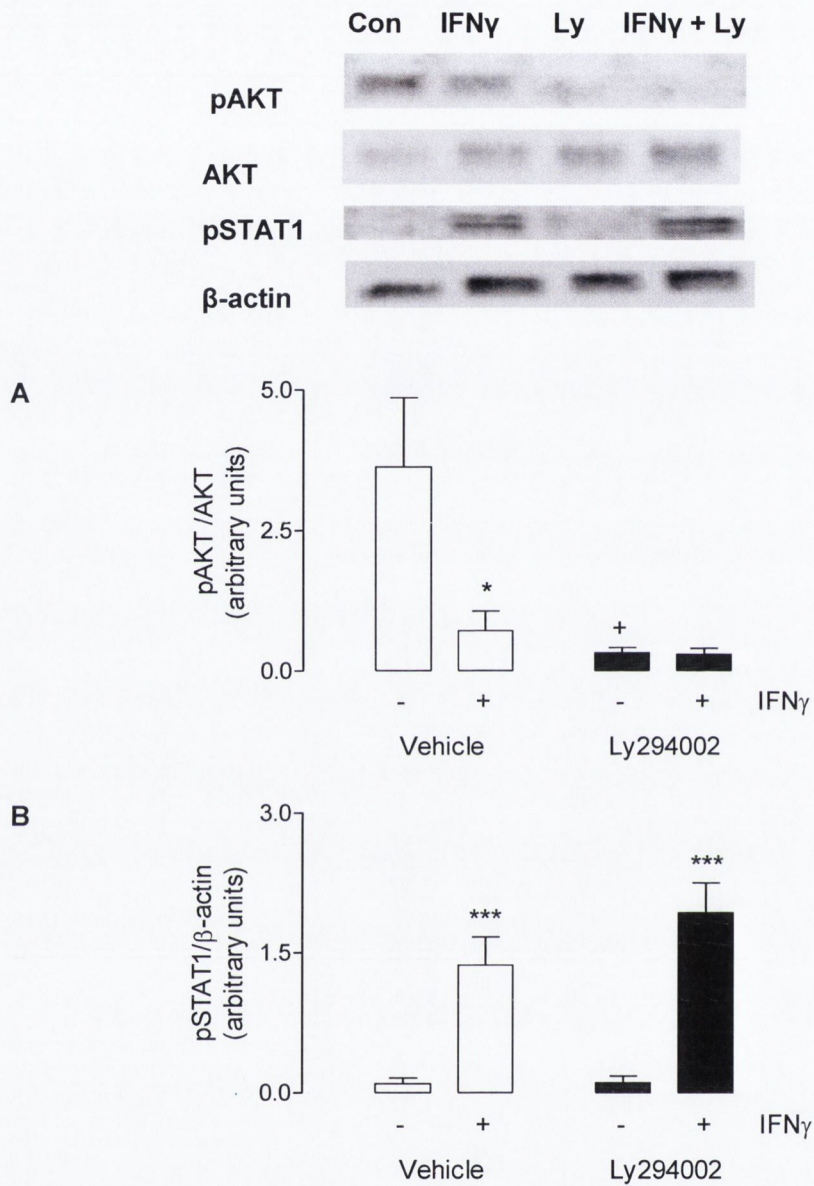
**Figure 5.13. The effect of Ly294002 on IFN $\gamma$ -induced MHCII and ICAM1 mRNA in BMDMs**

BMDMs were treated with IFN $\gamma$  (50ng/ml; 24 hours) alone or in the presence of Ly294002 (10 $\mu$ m). IFN $\gamma$  increased MHCII and ICAM1 mRNA (\*\*\*) $p$ <0.001; ANOVA; A and B respectively). In the presence of Ly294002, IFN $\gamma$ -induced MHCII and ICAM1 mRNA was significantly increased (\*\*\*) $p$ <0.001; ANOVA; A and B respectively). Values are presented as means ( $\pm$ SEM;  $n$ =5) and expressed as a ratio to  $\beta$ -actin mRNA.



**Figure 5.14. The effect of Ly294002 on IFN $\gamma$ -induced CD40 and NOD2 mRNA in BMDMs**

BMDMs were treated with IFN $\gamma$  (50ng/ml; 24 hours) alone or in the presence of Ly294002 (10 $\mu$ m). IFN $\gamma$  increased CD40 and NOD2 mRNA (\*\*\*) $p$ <0.001; ANOVA; A and B respectively). Ly294002 treatment did not alter IFN $\gamma$ -induced CD40 and NOD2 mRNA expression. Values are presented as means ( $\pm$ SEM;  $n=5$ ) and expressed as a ratio to  $\beta$ -actin mRNA.



