



Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

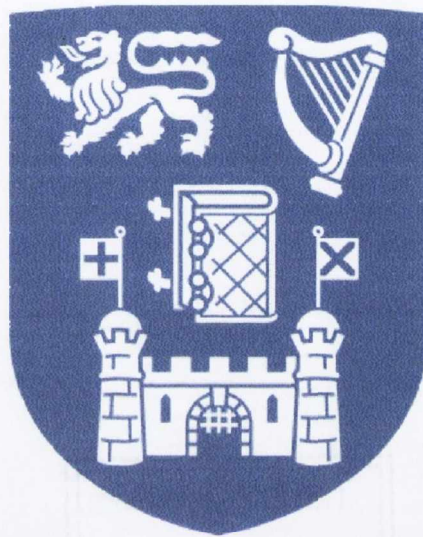
By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

Analysis of the Metabolic Shift that Accompanies Differential Activation of Microglia



Robert Holland

BA (Mod) Physiology

MSc Immunology

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

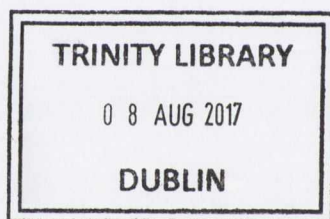
Supervisor: Prof. Marina Lynch

Trinity College Institute of Neuroscience,

Department of Physiology,

School of Medicine

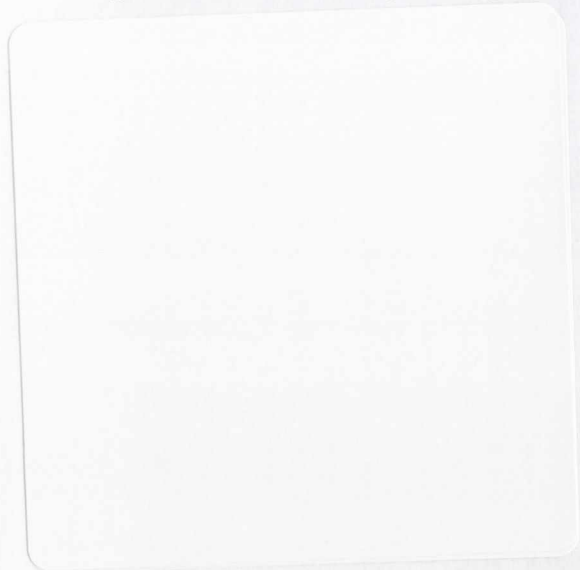
2017



Thesis 11414

Declaration of Authorship

This thesis is submitted by the undersigned for the degree of Doctor of Philosophy at the University of Dublin. 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. 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.



Declaration of Authorship

This thesis is submitted by the undersigned for the degree of Doctor of Philosophy at the University of Dublin. I declare that this thesis is entirely my own work. I have read and I understand the plagiarism provisions in the General Regulations of the University Calendar for the current year, found at <http://www.tcd.ie/calendar>. This work has not been submitted in whole or part to this or any other university for any other degree. The author gives permission to the library to lend or copy this work upon request.

Abstract

Microglia, the resident immune cells of the central nervous system (CNS), adopt different phenotypes upon activation by different stimuli. The primary role of microglia is protection and maintenance of homeostasis, and any threat to homeostasis results in activation of cells. Under normal circumstances, cells switch back to their resting state once the threat is removed, but this ability to switch is decreased with age, whereupon cells become persistently activated. Dysfunctional, chronic activation of inflammatory microglia has been associated with most neurodegenerative disorders.

In recent years, it had become evident that immune cell function and metabolism are intrinsically linked, with pro-inflammatory cells demonstrating increased glycolysis while anti-inflammatory cells are associated with increased oxidative metabolism. However, this metabolic reprogramming has not yet been investigated in the CNS. The aims of this study were to characterise microglial activation states in terms of their metabolic and iron handling profiles *in vitro* and to determine whether these changes accompany the inflammation that occurs in microglia in the brains of aged mice and APP/PS1 mice.

IFN- γ and IL-4 have been shown to induce the so-called M1- and M2-like activation states in macrophages and microglia. The M1 activation state is generally considered pro-inflammatory, while the M2 activation state is associated with the resolution of inflammation and repair. Here, the increased mRNA expression of TNF α and iNOS, which typify the M1 activation state, in macrophages and microglia was accompanied by increased glycolysis. In contrast, IL-4-induced expression of M2 activation state markers, Arg-1 and MRC1, was accompanied by increased oxidative metabolism. The ratio between expression of PFKFB3 and PFKFB1 was found to be a reliable proxy marker for the metabolic signatures of polarised cells *in vitro*, as it was increased in M1-like cells and decreased in M2-like cells. IFN- γ increased ferritin expression in microglia, while IL-4 upregulated the expression of transferrin receptor, and these findings suggest that M1-like cells are iron retentive whereas M2-like cells recycle iron. These data suggest that molecules involved in metabolism and iron handling may be used as novel markers to characterise microglial activation.

Genotype- and age-associated increases in TNF α mRNA expression were observed in the hippocampus of aged mice and APP/PS1 mice and microglia isolated from the brains of these mice showed similar changes; these changes are reflective of the neuroinflammatory environment associated with these conditions. Analysis of the metabolic profile of microglia from these mice revealed an age-related increase in oxidative metabolism, and a genotype-related increase in maximal respiration. An age-related increase in PFKFB3:PFKFB1 in microglia, indicative of a pro-inflammatory, glycolytic phenotype was observed in these cells. An iron retentive phenotype was observed in microglia with age and genotype, paralleling the IFN- γ -induced changes observed *in vitro*.

These data suggest that activated microglia can be characterised by their metabolic signature and iron handling functions and suggests these changes are evident in neuroinflammatory conditions.

Acknowledgements

I would firstly like to thank my supervisor Professor Marina Lynch for giving me the opportunity to carry out this work. Thank you for your patience, guidance and persistence throughout. I am incredibly lucky to have had such a PI. I have learnt much more than physiology over the past three years and I am truly grateful for that experience.

I owe a big thank you to everyone I have shared time with in the lab, all of whom have helped me in some way shape or form. To Derek, Aedín, Raasay, James, Roisín, Niamh, Colin, Matheus, Claire, Ana, Karen, Orla and Hannah (you've got your own office now!). To Kieran, Alice and the rest of the Physiology Department as well as to the staff in TCIN. To Jamal and Barry for help with FACS. To Gavin for help with confocal. To Joern for help with ICC. I owe a lot of people a lot of thanks.

Thank you to SFI for providing funding for my project.

I would like to thank my parents for the unwavering support and belief they showed me. Thank you for putting up with me through the tougher times. And finally, Sarah, thanks for just being you.

Table of Contents

Declaration of Authorshipi

Abstract ii

Acknowledgements iv

Table of Contentsv

List of Figures..... ix

List of Tables.....xiii

List of Abbreviations xiv

Chapter 1: Introduction 1

 1.1 Macrophages.....2

 1.1.1 Origins.....2

 1.2 Microglia3

 1.2.1 Origin4

 1.3 Microglial activation4

 1.3.1 Characterisation of microglial activation6

 1.3.2 The M1 activation state7

 1.3.3 The M2 activation state 10

 1.3.4 Modulation of microglial activation 11

 1.3.5 Activation states and iron handling 11

 1.3.6 Microglial phagocytosis and antigen presentation 14

 1.4 Astrocytes 14

 1.5 Metabolic signature of immune cells 16

 1.5.1 Overview of metabolic pathways 16

 1.5.2 HIF-1α induced metabolic reprogramming 18

1.5.3 Metabolic reprogramming of T cells	21
1.5.4 Metabolic profiles of polarised macrophages	22
1.5.5 Metabolic reprogramming in microglia	24
1.6 Neuroinflammation & AD	25
2.2.2 Removal of brain tissue	25
1.6.1 Alzheimer's disease	26
1.6.2 A β and the amyloid hypothesis	26
1.6.3 Neuroinflammation & AD	27
1.6.4 APP/PS1 mouse model of AD	29
1.7 Aims.....	30
Chapter 2 Methods	31
2.1 Animals.....	32
2.2 Sacrifice and tissue harvesting	32
2.2.1 Intracardial saline perfusion	32
2.2.3 <i>Ex vivo</i> cell isolation	33
2.3 Cell culture	35
2.3.1 Aseptic techniques.....	35
2.3.2 Preparation of primary cortical microglia	35
2.3.3 Preparation of primary cortical astrocytes	36
2.3.4 Preparation of mixed glial cultures	37
2.3.5 Maintenance of L929 cell line	37
2.3.6 Isolation and culture of murine BMDMs	37
2.3.7 <i>In vitro</i> polarisation of M1/M2 cells	38
2.3.8 Preparation of glia:T cell co-culture for CTV proliferation assay	38

2.3 Assessment of glial phagocytosis	40
2.3.1 Immunocytochemistry	41
2.4 Flow cytometry	42
2.4.1 Extracellular staining	42
2.4.2 Intracellular staining	42
2.4.3 Flow cytometric analysis	43
2.5 Using the SeaHorse XF Analyser	44
2.6 Analysis of protein secretion by ELISA	46
2.8 Analysis of mRNA by RT-PCR	47
2.8.1 RNA extraction	47
2.8.2 cDNA Synthesis	48
2.8.3 Reverse Transcription-Polymerase Chain Reaction	48
2.8.4 PCR quantification	50
2.9 Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis ..	51
2.9.1 Sample preparation for SDS-PAGE	51
2.9.2 Pierce® BCA protein assay	51
2.9.3 SDS-PAGE	52
2.9.4 Western transfer protocol	52
2.9.5 Western immunoblotting	52
2.9.6 Analysis	54
2.10 Statistical analysis	54
Chapter 3: Metabolic reprogramming in polarised BMDMs	55
3.1 Introduction	56
3.2 Methods	59

3.3 Results	60
3.4 Discussion	70
Chapter 4: Characterisation of the activation states of polarised glia	77
4.1 Introduction	78
4.2 Methods	81
4.3 Results	82
4.4 Discussion	107
Chapter 5: Functional consequences of glial activation.....	115
5.1 Introduction	116
5.2 Methods	118
5.3 Results	119
5.4 Discussion	134
Chapter 6 Characterisation of age- and genotype-related effects on microglial activation	139
6.1 Introduction	140
6.2 Methods	142
6.2.1 Modified SeaHorse mitochondrial stress test for isolated microglia	142
6.3 Results	144
6.4 Discussion	161
Chapter 7 General discussion	166
7.1 General discussion	167
Chapter 8: References	176
Appendix I	204
Solutions	205

List of Figures

Figure 1.1 Summary of M1 and M2 activation states of macrophages and microglia7

Figure 1.2 Overview of metabolic pathways 17

Figure 1.3 HIF-1 α -induced metabolic reprogramming 19

Figure 1.4 Metabolic reprogramming of M2 macrophages 23

Figure 2.1 Isolation of cells by Percoll gradient 34

Figure 2.2 Example SeaHorse graph for the mitochondrial stress test 45

Figure 2.3 Example SeaHorse graph for the glycolytic flux test 46

Figure 3.1 IFN- γ increased expression of M1 activation state markers in BMDMs 62

Figure 3.2 IL-4 increased expression of the M2 activation state markers in BMDMs 63

Figure 3.3 IFN- γ significantly increased glycolysis in BMDMs 64

Figure 3.4 IL-4 significantly increased mitochondrial respiration in BMDMs 65

Figure 3.5 IFN- γ significantly increased PFKFB3:PFKFB1 in BMDMs 66

Figure 3.6 IL-4 significantly increased TfR in BMDMs 67

Figure 3.7 IL-4 and IFN- γ decreased ferroportin in BMDMs 68

Figure 3.8 IL-4 and IFN- γ decreased the mRNA expression, but increased the protein expression of ferritin in BMDMs 69

Figure 4.1 IL-4 and IFN- γ increased the mRNA expression of markers of microglial activation.....	87
Figure 4.2 IFN- γ increased the mRNA expression of markers of the M1 activation state in microglia.....	88
Figure 4.3 IL-4 increased the mRNA expression of markers of the M2 activation state in microglia.....	89
Figure 4.4 IFN- γ significantly increased glycolysis in microglia.....	90
Figure 4.5 IL-4 significantly increased mitochondrial respiration in microglia	91
Figure 4.6 IFN- γ decreased OCR/ECAR and increased ECAR/OCR ratio in microglia, while IL-4 had the opposite effect	92
Figure 4.7 IFN- γ significantly increased PFKFB3 mRNA in microglia	93
Figure 4.8 IFN- γ significantly decreased the PFKFB1 mRNA in microglia	94
Figure 4.9 IFN- γ significantly increased PFKFB3/PFKFB1 in microglia	95
Figure 4.10 HIF-1 α expression was induced by IFN- γ , but this had no effect on PDK1 expression in microglia	96
Figure 4.11 Polarised microglia did not alter pAMPK & PGC1 β expression.....	97
Figure 4.12 Polarised microglia did not alter GLUT1 expression in microglia	98
Figure 4.13 IL-4 and IFN- γ had opposite effects on TfR mRNA expression in microglia	99
Figure 4.14 IL-4 and IFN- γ decreased the mRNA expression of ferroportin in microglia	100

Figure 4.15 IL-4 and IFN- γ had contrasting effects on ferritin mRNA expression in microglia	101
Figure 4.16 IFN- γ increased the mRNA expression of GLAST in astrocytes	102
Figure 4.17 IFN- γ increased the mRNA expression of markers of the M1 activation state in astrocytes	103
Figure 4.18 IL-4 increased M2 activation state markers in astrocytes	104
Figure 4.19 IL-4 and IFN- γ significantly increase the glycolytic rate in astrocytes	105
Figure 4.20 IL-4 or IFN- γ did not affect oxidative metabolism in astrocytes	106
Figure 5.1 Fluorescently-labelled latex beads were taken up by microglia, but not astrocytes <i>in vitro</i>	123
Figure 5.2 Microglia were shown to engulf FITC-conjugated A β	125
Figure 5.3 Neither IL-4 nor IFN- γ affected phagocytosis of fluorescently-labelled latex beads	127
Figure 5.4 IFN- γ and IL-4 increase the mRNA expression of CD68 and decrease the expression of CD33 in microglia	128
Figure 5.5 IFN- γ increases the mRNA expression of MHC II and CD86 in microglia	129
Figure 5.6 Neither IL-4 or IFN- γ affect microglial capacity to induce T cell proliferation	130
Figure 5.7 IFN- γ and IL-4 stimulated microglia induce the proliferation of IFN- γ ⁺ T cells.....	131

Figure 5.8 IFN- γ significantly increases microglial secretion of chemokines	132
Figure 5.9 IL-4 induces microglial secretion of BDNF and IGF1	133
Figure 6.1 Increased TNF- α mRNA was observed in hippocampus of APP/PS1 mice	148
Figure 6.2 Microglial expression of TNF- α and iNOS mRNA was affected by age and genotype	149
Figure 6.3 Maximal respiration was decreased in aged WT mice compared with younger controls	150
Figure 6.4 There was no change in respiration in microglia from 9 month-old APP/PS1 mice compared with WT controls	151
Figure 6.5 Respiration was higher in aged APP/PS1 mice compared with younger controls.....	152
Figure 6.6 Maximal respiration was increased in aged APP/PS1 microglia	153
Figure 6.7 Age- and genotype-effects on microglial respiration.....	155
Figure 6.8 PFKFB3:PFKFB1 was increased in an age-dependent manner	157
Figure 6.9 Ferroportin mRNA expression was decreased in microglia from aged APP/PS1 mice	158

List of Tables

Table 2.1 Antibodies used for immunocytochemistry 41

Table 2.2 Antibodies used for flow cytometry 43

Table 2.3 List of primers for RT-PCR 50

Table 2.4 Antibodies used for Western immunoblotting 54

Table 6.1 Neither age nor genotype affected the mRNA expression of PFKFB
isozymes in hippocampal tissue, but age and genotype affected ferritin and
ferroportin expression respectively 159

Table 7.1 Summary of changes in metabolism and iron handling profile .. 168

List of Abbreviations

AD	Alzheimer's disease
AICD	Amyloid precursor protein intracellular domain
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
APC	Antigen presenting cell
APP/PS1	Amyloid precursor protein/presenilin 1 double mutation
APS	Ammonium persulphate
Arg-1	Arginase
ATP	Adenosine triphosphate
Aβ	Amyloid beta
BBB	Blood-brain barrier
BDNF	Brain-derived growth factor
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
cDMEM	Complete Dulbecco's modified Eagle medium
cDNA	Complementary deoxyribonucleic acid
Chi3L3	Chitinase 3-like 3
CNS	Central nervous system
CTV	Cell trace violet
CXCL	C-X-C motif chemokine
DAMP	Damage-associated molecular pattern
DAPI	4'-6-Diamidino-2-phenylindole

DC	Dendritic cell
DD	Death domain
df	Degrees of freedom
dH₂O	Distilled water
DMEM	Dulbecco's modified Eagle medium
DMT	Divalent metal ion transporter
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune Encephalomyelitis
ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FAD	Familial-associated Alzheimer's disease
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FIZZ1	Found in inflammatory zone protein 1
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate aspartate transporter
GLUT-1	Glucose transporter 1
GM-CSF	Granulocyte macrophage colony stimulating factor
HBSS	Hank's balanced salt solution
HIF-1α	Hypoxia-inducible factor 1-alpha
Iba1	Ionized calcium binding adaptor molecule 1
IFN-γ	Interferon gamma
IFNγR	Interferon gamma receptor

IGF1	Insulin-like growth factor 1
IL	Interleukin
IL-1RI	IL-1 receptor I
IL-4R	IL-4 receptor
iNOS	Inducible nitric oxide synthase
IP-10	IFN- γ induced protein 10
JAK	Janus kinase
LPS	Lipopolysaccharide
MACS	Magnetic-activated cell sorting
MCP1	Monocyte chemotactic protein 1
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MIP1α	Macrophage inflammatory protein 1 alpha
MRC1	Mannose receptor
mRNA	Messenger ribonucleic acid
MS	Mean squared
MS	Multiple sclerosis
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NK	Natural killer
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NOS2	Nitric oxide synthase 2
OCR	Oxygen consumption rate
PAMP	Pathogen-associated molecular pattern

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PB-T	Phosphate buffered saline-tween
PCR	Polymerase chain reaction
PD	Parkinson's disease
PDK	Pyruvate dehydrogenase kinase
PE	Phycoerythrin
PE-Cy	Phycoerythrin-cyanine
PFKFB	6-phosphofructo-2-kinase/fructose-2,6-bisphosphate
PGC	PPAR- γ coactivator
PPAR	Peroxisome proliferator activated receptor
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation end products
RANTES	Regulated and normal T cell expressed and secreted
RBC	Red blood cell
RIG-1	Retinoic acid-inducible gene 1
RNA	Riboxynucleic acid
RPMI	Roswell park memorial institute
RT-PCR	Real time polymerase chain reaction
SCA	Scavenger receptor A
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SS	Sum of squares
STAT	Signal transducer and activator of transcription

TBS	Tris buffered saline
TBS-T	Tris buffered saline-tween
TfR	Transferrin receptor
TGF-β	Transforming growth factor beta
T_h	T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	TNF receptor
T_{reg}	T regulatory
TREM2	Triggering receptor expressed on myeloid cells 2
WT	Wild type

Chapter 1: Introduction

1.1 Macrophages

Macrophages are large, phagocytic cells of the innate immune system that are found in almost all tissues and carry out a remarkably diverse array of functions (Wynn and Barron, 2010). They were first identified by Elie Metchnikoff in the late 19th century, when he described their recruitment to the site of a thorn wound in starfish. They were shown to engulf cellular debris, a phenomenon termed phagocytosis a year later by Carl Claus (Cavaillon, 2011). Along with their immune activity, macrophages play a vital role in the maintenance of homeostasis. As professional phagocytes, macrophages are responsible for the uptake and clearance of apoptotic cells and cellular debris that may arise during tissue remodelling. They express a suite of receptors which facilitate this clearance including scavenger receptors, phosphatidyl serine receptors, the thrombospondin receptor, integrins, and complement receptors (Erwig and Henson, 2007). Macrophages remove approximately 2×10^{11} erythrocytes each day from the circulation and are responsible for recycling iron from these cells.

1.1.1 Origins

Despite continuing advances, our understanding of the origin of macrophages is still incomplete. Until recently, the mononuclear phagocyte system has acted as a foundation dogma in immunology; Ralph van Furth and Zanvil Cohn proposed that major populations of macrophages were derived from circulating monocytes (van Furth and Cohn, 1968). This is a linear model which proposes that bone marrow precursors give rise to circulating monocytes which, in turn, migrate and differentiate into tissue macrophages in order to replenish tissue macrophage populations. Although monocytes can give rise to macrophages under certain circumstances, such as inflammation, it has been shown that monocytes do not contribute substantially to tissue macrophage populations, and macrophages themselves in tissues undergo proliferation to replenish their number (Sawyer et al., 1982). Recent technologies, such as fate-mapping, have shown that some tissue macrophages are of embryonic origin, seeded prior to birth, and not derived from the bone marrow (Ginhoux et al., 2010).

In contrast to the mononuclear phagocyte system and model of macrophage differentiation from bone marrow progenitors, the current understanding of macrophage origin is that macrophages are of embryonic origin and seeding of cells in tissue occurs in two stages. Initially, myeloblastosis-independent haematopoiesis in the yolk sac gives rise to early tissue macrophages. This is called primitive haematopoiesis and begins at E7.5, with cells colonising tissues from E9. Myeloblastosis-dependent, or definitive, haematopoiesis begins later at E11 in the foetal liver and gives rise to monocytes which invade embryonic tissues at E14.5, a few days after yolk sac derived macrophage colonization (Cumano and Godin, 2007). Once inside the tissue, these cells differentiate into macrophages and proliferate. However, in most adult tissue, macrophages are of foetal liver origin, meaning that early yolk sac derived cells are replaced by those from the foetal liver (Ginhoux and Jung, 2014). This is true for alveolar macrophages (Guilliams et al., 2013) and for heart macrophages (Epelman et al., 2014). Dual origin has been shown for Langerhans cells in the skin, although the vast majority arise from the foetal liver (Hoeffel et al., 2012). The only tissue resident macrophages that are entirely of yolk sac origin are the microglial cells in the brain (Ginhoux et al., 2010).

1.2 Microglia

Microglia were first identified in 1880 by Franz Nissl and their activation was witnessed by Victor Babeş in 1897 while studying viral infections of the brain (Babes, 1892). Pio del Rio-Hortega is known as the “Father of Microglia” for his extensive work in characterising their activation using silver carbonate staining, and for coining their name (Rezaie and Male, 2002). Microglia constitute 10-12% of the total cells in the brain (Block and Hong, 2005) and are the resident immune cells of the CNS. They are responsible for the induction, regulation and resolution of inflammation (Lyman et al., 2014) and tissue repair (Harry and Kraft, 2012). Their functions are similar to macrophage functions and include phagocytosis, antigen presentation and the production of chemokines and cytokines (Benveniste, 1998). As well as responding to insult

and injury, microglia are also supportive glia that play an important role in maintaining homeostasis in the CNS. They interact with other glial cells and neurons to fulfil neurotrophic roles (Vilhardt, 2005) and are crucial for synapse pruning and remodelling during the maturation of the brain (Tremblay et al., 2011).