**Figure 5.15. The effect of Ly294002 on IFN $\gamma$  signalling in BMDMs.**

BMDMs were treated with IFN $\gamma$  (50ng/ml; 24 hours) alone or in the presence of Ly294002 (10 $\mu$ m). IFN $\gamma$  treatment decreased pAKT expression in BMDMs (\* $p$ <0.05; ANOVA; A). Ly294002 treatment induced a significant decrease in pAKT expression (\* $p$ <0.05; ANOVA: A). BMDMs treated with IFN $\gamma$  exhibited a significant increase in pSTAT1 expression (\*\*\* $p$ <0.001; ANOVA; B), Ly294002 had no effect on IFN $\gamma$ -induced pSTAT1. Values are presented as means ( $\pm$ SEM;  $n$ =5) and expressed as pAKT/AKT or pSTAT1/ $\beta$ -actin.

## 5.4. Discussion

One of the significant findings of this study is that the PI3K/AKT pathway is activated in macrophages following IL-4 stimulation and inhibition of PI3K results in macrophages exhibiting a decreased response to IL-4. Therefore it can be concluded that activation of the PI3K/AKT pathway is crucial to enable the cells to adopt the M2 activation state. The second significant finding of this study is that IFN $\gamma$  decreases pAKT expression in BMDMs and, in the presence of the PI3K inhibitor Ly290042, certain IFN $\gamma$ -induced changes are enhanced in BMDMs. These findings suggest that activation of the PI3K/AKT signalling pathway may play a role in the polarisation of macrophages into the M1 or M2 state.

It has been well documented that IL-4 treatment results in the phosphorylation of STAT6 (Gordon & Martinez, 2010) and it was concluded several years ago that STAT6 activation was essential in driving IL-4 (and IL-13)-induced changes in peritoneal macrophages (Takeda *et al.*, 1996). In the present study, enhanced STAT6 activation was observed in IL-4-treated BMDMs as early as 30 minutes and this persisted for the duration of the study although a decrease in activation was evident from 4 hours. This is similar to work carried out by others who reported that IL-4 induced pSTAT6 expression in BMDMs in the same time frame (Haque *et al.*, 1998). Importantly, total STAT6 expression was unaffected by IL-4 treatment, indicating that the increase in activated STAT6 was independent of a change in total protein. A role for IL-4 in modulating microglial activation has also been described (Loane *et al.*, 2009) and evidence from this laboratory reported a decrease in IL-4 concentration and decreased STAT6 signalling in the brains of aged rats, and this was accompanied by evidence of inflammation (Nolan *et al.*, 2005).

In addition to activating STAT6, IL-4 impacts on other signalling pathways. For example it activates IRS-2 (Heller *et al.*, 2008) and consequently impacts on signalling through PI3K and the RAS-MAPK pathway, which activate numerous cell processes including cell proliferation (Gordon & Martinez, 2010). A defect in this IL-4-induced signalling cascade has been identified in type 2 diabetes and has been associated with decreased IL-1ra production in peritoneal macrophages (O'Connor *et al.*, 2007). However, in this study, there was no evidence of an effect of IL-4 on PI3K activation at any time point between 30 minutes and 24 hours, although it has been reported to be required for

macrophage proliferation (Ruckerl *et al.*, 2012), Induction of IL-4R $\alpha$ -dependent microRNAs identifies PI3K/Akt signaling as essential for IL-4-driven murine macrophage proliferation in vivo (Ruckerl *et al.*, 2012). Previous evidence has also indicated that inhibition of PI3K by wortmannin decreases the IL-4-induced expression of Fizz1, but not Arg1, in murine macrophages (Heller *et al.*, 2008). It is clear that further investigation relating to the role of PI3K activation in IL-4-induced markers of M2 activation in macrophages is necessary.

Even though IL-4 did not induce activation of PI3K, pAKT expression was significantly enhanced in BMDMs after 24 hours incubation with IL-4. The PI3K/AKT pathway has been shown to promote anti-inflammatory effects and evidence indicates that it modulates LPS-induced cytokine release (Luyendyk *et al.*, 2008). Specifically, phosphatase and tensin homologue (PTEN) is a negative regulator of the PI3K/AKT pathway (Naguib & Trotman, 2013), and Luyendyk and colleagues reported that macrophages from PTEN-deficient mice exhibited increased pAKT expression (Luyendyk *et al.*, 2008). LPS-induced TNF $\alpha$  and IL-6 production was decreased in macrophages from PTEN<sup>-/-</sup> mice and this was attributed to the increased activity of the PI3K/AKT pathway. This evidence led to the hypothesis that some of the anti-inflammatory effects of IL-4 on macrophages may be mediated through activation of the PI3K/AKT.

Analysis of time-related IL-4-induced changes in expression of markers of M2 activation indicated that maximal upregulation of MRC1 mRNA in BMDMs occurred at 1 hour, closely following the activation of STAT6 which was observed at 30 minutes. In contrast, the induction of Arg1, Chi3li3 and Fizz1 was much later than MRC1 and this is similar to findings reported by Heller and colleagues (Heller *et al.*, 2008). This group showed that IL-4 and IL-13 activated STAT6 in a similar manner but that IL-4 increased the expression of Arg1, Chi3li3 and Fizz1 in a more robust fashion than IL-13. However the effect of IL-4 and IL-13 on MRC1 mRNA expression was found to be similar. This may be explained by the finding that activation of type I, but not type II, IL-4R can activate the PI3K/AKT pathway (Gordon & Martinez, 2010) and the evidence indicates that IL-4, but not IL-13, signals through this receptor, activating IRS-2 and subsequently PI3K (Heller *et al.*, 2008; Gordon & Martinez, 2010). These findings, combined with the data from the present study, suggest that activation of the PI3K/AKT pathway mediates part of the IL-4 response which is independent of STAT6 activation.