1.2.1 Origin

Unlike neurons and other glial cells, all of which originate in the neuroectoderm, microglia are of mesodermal origin, like other myeloid progenitor cells. Microglial cells are ontogenically distinct from monocytes and other tissue-resident macrophages as the early yolk sac-derived populations of cells are not replaced by cells of foetal liver origin. Foetal liver-derived monocytes might lack the intrinsic potential to differentiate into microglia, or they may be restricted in their access to the brain (Ginhoux and Jung, 2014). In any case, there is no recruitment of foetal liver-derived monocytes to the brain rudiment from day E13.5, when they seed other tissues. This may be because the blood-brain barrier (BBB) begins to develop around this time (Ginhoux et al., 2010), restricting the access of cells. In adult mice monocytes only enter the brain under inflammatory conditions when the integrity of the BBB is impaired. Instead, self-renewal of CNS-resident cells is responsible for sustaining microglial populations and the expansion of microglial numbers in response to insult or stress, called microgliosis, has been shown to be independent of infiltrating cells and due to the expansion of local populations (Ajami et al., 2007).

1.3 Microglial activation

Microglia are best known for their immune effector functions which can be triggered upon detection of stimuli that trigger an immune response. Due to their functional similarities, much of our understanding of microglial activation has arisen from studies carried out on macrophages.

At rest, microglia exist in a quiescent, highly sensitive, monitoring state but are readily activated in response to immunological stimuli, infection or injury (Liu and Hong, 2003). In order to maximise their effectiveness, microglia express a plethora of receptors that are capable of detecting a wide range of molecules that can bring about cell activation (Aloisi, 2001). Pattern recognition receptors (PRRs) are expressed by microglia and detect pattern-associated molecular patterns (PAMPs). PAMPs are conserved molecular structures that are essential for the integrity or virulence of microorganisms and are therefore difficult for the pathogen to alter. Because they are not present in the host under normal circumstances, PAMPs can be recognised as markers of infection by PRRs and lead to activation (Turvey and Broide, 2010). PRRs can be membrane-bound or cytosolic and include Toll-like receptors (TLRs) (Akira and Takeda, 2004), NOD-like receptors (Chen et al., 2009) and RIG-1-like receptors (Loo and Gale, 2011) which, upon ligation, induce signalling cascades culminating in the production of immunological factors.

Microglia can also recognise endogenous damage associated molecular patterns (DAMPs) that may be present in the extracellular matrix following necrosis of cells as a result of trauma or stress. These danger signals include heat-shock proteins, nuclear proteins, histones, DNA and other nucleotides (Zhang and Mosser, 2008). Adenosine triphosphate (ATP) is an example of a DAMP; it binds to the purinergic receptor P2X7 and brings about the activation of macrophages and microglia, resulting in the production of cytokines via the assembly of NLRP3 inflammasome (Franceschini et al., 2015).

Upon activation, microglia undergo morphological changes that have been recognised for over a century (Kreutzberg, 1996). Quiescent cells exhibit in a ramified, branched morphology, possessing long processes that sample the microenvironment. These processes increase the surface area of the cell, allowing for greater receptor expression and therefore increasing their range of immunosurveillance. Upon detection of an activating stimulus, these processes

retract and cells take on an amoeboid shape with a bushy appearance (Benveniste, 1998).

1.3.1 Characterisation of microglial activation

In an attempt to characterise and understand microglial activation, populations have been divided into two main phenotypes mirroring those described in macrophage literature (Colton and Wilcock, 2010). In this paradigm, cells are categorised as being in one of two functional activation states (Figure 1.1). Cells in the M1 activation state are regarded as classically-activated cells, while cells adopting the M2 phenotype are described as alternatively-activated (Gordon, 2003b). However, the idea that a simple dichotomy of activation states exists is considered to be outdated (Murray et al., 2014). It is accepted that this *in vitro* classification is unlikely to be replicated *in vivo*, where cells are exposed to multiple stimuli and cell-cell interactions. The plastic nature of microglia suggest that they are capable of concurrently attaining multiple functional attributes and can readily modify their phenotype (Town et al., 2005, Heppner et al., 2015). In the current experimental paradigm, distinct activation states are considered two extremes on a scale but cells are more likely to fall into a spectrum of activation (Mosser and Edwards, 2008). With this in mind, it is necessary to further understand the properties of pro-inflammatory and anti-inflammatory cells based on alternative measures to the expression of particular molecular markers.

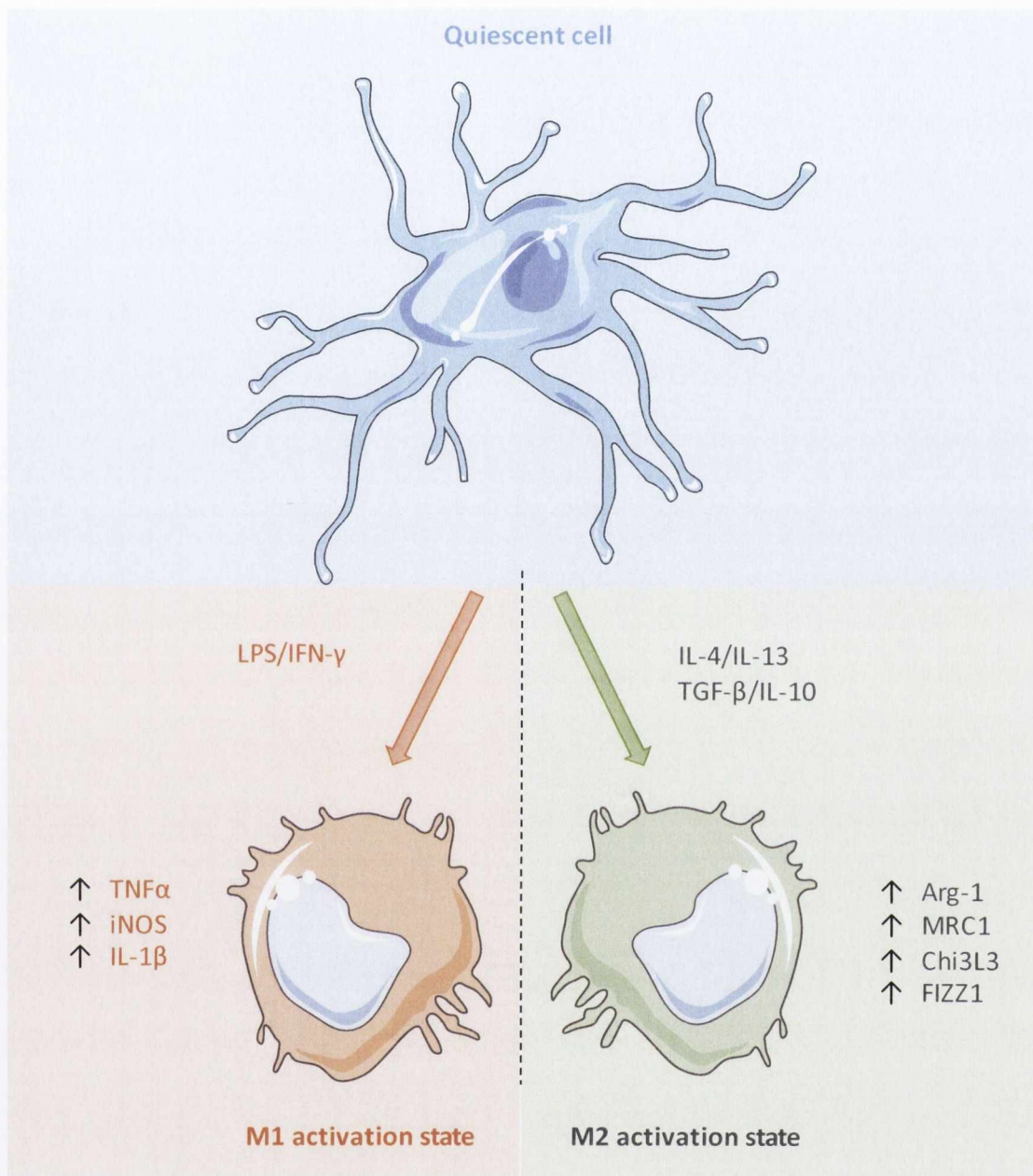


Figure 1.1 Summary of M1 and M2 activation states of macrophages and microglia. IFN- γ and LPS induce the pro-inflammatory M1 activation state characterised by increased expression of TNF α , iNOS, and IL-1 β . IL-4 and IL-13 induce the anti-inflammatory M2 activation state, characterised by increased expression of Arg-1, MRC1, Chi3L3, and FIZZ1.

1.3.2 The M1 activation state

The M1, or classical activation, state was originally used to describe macrophages activated by tumor necrosis factor α (TNF α) and interferon- γ (IFN-

γ) (Nathan et al., 1983). Cells displaying this phenotype are pro-inflammatory and mount an immune response to protect the host from infection and injury. Although initial *in vitro* studies designed to explore these activation states used a combination of TNFα and IFN-γ, a wide range of stimuli are known to induce the M1 activation state in microglia including PAMPs, DAMPs and pro-inflammatory cytokines.

TLRs are type I transmembrane glycoproteins and are the most widely studied of the PRRs. These receptors are expressed on the plasma membrane and on the membranes of cytosolic compartments of many cells, including microglia (Kielian, 2006), and respond to a variety of motifs associated with pathogenic infection. TLR4 and TLR2 are expressed on the plasma membrane and respond to lipopolysaccharide (LPS) (Poltorak et al., 1998) and lipotechoic acid (Schwandner et al., 1999) respectively. LPS is a component of the cell wall of Gram-negative bacteria and lipotechoic acid is a major constituent of the cell wall of Gram-positive bacteria. In contrast, TLR3 and TLR9 are expressed in the membranes of cellular compartments where they detect double-stranded RNA (Alexopoulou et al., 2001) and unmethylated CpG oligodeoxynucleotide DNA (Bauer et al., 2001), arising from viral and bacterial infection. Upon detection of these exogenous ligands, TLRs initiate signalling cascades through cytosolic adaptor proteins, culminating in the activation of transcription factors such as nuclear factor κB (NF-κB) and interferon regulatory factors 3 and 7 and the transcription of pro-inflammatory cytokines, chemokines and interferons (Akira and Takeda, 2004).

IFN-γ polarises microglia to an M1 phenotype (Colton, 2009). IFNs are a family of cytokines that were first characterised in 1957 for their ability to disrupt viral replication (Isaacs and Lindenmann, 1957). IFN-γ, the only type II IFN, is a homodimer protein that signals through the IFN-γ receptor (IFNγR) that consists of two ligand binding IFNγRI subunits and two signal-transducing IFNγRII subunits (Pestka et al., 2004). IFN-γ is primarily produced by T helper (Th) 1 cells, CD8⁺ cytotoxic T cells and natural killer (NK) cells in response to

exposure to interleukin (IL) 12, -18 and antigen presentation. The signalling cascade initiated by ligation of IFN γ R culminates in the activation of the transcription factor signal transducer and activator of transcription 1 (STAT1) and the transcription of immune effector genes (Schroder et al., 2004). This facilitates cells to execute effector functions, and induces the upregulation of pro-inflammatory cytokines, chemokines and molecules involved in antigen presentation (Lynch, 2009). The source of IFN- γ in the CNS is much debated. Its origin has been attributed to infiltrating peripheral immune cells, such as T cells or macrophages, that can migrate through a permeable BBB under chronic inflammatory conditions (Minogue et al., 2014b). However, there are a few sporadic reports suggesting that microglia are capable of producing IFN- γ (Kawanokuchi et al., 2006), which would suggest that the generation of M1 microglial populations may occur in the absence of infiltrating peripheral immune cells.

The M1 phenotype is characterised by the expression of a number of archetypal markers. Specifically, the upregulation of expression of TNF α , inducible nitric oxide synthase (iNOS) and IL-18 (Figure 1.1) (Chhor et al., 2013a). TNF α is a potent pro-inflammatory cytokine that exerts pleiotropic effects on various cell types. It is a type II transmembrane protein that can exist in a membrane-bound and a cleaved, soluble form, with the TNF α -converting enzyme responsible for its cleavage and release (Black et al., 1997). The interaction of TNF α with its receptors TNF-receptor (TNF-R) 1 and TNF-R2, recruits cytoplasmic adaptor proteins TNF receptor-associated factors and death domain (DD) molecules (Chen and Goeddel, 2002). Recruitment of TNF receptor-associated factors leads to the transcription of pro-inflammatory genes via c-jun-N-terminal kinase (JNK) and NF- κ B activation, while DD proteins such as Fas-associated DD and TNFR associated DD lead to the activation of caspases and cell death (Locksley et al., 2001).

M1 microglia exhibit an increased production of nitric oxide (NO), mediated by increased iNOS expression (Murphy, 2000). iNOS catalyses the oxidation of L-

arginine into NO and L-citrulline (Lowenstein and Padalko, 2004). NO free radicals are highly diffusible and have antimicrobial and antiviral properties, as they act as a toxin to disrupt DNA (Bogdan, 2001).

IL-1 β is another cytokine that contributes to the pro-inflammatory response of M1 activated microglia. A member of the IL-1 family of cytokines, it is upregulated by microglia in response to insult or stress (Murray and Lynch, 1998) and is a key mediator in the immune response. However, IL-1 β is not transiently expressed in microglia, but polarisation to the M1 activation state by LPS has been shown to increase its expression (Henry et al., 2009). IL-1 β amplifies the immune cascade by inducing the secretion of other pro-inflammatory cytokines such as TNF α and IL-6 (Mills and Dunne, 2009). IL-1 receptor type I (IL-1RI) is expressed on almost all cells while IL-1 receptor II (IL-1RII) acts as a competitive antagonist of IL-1 β responses (O'Neill, 2008). The widespread expression of IL-1 β receptors highlights the extent of its activity.

1.3.3 The M2 activation state

The M2, or alternative activation, state is considered to be anti-inflammatory and the antithesis of the classical M1 activation state. First identified by Siamon Gordon and colleagues in macrophages following IL-4 and IL-13 stimulation, cells adopting this phenotype are involved in tissue repair and remodelling and the resolution of inflammation (Stein et al., 1992). IL-4 is the primary cytokine associated with the induction of an M2-like activation state in microglia (Ponomarev et al., 2007), and can be produced in the CNS by microglia, astrocytes and even neurons (Colton, 2009). IL-4 receptor (IL-4R) dimerization occurs following ligation by the cytokine and induces Janus kinase (JAK) activation and signal transducer and activator of transcription (STAT6) phosphorylation (Mikita et al., 1996).

This activation state is characterised by the expression of Arg-1 and MRC1, as well as Chi3L3 and FIZZ1 (Chhor et al., 2013b). Arg-1 is a cytosolic enzyme that

competes with iNOS for the same substrate, L-arginine, and converts it to L-orthinine. This acts as a precursor for polyamines and L-proline, which are used for cell proliferation and collagen production associated with tissue healing and remodelling. MRC1 is a scavenger receptor involved in the uptake of mannosylated glycoproteins by macrophages (Stahl et al., 1980), however its function in the CNS is not well understood. Similarly, although strongly linked with the M2 phenotype (Cameron and Landreth, 2010), the activity of Chi3L3 and FIZZ1 in the brain is not well characterised.

Another subdivision of the M2 activation state, termed acquired deactivation, can be induced by IL-10 and TGF- β (Saijo and Glass, 2011a). These cytokines can be secreted by microglia and astrocytes in the CNS (Pratt and McPherson, 1997) and diminish pro-inflammatory activity of cells, switching them back to a quiescent state.

1.3.4 Modulation of microglial activation

Under normal physiological conditions, microglial activation is modulated by a number of factors expressed by neurons and other glial cells. This regulation of activation is important to protect the CNS against unnecessary inflammation, as chronic inflammation is associated with most, if not all, neurodegenerative disorders (Lynch, 2014). For example, CD200 and CXCL3, or fractalkine, are proteins expressed by neurons which interact with their receptors on microglia and modulate microglial activity (Cardona et al., 2006, Hoek et al., 2000). Decreased expression of these molecules results in microglial activation and the induction of an inflammatory response and is associated with neurodegeneration (Lyons et al., 2007a, Mizuno et al., 2003).

1.3.5 Activation states and iron handling

Polarisation induces differential iron handling properties in macrophages; the M1 phenotype is associated with increased iron storage and decreased transmembrane iron flux, while the M2 phenotype is associated with increased iron recycling (Cairo et al., 2011). Iron is a crucial growth factor for most

bacteria, and iron sequestration by pro-inflammatory macrophages is viewed as a bactericidal mechanism to starve pathogens of the essential metal (Ganz, 2009). In contrast, M2 macrophages, by transporting iron from the cell, can increase iron concentrations extracellularly, which contributes to tissue repair and wound healing (Corna et al., 2010). Macrophages achieve these differential iron handling profiles by altering the expression of a number of proteins involved in iron transport and sequestration, such as transferrin receptor (TfR), ferroportin, and ferritin.

TfR is a cell membrane protein responsible for transferrin-bound iron influx into the cell (Pearse and Robinson, 1990). The receptor undergoes clathrin-mediated endocytosis following ligation and releases iron into the cytosol before being trafficked back to the cell membrane (Mayle et al., 2012). TfR expression is significantly decreased in IFN- γ -stimulated macrophages (Byrd and Horwitz, 1993), which has been shown to negatively impact on the multiplication of intracellular pathogens such as *Legionella pneumophila* (Byrd and Horwitz, 1989). It has been suggested that this IFN- γ -induced reduction in TfR is mediated by NO production (Kim and Ponka, 2000).

Ferroportin, the only known cellular exporter of non-heme-associated iron (Donovan et al., 2000), is decreased in M1 polarised macrophages (Cairo et al., 2011). This is accompanied by increased hepcidin expression, which is transcriptionally induced by pro-inflammatory stimuli, such as LPS and IL-6 (Kemna et al., 2005, Nemeth et al., 2004). Hepcidin binds to ferroportin and mediates its internalisation and degradation, leading to diminished cellular iron export (Ganz, 2007).

Ferritin chelates and stores iron in a non-toxic form and inhibits bacterial survival within the cell (Gozzelino et al., 2010). Ferritin expression is increased in M1 macrophages (Cairo et al., 2011) and its sequestration of iron can contribute to the inflammatory phenotype by inducing HIF-1 α stabilisation, iNOS expression and TLR4 signalling (Siegert et al., 2015, Weiss et al., 1994, Wang et al., 2009). The retention and sequestration of iron by M1 macrophages,

via regulation of ferroportin-mediated iron efflux and ferritin-associated iron chelation, is important in determining the fate of a number of intracellular bacteria, such as *Salmonella typhimurium* and *Salmonella enterica* (Nairz et al., 2008, Chlosta et al., 2006, Paradkar et al., 2008).

In contrast to M1 macrophages, M2 macrophages are associated with decreased iron retention and increased iron recycling, mediated by increased TfR and ferroportin, and decreased or unaltered ferritin expression (Recalcati et al., 2010, Valletian et al., 2010, Corna et al., 2010, Cairo et al., 2011). Increased TfR is usually accompanied with increased ferritin expression, but this does not occur in M2 macrophages (Recalcati et al., 2010). Instead iron is cleared from the cell by increased ferroportin expression, which is induced at the transcriptional level by M2-polarising stimuli, such as glucocorticoids and IL-4 (Schaer et al., 2008, Recalcati et al., 2010).

Low intracellular iron concentration in M2 macrophages is associated with reduced IL-6 and TNF α secretion, and LPS-induced cytokine expression, consistent with the anti-inflammatory properties of this phenotype (Wang et al., 2008, Wang et al., 2009). Increased iron efflux could contribute to M2 macrophages fulfilling their role in tissue repair, as increased extracellular iron can be endocytosed by adjacent cells and used for a range of purposes such as cell growth and respiration (Cairo et al., 2011).

Although the iron handling properties of polarised macrophages are well defined, this is not the case for microglia. Determining microglial iron handling properties is important, as iron accumulation occurs in the CNS of aged animals, as well as in a number of neuroinflammatory disorders (Rouault, 2013). This is accompanied by increased ferritin expression and data suggest that iron metabolism plays an important role in normal physiological functions of the CNS (Jellinger et al., 1990). It has been suggested that ferritin levels in the cerebrospinal fluid (CSF) predict Alzheimer's disease (AD) outcomes, with increased ferritin expression being strongly associated with AD risk allele *APOE- ϵ 4* (Ayton et al., 2015). However, it is unknown whether defective iron

metabolism is a cause or consequence of neurodegeneration, and must be examined further.

1.3.6 Microglial phagocytosis and antigen presentation

As the primary phagocytes of the CNS, microglia are responsible for the clearance of cellular debris (Neumann et al., 2009), and can engulf material following binding of receptors such as TLRs (Doyle et al., 2004) or TREM2 (Takahashi et al., 2005). Internalised material is digested in lysosomal compartments and any insulting agents can be presented on the cell surface by major histocompatibility complex (MHC) II. MHC II interacts with T cell receptors (TCR) and induces T cell activation (Smith-Garvin et al., 2009). However, the presence of co-stimulatory molecules CD80 and CD86 are also required to bind to CD28 and cytotoxic T lymphocytes antigen 4 respectively on T cells to mediate full activation (Bhatia et al., 2006), and prevent T cell anergy (Schwartz, 2003). The differentiation of T cells is dependent on the activation state of the antigen presenting cell (APC), as the cytokines produced by the APC are considered the third stimulus necessary for inducing T cell activation (Curtsinger et al., 1999).

The expression of MHC II on microglia is associated with the M1 phenotype, as it is upregulated in response to IFN- γ , LPS, and amyloid- β (A β) (Lyons et al., 2007b, McQuillan et al., 2010, Buttini et al., 1996). CD80 and CD86 expression are also increased in inflammatory conditions (Griffin et al., 2006, Hanisch, 2002), suggesting that M1-like microglia are capable of inducing the differentiation and proliferation of T cells.

1.4 Astrocytes

Astrocytes are the most abundant cells in the CNS. These star-shaped macroglia are responsible for a diverse array of functions that maintain homeostasis in the brain. They support neurons both structurally and metabolically, and control ion concentrations necessary for the firing of action potentials (Kimelberg and Nedergaard, 2010). They are also capable of modulating synaptic activity and

are an integral part of the tripartite synapse, where they are involved in uptake of neurotransmitters and iron, thereby maintaining homeostasis, and where they release gliotransmitters such as glutamate and GABA (Newman, 2003). Other functions of astrocytes include pH balance, fluid homeostasis, blood flow regulation, and the secretion of growth factors such as brain-derived neurotrophic factor (BDNF), which is important in the regulation, differentiation and survival of neurons (Dong and Benveniste, 2001). Astrocyte end-feet together with pericytes and endothelial cells form the BBB, the physical barrier surrounds the vasculature in the CNS and limit infiltration of plasma components, red blood cells and leukocytes into the brain.

Although microglia were previously thought to be the sole immune-competent cells of the CNS, there is considerable evidence suggesting that astrocytes also play an important role in the immune response in the brain (Dong and Benveniste, 2001). CNS injury or disease results in increased proliferation and activation of astrocytes, characterised by an upregulation of glial fibrillary acidic protein (GFAP), termed “reactive astrogliosis”. Astrocytes also express a number of TLRs (Ransohoff and Brown, 2012, Carpentier et al., 2005) and secrete cytokines and chemokines in response to TLR stimulation, but also in response to other stimuli like IFN- γ (Farina et al., 2007, Ransohoff and Brown, 2012). Astrocytes have been shown to possess phagocytic capabilities and express a number of receptors such as receptor for advanced glycation end products (RAGE), CD36 and CD47, implicated in the phagocytosis of A β (Jones et al., 2013).