To further investigate the effect of PI3K/AKT on the induction of markers of M2 activation, BMDMs were treated in the presence or absence of IL-4 and the PI3K inhibitor, Ly290042. The significant finding is that the IL-4-induced upregulation of Arg1, Chi3li3 and Fizz1 mRNA was significantly decreased by Ly290042; this confirms the findings of previous studies which reported that inhibiting PI3K/AKT signalling in IL-4- stimulated macrophages attenuated Arg1, Chi3li3 and Fizz1 mRNA expression (Heller *et al.*, 2008; Ruckerl *et al.*, 2012). Therefore PI3K/AKT appears to be crucial in the induction of at least these M2 activation markers as indicated by the present findings and others. The effect of the Sh2-containing inositol phosphatase (SHIP) on PI3K activity further highlights the role of the PI3K/AKT pathway in the induction of M2 activation; SHIP is a negative regulator of PI3K activity, and Weisser and colleagues reported that macrophages from SHIP-deficient mice exhibit an exaggerated response to IL-4 treatment (Weisser *et al.*, 2011). It should be noted that the concentration of Ly290042 used in this study did not completely block IL-4-induced changes in Arg1, Chi3li3 and Fizz1 mRNA expression; the effect may be concentration-dependent but it may also indicate that other signalling pathways play a role and analysis of the effect of inhibiting STAT6 would be of particular interest.

In complete contrast to the effect of Ly290042 on IL-4-induced changes in Arg1, Chi3li3 and Fizz1 mRNA, IL-4-induced upregulation of MRC1 mRNA was significantly enhanced by Ly290042. Interestingly, Heller and colleagues also reported that inhibition of PI3K/AKT signalling did not attenuate the change in MRC1 mRNA (Heller *et al.*, 2008), although this group did not report an exacerbated response. It must be concluded that expression of the markers of M2 activation in BMDMs are differentially controlled and it remains to be established whether activation of STAT6 is primarily involved in modulation of the expression of MRC1.

It is well documented that IFN $\gamma$  induces STAT1 activation; in BMDMs, IFN $\gamma$ -induced STAT1 activation was evident at the earliest time point of 30 minutes, persisted for 1 hour and returned almost to baseline by 4 hours. This early activation has already been reported in RAW 264.7 cells where an increase in STAT1 activation was found as early as 10 minutes following IFN $\gamma$  treatment (Luo *et al.*, 2011). IFN $\gamma$  also increased total STAT1 but this change was not observed until after 24 hours.

A major objective of this study was to investigate the differential effects of IL-4 and IFN $\gamma$  on the PI3K/AKT pathway and the data indicated that IFN $\gamma$  exerted no effect on PI3K activity. However, a role for PI3K in IFN $\gamma$ -induced changes has been implied by the finding that PI3K inhibitor attenuated IFN $\gamma$ -induced NO production in BV2 cells (Hwang *et al.*, 2004). In addition, peritoneal macrophages, deficient in PI3K, produced less NO than wildtype cells when stimulated with IFN $\gamma$  together with LPS (Sakai *et al.*, 2006). It is possible that the signalling cascades triggered by IFN $\gamma$  are cell-specific and it is also possible that a more extensive time-related study might uncover a direct effect of IFN $\gamma$  on PI3K activation in BMDMs.

Analysis of AKT activation revealed an inhibitory effect of IFN $\gamma$  only after 24 hours incubation; given the lack of change in PI3K, it must be concluded that the effect of IFN $\gamma$  on pAKT expression occurs through another signalling cascade. In contrast to these findings in BMDMs, an IFN $\gamma$ -induced increase has been observed in other cell types including T98G human glioblastoma cells, human fibrosarcoma 2FTGH cells, the derivative U4A cell line and BV2 cells (Nguyen *et al.*, 2001; Hwang *et al.*, 2004) and, at least in BV2 cells the increase in AKT activation was maximal at 30 minutes. Consistently, Ly290042 inhibited IFN $\gamma$ -induced NO production, NOS2 mRNA and cell death in BV2 cells (Hwang, Jung *et al.*). Although no significant effect of Ly290042 was observed in the present study, the evidence suggests that at least in some cell types AKT activation is an important upstream event in IFN $\gamma$ -induced STAT1 activation (Nguyen *et al.*, 2001; Hwang *et al.*, 2004). In contrast to these reports and broadly consistent with the present findings, IFN $\gamma$  has been shown to attenuate the TLR2-induced increase in pAKT expression in human macrophages prepared from peripheral blood mononuclear cells (Hu *et al.*, 2006). These data suggest that IFN $\gamma$ -induced pAKT may be cell-specific.