A recent study has suggested that astrocytes can be polarised to M1-like and M2-like phenotypes, with evidence of an upregulation of TNF α , iNOS and IL-1 β in astrocytes in response to stimulation with a combination of LPS and IFN- γ *in vitro*, and upregulation of Arg-1, MRC1, Chi3L3 and FIZZ1 in response to a combination of IL-4 and IL-13 (Jang et al., 2013).

1.5 Metabolic signature of immune cells

1.5.1 Overview of metabolic pathways

Cells of the CNS derive their energy like other cells, from glycolysis, the tricarboxylic acid (TCA) cycle, β -oxidation of fatty acids (FA), and oxidative phosphorylation (Figure 1.2).

Glycolysis involves the metabolism of glucose, generates 2 molecules of ATP per molecule of glucose and yields pyruvate molecules which can then translocate into mitochondria to be converted to acetyl coenzyme A (acetyl CoA) which is metabolised to CO_2 in the TCA cycle. The TCA cycle generates NADH and FADH_2 which fuel oxidative phosphorylation by the electron transport chain, and generate up to 36 molecules of ATP per molecule of glucose. β -oxidation of fatty acids can also provide acetyl CoA for the TCA cycle and oxidative phosphorylation.

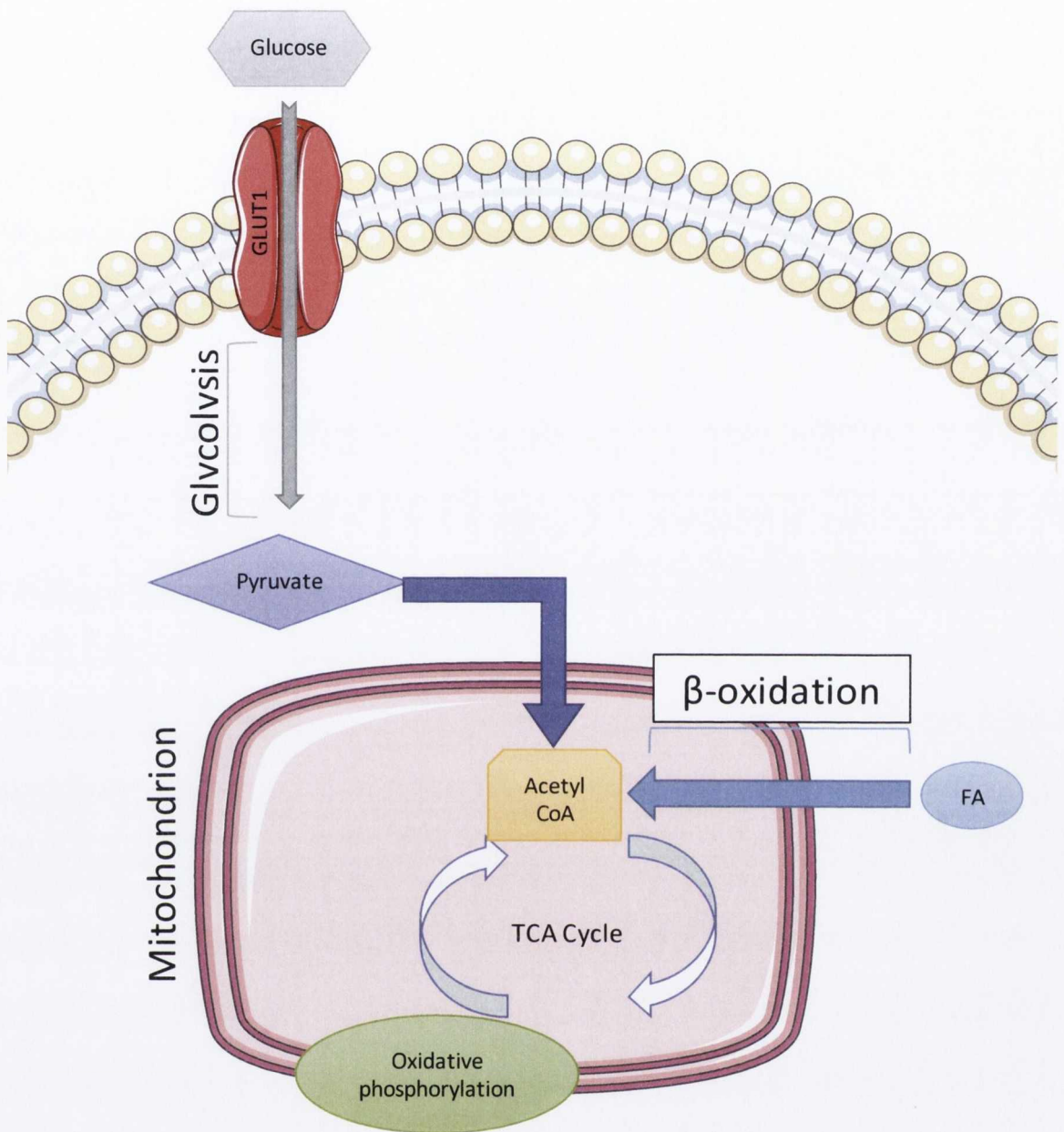


Figure 1.2 Overview of metabolic pathways. Glucose enters the cytosol through glucose transporter 1 (GLUT1) and glycolysis converts it to pyruvate. Acetyl CoA is produced from pyruvate or β -oxidation of fatty acids (FA) and feeds the TCA cycle in the mitochondria. This produces NADH and FADH₂, which fuel oxidative phosphorylation and the electron transport chain.

1.5.2 HIF-1 α induced metabolic reprogramming

Under hypoxic conditions, cells undergo a shift in metabolism whereby pyruvate generated in glycolysis is fermented to lactate, reducing oxidative metabolism, and in this case, the primary source of ATP becomes glycolysis. The molecular mechanism mediating this metabolic switch is the switch of hypoxia-inducible factor 1 α (HIF-1 α). HIF-1 α is a heterodimeric transcription factor consisting of a constitutively expressed β subunit and an inducible α subunit. The α subunit undergoes rapid proteasomal degradation in the presence of oxygen due to prolyl 4-hydroxylase-dependant modifications that target it for destruction by E3 ubiquitin ligases (Semenza, 2007). However, prolyl hydroxylase activity is severely inhibited in hypoxic conditions, resulting in the accumulation of HIF-1 α and the formation of the functionally active HIF-1 dimer (Bruick and McKnight, 2001). The induction of HIF-1 activity enables cells to survive in the absence of oxygen by discarding the oxidative respiratory pathways and prioritising the oxygen-independent pathway of glycolysis. HIF-1 α expression is associated with the upregulation of a number of proteins associated with mediating the metabolic switch, such as 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB) 3, glucose transporter (GLUT) 1 and pyruvate dehydrogenase kinase (PDK) 1 (Iyer et al., 1998, Kim et al., 2006b, Minchenko et al., 2003) (Figure 1.3).

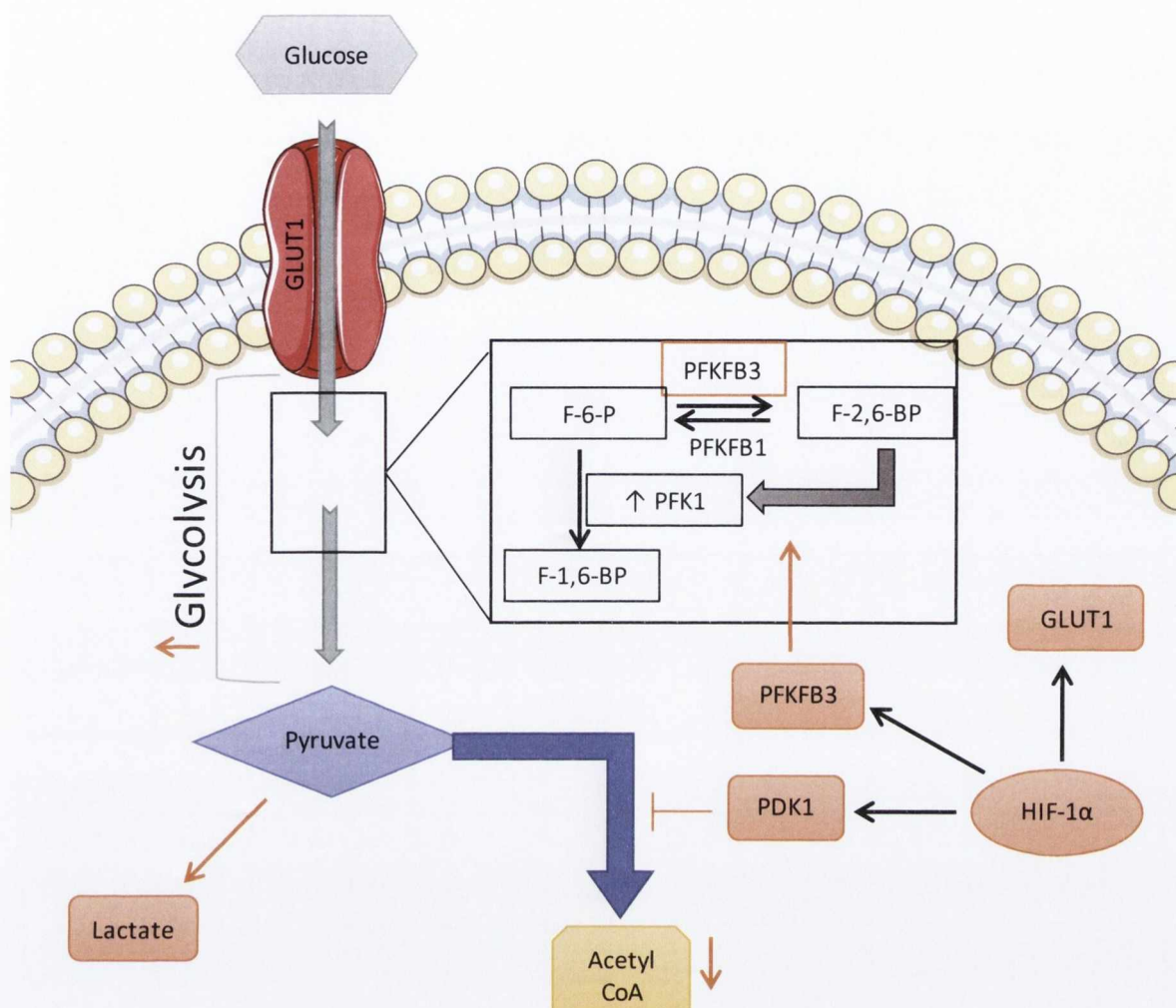


Figure 1.3 HIF-1 α induced metabolic reprogramming. HIF-1 α increases the expression of glucose transporter 1 (GLUT1), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), and pyruvate dehydrogenase 1 (PDK1). GLUT1 increases glucose uptake, while PFKFB3 increases PFK1 activity via increased fructose 2, 6-bisphosphate (F-2, 6-BP) production. PDK1 inhibits the conversion of pyruvate to acetyl CoA, resulting in the production of lactate instead.

The enzymes PFKFB1 and PFKFB3 are members of the PFKFB family of bifunctional isozymes that are central to facilitating increased glycolysis. They contain both a phosphatase and a kinase domain and catalyse the same reactions at different rates. Using fructose-6-phosphate as substrate, the kinase domain of the enzymes generates fructose-2, 6-bisphosphate, which

allosterically activates 6-phosphofructo-1 kinase (PFK1) (Pilkis et al., 1981). PFK1 converts fructose-6-phosphate to fructose-1, 6-bisphosphatate, which is the rate limiting step of the glycolytic pathway. The phosphatase domain reverses this reaction, slowing glycolysis, and the balance depends on concentrations of substrate in the cytosol (Lee et al., 2003).

The PFKFB1 kinase:phosphatase activity ratio is approximately 1:1 (REF), whereas the phosphatase domain of PFKFB3 is functionally impaired due to a mutation in a phosphatase active site resulting in a kinase:phosphatase activity ratio of approximately 740:1 (Sakakibara et al., 1997). Therefore PFKFB3 drives glycolysis by increasing PFK1 activity and is upregulated under hypoxic conditions by HIF-1 α (Bobarykina et al., 2006, Minchenko et al., 2002). Interestingly, PFKFB3 is the first metabolic enzyme to be identified with multiple AUUUA instability motifs in its mRNA transcripts (Chesney et al., 1999). These AU-enriched regions are usually observed in genes encoding for inflammatory mediators or other potentially damaging proteins such as TNF α , IL-1 β and IFN- γ to protect against aberrant expression (Stumpo et al., 2010). This suggests that the induction of the enzyme is tightly regulated due the potential negative impact its activity may have on cell survival.

This metabolic switch to glycolysis can occur in the presence of oxygen, a phenomenon termed “aerobic glycolysis”. This was first observed in 1924 by Otto Warburg in tumour cells and is described as the Warburg effect (Warburg, 1924, Warburg, 1956). Aerobic glycolysis also occurs during proliferation; mouse lymphocytes (Wang et al., 1976), rat thymocytes (Brand, 1985) and mitogen-stimulated human lymphocytes (Hedekov, 1968) all demonstrate aerobic glycolysis, mediated by PFKFB3 upregulation (Yalcin et al., 2014). Although less efficient than oxidative metabolism in terms of ATP generated per molecule of glucose, it has been suggested that the rapid nature of ATP production through glycolysis provides for high-energy processes such as cell proliferation, provided there is an abundant supply of glucose (Pfeiffer et al., 2001).

1.5.3 Metabolic reprogramming of T cells

Metabolic reprogramming of immune cells is a recent development in our understanding of the immune response. Immunometabolism refers to the metabolic reprogramming that occurs in activated immune cells that is intrinsically linked to their functional state. As with proliferating cells, immune cell activation requires robust and rapid generation of ATP to meet increased energy demands.

Activation and differentiation of T cells is associated with metabolic reprogramming (Buck et al., 2015). Effector CD4⁺ T cells, such as T_h1, T_h2, and T_h17 cells exhibit a robustly glycolytic metabolic phenotype (Michalek et al., 2011). The mammalian target of rapamycin (mTOR) complex (mTORC) is a serine/threonine kinase involved in controlling metabolism in T cells and is considered central to the differentiation of effector T cells, as genetic deletion of mTORC1 mitigates differentiation of these subsets (Delgoffe et al., 2011, Delgoffe et al., 2009). The relationship between metabolic profile and function in T cells is highlighted by reports demonstrating that inhibition of glycolysis by addition of the competitive antagonist 2-DG profoundly attenuates IFN- γ secretion from T_h1 cells (Zheng et al., 2009), and T cell activity can be modulated by targeting metabolism. For example, the addition of exogenous FA to cultures suppresses T_h1 and T_h17 development (Michalek et al., 2011), and peroxisome proliferator-activated receptor (PPAR) γ activation, which increases FA oxidation, significantly decreases IL-2 and IFN- γ secretion from CD4⁺ T cells.

In contrast to the glycolytic profile of effector T cells, T_{reg} and memory T cells derive their energy from FA oxidation and oxidative phosphorylation (Waickman and Powell, 2012, Michalek et al., 2011). Treatment of CD4⁺ T cells with 2-DG enhances the development of T_{reg} cells, while inhibition of FA oxidation antagonises their development (Michalek et al., 2011). The evidence suggests that effector T cells increase aerobic glycolysis to rapidly produce ATP that is required for immediate action, while the prolonged life of T_{reg} or memory cells is associated with the sustainable generation of ATP by oxidative means.

1.5.4 Metabolic profiles of polarised macrophages

Macrophage activation is also accompanied by metabolic reprogramming, with differentially polarised cells adopting different metabolic profiles. Pro-inflammatory M1 cells are associated with increased aerobic glycolysis and impaired oxidative metabolism, which is tightly linked to HIF-1 α expression (Galvan-Pena and O'Neill, 2014). However, although HIF-1 α does induce PFKFB3 and iNOS expression associated with the M1 phenotype, these changes can also occur independent of HIF-1 α activity (Rodriguez-Prados et al., 2010, Takeda et al., 2010, Peyssonnaud et al., 2005).

The metabolic reprogramming of M1 macrophages not only facilitates the rapid production of ATP necessary for immune functions, but also provides cells with pro-inflammatory mediators. For example, attenuation of the electron transport chain results in the production of reactive oxygen species (ROS) for bactericidal activity (Galvan-Pena and O'Neill, 2014), and inhibition of the TCA cycle leads to the accumulation of intermediates such as succinate and citrate, which have pro-inflammatory effects; succinate enhances the production of IL-1 β in a HIF-1 α -dependent manner, which can be attenuated by 2-DG inhibition of glycolysis, and citrate induces NO and PG production (Tannahill et al., 2013, O'Neill, 2011, Ricciotti and FitzGerald, 2011).

In contrast, the M2 phenotype is associated with increased FA oxidation and oxidative respiration (Figure 1.4), similar to T_{reg} cells (Mills and O'Neill, 2016). It has been suggested that M1 macrophages utilise glycolysis to generate the significant quantities of ATP required to respond to immediate threats against the host, whereas M2 macrophages are involved in longer term activities, such as fighting parasitic infections and tissue repair, which require sustained production of ATP. PPAR γ -coactivator-1 β (PGC-1 β) is considered the key player responsible for the metabolic switch in M2 macrophages and is increased in a STAT6-dependent manner in response to IL-4 (Wu et al., 1999). PGC-1 β increases mitochondrial respiration and mitochondrial biogenesis to increase oxidative respiration and ATP production (St-Pierre et al., 2003, Shao et al.,

2010). However, some reports suggest that IL-4-induced M2 activation can occur in the absence of PGC-1 β upregulation (Namgaladze and Brune, 2014). M2 macrophages have high adenosine monophosphate-activated protein kinase (AMPK) activity (Sag et al., 2008), which also induces oxidative phosphorylation and FA oxidation (O'Neill and Hardie, 2013) and can increase the expression of PGC-1 β .

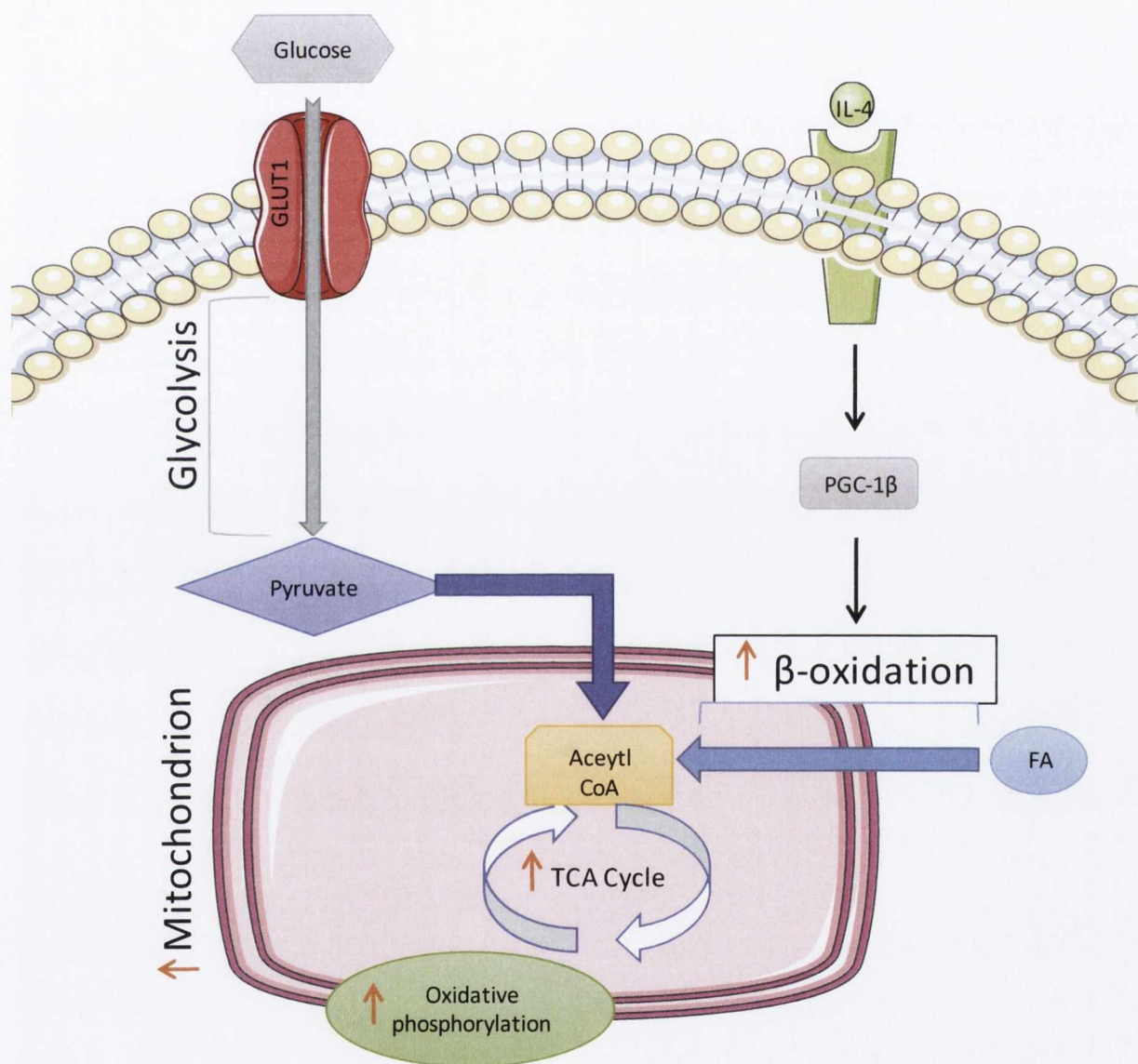


Figure 1.4 Metabolic reprogramming of M2 macrophages. IL-4 induces the expression of PPAR γ coactivator (PGC)-1 β , which increases oxidative metabolism via increased mitochondrial biogenesis and increased oxidation of fatty acids (FA).

It has been shown that targeting metabolic components can influence macrophage activation state. For example, enhancing PGC-1 β -mediated oxidative metabolism in M1 macrophages potentiates an M2 phenotype by attenuating IL-12 and IL-6 secretion and increasing Arg-1 activity (Vats et al., 2006). Similarly, PFKFB3 siRNA attenuates iNOS and COX2 expression in M1 macrophages (Rodriguez-Prados et al., 2010) and treatment of macrophages with pyruvate kinase M2 activators DASA-58 and TEPP-46, and the AMPK activator AICAR, have also been shown to mediate a phenotype switch in macrophages to an anti-inflammatory state (Palsson-McDermott et al., 2015, Mounier et al., 2013). Furthermore genetic manipulation of AMPK results in increased secretion of pro-inflammatory cytokines TNF α , IL-1 β and IL-6 from macrophages, as well as decreased oxidative metabolism (Carroll et al., 2013, Galic et al., 2011). These data demonstrate the connection between metabolic profile and macrophage phenotype.

The advent of recent technologies such as the SeaHorse flux analyser allows measurement of the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) in cell cultures, providing a measure of glycolysis and oxidative metabolism respectively. LPS stimulation has been shown to increase ECAR and decrease OCR in bone marrow-derived macrophages (BMDMs), as well as in RAW264.7 and J774.1A macrophage cell lines (Menon et al., 2015, Haschemi et al., 2012b, Nemeth et al., 2016).