Data from numerous studies have indicated that activation of the PI3K/AKT pathway promotes anti-inflammatory effects and, consistently, it has been shown to attenuate TLR-induced cytokine production (Luyendyk *et al.*, 2008; Medina *et al.*, 2010). Similarly decreased signalling through this pathway has been associated with an increased response to inflammatory stimuli; specifically Luyendyk and colleagues demonstrated that macrophages in which PI3K signalling was decreased exhibit an enhanced response to LPS stimulation (Luyendyk *et al.*, 2008). In contrast, they reported that macrophages cultured from PTEN<sup>-/-</sup> mice exhibit enhanced AKT activation and this

was associated with a decrease in responsiveness to LPS stimulation. Similar to PTEN, SHIP also inhibits the activity of PI3K, and LPS-induced cytokine release is also impaired in macrophages prepared from SHIP-deficient mice (Weisser *et al.*, 2011). Thus the PI3K/AKT pathway plays a part in restricting inflammatory events, and evidence from *in vivo* experiments have demonstrated that inhibition of PI3K increased inflammation in mouse models of endotoxemia and sepsis (Schabbauer *et al.*, 2004; Williams *et al.*, 2004).

The present data indicate that Ly290042 exacerbated some of the IFN $\gamma$ -induced effects in BMDMs and an upregulation in expression of 2 markers of activation, MHCII and ICAM-1, was observed. However IFN $\gamma$ -induced expression of the markers of classical activation, NOS2 and NOD2, was similar in IFN $\gamma$ -macrophages irrespective of the presence of Ly290042.

The main finding of the current study is that IL-4 and IFN $\gamma$  treatments have differential effects on pAKT expression. Activation of the PI3K/AKT pathway is involved in the induction of the M2 markers Arg1, Chi3li3 and Fizz1 and inhibition of the PI3K/AKT pathway leads to a dramatic decrease in IL-4-induced expression of these markers. Previous work has shown that the PI3K/AKT pathway is involved in the regulation of inflammatory events; the present data demonstrate that manipulation of this pathway may impact on the polarisation of macrophages into the M1 or M2 state.

Chapter 6  
General Discussion

## 6.1. General Discussion

It has been acknowledged for over a decade that macrophages adopt different phenotypes in response to different stimuli. In the past few years, it has become apparent that macrophages infiltrate the brain under certain circumstances and infiltrating cells have been shown in the brain of aged animals and animal models of neurodegenerative disorders (Blau *et al.*, 2012; Kelly *et al.*, 2013). However, there is significant debate concerning their role upon entering brain parenchyma. At this point, the evidence that neuroinflammatory changes develop with age, and that most, if not all, neurodegenerative conditions are associated with neuroinflammatory changes, is overwhelming. Furthermore, a number of neuroinflammatory conditions, including AD, are associated with BBB disruption (Avison *et al.*, 2004) and transendothelial migration of peripheral leukocytes (Togo *et al.*, 2002). In the context of the present study, the primary question was how macrophages that infiltrate the brain of aged rats, or APP/PS1 mice, respond to the inflammatory environment that they encounter. Therefore, the primary aim of this study was to compare the responsiveness of macrophages from aged and young rats, and WT and APP/PS1 mice, to the polarizing stimuli, LPS, IFN $\gamma$  and IL-4 and, on the basis of the findings, predict what their responses may be when they infiltrate the brain. In addition to this, the possibility that macrophage activation might be modulated by manipulating the PI3K/AKT pathway was considered.

The most significant findings from these studies were that BMDMs from aged rats and APP/PS1 mice are more responsive to pro-inflammatory (summarised in tables 6.1. and 6.2.), rather than anti-inflammatory stimuli and that these cells are capable of infiltrating the brain, as evidenced by their presence in brain tissue prepared from aged rats and APP/PS1 mice. Therefore, given the underlying pro-inflammatory environment that exists in the brain of aged rats and APP/PS1 mice, it seems likely that infiltrating macrophages may be stimulated to respond in a manner that would exacerbate inflammation and may be detrimental to neuronal health. It has been hypothesised that macrophage phenotype may be controlled by molecular switches, and the present study demonstrates that inhibition of the PI3K/AKT pathway results in exacerbated responses to IFN $\gamma$  and impaired induction of the M2 state. These findings suggest that targeting the PI3K/AKT pathway may prove to be therapeutically beneficial in inflammatory disorders.

A comprehensive study by Siamon Gordon and colleagues in 1992 revealed that when macrophages were stimulated with the anti-inflammatory cytokine IL-4, they adopted a phenotype which did not resemble the more familiar activation state induced by inflammatory cytokines, and this state was described as the alternative activation state (Stein *et al.*, 1992). This finding was confirmed by others (Doyle *et al.*, 1994) and it is now accepted that macrophages respond to various stimuli differently (Gordon, 2003; Mosser, 2003; Gordon & Martinez, 2010). Activation states can be classified into two major subsets, and further intermediate ones, with discrete molecular phenotypes and effector functions. M1 or classical activation, stimulated by IFN $\gamma$ , is identified by upregulation of TNF $\alpha$  and NOS2 and is associated with inflammation, ECM destruction and, in some cases, apoptosis (Mosser & Edwards, 2008). M2 or alternative activation, stimulated by IL-4, is associated with cell and tissue repair and return to homeostasis (Mosser, 2003). It is identified by markers like MRC1 and Arg1 which decrease inflammatory activity, and Chi3li3 and Fizz1 which play a role in repair of the ECM.