1.5.5 Metabolic reprogramming in microglia

The study of metabolic changes in microglia is in its earliest stages. However, LPS and IFN- γ -induced M1 polarisation of the immortalised BV2 microglial cell line is accompanied by increased glucose uptake and expression of glycolytic enzymes, suggesting this phenotype is supported by enhanced glycolytic activity (Gimeno-Bayón et al., 2014). Specifically, LPS increases ECAR and decreases OCR in BV2 cells (Voloboueva et al., 2013, Orihuela et al., 2016), consistent with a metabolic switch in M1 microglia. Orihuela and colleagues (2016) demonstrated that these modifications also occur in primary microglial cultures,

although the LPS-induced decrease in OCR was not consistently observed. A combination of IL-4 and IL-13 was used to induce the M2 phenotype, but it did not significantly affect OCR in BV2 cells, nor in primary microglial cultures (Orihuela et al., 2016). Although cell lines are less expensive and grow more rapidly than primary cell cultures, BV2 cells are not always representative of primary microglia, especially in terms of their inflammatory response (Stansley et al., 2012).

1.6 Neuroinflammation & AD

Acute activation of microglia to the M1 phenotype is an important immunological process but aberrant or chronic activation is associated with inflammation in the CNS and leads to neuronal cell death. Indeed, chronic inflammation is associated with most, if not all, neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Lynch, 2014, Wang et al., 2015). TNF α has been shown to play a critical role in the pathogenesis of chronic inflammatory diseases, such as AD (Perry et al., 2001). Chronic expression of iNOS can also have tissue-damaging effects, as NO can cause necrosis and apoptosis of parenchymal cells in the CNS (Lucas et al., 2006). IL-1 β can also trigger neuronal cell death and it is associated with a wide range of inflammatory diseases (Church et al., 2008). Increased caspase-1 activity and IL-1 β expression has been observed in aged animals (Murray and Lynch, 1998) and are associated with loss of neuronal function.

2.2.2 Removal of brain tissue

The animal was placed on its abdomen and the mouse was decapitated with one clean incision. The skin was removed and the skull was cut along the medial longitudinal fissure and carefully pulled away on each side using a forceps. The brain was gently removed from the cranial cavity anterior to the olfactory bulbs and was placed on top of a Petri dish (Sarstedt, Ireland) containing ice. The cerebellum was removed using the curved side of the forceps and the brain was dissected along the medial longitudinal fissure using a scalpel (Schwann-Mann,

UK). The left hemisphere of the brain was placed with the medial side facing up. The tissue covering the hippocampus was removed using a pair of forceps and the hippocampus was gently rolled out from the cortex. The hippocampus was divided in two, and one half was placed in a labelled 1.5mL Eppendorf tube (Sarstedt, Ireland) and flash frozen in liquid N₂.

1.6.1 Alzheimer's disease

AD is an age-related, chronic neurodegenerative disease that affects 36.5 million people worldwide, and with an ageing global population, is considered one of the largest medical challenges for future generations (Ittner and Gotz, 2011). Patients initially present with short-term memory loss, before the disease progresses over time and neuronal degeneration inflicts severe functional failures, leading to cognitive impairment and eventually death (Bekris et al., 2010). AD is characterised by substantial neuronal and synaptic loss, particularly in the hippocampus, cerebral cortex, and basal forebrain, which are areas associated with memory and learning (Nagele et al., 2004). This is accompanied by deposition of senile plaques in the extracellular matrix comprised of amalgamated A β peptides and intracellular neurofibrillary tau tangles in neurons comprised of hyperphosphorylated tau. Approximately 5% of cases are genetic, termed familial AD (FAD) but the underlying cause or causes late-onset sporadic AD have not yet been elucidated.

1.6.2 A β and the amyloid hypothesis

The deposition of A β plaques was first described 1906 by Alois Alzheimer upon post-mortem examination of AD brain. A β is a small, 4kDa peptide of 37-43 amino acids in length that is produced upon the cleavage of amyloid precursor protein (APP). APP is a type I transmembrane glycoprotein that is highly expressed at neuronal synapses. Its expression is upregulated during neuronal differentiation and after neuronal injury suggesting a role in synapse formation and neuronal plasticity (Priller et al., 2006). Proteolytic cleavage of APP can occur in two ways, the non-amyloidogenic pathway or the amyloidogenic pathway. The non-amyloidogenic pathway involves the cleavage of APP by α -

secretase and γ -secretase, resulting in the production of non-pathogenic sAPP α , P3 and the amyloid precursor protein intracellular domain (AICD). In contrast, the amyloidogenic pathway is mediated by β -secretase (also known as BACE1) cleavage of APP, rather than α -secretase, and the cleavage of the resulting intracellular peptide by γ -secretase. This results in the production of sAPP β , AICD and A β peptides. A β peptides can vary in length between 37 and 43 amino acids in length because γ -secretase can cleave the membrane bound APP fragment at number of sites (Newman et al., 2007). These peptides form dimer, trimers and oligomers and eventually aggregate to form insoluble fibrils arranged as β -pleated sheets which constitute the senile plaques found in the AD brain (Querfurth and LaFerla, 2010, Nag et al., 2011).

The amyloid hypothesis states that increased production and/or decreased clearance of A β leads to aggregates that chronically activate glia and induce an unsurmountable inflammatory response that leads to neurotoxicity, cell death and cognitive decline (Karran et al., 2011).

1.6.3 Neuroinflammation & AD

Like most, if not all, neurodegenerative diseases, chronic inflammation has been associated with disease progression and neuronal loss in both FAD and sporadic AD (McGeer and McGeer, 2003, Heneka et al., 2015). It has been proposed that this inflammation is not just a consequence of the disease, but that it contributes to the pathophysiology of disease progression (Zhang et al., 2013). Evidence for this comes from studies which demonstrate that treatment with non-steroidal anti-inflammatory drugs lowers the incidence of AD, and decreases microglial activation in animal models of AD (Bamberger and Landreth, 2001).

Activated microglia and hypertrophic reactive astrocytes accumulate around A β deposits in the brains of AD patients (Medeiros and LaFerla, 2013, McGeer et al., 1993) and in mouse models of AD (Bornemann et al., 2001, Olabarria et al., 2010). A β is known to induce a pro-inflammatory microglial phenotype; A β increases microglial expression of pro-inflammatory cytokines, chemokines and APC molecules *in vitro* (Lue et al., 2001, Smits et al., 2002, McQuillan et al.,

2010) and *in vivo* (Clarke et al., 2007, Ishizuka et al., 1997, Vodovotz et al., 1996). A number of receptors have been associated with binding A β and mediating these effects, such as TLR2, TLR4, RAGE, CD47, CD36 and SRA (Jana et al., 2008, Bamberger et al., 2003, Reed-Geaghan et al., 2009).

Microglia phagocytose monomeric, oligomeric and fibrillary A β *in vitro* (Lee and Landreth, 2010) and contain intracellular A β fragments *in vivo* (Lewandowska et al., 2004). Under normal conditions, microglia also phagocytose and degrade potentially insulting agents, such as cell debris and apoptotic cells, and return to a quiescent state. However, in AD it has been suggested that receptor-mediated phagocytosis of A β is not followed by adequate degradation, due to lysosomal acidity levels being too low (Majumdar et al., 2007). The failure to effectively remove A β from the CNS leads to chronic activation of microglia and the persistent release of pro-inflammatory mediators that negatively impact neuronal health (El Khoury and Luster, 2008). Enhancing microglial phagocytosis of A β has been achieved by administration of anti-A β antibodies and LPS stimulation and is associated with increased A β clearance in transgenic mice (Wilcock et al., 2004, Herber et al., 2007). In AD, the persistence of A β and the chronic inflammatory environment has been shown to negatively affect neuronal function and lead to cell death associated with the disease (Palop and Mucke, 2010, Kadowaki et al., 2005).

Age is the largest risk factor associated with AD, with incidence doubling every 5 years in the over 65 population, reaching a prevalence greater than 25% in people over the age of 90 (Qiu et al., 2009). Microglia from the aged brain are more responsive to pro-inflammatory stimuli (Perry and Teeling, 2013, Jones et al., 2015) and it has been suggested that age-related decreases in neuronal-glial interactions can contribute to this microglial priming (Biber et al., 2007), such as the CD200-CD200R interaction which is decreased in aged mice (Lyons et al., 2007a). Microarray analysis of tissue from young and aged mice have revealed that the aged brain is associated with an increased inflammatory profile, demonstrated by increased expression of microglial activation markers, pro-

inflammatory cytokines, chemokines and co-stimulatory molecules, such as CD40, CD68, TNF α , IL-1 β , MHC II, CD80 and CD86 (Prolla, 2002b, Lee et al., 2000, Lynch et al., 2010, Lynch, 2009). These factors may contribute to the development of a chronically inflamed CNS and pave the way for progression of AD pathology.

1.6.4 APP/PS1 mouse model of AD

Murine models of AD have been used in an attempt to elucidate the molecular mechanisms underlying the disease and they serve as tools through which therapeutic interventions can be trialled. Animal models have been generated through manipulation of genes associated with FAD. These genes include APP, presenilin (PS) 1, and PS2. A double mutation in APP was first identified in a Swedish family (APP^{swe}) and is associated with enhanced β -secretase cleavage of APP, leading to an 8-fold increase in A β production *in vitro* (Haass et al., 1995, Citron et al., 1992). PS1 and PS2 are catalytic subunits of γ -secretase, and mutations in these genes leads to accelerated production of the pathogenic A β ₁₋₄₂ subtype (Higgins and Jacobsen, 2003, Duff et al., 1996). The APP^{swe}/PS1dE9 mouse model, used in this study, possesses a transgene encoding a chimeric APP with a double K595N M596L mutation (the Swedish mutations), and another encoding PS1 with a DeltaE9 mutation. These mice develop large numbers of A β plaques, which are evident in the cortex and hippocampus at 6 months of age and suffer progressive memory loss (Garcia-Alloza et al., 2006, Cao et al., 2007). However, neuronal loss is not prevalent in the APP/PS1 mouse model, although synaptic loss and cognitive impairment do occur (Viana da Silva et al., 2016).

Other models of AD which target the expression of APP include the platelet-derived growth factor-promoter driving a human APP770 minigene with a V717F mutation (PDAPP), which was the first model of AD displaying enhanced A β deposition along with memory loss and gliosis (Games et al., 1995), and the Tg2576 mouse, which was generated through expression of human APP695 with a K670N M671L double mutation (Hsiao et al., 1996). A β deposition in these models are not accompanied by the neurofibrillary tangles associated with

hyperphosphorylated tau seen in AD, but transgenic mice that possess both tau tangles and A β plaques are paralyzed and thus unsuitable for research purposes (Ashe, 2001). The APPSwDI/NOS2(-/-) mouse model is a more recent development, which has been shown to display A β pathology as well as hyperphosphorylated and aggregated tau, which is accompanied by neuronal loss and cognitive impairment (Wilcock et al., 2008).

1.7 Aims

The primary of this study were:

- To characterise microglial activation in terms of metabolic profile and iron handling properties and identify novel markers of activation.
- To examine the functional consequences of microglial activation in terms of phagocytic, antigen presentation, and secretory capabilities.
- To investigate the metabolic profile and iron handling properties of microglia isolated from the brains of APP/PS1 and aged mice.

Chapter 2 Methods

2.1 Animals

Animals were maintained under veterinary care in a specific pathogen free (SPF) environment at 22-23°C. A 12h light/dark cycle was employed with lab chow and water available *ab libitum*. Experiments were carried out under licence from the Irish Medicine Board under the Cruelty to Animals Act 1876, the European Community Directive, 86/609/EEC, and in agreement with the experimental procedures of Trinity College Research Ethics Committee.

C57/BL6 mice from the Comparative Medicine Unit, Trinity College Dublin were used for *in vitro* experiments.

Male and female WT and APP/PS1 mice were used in these studies. APP/PS1 mice contain 2 transgenes inserted at a single locus: a chimeric APP and a deltaE mutation of human PS1. Mice were initially purchased from Jackson Laboratory (USA) and bred with in house C57/BL6 mice. Mice were sacrificed by decapitation while under anaesthesia induced by urethane (1.5g/kg; Sigma Aldrich; UK).

2.2 Sacrifice and tissue harvesting

2.2.1 Intracardial saline perfusion

Groups of young (9 months; n=15) and aged (15-17 months; n=14) wildtype and APP/PS1 mice were used for this study. Animals were anaesthetized by intraperitoneal injection of urethane (1.5g/kg; Sigma-Aldrich, UK) and full anaesthesia was confirmed by the absence of a pedal reflex. Mice were placed on their backs on a styrofoam board with their limbs pinned down. The abdomen was sprayed with 70% ethanol and, using a forceps and scissors, the abdomen skin was tented and an incision was made. The diaphragm was disconnected and 2 vertical cuts along the ribcage enabled it to be pinned back, allowing access to the thoracic cavity. The right atrium was snipped and a 23 gauge needle (BD Microlance, USA) on a 20mL syringe (BD Plastipak, USA) filled with ice cold phosphate-buffered saline (PBS; Appendix I) was inserted into the left

ventricle. The PBS was slowly passed through the vasculature, with the liver colour being indicative of successful perfusion. Spleens were removed from young WT mice and placed in warm complete Roswell Park Memorial Institute media (cRPMI; Appendix I) for isolation of CD4⁺ T cells as described in section 2.3.8. Legs were removed, sprayed liberally with ethanol, and transferred to a laminar flow cell culture hood (Advanced Biosafety Cabinet Class II; AGB Scientific Ltd., Ireland) for the isolation of bone marrow-derived macrophages (BMDMs) as described in section 2.3.6.

2.2.3 *Ex vivo* cell isolation

Prior to separation procedure, a stock isotonic Percoll (SIP) solution was prepared by mixing 9 volumes of Percoll (Sigma Aldrich, UK) with 1 volume of 10X Hank's balanced salt solution (HBSS; Gibco, UK). Two ice cold Percoll solutions (75% and 25%) were prepared from SIP using PBS.

Brain tissue was cross-chopped in medium A (Appendix I) in the lid of a Petri dish and placed in a glass hand held homogeniser, where it was mechanically dissociated. The resulting cell suspension was passed through a 70µm filter (Becton Dickinson Labware, France) into a 50mL Falcon tube (Sarstedt, Ireland), brought to 25mL with medium A and centrifuged (200g; 10min; 4°C, no brake; Sovall Legend RT). The resulting pellet was resuspended in 75% Percoll solution (10mL) and carefully overlaid with 25% Percoll solution (10mL) and PBS (6mL). The gradient was then centrifuged (800g; 30min; 4°C, no brake), resulting in the separation of the myelin and mononuclear cells (Figure 2.1). The myelin from the 0-25% interface was discarded using a Pasteur pipette (Sarstedt, Ireland) and the enriched microglial population was removed from the 25-75% interface and added to new 50mL tubes. Microglia were washed with PBS (30mL), centrifuged (200g; 10min; 4°C, no brake) and the supernatant was collected. The pellet was resuspended in SeaHorse mitochondrial stress test assay media (200µL; Appendix I) and the metabolic profile of the isolated microglia was assessed as described in section 2.6.2. The supernatant was centrifuged (800g; 10min; 4°C) and the resulting pellet was resuspended in RA1

buffer (Macherey-Nagel, Germany) and β -mercaptoethanol (Sigma-Aldrich, UK; 100:1; 350 μ L) and assessed for mRNA expression by RT-PCR as described in section 2.9.

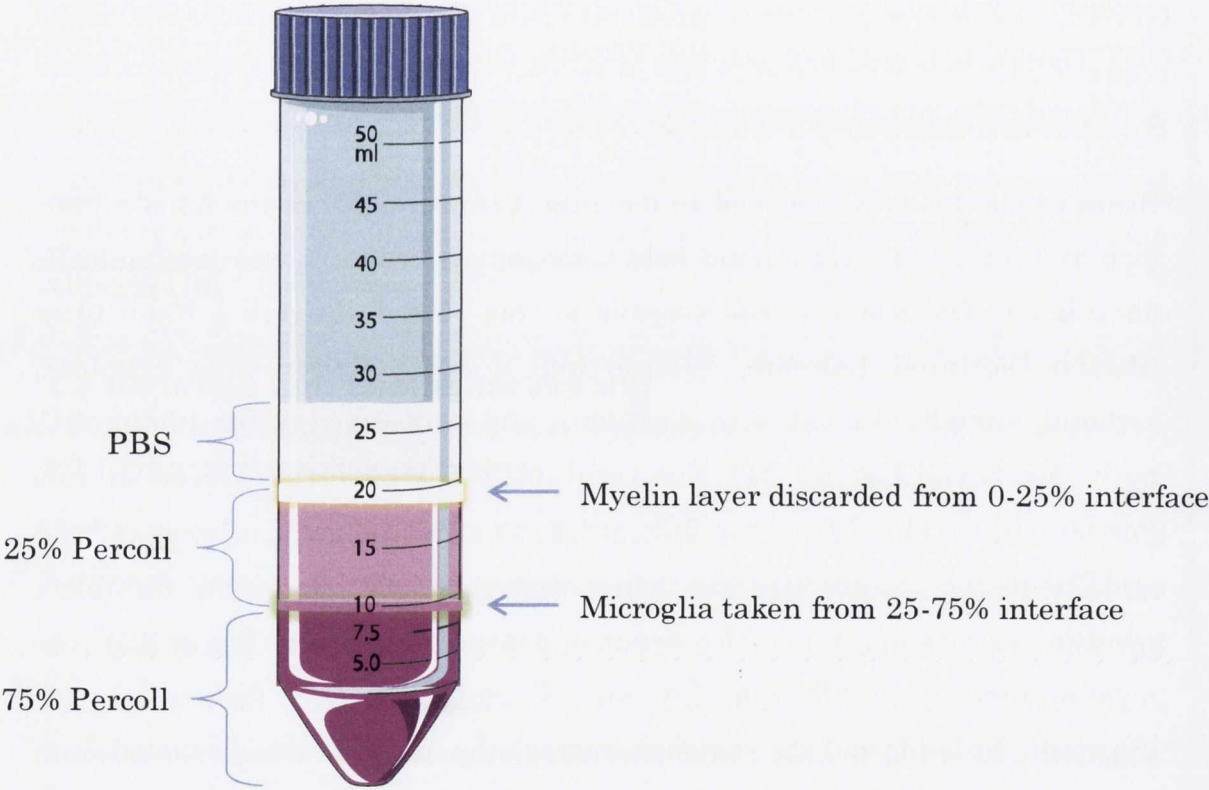


Figure 2.1 Isolation of cells by Percoll gradient. Microglia were extracted from the interface between the 25% and 75% Percoll solutions.

2.3 Cell culture

2.3.1 Aseptic techniques

All cell culture work was carried out in a laminar flow cell culture hood. The cell culture hood was sprayed liberally with 70% ethanol before and after use and was exposed to ultraviolet light overnight. All materials used were sterile and doused in 70% ethanol prior to entering the cell culture hood. Disposable latex powder-free gloves were worn at all times and sprayed with 70% ethanol. Dissection instruments were sterilised by thorough cleaning with Virkon and baked overnight in an oven at 200°C following use.

2.3.2 Preparation of primary cortical microglia

Primary cortical glial cells were procured from P0 neonatal C57BL/6 mouse pups (Comparative Medicine Unit, Trinity College, Dublin, Ireland). The mice were decapitated using a sterile scissors, the cortices were dissected free and the meninges removed. The cortices were cross-chopped using a sterile scalpel in the lid of a sterile Petri dish containing a few drops of warm cDMEM (Appendix I). Tissue was placed in 15mL Falcon tubes and was titrated using a sterile plastic Pasteur pipette. The suspension was passed through a sterile 40µm nylon mesh (BD Plastipak, USA) into 50mL Falcon tubes and centrifuged (1200rpm, 5min, 20°C). The supernatant was discarded and the pellet was resuspended in warm cDMEM (1mL per brain). The resuspended mixed glia were added to labelled T25 flasks (1mL/flask; Fisher Scientific, UK) and incubated for 2h at 37°C before the addition of warm cDMEM to each flask (8mL).

For primary microglial cultures, cDMEM, supplemented with macrophage colony stimulating factor (M-CSF; 100ng/mL; R&D Systems, UK, Appendix I) and granulocyte macrophage colony stimulating factor (GM-CSF; 100ng/mL; R&D Systems, UK, Appendix I), was added to each flask (1mL/flask) the following day and cells were grown at 37°C in a humidified 5% CO₂; 95% air environment for 10-12 days. At this point, the neck and cap of each flask was wrapped with parafilm (Sigma-Aldrich, UK) to make them air-tight and they

were placed on an orbital shaker for 2h (110rpm, 20°C; Labnet International). The flasks were returned to the sterile flow hood and tapped 10 times to separate the non-adherent microglia, which were decanted into 50mL Falcon tubes which were centrifuged (1200rpm, 5min, 20°C). The supernatant was removed and the resulting microglial pellet was resuspended in cDMEM (1mL). Viable microglial cells were counted under a light microscope (Olympus 1X51, Olympus Ltd, UK) using a haemocytometer (Hycor Biomedical, UK), equalised to a density of 12×10^5 cells/mL with cDMEM and seeded (250 μ L/well) in labelled 6-well plates (Fisher Scientific; UK) at a density of 3×10^5 cells/well. For immunocytochemistry, cells were seeded (30 μ L) onto poly-D-lysine coated coverslips (Appendix I) in 24-well plates at a density of 3×10^4 cells/coverslip. Plates were incubated for 2h at 37°C before wells were topped up with warm cDMEM (750 μ L for 6-well; 270 μ L for 24-well). Microglia were grown at 37°C in a humidified 5% CO₂; 95% air environment and treated 2 days after seeding.

2.3.3 Preparation of primary cortical astrocytes

For primary astrocytic cultures, cells were grown at 37°C in a humidified 5% CO₂; 95% air environment for 10-12 days in the absence of growth factors. Flasks were shaken for 1h as described and microglia were removed. The astrocytes which remained adhered to the surface of the flasks were rinsed with sterile PBS (5mL; Gibco, UK) and incubated for 5min at 37°C with trypsin-ethylenediaminetetraacetic acid (EDTA; 3mL per flask; Gibco, UK). Trypsin activity was halted by adding warm cDMEM (5mL) to each flask and a cell scraper (Sarstedt, Ireland) was used to ensure all cells were collected. The astrocytic cell suspension was transferred into 50mL Falcon tubes and centrifuged (1200rpm, 5min, 20°C). The supernatant was discarded and the pellet was resuspended in DMEM (1mL). Viable astrocytes were counted under a light microscope using a haemocytometer, equalised to a density of 12×10^5 cells/mL with DMEM and seeded (250 μ L/well) in labelled 6-well culture plates at a density of 3×10^5 cells/well. For immunocytochemistry, cells were seeded (30 μ L) onto poly-D-lysine coated coverslips in 24-well plates at a density of 3×10^4 cells/coverslip. Plates were incubated for 2h at 37°C before wells were

topped up with warm cDMEM (750 μ L for 6-well; 300 μ L for 24-well). Astrocytes were grown at 37°C in a humidified 5% CO₂; 95% air environment and treated 2 days after seeding.

2.3.4 Preparation of mixed glial cultures

Primary mixed glial cultures were prepared in the same way as microglia in section 2.3.2, except cells were cultured in 6-well plates in the absence of growth factors and media was changed every 4 days.

2.3.5 Maintenance of L929 cell line

L929 cells were kindly donated by Professor Luke O'Neill. L929 cells are a murine fibroblast cell line used as a source of M-CSF for the culture of BMDMs. Cells were grown in cDMEM in T175cm² flasks (Fisher Scientific, UK) until confluent and harvested using heat-activated trypsin-EDTA. The cells were centrifuged (850g; 4min) and the resulting pellet was resuspended in cDMEM. Cells were counted and re-seeded in T175cm² flasks (40mL) at a density of 0.5x10⁶cells/mL. L929 cells were grown at 37°C in a humidified 5% CO₂; 95% air environment for 7 days, at which time the L929 conditioned media was removed and cells were split once more. To ensure no L929 cells remained in the L929 conditioned media, samples were centrifuged (850g; 4min) and the top 80% of supernatant was collected and filter sterilised using a cellulose acetate membrane filter.

2.3.6 Isolation and culture of murine BMDMs

BMDMs were generated from the femurs and tibiae of mice. The legs were dissected and the muscle removed from the bones using a scalpel. In a laminar flow hood, bones were cut at either end and bone marrow was flushed with cDMEM using a 20mL syringe and a 23 gauge needle and collected into a 50mL Falcon tube. Cells were titrated using a Pasteur pipette and passed through a 40 μ m nylon mesh filter. The cell suspension was centrifuged (1250rpm; 5min) and the resulting pellet resuspended in sterile red blood cell (RBC) lysis buffer (1.5mL; Appendix I). Cells were incubated for 3min at room temperature,

centrifuged (1250rpm; 5min) and the pellet resuspended in freezing buffer (4mL; Appendix I). This was split across 4 cryovials (Nalgene, USA) and placed in a Mr.Frosty™ freezing container (Thermo Scientific, USA) at -80°C for 24h before being transferred to liquid nitrogen storage. When required, cells were thawed rapidly, diluted in cDMEM (1:10) and centrifuged (1250rpm; 5min). The cells were resuspended in cDMEM supplemented with 20% L929 conditioned media and seeded in 6-well plates at a density of 2.5×10^5 cells/mL. Cells were grown at 37°C in a humidified 5% CO₂; 95% air environment for 2 days prior to treatment.

2.3.7 *In vitro* polarisation of M1/M2 cells

After the 48h plating period, medium was removed from microglia, astrocytes, and BMDMs and replaced with cDMEM alone, cDMEM containing IFN- γ (50ng/mL; R&D Systems, UK, Appendix I) or cDMEM containing IL-4 (20ng/mL; R&D Systems, UK, Appendix I). Cells were incubated in these conditions at 37°C in a humidified 5% CO₂; 95% air environment for 24h before harvesting for various experimental analyses. Supernatants were aspirated and transferred into 1.5mL Eppendorf tubes for analysis by enzyme-linked immunosorbent assay (ELISA) as described in section 2.7.

2.3.8 Preparation of glia:T cell co-culture for CTV proliferation assay

A 96-well round bottomed tissue culture plate (Fisher Scientific, UK) was coated with anti-CD3e (1 μ g/mL in cRPMI) and incubated for at least 2h at 37°C prior to CD4⁺ T cell isolation. After this time, the plate was washed twice with PBS.

T helper cells were isolated from the spleens of 12 month old green fluorescent protein (GFP) mice using a Miltenyi Biotec CD4⁺ T cell isolation kit (Miltenyi Biotec, Germany). After intracardial perfusion, as described in section 2.2.1, the spleens were removed and mechanically dissociated in cRPMI using the plunger of a 1mL syringe and a 40 μ m nylon mesh filter to create a single cell suspension. This cell suspension was centrifuged (1250rpm; 5min; 20°C) and the supernatant was discarded. The pellet was resuspended in RBC lysis buffer (2mL/spleen) and incubated at room temperature for 3min in the fume hood in

order to eliminate RBCs and purify the lymphocytes. Following RBC lysis, PBS (3mL) was added and the cells were centrifuged (1250rpm; 5min; 20°C). The supernatant was discarded and the pellet resuspended in PBS (1mL) and cells were counted under a light microscope using a haemocytometer. The cell suspension was centrifuged (1250rpm; 5min; 20°C) and the pellet resuspended in magnetic-activated cell sorting (MACS) buffer (40µL/10⁷ cells; Appendix I). Biotin-antibody cocktail was added (10µL/10⁷ cells; Miltenyi Biotec, Germany) and the cell suspension was mixed and incubated for 5min at 4°C. After this incubation period, MACS buffer (30µL/10⁷ cells) and anti-biotin microbeads (20µL/10⁷ cells; Miltenyi Biotec, Germany) were mixed with the cell suspension. The cell suspension was incubated for 10min at 4°C.

A MACS LS separation column (Miltenyi Biotec, Germany) was mounted in the magnetic field of a MACS separator (Miltenyi Biotec, Germany) and rinsed with MACS buffer (3mL). The cell suspension was applied to the column and magnetically labelled CD4⁺ cells were arrested in the column. The column was then removed from the magnetic field and the effluent containing CD4⁺ T cells was collected in a 15mL Falcon tube. This was passed through the column again to ensure maximal yield. The CD4⁺-enriched cell suspension was centrifuged (1250rpm; 5min; 20°C) and the resulting pellet resuspended in warm cRPMI (1mL). Cells were counted under a light microscope using a haemocytometer and centrifuged (1250rpm; 5min; 20°C). The resulting pellet was resuspended in warm PBS (11mL) and 1mL was removed to a new 15mL Falcon tube and centrifuged (1250rpm; 5min; 20°C). The pellet was resuspended in cRPMI to act as a non-stained control. The remaining T cell suspension was stained with cell trace violet (CTV; 2µL; Thermo Fisher, UK) for 10min at 37°C. CTV staining was stopped by submerging the Falcon tube in ice until cold. The cell suspension was centrifuged (1250rpm; 5min; 20°C) and the pellet was resuspended in 1mL cRPMI. CTV diffuses into the cell and covalently binds intracellular amines without affecting morphology or physiology, allowing for the tracing of generations or divisions.

CD4⁺ T cells were counted and brought to a density of 5×10^5 cells/mL in cRPMI. This cell suspension was added to anti-CD3e coated 96 well plates (100 μ L/well) to give a density of 0.5×10^5 cells/well. Glia, which had been polarised as described in section 2.3.7, were gently scraped from 6-well plates and centrifuged (1200rpm; 5min; 20°C). The resulting pellets were resuspended in cRPMI (1mL) and cells were γ -irradiated (45Gy; Gammacell® 3000 Caesium¹³⁷ Irradiator, Canada). After this, cells were counted and brought to a density of 1×10^6 cells/mL with cRPMI and added to the T cells in the 96-well plate (100 μ L/well) to give a density of 1×10^5 cells/well, generating a 2:1 ratio of glial cells to T cells. Co-cultures were grown at 37°C in a humidified 5% CO₂:95% air environment for 7 days before being analysed by FACS (section 2.5). CTV stain is detected on the Brilliant Violet 421 channel on the flow cytometer and its intensity decreases in daughter cells. Measuring the decrease in staining gives an indication of the rate of proliferation of the cells.

2.3 Assessment of glial phagocytosis

Phagocytosis was assessed using immunocytochemistry and flow cytometry. For analysis by flow cytometry, cells were harvested, transferred to FACS tubes and centrifuged (1200rpm; 5min; 20°C). The supernatant was discarded and tubes were dabbed on clean tissue to remove any residual liquid. Cells were resuspended in cDMEM alone or cDMEM containing HiLyte Fluor™ 488-labelled amyloid- β (Abs/Em = 503/528nm; 0.5 μ M; Appendix I) or fluorescently-labelled yellow/green latex beads (Abs/Em = 470/505nm; 1:200 dilution; Sigma-Aldrich, UK) and were incubated for 2h at 37°C. After the 2h incubation period, tubes were centrifuged (1200rpm; 5min; 20°C) and the resulting pellet was resuspended in 0.4% trypan blue solution (Sigma-Aldrich, UK) to quench extracellular fluorescence. Cells were washed 3 times with FACS buffer (1mL; Appendix I) and normal extracellular staining protocol was carried out as described in section 2.4.1.

2.3.1 Immunocytochemistry

Microglia or mixed glia were seeded onto poly-D-lysine coated coverslips as described in section 2.3.2 and 2.3.4 respectively. Media was removed 24h after seeding and replaced with cDMEM alone or cDMEM containing HiLyte Fluor™ 488-labelled amyloid-β (Abs/Em = 503/528nm; 0.5μM) or fluorescently-labelled yellow/green latex beads (Abs/Em = 470/505nm; 1:200 dilution) and cells were incubated for 2h at 37°C. Following this, the media was removed and cells were fixed cells with 4% PFA (500μL; Appendix I) for 30min. Coverslips were washed 3 times with PBT (500μL; Appendix I) and blocked with 10% horse serum (500μL; in PBT; Vector Laboratories, USA) for 1h at room temperatyre. Primary antibodies (250μL; Table 2.5; in 10% horse serum) were added to the coverslips and incubated overnight on a rocker at 4°C in the dark. The next day, coverslips were washed 3 times with PBT (500μL) and secondary antibody (500μL; 1:1000 in PBT; Table 2.5) was added. Coverslips were incubated in the dark for 2h at room temperature before being washed twice with PBT (500μL) and once with PBS (500μL). Coverslips were mounted onto clean glass slides using Vectashield mounting medium with DAPI (Vector Laboratories, USA) and cells were imaged using fluorescent (Axio Imager.Z1, ApoTome Zeiss, Germany) and confocal (Leica TCS SP8, Leica Microsystems, Germany) microscopes.

	Antibody	Company	Dilution
Primary	Rabbit anti-mouse Iba-1	AbD Serotec (MCA2759)	1:200
	Rabbit anti-GFAP	Dako (Z0334)	1:2000
Secondary	Alexa-Fluor®-633 donkey anti-rabbit	Life technologies (A21207)	1:1000

Table 2.1 Antibodies used for immunocytochemistry

2.4 Flow cytometry

2.4.1 Extracellular staining

Cells were transferred to FACS tubes and washed 3 times with FACS buffer (1mL). Cells were centrifuged (1200rpm; 5min; 20°C) and resuspended in FACS buffer containing rat anti-mouse CD16/CD32 FcγRIII (50μL; 1:100; Mouse BD Fc Block™, BD Biosciences, USA). Cells were incubated in this solution for 10min at room temperature to block low-affinity IgG receptors. Cells were then incubated in with appropriate antibodies (50μL; Table 2.1) for 30min at room temperature in the dark. Cells were washed 3 times and following the final wash, cells were resuspended in FACS buffer (300μL).

2.4.2 Intracellular staining

Cells were prepared for intracellular staining using the FIX & PERM® kit (Invitrogen, UK). Cells were transferred to FACS tubes and stimulated with phorbol myristate acetate (PMA; 10ng/mL; Sigma Aldrich, UK), ionomycin (1μg/mL; Sigma Aldrich, UK), and brefeldin-A (BFA; 5μg/mL; Sigma Aldrich, UK) for 5h at 37°C in order to enhance detection of intracellular cytokines. BFA inhibits the intracellular transport of cytokines upregulated by PMA and ionomycin, thus allowing for their detection within the cell. Following this, cells were washed 3 times with PBS, centrifuged, and resuspended in Fixable Viability Dye eFluor506 (500μL; 1:1000; eBiosciences, USA). Cells were vortexed thoroughly and incubated at 4°C for 30min. Cells were washed 3 times with FACS buffer, blocked and the extracellular staining protocol was carried out as described in section 2.5.1. Fixation medium A (50μL; Invitrogen, UK) was added to each tube for 15min at room temperature. Cells were washed 2 times with FACS buffer, centrifuged (1200rpm; 5min; 20°C), and resuspended in permeabilisation media B (50μL; Invitrogen, UK) containing intracellular antibodies (Table 2.1) and incubated with cells in the dark for 15min at room temperature. Cells were washed 3 more times and following the final wash, cells were resuspended in FACS buffer (300μL).

Target	Antibody	Dilution	Supplier (Cat #)
CD11b	Anti-mouse CD11b PE-eFluor® 610	1:100	eBioscience (83-0451-41)
IFN- γ	Anti-mouse IFN- γ PE-Cy7	1:100	eBioscience (25-7311-41)
IL-4	Anti-mouse IL-4 FITC	1:100	eBioscience (11-7042-41)
IL-17	Anti-mouse IL17A PE	1:100	BD Pharmingen (559502)

Table 2.2 Antibodies used for flow cytometry

2.4.3 Flow cytometric analysis

With the exception of Fixable Viability Dye and BV421, compensation beads (BD Bioscience, USA) were used for each colour to correct for spectral overlap in fluorochrome detection. Positive and negative compensation beads were added to FACS tubes and diluted with PBS to ensure adequate volume in the tube (50 μ L). Appropriate antibody was added to the tube at the same concentration used in staining. Fixable Viability Dye eFluor506 and BV421 positively and negatively stained cells were used for compensation controls. Fluorescence minus one controls were used to identify gating boundaries. These controls consisted of a tube containing every other fluorochrome except for the one under investigation. Flow cytometric analysis was carried using the Fortessa flow cytometer (Becton-Dickinson, USA) and analysed using FlowJo v7.6.5 software (Tree Star Inc., USA).

2.5 Using the SeaHorse XF Analyser

The SeaHorse Extracellular Flux (XF24) Analyser (SeaHorse Bioscience, USA) was used to carry out bioenergetic analysis of cells by measuring glycolytic activity and mitochondrial respiration. Microglia, astrocytes and BMDMs were isolated as per sections 2.2.2, 2.2.3 and 2.2.6 and seeded (100 μ L/well) in SeaHorse cell culture microplates at densities of 1x10⁵cells/well, 8x10⁴cells/well and 2x10⁵cells/well respectively, with care being taken not to seed cells in background correction wells. The plate was left in the hood for 1h before being incubated for a further 1h at 37°C. Wells were topped up with cDMEM (150 μ L) and incubated at 37°C in a humidified 5% CO₂; 95% air environment for 24h. Cells were treated with cDMEM alone, cDMEM containing IFN- γ (50ng/mL) or IL-4 (20ng/mL), which were made up at 6X concentrations and added (50 μ L) to the medium in the SeaHorse plates to give a final volume of 300 μ L/well. The sensor cartridge was hydrated by adding SeaHorse XF Calibrant solution (1mL; SeaHorse BioScience, USA) to each well of the utility plate and was left overnight in a CO₂-free incubator at 37°C.

On the day of the assay, specific assay media was made up for the mitochondrial stress test or the glycolytic flux test (Appendix I). The cells were washed twice by very gently removing all but 50 μ L of media from the wells and replacing with assay medium (1mL). After the final wash, assay media was very gently added to each well (425 μ L) to give a final volume of 475 μ L/well and the plate was placed in a no-CO₂ incubator for 1h. The absence of CO₂ is important to ensure acidification and changes in pH do not occur, as this is an important measure for the SeaHorse XF Analyser.

For the mitochondrial stress test, stock solutions of oligomycin (Abcam, UK), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; Sigma-Aldrich, UK) and antimycin A (Sigma-Aldrich, UK) were diluted to working concentrations of 20 μ M, 20 μ M, and 40 μ M in mitochondrial stress test assay media and loaded into ports A (56 μ L), B (62 μ L) and C (69 μ L) of the cartridge respectively. For the glycolytic flux test, glucose (Sigma-Aldrich, UK),

oligomycin and 2-deoxy-D-glucose (2-DG, Sigma-Aldrich, UK) were brought to working concentrations of 10mM, 20μM, and 500mM in glycolytic flux assay media (Appendix I) and loaded into ports A (56μL), B (62μL) and C (69μL) of the cartridge respectively. The cartridge was loaded into the SeaHorse XF Analyser, allowing it to be calibrated. Following calibration, the utility plate was removed and replaced with the cell culture plate and the assay was begun.

Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured every 8min for 96min. Baseline measurements were taken at 0, 8 and 16min and compounds were injected sequentially after every 3 time points. ECAR and OCR were automatically calculated by the SeaHorse XF24 software and each point represents an average of 4-6 different wells. Sample ECAR and OCR plots can be seen in Figures 2.3 and 2.4.

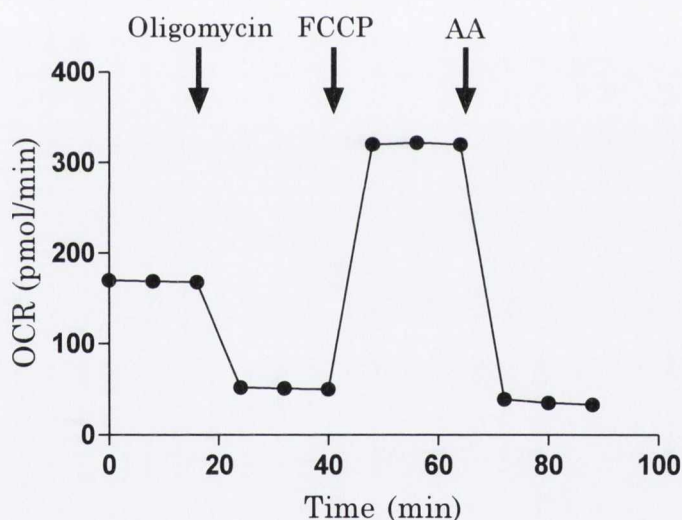


Figure 2.2 Example SeaHorse graph for the mitochondrial stress test. The basal respiration is assessed from the OCR readout by calculating the difference between the average of the first 3 measurements and the final 3 measurements i.e. after the addition of antimycin A. The maximal respiration is found by calculating the difference between the average of the first 3 measurements and the third 3 measurements i.e. after the addition of FCCP. ATP production is

calculated by finding the difference in OCR between the first 3 measurements and the second 3 measurements.

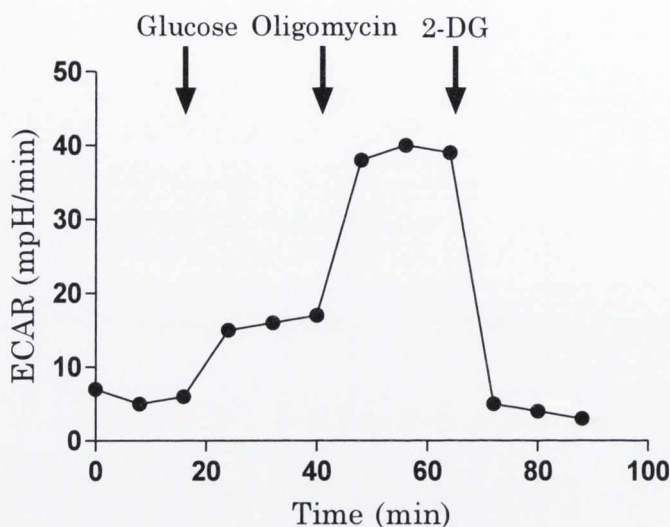


Figure 2.3 Example SeaHorse graph for the glycolytic flux test. Glycolysis is assessed from the ECAR readout by calculating the difference between the average of the first and the second 3 measurements i.e. after the addition of glucose to the cells. The glycolytic capacity of cells is determined by calculating the difference between the average of the first 3 measurements and the third 3 measurements. Glycolytic reserve is found by measuring the difference between the average of the second 3 measurements and the third 3 measurements.

2.6 Analysis of protein secretion by ELISA

The concentrations of the chemokines CCL2 (Mouse CCL2/MCP1 DuoSet ELISA, R&D Systems, Cat # DY479), CCL3 (Mouse CCL3/MIP1 α DuoSet ELISA, R&D Systems, Cat# DY450), and CCL5 (Mouse CCL5/RANTES DuoSet ELISA, R&D Systems, Cat # DY478) were assessed by ELISA in supernatant samples. ELISA plates were coated with capture antibody (100 μ L per well), covered with an adhesive plate sealer (Cruinn, Ireland) and incubated overnight at room temperature. Plates were washed 3 times with ELISA wash buffer (Appendix I) and blocked with reagent diluent (300 μ L/well; 1% bovine serum

albumin [BSA; Sigma-Aldrich, UK] in PBS) by incubating at room temperature for at least 1h on an orbital shaker (Stuart Scientific, UK). Plates were washed 3 times and standards and samples were diluted in cDMEM and added to the plate in triplicate (100 μ L/well). Plates were returned to the orbital shaker for 2h and washed 3 times with wash buffer and the addition of detection antibody (100 μ L/well). The plate was sealed and incubated for a further 2h at room temperature on an orbital shaker. Plates were washed 3 times with wash buffer and horseradish peroxidase-conjugated streptavidin was added (1:40 dilution in reagent diluent; 100 μ L/well). The plate was covered with an adhesive plate sealer and tinfoil and incubated on an orbital shaker for 20min. The wells were washed 3 times with wash buffer and substrate solution was added (1:1 mixture of hydrogen peroxide and 3, 3', 5, 5'-tetramethylbenzidine; 100 μ L/well). The plate was sealed and incubated in the dark for at least 20min. The reaction was stopped by adding 1M H₂SO₄ (50 μ L/well) and absorbance was read at 450nm using a plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek, USA). A standard curve was produced using Prism software and the concentrations of samples were extrapolated from this.

2.8 Analysis of mRNA by RT-PCR

2.8.1 RNA extraction

A Nucleospin® RNA II kit (Macherey-Nagel, Germany) was used to isolate mRNA from cell and tissue lysates for assessment by reverse transcriptase-polymerase chain reaction (RT-PCR). To harvest cells from 6-well plates, supernatants were aspirated and RA1 buffer (Macherey-Nagel, Germany) with β -mercaptoethanol (Sigma-Aldrich, UK) was added (100:1; 350 μ L/well) to lyse cells. A cell scraper was used to remove cells from the wells. Hippocampal tissue samples were thawed on ice and lysis buffer (350 μ L) was added and tissue was homogenised using a hand held homogeniser.

The lysate was passed through a Nucleospin filter placed in a 2mL Eppendorf collection tube and centrifuged (11,000g; 1min). The filters were discarded and

ethanol (70% in RNase free H₂O; 350µL) was added to the cell lysate and mixed by pipetting up and down 8 times. This was loaded onto Neucleospin RNA II columns and samples were centrifuged (11,000g; 30s) to allow the RNA to bind to the columns. Membrane desalting buffer (350µL) was added to each sample and the samples were centrifuged (11,000g; 1min). DNA was digested by adding DNase reaction mixture (95µL) directly onto the silica membrane column of each sample and incubating at room temperature for 15min. The reaction was stopped by adding RA2 buffer (200µL) and centrifuging (11,000g; 1min) to dry the column. The column was washed with RA3 buffer (600µL) and centrifuged (11,000g; 30s). A final wash with RA3 buffer (250µL) was carried out and the silica membrane dried by centrifuging (11,000g; 2min). RNA was eluted from the column by adding nuclease-free water (60µL) and centrifuging (11,000g; 1min). mRNA was collected in nuclease free 1.5mL Eppendorf tubes and stored at -20°C.

2.8.2 cDNA Synthesis

Samples were kept on ice for the duration of the reverse transcription process to prevent their degradation and all surfaces were cleaned thoroughly with RNase away. The RNA concentration of samples was determined using a NanoDrop Spectrophotometer (ND-1000 v3.5, NanoDrop Technologies Inc., USA). Samples were equalised with nuclease-free water and reverse transcribed to cDNA using high-capacity cDNA archive kit (Applied Biosystems, Germany). A mastermix was prepared with 10X RT buffer (6µL), 25X dNTPs (2.4µL), 10X random primers (6µL), multiscribe reverse transcriptase (3µL) and nuclease-free water (12.6µL) per sample. The mastermix (30µL) was mixed with each RNA sample (30µL) in labelled PCR tubes. These tubes were centrifuged and subjected to a 10min 25°C cycle and a 2h 37°C cycle in a thermal cycler (PTC-200, Peltier Thermal Cycler, USA). cDNA samples were stored at -20°C until amplification.