Increased inflammatory activity has been observed in the brain of aged animals and models of neurodegenerative disorders, and the present data demonstrate that macrophages prepared from aged rats and APP/PS1 mice exhibit an enhanced response to pro-inflammatory stimuli and that these cells infiltrate the brain. Some studies have suggested that infiltration of macrophages may be beneficial and that their phagocytic potential is greater than that of microglia (Simard *et al.*, 2006; El Khoury *et al.*, 2007). Others have suggested that macrophages may have detrimental effects within the brain (Denieffe *et al.*, 2013; Girard *et al.*, 2013); specifically, it has been shown that infiltration of macrophages into the brain was associated with increased inflammation, particularly in CD200-deficient mice perhaps as a result of the release of IFN $\gamma$  from these cells (Denieffe *et al.*, 2013). Whereas recent evidence suggests that the entry of M2 macrophages may be beneficial after spinal cord injury (Shechter *et al.*, 2013), it may prove impossible for macrophages to maintain the M2 state in brains where a pro-inflammatory environment prevails. In this case infiltrating macrophages may exacerbate inflammatory conditions; the possibility that the phenotype of cells which enter the brain changes in response to endogenous signals requires further investigation.

The present study shows that BMDMs from aged rats exhibited an exaggerated response to TLR4 activation and the significance of this lies in the finding that

hippocampal concentration of the TLR4 agonist HMGB1 was increased in aged, compared with young, rats, and it may trigger an enhanced inflammatory response from infiltrating cells. Similarly, BMDMs prepared from aged rats responded more profoundly to IFN $\gamma$  than BMDMs from young rats. It has been reported that IFN $\gamma$  concentration is increased in brain tissue prepared from aged, compared with young, rats (Lyons *et al.*, 2011) and therefore the possibility is that infiltrating cells might respond not only to HMGB-1, but also to IFN $\gamma$ ; in this case a marked increase in inflammation might be predicted as the cells adopt the M1 state. M1 activation of macrophages is also associated with a decrease in the expression of markers associated with M2 activation; IFN $\gamma$  treatment induces a decrease in the expression of MRC1 (Stein *et al.*, 1992) and attenuates the IL-4-induced expression of Arg1, Chi3li3 and Fizz1 (Raes *et al.*, 2002a). Conversely, M2 activated macrophages exhibit an inability to produce NO and secrete anti-inflammatory mediators such as IL-1RA and IL-10 (Mantovani *et al.*, 2002; Edwards *et al.*, 2006). Therefore, an increase in the number of M1-activated macrophages, accompanied by a decrease in M2-activated cells, may exacerbate tissue damage. In this study, in contrast to the increased responses of BMDMs from aged rats to LPS and IFN $\gamma$ , there was no evidence of a similar change in response to IL-4. This, coupled with the evidence that IL-4 concentration is decreased in brain tissue prepared from aged rats (Nolan *et al.*, 2005; Downer *et al.*, 2009; Loane *et al.*, 2009) makes it unlikely that infiltrating BMDMs will adopt the M2 activation state.

Similar to aged rats, macrophages from APP/PS1 mice exhibit an exaggerated response to pro-inflammatory stimuli and a greater LPS-induced increase in NOS2 mRNA and TNF $\alpha$  mRNA was observed. Similarly, an increased response to IFN $\gamma$  was observed which may, in part, be explained by the accompanying increased expression of IFN $\gamma$ R on cells. This enhanced response is significant because hippocampal concentration of IFN $\gamma$  is increased in APP/PS1 mice and because there is an increase in infiltration of peripheral cells (Minogue *et al.*, personal communication), including macrophages as shown here, into the brain of APP/PS1 mice. There was no evidence of a genotype-related change in the response of BMDMs to IL-4. It can be concluded that macrophages from aged rats and APP/PS1 mice gain entry to the brain and encounter inflammatory stimuli which induce the cells to adopt the M1 phenotype.

The present findings which reveal that macrophages from APP/PS1 mice are more likely to adopt an inflammatory phenotype may explain why macrophages from AD patients exhibit a decreased ability to clear A $\beta$  (Fiala *et al.*, 2005). The majority of evidence indicates that M2 macrophages/microglia exhibit enhanced phagocytic activity compared with M1 macrophages/microglia (Koenigsnecht-Talboo & Landreth, 2005; Townsend *et al.*, 2005; Chinetti-Gbaguidi *et al.*, 2011). M2 macrophages express high levels of MRC1 which is involved in the clearance of dying cells (Nauta *et al.*, 2003) and M2 activated microglia exhibit greater uptake of A $\beta$  than M1 microglia (Jimenez *et al.*, 2008). Conversely, treatment of microglia with CD40 ligand induced M1 activation and this was associated with a decrease in the uptake of FITC-labelled A $\beta$  (Townsend *et al.*, 2005) and injection of A $\beta$ -specific Th1 cells into APP/PS1 mice increased neuroinflammation and also A $\beta$  accumulation (Browne *et al.*, 2013b) while injection of Th2 cells increased A $\beta$  clearance (Cao *et al.*, 2009). Similarly, when macrophages were stimulated with LPS+IFN $\gamma$  their ability to phagocytose A $\beta$  was decreased whereas when they were stimulated with IL-4+IL-13 the cells were more effective phagocytes (Durafourt *et al.*, 2012).