2.8.3 Reverse Transcription-Polymerase Chain Reaction

A mastermix consisting of RNase-free water (8µL), Sensimix Probe II (12.5µL; Bioline, UK), β-actin primer as an endogenous control (1.25µL) and the target

primer (1.25µL; TaqMan Gene Expression Assays, Applied Biosystems, Germany; listed in table 2.1) was prepared per sample and added (23µL) to each well of a 96-well plate (Thermo Scientific, UK). cDNA samples (2µL) were added to the appropriate wells and the plate was sealed and centrifuged (1200rpm; 1min; 20°C). Real-time PCR (60 cycles with 2min at 50°C, 10min at 95°C, 15s at 95°C and 1min at 60°C) was carried out using Applied Biosystems ABI Prism 7300 Fast Track Sequence Detection System v1.4.1.

Gene Name	TaqMan Gene Expression Assay Number
Arginase-1	Mm00475988_m1
β-actin	Mm00407939_s1
BDNF	Mm04230607_s1
CCL2	Mm00441242_m1
CCL3	Mm00441259_g1
CCL5	Mm01302427_m1
Chi3L3	Mm00657889_mH
CD11b	Mm00434455_m1
CD29	Mm01253230_m1
CD33	Mm00491152_m1
CD40	Mm00441891_m1
CD68	Mm03047343_m1
CD80	Mm00711660_m1

CD86	Mm00444543_m1
Ferritin	Mm00850707_g1
Ferroportin	Mm01254822_m1
IGF1	Mm00439560_m1
IL-1 β	Mm00434228_m1
iNOS	Mm00440502_m1
GFAP	Mm01253033_m1
GLAST	Mm00600697_m1
MRC1	Mm00485148_m1
MHCII	Mm00482914_m1
NGF	Mm00443039_m1
PFKFB1	Mm01256237_m1
PFKFB3	Mm00504650_m1
TNF- α	Mm00443258_m1
TREM2	Mm04209424_g1

Table 2.3 List of primers for RT-PCR.

2.8.4 PCR quantification

RT-PCR analysis was carried out using the delta CT method to determine gene expression. This method allows the user to determine the levels of target gene expression compared with their endogenous controls (β -actin). Values were

normalised to control sample and the fold changes in gene expression is compared between groups.

2.9 Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis

2.9.1 Sample preparation for SDS-PAGE

To harvest cells from 6-well plates for Western immunoblotting, supernatant samples were aspirated from wells and cells were gently rinsed with PBS, followed by a 10min incubation with lysis buffer (Appendix I; 60 μ L/well). A cell scraper was used to harvest cells, which were centrifuged (15,000g; 10min; 4°C) and the supernatant (50 μ L) was carefully collected and prepared for Western immunoblotting.

2.9.2 Pierce® BCA protein assay

A Pierce® BCA protein assay (Fisher Scientific, UK) was used to determine the protein concentration of samples. A standard curve was produced by carrying out serial dilutions of stock BSA prepared from stock (2mg/mL in dH₂O) which were in dH₂O to create a range of concentrations up to 2000 μ g/mL which were added in duplicate (25 μ L/well) to designated wells. Samples were diluted in dH₂O (1:5) and added to in duplicate (25 μ L/well) to designated wells. Pierce® BCA reagents A and B (Fisher Scientific, UK) were prepared in a 49:1 ratio (v:v) and added to each well (200 μ L/well). The plate was incubated in the dark for 30min at 37°C to develop colour and the absorbance was read at a wavelength of 562nm using a BioTek Synergy HT Multi-Mode Microplate Reader.

A standard curve was produced using Prism software (v5.0, Graphpad, USA) and protein concentrations were determined using regression analysis ($r^2 > 0.98$). Protein concentrations were equalised with ice cold lysis buffer. Samples were diluted with 4X sample buffer (Appendix I) and heated for 5min at 70°C in order to denature proteins. Samples were stored at -20°C.

2.9.3 SDS-PAGE

Polyacrylamide separating gels (1mm thick) with a monomer of 7-15% (Appendix I) were cast between 2 glass plates (10cm; BioRad Laboratories, USA) and overlaid with 4% stacking gels (Appendix 1). A comb was inserted to construct separate loading wells. Gels were mounted in an electrophoretic unit (BioRad Laboratories, USA) and electrode running buffer (Appendix I) was added to the inner and outer reservoirs. Samples were thawed rapidly, heated for 5min at 70°C and loaded (10µg or 20µg protein/well) into designated wells of the gels. A pre-stained molecular marker (10µL; Spectra, Fisher Scientific, UK) was added to each gel for identification of bands by molecular weight. The proteins were separated by the application of 100V across the unit until the molecular marker indicated that the samples had run the appropriate amount.

2.9.4 Western transfer protocol

Filter paper (Sigma-Aldrich, UK) and labelled nitrocellulose membranes (Sigma-Aldrich, UK) were cut to the size of the gel and soaked in transfer buffer (Appendix I) along with 2 blotting pads (BioRad Laboratories, USA). Once the electrophoretic unit was switched off, the gel was removed and the stacking gel was carefully detached and disposed of. The resolving gel was soaked in transfer buffer and placed on the sheet of nitrocellulose paper sandwiched between filter paper and blotting pads. Air bubbles were removed by gently rolling a glass pipette (Fisher Scientific, UK) along the surface of the gel. The sandwich was placed in a blotting unit (BioRad Laboratories, USA) which was filled with transfer buffer and transferred at 220mA for 60min.

2.9.5 Western immunoblotting

Nitrocellulose membranes were blocked for 1h with 5% Marvel milk or BSA in TBS-T (w/v; 10mL; Appendix I) for non-specific binding of protein and probed with an antibody raised against the protein of interest. See-saw rockers (SSL4, Stuart Scientific, UK) were used throughout to ensure that complete coverage of the membrane by solutions was achieved. Primary antibodies (10mL) were diluted as summarised in table 2.2 and applied for 2h at room temperature or

overnight at 4°C. The membranes were washed 3 times with TBS-T (15min) and the appropriate secondary antibodies (Goat anti-mouse IgG or goat anti-rabbit IgG; 1:5000 in 5% Marvel milk or BSA in TBS-T; 10mL) conjugated to horseradish peroxidase (HRP; Jackson ImmunoResearch Laboratories, USA) were applied for 1h at room temperature. After washing 3 times with TBS-T (15min), the membranes were incubated with a chemiluminescent detection solution (3min; Immobilon Western, Millipore, USA) to detect the HRP-linked antibodies and visualise protein complexes. Protein bands were imaged using FujiFilm LAS-4000 imaging system (Fuji, UK). Membranes were stripped with Re-Blot Plus strong solution (10mL; Merck Millipore, Germany) for 5min and re-probed for β -actin and imaged as described above.

Protein (Molecular Weight)	Primary Antibody (Supplier)	Dilution
pAMPK (62kDa)	Rabbit anti-phospho-AMPK α (Thr172) IgG (Cell Signalling cat # 2535)	1:1000 in 5% Marvel/TBS-T
tAMPK (62kDa)	Rabbit anti-AMPK α 1 + AMPK α 2 IgG (Abcam cat # Ab80039)	1:1000 in 5% Marvel/TBS-T
Ferritin (20kDa)	Rabbit anti-ferritin IgG (Abcam cat # Ab183781)	1:1000 in 5% Marvel/TBS-T
Ferroportin (63kDa)	Rabbit anti-SLA40A1 IgG (Abcam cat # Ab85370)	1:1000 in 5% Marvel/TBS-T
GLUT1 (55kDa)	Rabbit anti-GLUT1 IgG (Abcam cat # Ab652)	1:1000 in 5% Marvel/TBS-T
HIF-1 α (130kDa)	Rabbit anti-HIF-1-alpha IgG (Abcam cat # Ab179483)	1:1000 in 5% Marvel/TBS-T

PDK1 (49kDa)	Rabbit anti-PDK1 IgG (Abcam cat # Ab202468)	1:2000 in 5% Marvel/TBS-T
PFKFB1 (55kDa)	Rabbit anti-PFKFB1 IgG (Abcam cat # Ab71626)	1:500 in 5% Marvel/TBS-T
PFKFB3 (55kDa)	Rabbit anti-PFKFB3 IgG (Abcam cat # Ab181861)	1:1000 in 5% Marvel/TBS-T
PGC-1β (113kDa)	Rabbit anti-PGC1 beta IgG (Abcam cat # Ab176328)	1:1000 in 5% Marvel/TBS-T
Transferrin receptor (98kDa)	Rabbit anti-Transferrin Receptor IgG (Abcam cat # Ab84036)	1:1000 in 5% Marvel/TBS-T
β-Actin (42kDa)	Mouse anti- β -actin IgG (Sigma-Aldrich)	1:5000 in 5% Milk/TBS-T

Table 2.4 Antibodies used for Western immunoblotting

2.9.6 Analysis

Protein bands were analysed and quantified by densitometric analysis using ImageJ (Rasband W.S., National Institutes of Health, USA) and values are expressed as a ratio of total protein of interest or β -actin as a loading control.

2.10 Statistical analysis

All statistical analysis was carried out using Prism 5 (GraphPad Prism, California, USA). Data were analysed, where appropriate, using two-tailed Student's t-tests, one-way analyses of variance (ANOVA) with Dunnett's post-hoc tests, or two-way ANOVA with Bonferroni post-hoc tests to determine whether significant differences existed between groups. Significance was conferred when $p < 0.05$.

Chapter 3: Metabolic reprogramming in polarised BMDMs

3.1 Introduction

Macrophages are the primary cells of the innate immune system and can adopt phenotypically distinct activation states in response to stimuli encountered in the microenvironment (Mosser, 2003). The classical or M1 activation state is pro-inflammatory and is characterised by increased iNOS and TNF α expression and the secretion of IL-1 β . iNOS is an important enzyme responsible for the production of anti-microbial NO from L-arginine, while TNF α and IL-1 β are pro-inflammatory cytokines that propagate the immune response, further exacerbating inflammation. This response is important for combating viral and bacterial pathogens and is induced by various stimuli including PAMPs, DAMPs, and pro-inflammatory cytokines such as IFN- γ (Gordon and Taylor, 2005). The alternative or M2 activation state is the antithesis of this and is associated with functions pertaining to the resolution of inflammation and tissue repair (Hume, 2015). The M2 phenotype is induced by anti-inflammatory cytokines, such as IL-4 and IL-13, and is characterised by expression of Arg-1, which competes with iNOS for L-arginine, as well as MRC1, Chi3L3 and FIZZ1 (Stein et al., 1992).

Although acute activation of the M1 phenotype is an important process in protection from infection, chronic activation of macrophages to a classical pro-inflammatory cells is associated with a plethora of inflammatory diseases such as RA (Li et al., 2012), while inhibition of inflammation has proven an effective treatment strategy (Feldmann, 2002). The M1/M2 classification of activation states, which is derived from *in vitro* experiments, has become outdated in recent years, in particular as it relates to the *in vivo* situation where cells are more likely to possess attributes of multiple phenotypes simultaneously (Mosser and Edwards, 2008). The M1/M2 classification also lacks the flexibility to reflect the diversity of macrophage activation. Therefore research has shifted towards identifying inflammatory cells in terms of functional outcomes, rather than their expression of particular molecular markers. A current area of research that is highly dynamic in this regard is immunometabolism.

Under normal physiological conditions, energy is produced largely when pyruvate, generated from glycolysis, feeds into the TCA cycle and oxidative phosphorylation. Oxidative phosphorylation generates 36 molecules of ATP per molecule of glucose in the process (Dashty, 2013). However, under hypoxic conditions, oxidative phosphorylation is prevented and glycolysis, which becomes the predominant metabolic pathway, yields lactic acid or ethanol and is less efficient, producing only 2 molecules of ATP per molecule of glucose. In order to generate the sufficient ATP to support cellular activity, the rate of glycolysis is increased during hypoxia. This shift in metabolic profile has also been shown to occur in the presence of oxygen under certain circumstances; a phenomenon described by Otto van Warburg in cancer cells in the 1920s termed “aerobic glycolysis” (Warburg, 1924).

Although less efficient, cells utilise aerobic glycolysis to meet metabolic demands when rapid bursts of activity are required, such as during cell proliferation (Lunt and Vander Heiden, 2011). Polarisation of macrophages is accompanied by alterations in the metabolic pathways employed by the cell, and the rapid burst of energy associated with aerobic glycolysis is utilised by macrophages adopting an M1 phenotype. The evidence suggests that the increase in glycolysis is mediated by an upregulation of PFKFB3 induced by HIF-1 α or NF- κ B, which increases fructose-2,6-bisphosphate accumulation and drives glycolysis (Obach et al., 2004). Macrophages adopting the M2 activation state meet the energy demands of activation by increasing oxidative metabolism, a more sustainable metabolic state (Galvan-Pena and O'Neill, 2014).

Changes in iron handling by cells have also been identified in the M1 and M2 phenotypes. Increased expression of the iron chelating protein, ferritin, which limits iron availability to intracellular bacteria, has been described in M1 cells while there is a decrease in expression of ferroportin to starve extracellular pathogens of the essential metal and induce T_h1 cell responses (Weiss et al., 1995). Conversely, ferroportin expression and iron recycling activity are

identified in M2 macrophages to provide for healing tissue (Brunelli and Rovere-Querini, 2008).

The aims of this study were to:

1. Investigate the metabolic profiles of M1 and M2 activated BMDMs and identify markers of this metabolic signature.
2. Investigate the iron handling properties of M1 and M2 activated BMDMs.

3.2 Methods

BMDMs were incubated *in vitro* with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h to induce polarisation of cells. The metabolic profile of cells was assessed using the SeaHorse XF analyser. Stimulated cells were also harvested for RT-PCR and Western immunoblotting analysis of mRNA and protein expression respectively. These methods are described in detail in Chapter 2.

3.3 Results

BMDMs were incubated with media alone, IFN- γ or IL-4 for 24h in vitro to induce the polarisation of cells to M1 and M2 activation states respectively and markers of these activation states were assessed by RT-PCR. IFN- γ significantly increased the M1 activation state markers TNF α , iNOS and IL-1 β (* p <0.05; *** p <0.001; one-way ANOVA with Dunnett's post hoc analysis; Figure 3.1A, B, C), while IL-4 significantly decreased expression of TNF α (* p <0.05; one-way ANOVA with Dunnett's post hoc analysis; Figure 3.1A), but had no effect on the expression of iNOS or IL-1 β (Figure 3.1B, C).

IL-4 increased expression of M2 activation state markers Arg-1, MRC1, Chi3L3 and FIZZ1 (** p <0.01; *** p <0.001; one-way ANOVA with Dunnett's post hoc analysis; Figure 3.2A, B, C, D). IFN- γ did not affect the expression of Arg-1, Chi3L3, or FIZZ1 (Figure 3.2A, C, D). However, the expression of MRC1 was significantly decreased following IFN- γ treatment (** p <0.01; one-way ANOVA with Dunnett's post hoc analysis; Figure 3.2B).

The SeaHorse XF analyser was used to determine the metabolic profiles of IFN- γ and IL-4 stimulated macrophages. A glycolytic stress test was carried out on stimulated BMDMs (Figure 3.3A) and the data revealed a significant increase in the glycolysis in IFN- γ stimulated BMDMs (** p <0.01; one-way ANOVA with Dunnett's post hoc analysis; Figure 3.3B) and IL-4 stimulated cells (* p <0.05; one-way ANOVA with Dunnett's post hoc analysis; Figure 3.3B), although the IL-4 induced change was not as significant as the IFN- γ induced change. A mitochondrial stress test was also carried out (Figure 3.4A) and the data indicate that IL-4 stimulated M2 macrophages exhibited a significantly higher basal level of oxygen consumption than unstimulated and M1 activated cells (** p <0.01; one-way ANOVA with Dunnett's post hoc analysis; Figure 3.4B).

The PFKFB family of isozymes have been implicated as having a pivotal role in the metabolic switch that occurs in inflammatory cells (Rider et al., 2004) and so expression of PFKFB1 and PFKFB3 was assessed in IL-4 and IFN- γ

stimulated BMDMs. The data revealed that IFN- γ significantly decreased PFKFB1 expression ($*p<0.05$; one-way ANOVA with Dunnett's post hoc analysis; Figure 3.5A), while IL-4 had no effect. In contrast, IFN- γ significantly increased PFKFB3 expression ($*p<0.05$; one-way ANOVA with Dunnett's post hoc analysis; Figure 3.5B). There was also a significant increase in the ratio of PFKFB3 to PFKFB1 observed in BMDMs stimulated with IFN- γ ($**p<0.01$; one-way ANOVA with Dunnett's post hoc analysis; Figure 3.5C).

Since the iron handling properties of polarised macrophages are reported to differ depending on the activation state of the cell (Recalcati et al., 2010). The expression of transferrin receptor (TfR), ferritin and ferroportin were assessed in M1 and M2 macrophages.

The data revealed IL-4 increased the expression of TfR mRNA ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 3.6A) and protein ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 3.6B). In contrast, IFN- γ significantly decreased receptor mRNA expression ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 3.6A), but did not affect protein expression (Figure 3.6B). IFN- γ and IL-4 significantly decreased ferroportin mRNA expression ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 3.7A). Although a decrease was also evident at the protein level, this did not reach statistical significance (Figure 3.7B). Both IL-4 and IFN- γ also decreased ferritin mRNA ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 3.8A). However, the data revealed a significant increase in the protein expression following stimulation with IFN- γ ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 3.8B), while IL-4 had no effect.

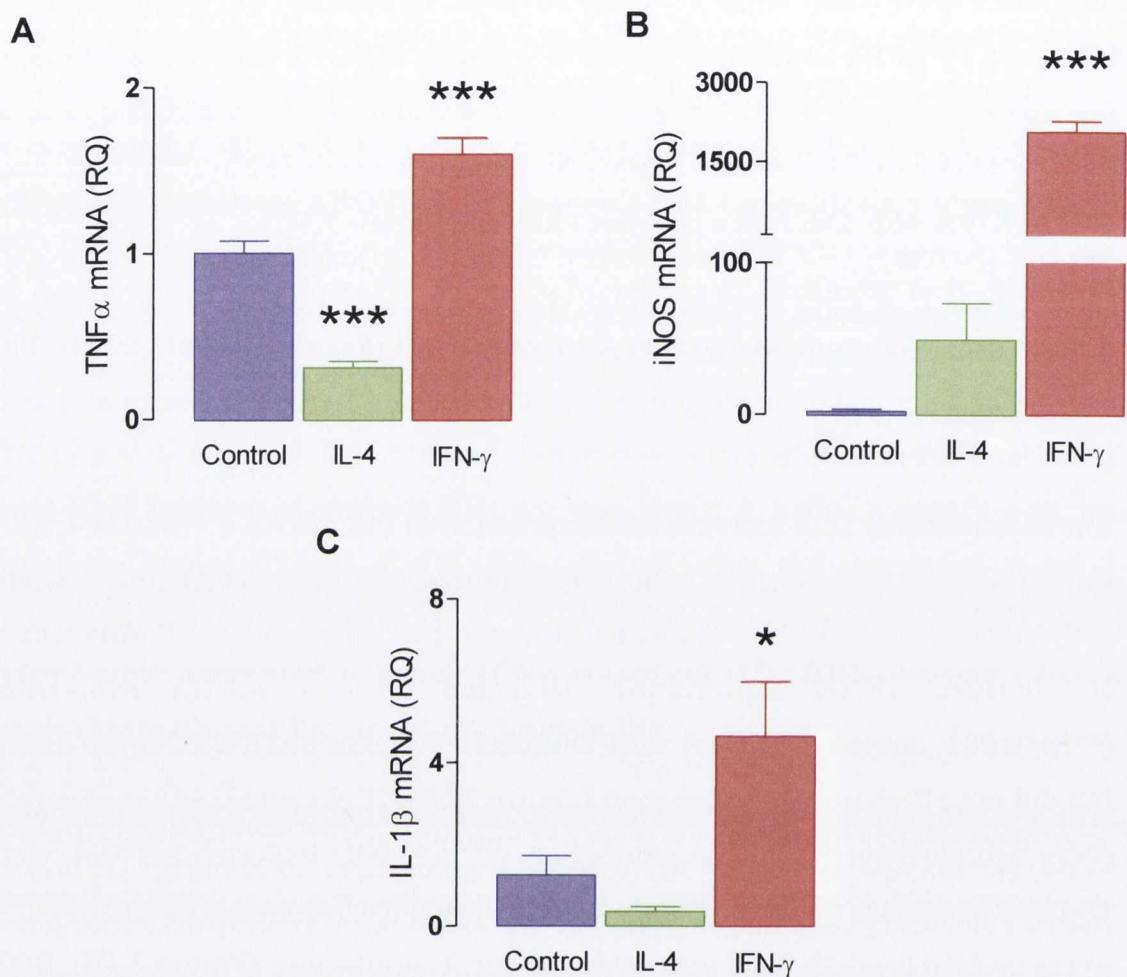


Figure 3.1 IFN- γ increased expression of M1 activation state markers in BMDMs

BMDMs were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA expression of M1 activation state markers. The data show that IFN- γ significantly increased the mRNA expression of TNF α (A), iNOS(B), and IL-1 β (C).. * p <0.05, *** p <0.001; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=4-5).

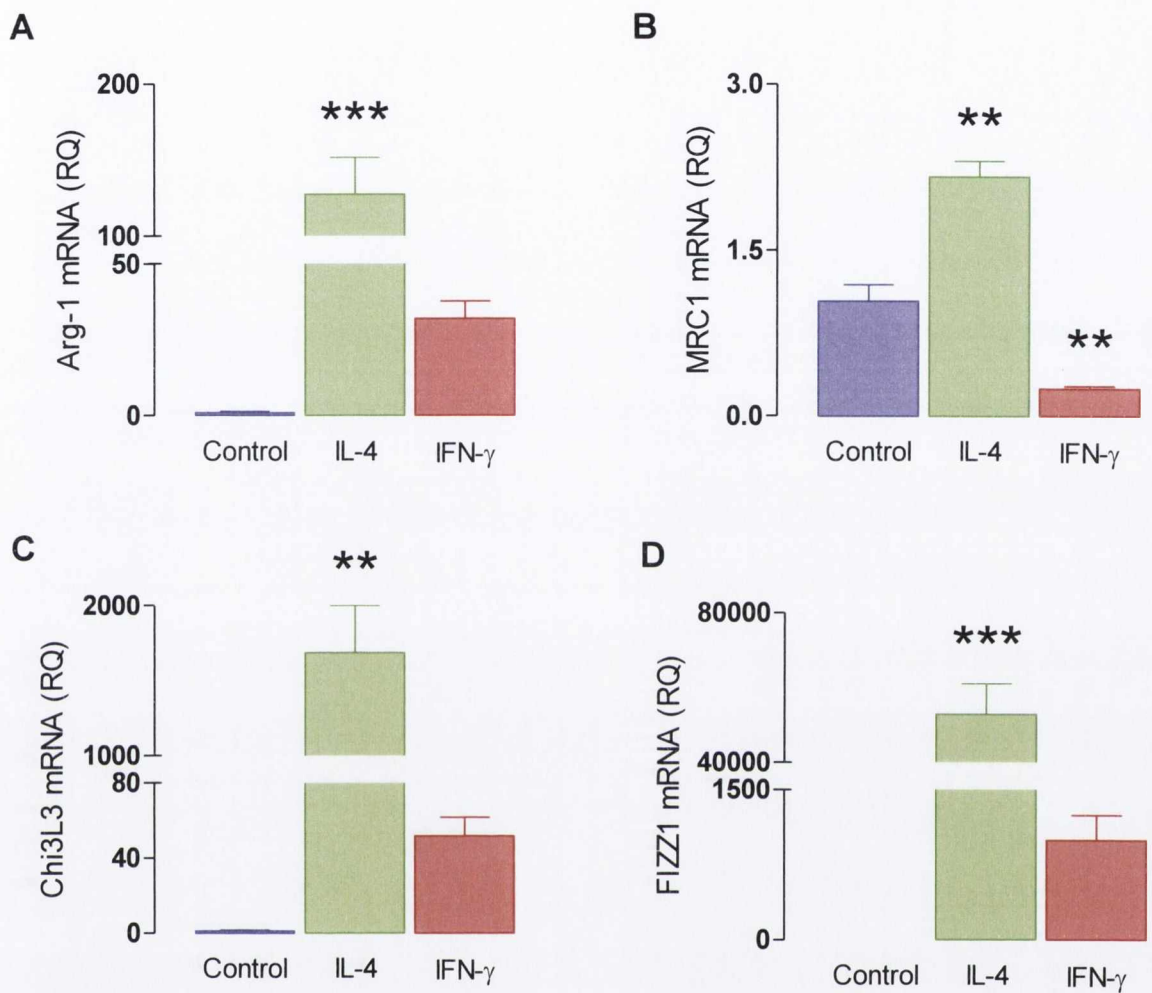


Figure 3.2 IL-4 increased expression of the M2 activation state markers in BMDMs

BMDMs were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA expression of M2 activation state markers. The data show that IL-4 significantly increased the mRNA expression of Arg-1 (A), MRC1 (B), CHI3L3 (C), and FIZZ1 (D). ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=4-5).

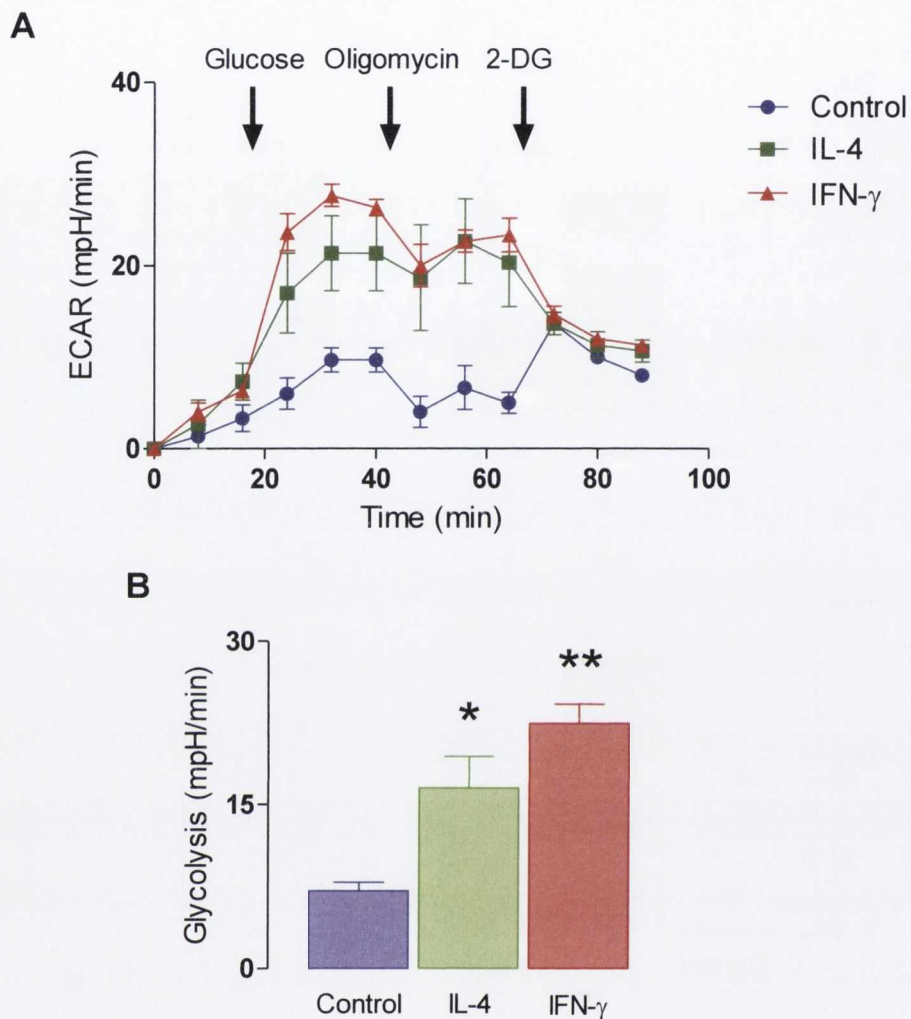


Figure 3.3 IFN- γ and IL-4 significantly increased glycolysis in BMDMs

BMDMs were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and a glycolytic flux assay was carried out to assess the metabolic signature of the cells. The Seahorse analyser was used to measure ECAR (A) and the data show that IFN- γ and IL-4 significantly increased the glycolytic rate of cells (B). * $p < 0.05$, ** $p < 0.01$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed as mean $\pm$ SEM (n=3).

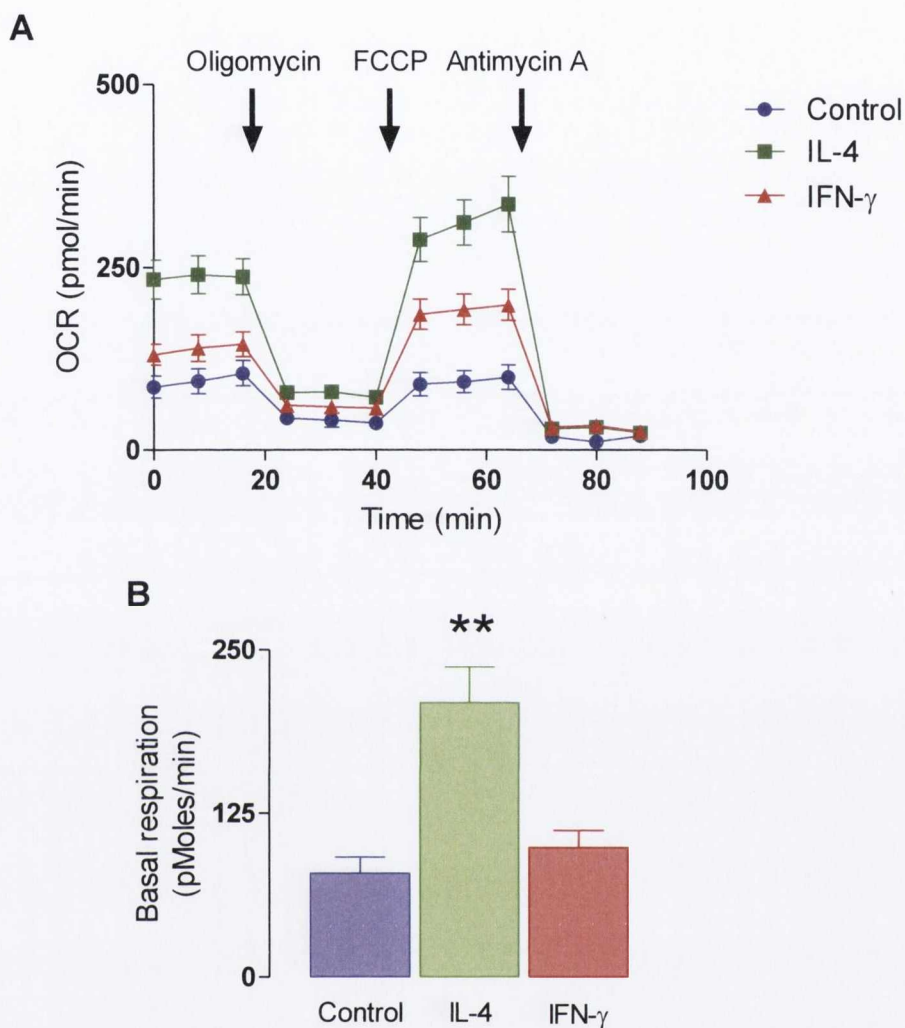


Figure 3.4 IL-4 significantly increased mitochondrial respiration in BMDMs

BMDMs were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and a mitochondrial stress test was carried out to assess the metabolic signature of the cells. The Seahorse analyser was used to measure OCR (A) and the data show that IL-4 significantly increased the basal respiration of cells (B). ** $p < 0.01$; one-way ANOVA with Dunnett's post hoc analysis s. Data are expressed as mean $\pm$ SEM (n=4).

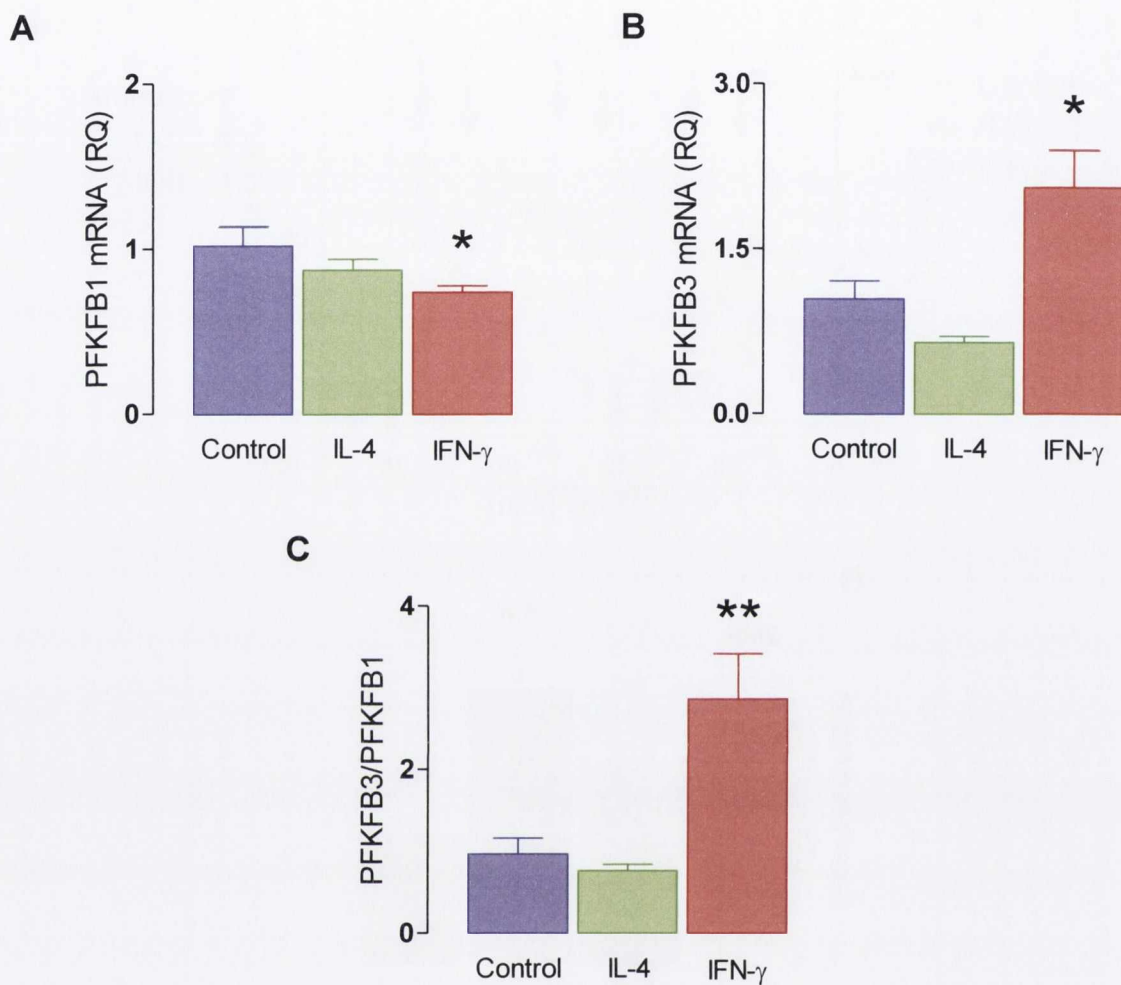


Figure 3.5 IFN-γ significantly increased PFKFB3:PFKFB1 in BMDMs

BMDMs were incubated with media alone, IL-4 (20ng/mL), or IFN-γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA expression of PFKFB1 and PFKFB3. The data show that the IFN-γ significantly decreased the mRNA expression of PFKFB1 (A), while PFKFB3 expression was significantly increased by IFN-γ (B). The PFKFB3:PFKFB1 expression ratio was significantly higher in IFN-γ stimulated BMDMs (C). * $p < 0.05$, ** $p < 0.01$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=5).

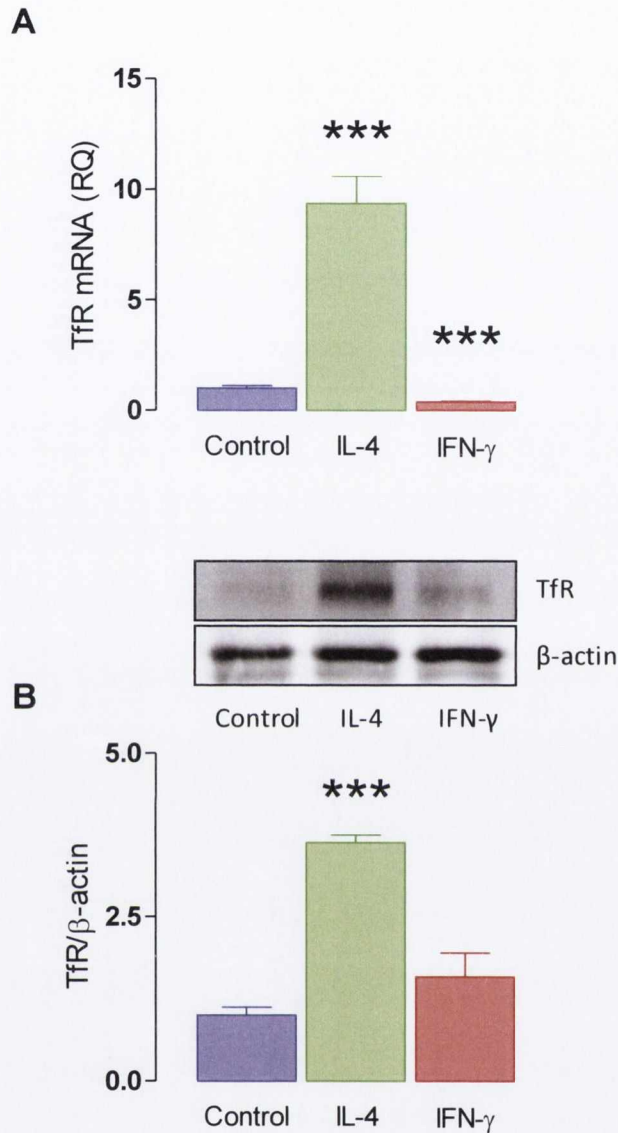


Figure 3.6 IL-4 significantly increased TfR in BMDMs

BMDMs were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA and protein expression of transferrin receptor. The data show that IL-4 significantly increased the mRNA expression of TrR while IFN- γ had the opposite effect (A). The protein expression is also significantly increased by IL-4 (B). Western immunoblotting data were normalised to β -actin and inserts illustrate representative blots for target proteins. *** $p < 0.001$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=3).

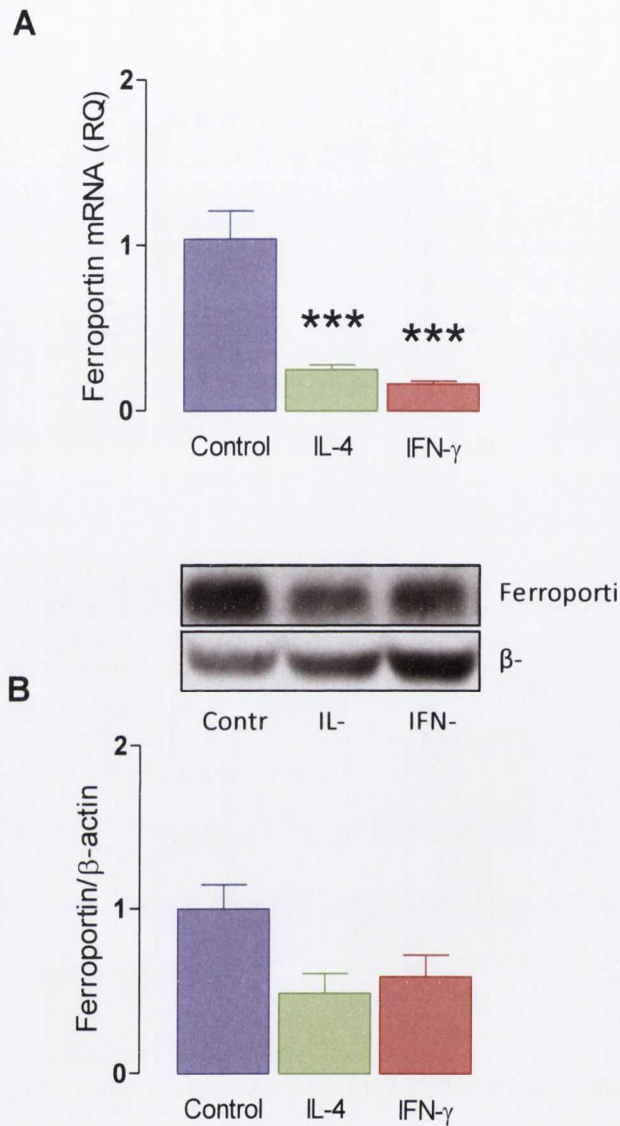


Figure 3.7 IL-4 and IFN- γ decreased ferroportin in BMDMs

BMDMs were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA and protein expression of ferroportin. The data show that the mRNA expression of ferroportin was significantly decreased by both IL-4 and IFN- γ (A), yet the protein expression was not significantly altered by either stimuli (B). Western immunoblotting data were normalised to β -actin and inserts illustrate representative blots for target proteins. ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=4-5).

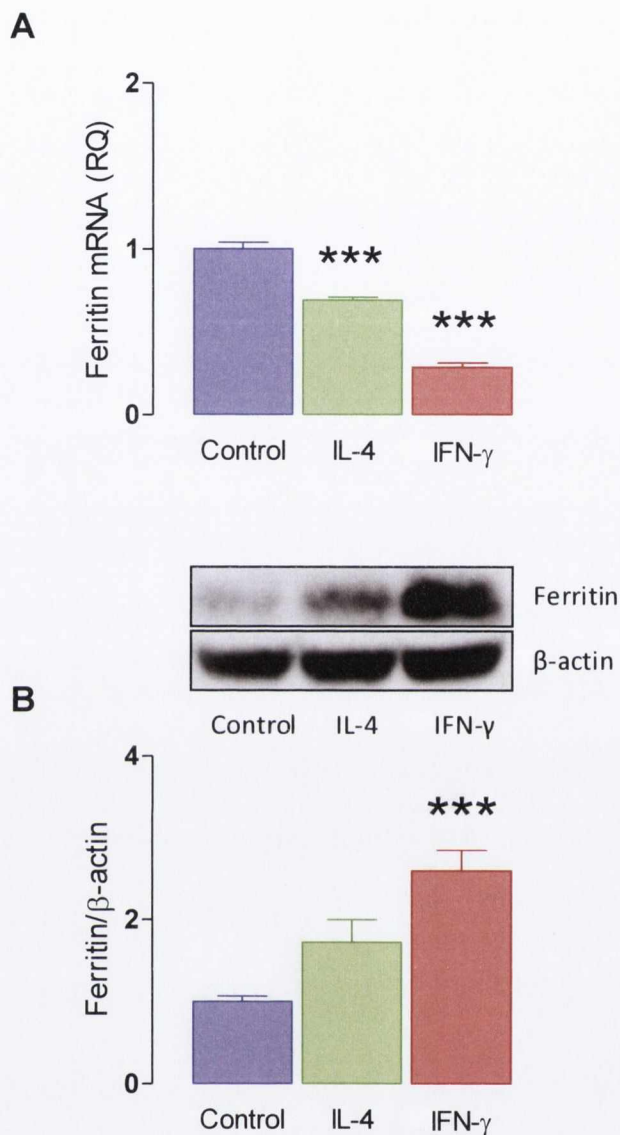


Figure 3.8 IL-4 and IFN- γ decreased the mRNA expression, but increased the protein expression of ferritin in BMDMs

BMDMs were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA and protein expression of ferritin. The data show that the mRNA expression of ferritin was significantly decreased by both IL-4 and IFN- γ (A), yet the protein expression was significantly increased by IFN- γ (B). Western immunoblotting data were normalised to β -actin and inserts illustrate representative blots for target proteins. * $p < 0.05$, *** $p < 0.001$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=4-6).

3.4 Discussion

The primary aim of this study was to characterise IFN- γ and IL-4 stimulation in BMDMs in terms of metabolic reprogramming and iron handling. The most significant findings are that IFN- γ , which predictably induced the M1 activation state, triggered an increase in glycolysis, correlating with increased expression of PFKFB3 and iron retention reflected by an increase in ferritin and decreased ferroportin and expression.

Macrophages are plastic cells that are capable of adopting different phenotypes in response to diverse activating stimuli. Classically- or M1-activated cells are considered pro-inflammatory, while alternatively- or M2-activated cells are associated with tissue repair and remodelling and are generally considered anti-inflammatory (Martinez et al., 2008). IFN- γ , the cytokine commonly used to induce the M1 activation state, was used here. Predictably, IFN- γ significantly increased the expression of the M1 activation state markers TNF α , iNOS and IL-1 β mRNA. LPS is often used in conjunction with IFN- γ to trigger M1 activation, but data have indicated that IFN- γ alone is capable of inducing classical activation of cells (Mosser, 2003).

Although a combination of IL-4 and IL-13 is often used to polarise cells to an M2-like phenotype (Scotton et al., 2005), IL-4 alone is also capable of inducing this state (Andrews et al., 2006). Here, IL-4 stimulation predictably increased the expression of Arg-1, MRC1, Chi3L3 and FIZZ1, which confirm previously described finding (Loke et al., 2002). IL-4 stimulated BMDMs also expressed significantly lower levels of TNF α mRNA expression than unstimulated cells, perhaps emphasising an anti-inflammatory phenotype.

As well as increasing the expression of M1 activation state markers in BMDMs, IFN- γ decreased MRC1 mRNA, which is consistently observed in the literature (Jablonski et al., 2015). In contrast to this, IFN- γ did not significantly affect the expression of Arg-1, Chi3L3 and FIZZ1, although

modest increases were observed. Macrophages upregulate the expression of iNOS in response to pro-inflammatory stimuli and it converts L-arginine to NO (Förstermann and Sessa, 2012), which is directly involved in microbial killing (Nathan and Hibbs, 1991). In contrast, Arg-1 expression is increased in IL-4 treated macrophages and competes with iNOS for L-arginine, hydrolysing it to L-ornithine which supports cell proliferation, collagen synthesis and tissue repair (Gordon, 2003a, Hesse et al., 2001). In the context of the present findings it is suggested that the relatively modest increase in Arg-1 expression in IFN- γ stimulated BMDMs would have a relatively small influence due to the very marked IFN- γ -induced increase in iNOS expression. However, in IFN- γ -stimulated BMDMs, expression of markers that are generally associated with the M2 activation state highlights the plasticity of these cells. The results are consistent with the view that absolute M1 or M2 activation state may be less reflective of reality, but that cells are capable of possessing multiple attributes simultaneously (Mosser and Edwards, 2008).