It has been suggested that macrophages are more effective phagocytes than microglia (Simard *et al.*, 2006) and, in a transgenic mouse model of AD, decreased recruitment of mononuclear cells into the CNS coincided with an increased plaque burden (El Khoury *et al.*, 2007). On the basis of the present data, it might be suggested that when macrophages enter the brain, they encounter an inflammatory microenvironment and react to the presence of endogenous TLR4 agonists and IFN $\gamma$  by adopting an M1 phenotype. One consequence of this is a decreased phagocytic ability which may contribute to increased A $\beta$  accumulation in APP/PS1 mice and in AD. A second consequence is that M1 macrophages may further increase inflammation and may have impact on neuronal function, since previous work has demonstrated the detrimental effects of inflammation on LTP (Vereker *et al.*, 2000; Costello *et al.*, 2011; Kelly *et al.*, 2013) and neuronal function (Browne *et al.*, 2013a; Gallagher *et al.*, 2013)

Evidence from studying macrophages led to the proposal that microglia might similarly adopt different activation states as discussed in detailed reviews by Colton and colleagues (Colton, 2009) and recent data has indicated that, at least *in vitro*, these activation states broadly reflect the macrophage phenotypes (Chhor *et al.*, 2013).



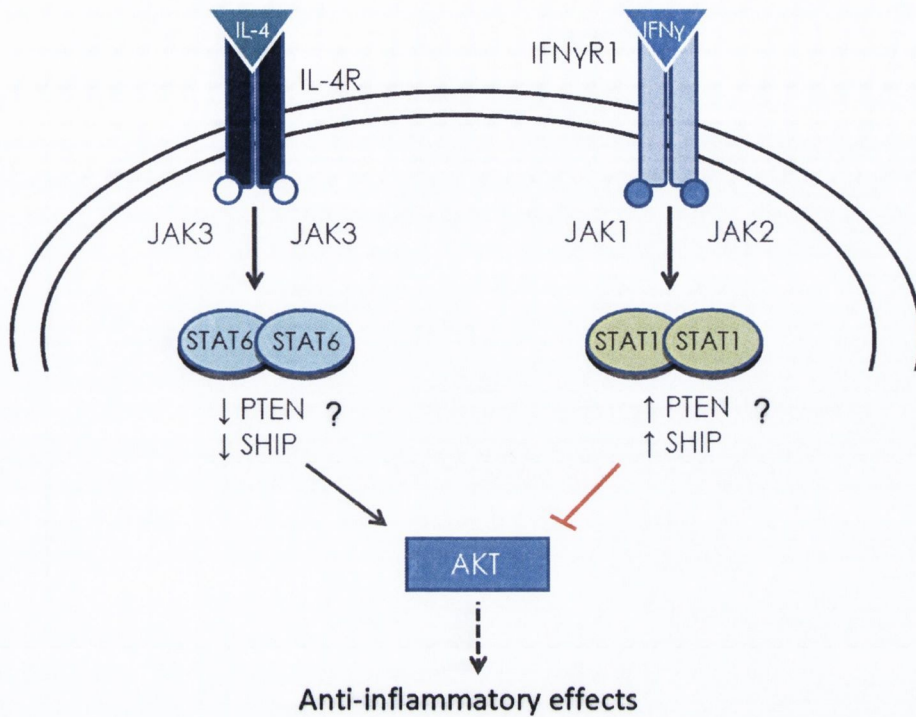
Although there are some studies which indicate that this may translate into the *in vivo* situation, this remains to be firmly established. The significance of this is that, in an inflammatory environment such as occurs with age and neurodegeneration, the response of microglia would be to adopt an M1 activation state and, together with infiltrating macrophages, potentially participate in a spiral of events which would not only be damaging to neurons, but would further activate microglia and astrocytes exacerbating the effects of inflammation.

A key role of macrophages is antigen presentation and the evidence indicates that M1 macrophages are more effective antigen presenting cells than M2 macrophages. MHCII expression is a hallmark of antigen-presenting cells and is robustly increased on BMDMs prepared from aged rats and, while its expression on cells from young rats is increased following IFN $\gamma$  treatment, the effect of IFN $\gamma$  is greater in cells from aged rats; this contrasts with the lack of effect of LPS on MHCII mRNA. M1 activation of macrophages is also associated with an increase in the expression of CD80, CD86 and CD40, which enable increased interaction with T cells. For example CD40 ligand is expressed on T cells, this receptor-ligand interaction leads to increased production of pro-inflammatory cytokines (Lynch, 2009), and inhibiting this CD40-CD40 ligand interaction has been demonstrated to be beneficial in an animal model of MS (Gerritse *et al.*, 1996). The present data also show that there is an age-related increase in expression of CD40 mRNA and a genotype-dependent response to IFN $\gamma$  which mirrors the change in MHCII mRNA. M2 macrophages are thought to be poor antigen presenting cells and exhibit low expression of costimulatory molecules (Edwards *et al.*, 2006). In support of this, it has been that while IFN $\gamma$  induced an increase in antigen presentation in the macrophage cell line J774A.1, the anti-inflammatory cytokine TGF- $\beta$  was shown to have an opposite effect (Delvig *et al.*, 2002).

In recent years attention has turned to investigating potential molecular switches that may be involved in polarisation of macrophages towards a M1 or M2 state, as they may be crucial to the development of new therapeutic strategies. Since IL-4 treatment results in downstream activation of AKT, it is not surprising that evidence is emerging suggesting a role for AKT in the induction of the M2 state (Heller *et al.*, 2008; Ruckerl *et al.*, 2012), and the present findings demonstrate that some of the effects of IL-4 are induced through activation of the PI3K/AKT pathway, whereas M1 activated macrophages

were associated with a decrease in PI3K/AKT pathway and the effects of IFN $\gamma$  treatment were exaggerated in the presence of the PI3K inhibitor, Ly294002. Consistent with these observations, it has been demonstrated that macrophages from SHIP<sup>-/-</sup> mice, which exhibit enhanced pAKT expression, are hyperresponsive to IL-4 treatment (Weisser *et al.*, 2011) whereas previous work from this lab has shown that fractalkine and FGL suppressed age-related neuroinflammation and markers of activation in LPS- and IFN $\gamma$ -stimulated glia (Downer *et al.*, 2009; Lyons *et al.*, 2009); these anti-inflammatory effects were found to be mediated in part through activation of AKT. The expression of pAKT has also been shown to modulate the response of macrophages to LPS; thus macrophages from PTEN<sup>-/-</sup> mice exhibit high levels of pAKT expression and this is associated with a decrease in LPS-induced cytokine production (Ruckerl *et al.*, 2012). In contrast, the same group found that LPS-induced IL-6 and TNF $\alpha$  were exaggerated in macrophages from mice that demonstrate impaired PI3K/AKT activity. The findings from this study combined with reports in the literature demonstrate that manipulation of molecular switches, such as AKT expression, may be a possible therapeutic target for the polarisation of macrophages. Figure 6.1. illustrates a possible mechanism by which AKT may modulate macrophage phenotype.

Overall the findings of this study show that macrophages from aged rats and APP/PS1 mice exhibit an enhanced response to inflammatory stimuli, and the most significant aspect of this is that these macrophages gain entry to the CNS. These data are important in the context of both normal aging and the progression of pathology as these cells encounter an inflammatory environment and may adopt an M1 state which could exacerbate the existing inflammatory condition. This study also proposes that it may be possible to modulate macrophage phenotype by manipulating the expression of molecular pathways such as the PI3K/AKT. The data presented here suggest that therapies that aim to modulate macrophage phenotype and restore the integrity of the BBB may be beneficial in neurodegenerative disorders.



**Figure 6.1. Proposed mechanism for AKT modulation on macrophage activation**

Binding of IL-4 and IFN $\gamma$  to their respective receptors leads to the induction of intracellular signalling cascade. IL-4 signalling enhances AKT activation, this may be mediated through decreased expression of PTEN and SHIP, both of which are negative regulators of AKT activation. The data presented here suggest that IFN $\gamma$  decreases AKT activity, one possible mechanism by which this may occur is the increased expression of proteins such as PTEN and SHIP.

	Young Control	Young LPS	Aged Control	Aged LPS	WT Control	WT LPS	APP/PS1 Control	APP/PS1 LPS
TNF- $\alpha$	-	↑	-	↑↑	-	↑	-	↑↑
NOS2	-	↑	-	↑	-	↑	-	↑
CD40	-	↑	-	↑↑	-	↑	-	↑
CD11b	-	↑	-	↑↑	-	↑	-	↑
IL-1 $\beta$	-	↑	-	↑↑				
IL-6	-	↑	-	↑				

**Table 6.1. Summary of mRNA changes observed following LPS stimulation (↑= increased; ↑↑=further increased)**

	Young Control	Young IFN $\gamma$	Aged Control	Aged IFN $\gamma$	WT Control	WT IFN $\gamma$	APP/PS1 Control	APP/PS1 IFN $\gamma$
MHCII	-	↑	↑	↑↑	-	↑	-	↑↑
TNF- $\alpha$	-	↑	-	↑↑	-	↑	-	↑↑
NOS2	-	↑	-	↑	-	↑	-	↑
NOD2					-	↑	-	↑↑
CD40	-	↑	-	↑↑				

**Table 6.2. Summary of mRNA changes observed following IFN $\gamma$  stimulation (↑= increased; ↑↑=further increased)**

## 6.2. Limitations and Future Studies

Although the data presented in this thesis suggest that BMDMs from aged rats and APP/PS1 mice exhibit an enhanced response to pro-inflammatory stimuli such as LPS and IFN $\gamma$ , no mechanism that may mediate this effect has been identified. Evidence from the literature demonstrates that enhanced responses to LPS and IFN $\gamma$  are associated with increased intracellular signalling, future studies should investigate the signalling pathways activated following LPS and IFN $\gamma$  stimulation of BMDMs from aged rats and APP/PS1 mice. This may lead to the identification of possible therapeutic targets that allow modulation of the inflammatory response.

Another area that may require further study is to assess whether changes in macrophage phenotype may be caused by circulating factors within the blood such as pro-inflammatory cytokines. To address this, BMDMs from young mice should be incubated in the presence of serum from WT and APP/PS1 mice, the hypothesis being that serum from APP/PS1 mice will induce the M1 phenotype. Further to this, analysis of the serum may lead to the identification of possible inflammatory markers that are responsible for the changes observed.

One of the main findings presented in this study suggests that AKT activity may play a role in the modulation of macrophage activation. The present data suggest that the effect of IL-4 and IFN $\gamma$  on pAKT expression is not mediated through PI3K activity. In light of these findings, one of the most important future studies is to identify how these effects are mediated. The proteins SHIP and PTEN are two possible candidates that may mediate these effects on pAKT expression, studies that focus on the effects of IL-4 and IFN $\gamma$  on these proteins may prove beneficial in modulating macrophage activation as they may develop into future therapeutic targets.

The data presented here suggest that macrophages gain entry to the brains of aged rats and APP/PS1 mice, however, the effect that infiltration of these cells have within the CNS remains largely unexplored. Infiltration of M1 macrophages may prove to be detrimental; in contrast M2 macrophages that migrate to the CNS may play a role in repair and resolution of inflammatory events. Future work that aims to determine the effect of infiltrating M1 and M2 macrophages should be a priority. This may be achieved by

polarising M1 and M2 macrophages and injecting these cells into the brains of WT and APP/PS1 mice. This work may give possible insights into the differential roles that M1 and M2 macrophages play within the CNS.

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## Appendix

## Solutions and buffers

<b>Electrode running buffer</b>	Tris base	25mM
	Glycine	192mM
	SDS	0.1% (w/v)
<b>FACS buffer</b>	PBS (1X)	500ml
	FBS	10ml
<b>Laemmli sample buffer (pH 6.8)</b>	Tris HCl	0.05M
	Glycerol	20% (v/v)
	SDS	2% (w/v)
	$\beta$ -mercaptoethanol	5% (v/v)
	Bromophenol blue	0.05% (w/v)
<b>Lysis buffer</b>	Tris HCl	10mM
	NaCl	50mM
	IGEPAL	1% (v/v)
	Protease inhibitor	1% (v/v)
	Sigma #P8340	
	Phosphatase inhibitors	1% (v/v)
	Sigma #P5726	
	Sigma #P0044	

<b>Medium A</b>	Glucose (45%)	650 $\mu$ l
	HEPES (1M)	750 $\mu$ l
	HBSS (1x)	50ml
<b>PBS (1X, pH 7.4)</b>	NaCl	137mM
	KCl	2.7mM
	Na <sub>2</sub> HPO <sub>4</sub>	8.1mM
	K <sub>2</sub> PO <sub>4</sub>	1.5mM
<b>RIPA Buffer</b>	NaCl	150 mM
	Tris-HCl (pH 7.4)	5 mM
	sodium deoxycholate	1% (w/v)
	Triton x-100	1% (v/v)
	EDTA	1 mM
	Protease inhibitor cocktail	1% (v/v)
	Sigma #P8340	
<b>Running gel (7%)</b>	Bis/Acrylamide (30% Stock)	23% (v/v)
	H <sub>2</sub> O	50% (v/v)
	Tris-HCl, 1.5M, (pH 8.8)	25% (v/v)
	10% SDS	1% (v/v)
	10% APS	0.5% (v/v)

	TEMED	0.5% (v/v)
<b>Running gel (10%)</b>	Bis/Acrylamide (30% Stock)	33% (v/v)
	H <sub>2</sub> O	40% (v/v)
	Tris-HCl, 1.5M, (pH 8.8)	25% (v/v)
	SDS	1% (v/v)
	APS	0.5% (v/v)
	TEMED	0.5% (v/v)
<b>Stacking gel (4%)</b>	Bis/Acrylamide (30% Stock)	13% (v/v)
	H <sub>2</sub> O	60% (v/v)
	Tris-HCl, 1.5M, (pH 8.8)	25% (v/v)
	10% SDS	1% (v/v)
	10% APS	0.5% (v/v)
	TEMED	0.5% (v/v)
<b>TBS (pH 7.4)</b>	Tris-HCL	20mM
	NaCl	150mM

**Transfer buffer**

Tris base	25mM
Glycine	192mM
MeOH	20%(v/v)
SDS	0.05% (w/v)