It has recently become apparent that metabolism and immune cell function are tightly linked (Rodriguez-Prados et al., 2010, Odegaard and Chawla, 2011, Olefsky and Glass, 2010b). Immune cells increase metabolism to support the energy demands of activation, with different metabolic pathways being employed under different circumstances. Generally, pro-inflammatory cells increase glycolysis and exhibit a disrupted TCA cycle to rapidly generate ATP and inflammatory mediators for acute activity (Liu et al., 2016). In contrast, anti-inflammatory cells display increased oxidative metabolism which is more efficient and sustainable for less urgent activity such as tissue repair (El Kasmi and Stenmark, 2015).

Metabolic reprogramming plays a role in facilitating the function of T cells (Buck et al., 2015, Man and Kallies, 2015), with effector CD4⁺ T cells exhibiting a robustly glycolytic phenotype to drive the inflammatory response (Michalek et al., 2011). mTOR has been identified as the primary mediator of this metabolic switch, as knockout of this kinase inhibits the differentiation

of effector T cells and instead leads to the generation of anti-inflammatory T_{reg} cells (Delgoffe et al., 2009). mTOR activity induces HIF-1 α expression in effector T cells (Shi et al., 2011), which promotes glycolysis through the upregulation of PFKFB3, GLUT1 and PDK1 activity (Iyer et al., 1998, Kim et al., 2006b, Minchenko et al., 2003). In contrast, T_{reg} cells display a much more oxidative metabolic profile, deriving energy from FAO and mitochondrial respiration (Michalek et al., 2011) in a PPAR- γ dependent manner (Hontecillas and Bassaganya-Riera, 2007).

Activated DCs also increase glycolysis to support their immune function (Pearce and Everts, 2015), demonstrated by increased ECAR in response to LPS (Everts et al., 2014), and similar metabolic reprogramming events have been reported in macrophages (Galvan-Pena and O'Neill, 2014). LPS induces increased HIF-1 α activity and PFKFB3 mRNA expression in atherosclerotic and peritoneal macrophages (Rodriguez-Prados et al., 2010, Tawakol et al., 2015), which facilitates increased glycolysis. Enhanced glycolysis has also been demonstrated by increased ECAR in LPS-stimulated BMDMs (Haschemi et al., 2012a). Increased oxidative metabolism in anti-inflammatory M2 BMDMs has been demonstrated by increased OCR (Nomura et al., 2016), which was found to be dependent on CPT system of mitochondrial β -oxidation of long chain fatty acids.

By targeting and manipulating metabolic pathways it is possible to manipulate the immune functions of cells. For example, inhibition of glycolysis by 2-DG profoundly attenuates IFN- γ secretion from T_h1 cells (Zheng et al., 2009), and inhibition of mTOR by rapamycin attenuates IL-17 secretion from T_h17 cells (Kopf et al., 2007). In macrophages, blocking glycolysis with dehydroepiandrosterone or oxamate attenuates the production of TNF α and IL-6 from LPS-stimulated RAW264.7 macrophages (Haschemi et al., 2012a), and siRNA knockdown of PFKFB3 attenuates iNOS and COX-2 mRNA expression in peritoneal macrophages (Rodriguez-Prados et al., 2010). In contrast, inhibition of oxidative metabolism via siRNA

targeting of PGC-1 β inhibits M2 activation in BMDMs, as measured by attenuated arginase activity, and potentiates the M1 phenotype (Vats et al., 2006). This highlights the potential for targeting metabolic changes as immunotherapies for the treatment of inflammatory diseases.

In the current study, IFN- γ -stimulated BMDMs displayed increased glycolysis, and IL-4 stimulated BMDMs exhibited increased oxidative metabolism. M2 activated cells also demonstrated an increase in glycolytic activity when compared with unstimulated cells, which has not been previously reported, but may be suggestive of a general increase in metabolic activity. Other studies have reported increases in ECAR in BMDMs stimulated with LPS (Haschemi et al., 2012a) or a combination of LPS and IFN- γ (Huang et al., 2014), but the present data suggest that IFN- γ alone is sufficient to induce metabolic changes in macrophages. Increases in basal respiration and maximal respiration in M2 activated BMDMs are consistent with changes reported in peritoneal macrophages polarised to an M2 phenotype in response to IL-4 (Huang et al., 2014) and the apolipoprotein A-I mimetic peptide 4F (Datta et al., 2015). However, the effect of IL-4 on oxidative metabolism is disputed, with RAW264.7 macrophages failing to respond to IL-4 stimulation by altering OCR (Haschemi et al., 2012a).

In the present study, M1 macrophages expressed increased levels of PFKFB3 and decreased levels of PFKFB1, correlating with the increased glycolytic rate demonstrated in these cells. In contrast, PFKFB3 expression in M2 macrophages was decreased. These bifunctional kinase/phosphatase enzymes play a role in controlling the rate of glycolysis by altering the rate of F2,6BP production (Kelly and O'Neill, 2015). This is a potent activator of PFK-1, the enzyme that mediates the rate-limiting step in the glycolytic pathway (Atsumi et al., 2005). The PFKFB1 isozyme is associated with respiration under normal physiological conditions and possesses a 1:1 ratio of kinase:phosphatase activity. However, the kinase:phosphatase activity for PFKFB3 is 740:1 due to a mutated phosphatase active site (Sakakibara et al.,

1997), leading to increased F2,6BP production and a heightened glycolytic rate. Interestingly, the *pfkfb3* gene possesses an AU-rich instability region, which is generally present in immune genes capable of inflicting damage, to deter aberrant activity (Chesney et al., 1999). The findings of the present study are consistent with reports demonstrating increased PFKFB3 expression in M1 macrophages (Tawakol et al., 2015, Ruiz-Garcia et al., 2011), but here, changes were induced by IFN- γ stimulation, while previous studies only described these changes following LPS stimulation. Although IL-4 did not induce expression of PFKFB1, this is consistent with the literature (Tawakol et al., 2015), and there was an IL-4-induced decrease in the PFKFB3:PFKFB1 expression in BMDMs. These data indicate that PFKFB3:PFKFB1 correlates with the metabolic signature demonstrated by analysis using the SeaHorse technology, and could be used as a proxy marker of metabolic reprogramming in activated BMDMs. While it is important to recognise that the assessment of enzymatic activity would be more beneficial to attest to metabolic switching than examining changes in mRNA expression, previous reports have demonstrated a correlation between PFKFB3 mRNA expression and F2,6BP production (Rodriguez-Prados et al., 2010).

IFN- γ decreased the mRNA expression of TfR and ferroportin in the present study, suggesting diminished iron cycling through transferrin-mediated iron influx and ferroportin mediated efflux. Assessing ferritin expression in M1 macrophages yielded conflicting results between mRNA and protein levels. An increase in protein was observed in IFN- γ stimulated BMDMs despite lower levels of mRNA expression. However, as protein is the functional entity, this suggests that M1 macrophages increase ferritin-mediated iron storage. In IL-4 stimulated BMDMs, an increase in TfR and decrease in ferroportin was observed. However, similar to M1 activated cells, changes in the mRNA and protein expression of ferritin in response to IL-4 revealed conflicting results, with the protein expression significantly increasing.

The decrease in TfR and ferroportin expression, and the increased ferritin expression observed in IFN- γ -stimulated BMDMs is consistent with the literature and highlight the importance of iron handling in M1 macrophages (Corna et al., 2010, Recalcati et al., 2010). M1 macrophages retain iron through low uptake, high storage, and low exportation of the metal, mediating bacteriostatic and tumourostatic activity (Biswas and Mantovani, 2012). Although the increased TfR expression observed in M2 BMDMs was consistent with the literature (Corna et al., 2010), the changes in ferritin and ferroportin was unexpected (Recalcati et al., 2010, Cairo et al., 2011). However, glucocorticoid treatment of blood-derived macrophages induces an M2 phenotype that correlates with increased ferritin and ferroportin expression (Vallelian et al., 2010), which suggests that differential expression of these markers in polarised macrophages is not definite. The iron handling properties of M2 macrophages are characterised high iron influx, low iron storage, and high iron efflux, facilitating immunoregulation, matrix remodelling, and cell proliferation (Biswas and Mantovani, 2012).

Due to the level of inconsistency, it may be beneficial to examine the differential expression of other proteins associated with modulating iron handling in BMDMs following polarisation. For example, divalent metal ion transporter 1 (DMT1) is another protein involved in iron uptake and sequestration, is increased in LPS treated PBMCs (Theurl et al., 2008). Hepcidin, which is upregulated by IFN- γ and IL-6 (Urrutia et al., 2013), modulates iron handling by inducing the internalisation of ferroportin, thus inhibiting ferroportin mediated iron efflux.. The expression of these proteins serves as a proxy marker of iron handling properties of polarised macrophages, but measurements of iron flux would be essential in definitely determining the iron homeostasis of these cells.

The data presented in this study demonstrate that that polarisation of M1 and M2 BMDMs by IFN- γ and IL-4 respectively is associated with metabolic changes that correlate with differential expression of PFKFB isozymes, and

altered iron handling properties, as demonstrated by differential expression of proteins involved in controlling iron homeostasis.

Chapter 4: Characterisation of the activation states of polarised glia

4.1 Introduction

Microglia are resident mononuclear phagocytes of the CNS. Although ontogenetically distinct (Ginhoux et al., 2013), they are oft termed macrophages of the brain due to the striking functional similarities the cells exhibit. Microglia are responsible for a range of regulatory processes important for maintaining homeostasis in the brain, including the clearance of pathogens, dying cells, debris and aberrant proteins. Similar to macrophages, microglia exist in a relatively quiescent state under normal physiological conditions, which is partly due to interactions with neurons and astrocytes. Upon detection of an activating agent, microglia can adopt different phenotypes, depending on the specific stimulus, to mount an appropriate immune response (Lynch, 2009).

The characterisation of microglial phenotypes *in vitro* mirrors that of macrophages. However, it is important to consider these cells as distinct and their responses are not necessarily the same. Similar to macrophages, the M1 activation state of microglia is induced by PAMPs, DAMPs and pro-inflammatory cytokines, resulting in increased expression of iNOS, TNF α and IL-1 β (Wu et al., 2009). Although acute activation of pro-inflammatory M1 microglia plays an important role in host defence, chronic M1 activation is associated with most, if not all neurodegenerative disorders (Lynch, 2014, Tang and Le, 2016). In contrast to this, the M2 activation state is anti-inflammatory, promoting tissue repair and the resolution of inflammation. This phenotype is induced by anti-inflammatory cytokines, such as IL-4 and IL-13, and is characterised by upregulation of activation state markers Arg-1, MRC1, ChiL3 and FIZZ1.

Although microglia are the primary immune cells of the CNS, astrocytes also display some immunological characteristics, as they have antigen presenting capabilities and are capable of phagocytosis (Jones et al., 2013) and the secretion of pro-inflammatory cytokines and chemokines (Choi et al., 2014). Like microglia, expression of activation state markers is upregulated in

response to polarising stimuli, and there is evidence that they are capable of adopting distinct phenotypes (Jang et al., 2013).

The limitations of identifying cells as M1 or M2 phenotypes have been discussed (Martinez and Gordon, 2014, Mosser and Edwards, 2008) and functionally defined microglia is the ideal. Similarly, defining cells by their metabolic state is relevant for our understanding of functional consequences of activation (Heppner et al., 2015), and the objective of the current study is to assess the metabolic signature of IFN- γ and IL-4 stimulated cells.

As described in Chapter 3, an increased glycolytic rate is a hallmark of rapidly activated immune cells, such as LPS-activated macrophages and DCs, NK cells, Th17, Th1, Th2, activated effector CD8⁺ T cells, and activated B cells (Pearce and Pearce, 2013). It is hypothesised that increasing glycolytic flux enables these cells to generate robust quantities of ATP rapidly to the meet the sizable increase in energy demands. In contrast to this, increased TCA cycle activity is associated with immune cells that are not inflammatory in nature and exhibit increased cellular lifespans, such as anti-inflammatory M2 macrophages, T_{reg} cells, and memory T cells (Pearce and Pearce, 2013). Metabolic reprogramming has been reported in BV2 microglial cells in response to LPS (Voloboueva et al., 2013, Orihuela et al., 2016), but descriptions of metabolic profiles in microglia are lacking.

Many factors are associated with the metabolic reprogramming of M1 activated macrophages. HIF-1 α plays a pivotal role in mediating this switch (Liu et al., 2016), inducing PFKFB3 expression which facilitates increased glycolytic flux (Obach et al., 2004), which was demonstrated in Chapter 3. HIF-1 α also increases PDK1 activity, which inhibits the conversion of pyruvate to acetyl-CoA thus inhibiting the TCA cycle and downregulating oxidative respiration (Kim et al., 2006a, Papandreou et al., 2006), and GLUT1 expression, necessary to provide the cell with adequate glucose (Freemerman et al., 2014). GLUT1-mediated glucose uptake is also induced by AMPK (Wu et al., 2013). However, AMPK activation antagonises HIF-1 α -induced

glycolysis in DCs (Krawczyk et al., 2010) and is associated with M2 polarisation of immune cells as it increases mitochondrial FA metabolism (Winder et al., 2000). PGC-1 β expression is induced by AMPK and IL-4-induced STAT6 signalling (Vats et al., 2006) which induces increased FA oxidation, TCA cycle activity, and oxidative phosphorylation (Shao et al., 2010). Knockdown of PGC-1 β impairs the metabolic profile and function of M2 macrophages (Vats et al., 2006), demonstrating the importance of metabolic reprogramming for immune functions.

It has been shown that iron retention is a characteristic of M1 macrophages, whereas M2 cells increase iron recycling. Iron handling by glia has not been examined in any detail but it is likely iron metabolism plays an important role in immunological function in the CNS, as iron deposition is observed in the chronically inflamed CNS (Andersen et al., 2014) and neurodegeneration is accompanied by iron and ferritin accumulation in microglia (Thomsen et al., 2015).

The aims of the current study are:

1. To establish whether there is altered expression of proteins that are associated with changes in metabolic profile and iron handling in M1 and M2 polarised microglia.
2. To determine whether astrocytes adopt M1-like and M2-like activation states and adopt metabolic profiles associated with these phenotypes.

4.2 Methods

Primary microglia and astrocytes were polarised to M1-like or M2-like and harvested for analysis of mRNA and protein expression by RT-PCR and Western immunoblotting respectively. The metabolic signature of cells was determined using glycolytic flux and mitochondrial stress tests with the SeaHorse XF analyser. These methods are described in detail in Chapter 2.

4.3 Results

Microglia were stimulated with IL-4 or IFN- γ and mRNA expression of markers of microglial activation were investigated by RT-PCR. IL-4 and IFN- γ significantly increased mRNA expression of CD11b ($*p<0.05$; $**p<0.01$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.1A) and CD40 ($**p<0.01$, $***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.1B).

To confirm IFN- γ - and IL-4-induced polarisation of microglia into M1-like or M2-like phenotypes respectively, activation state markers were assessed by RT-PCR. IFN- γ significantly increased TNF α ($**p<0.01$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.2A), iNOS ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.2B) and IL-1 β ($**p<0.01$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.2C) mRNA. IL-4 had no effect on TNF α , iNOS or IL-1 β mRNA expression (Figure 4.2A, B, C).

IL-4 induced the polarisation of microglia to an M2-like phenotype, as confirmed by significant increases in mRNA expression of Arg-1 ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.3A), MRC1 ($**p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.3B), Chi3L3 ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.3C) and FIZZ1 ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.3D). No significant IFN- γ -induced changes were observed in Arg-1, MRC1, Chi3L3 or FIZZ1 (Figure 4.3A, B, C, D). The metabolic signature of polarised microglia was investigated using the Seahorse XF flux analyser. A glycolytic stress test indicated that M1-like microglia have significantly higher rates of glycolysis than unstimulated ($**p<0.01$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.4B) and M2-like cells ($*p<0.05$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.4B). IL-4 had no effect on the rate of glycolysis (Figure 4.4B). The maximal glycolytic capacity and glycolytic reserve were unchanged in M1-like and M2-like microglia (Figure 4.4C, D).

Oxidative respiration was assessed using a mitochondrial stress test (Figure 4.5A) and the data revealed a significantly increased basal respiration in M2-like microglia (** $p < 0.01$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.5B). A significant increase in maximal respiration was also observed in these IL-4 stimulated cells (* $p < 0.05$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.5C), accompanied with a higher rate of ATP production (** $p < 0.05$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.5D). IFN- γ stimulation had no effect on the basal respiration, maximal respiration, or ATP production (Figure 4.5B, C, D) of microglia.

By assessing the ratio of ECAR to OCR, the SeaHorse technology provides an indication of the metabolic pathways being utilised by cells. A high OCR/ECAR indicates a preferential use of sustainable oxidative metabolism over anaerobic means of ATP production. In contrast, a higher ECAR/OCR suggests that the cells are utilising glycolysis in lieu of more sustainable oxidative metabolic pathways. OCR/ECAR was significantly decreased in M1-like microglia and increased in M2-like microglia (* $p < 0.05$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.6A). Conversely, ECAR/OCR was significantly increased in M1-like microglia and decreased in M2-like microglia (* $p < 0.05$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.6B).

The data in Chapter 3 suggested that increased glycolysis was associated with increased PKFB3 and, here, the possibility that this correlation also exists in microglia was assessed. IFN- γ significantly increased PFKFB3 mRNA expression (* $p < 0.05$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.7A), although no change was observed at the protein level as indicated by the data obtained from Western immunoblotting (Figure 4.7B). IL-4 did not affect PFKFB3 mRNA or protein. IFN- γ significantly decreased PFKFB1 mRNA expression (* $p < 0.05$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.7A), although this effect was not observed at the protein level (Figure 4.8B) and IL-4 exerted no effect on PFKFB1 mRNA or protein (Figure 4.8A, B). IFN- γ significantly increased

PFKFB3/PFBFB1 ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.9A), while IL-4 had no effect.

HIF-1 α is considered to be a crucial element responsible for the metabolic reprogramming of M1-polarised macrophages (Cramer et al., 2003) and analysis of HIF-1 α by Western immunoblotting showed that it was not significantly affected by IL-4 or IFN- γ , although IFN- γ increased its expression 2.5 fold (Figure 4.10A). HIF-1 α has been shown to decrease metabolism through the TCA because it activates PDK1 which, in turn, inhibits pyruvate dehydrogenase that is required to drive the TCA cycle (Kim et al., 2006a). Because of the marked, albeit insignificant, IFN- γ -induced increase in HIF-1 α , PDK1 expression was assessed. However, its expression was not affected by either IFN- γ or IL-4 (Figure 4.10B).

AMPK activity and PGC-1 β have been associated with the M2 polarisation of macrophages. AMPK is activated following phosphorylation, and so the expression of phospho-AMPK was assessed and analysed relative to the total AMPK expression. A small but insignificant increase in AMPK activation was observed in IL-4-stimulated microglia (Figure 4.11A). The expression of PGC-1 β was not altered in M1-like or M2-like microglia (Figure 4.11B).

GLUT1 is a plasma membrane protein that facilitates the influx of glucose into the cytosol that is upregulated by both AMPK and HIF-1 α to provide the cell with more glucose to meet the metabolic demands of activation. GLUT1 expression in microglia was not affected by IFN- γ or IL-4 (Figure 4.12A).

In Chapter 3, the M1 phenotype in BMDMs was associated with iron retention, while the M2 phenotype exhibited increased iron transport. Here, expression of TfR, ferritin, and ferroportin were examined in microglia treated with IFN- γ and IL-4. TfR mRNA expression was significantly increased in IL-4-stimulated microglia and significantly decreased in IFN- γ -stimulated cells ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.13A). However, neither stimulus induced changes in

expression at the protein level (Figure 4.13B). IFN- γ also decreased ferroportin mRNA ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.14A), similar to its effect on TfR, and this is consistent with its induction of iron sequestration. Consistent with this, IFN- γ increased ferritin mRNA ($**p<0.01$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.15A) and induced a marked, yet insignificant, increase in protein levels (Figure 4.15B). However IL-4 also decreased ferroportin mRNA ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.14A), while neither stimulus affected its protein levels (Figure 4.14B).

Astrocytes share some immune functions with microglia and here the effects of IFN- γ and IL-4 were assessed on expression of markers of activation in polarised astrocytes. IL-4 did not induce any changes in GFAP or GLAST expression (Figure 4.16A, B), but IFN- γ increases the expression of GFAP (Figure 4.16A), although this increase did not reach statistical significance, and GLAST ($**p<0.05$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.16B).

IFN- γ increased expression of TNF α and iNOS ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.17A, B) but not IL-1 β mRNA (Figure 4.17C). IL-4 decreased the expression of TNF α ($**p<0.01$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.17A), but did not induce any changes in the expression of iNOS or IL-1 β (Figure 4.17B, C). IL-4 increased the expression of Arg-1, MRC1, Chi3L3 and FIZZ1 ($**p<0.01$, $***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.18A-D), while IFN- γ did not affect the expression of these markers (Figure 4.18A, B, C D).

The metabolic profiles of M1-like and M2-like astrocytes were assessed using the SeaHorse XF flux analyser and the glycolytic flux test (Figure 4.19A) revealed that both IFN- γ and IL-4 increased glycolysis ($**p<0.01$; one-way ANOVA with Dunnett's post hoc analysis; Figure 4.19B). No changes were observed in the glycolytic capacity or glycolytic reserve with either stimulus (Figure 3.19C, D). A mitochondrial stress test was carried out to assess the

impact of IFN- γ and IL-4 on oxidative metabolism in astrocytes (Figure 4.20A), but this did not reveal any differences in basal metabolic rate, maximal respiratory capacity or ATP production (Figure 4.20B-D

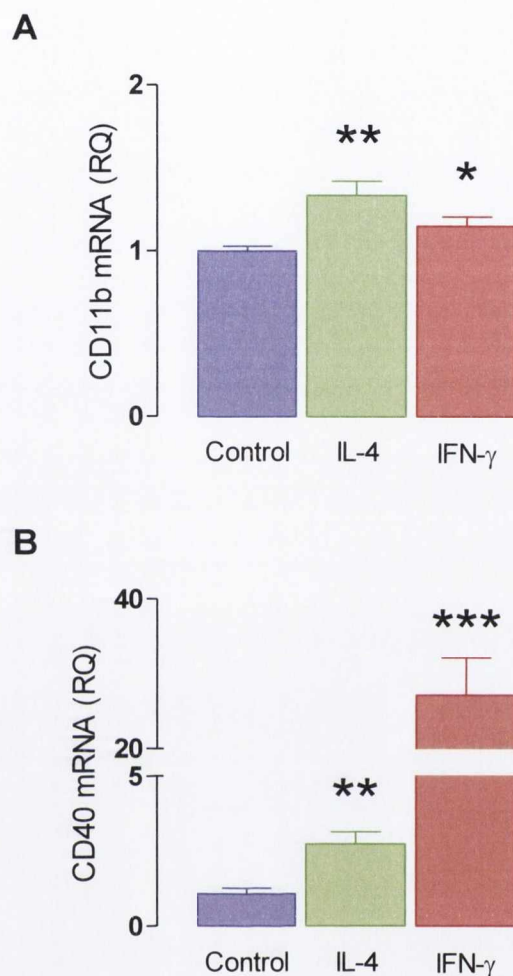


Figure 4.1 IL-4 and IFN- γ increased the mRNA expression of markers of microglial activation

Microglia were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA expression of markers of activation. The data show that IL-4 and IFN- γ induced significant increases in the the mRNA expression of CD11b (A), and CD40 (B). * p <0.05, ** p <0.01, *** p <0.001; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=5-6).

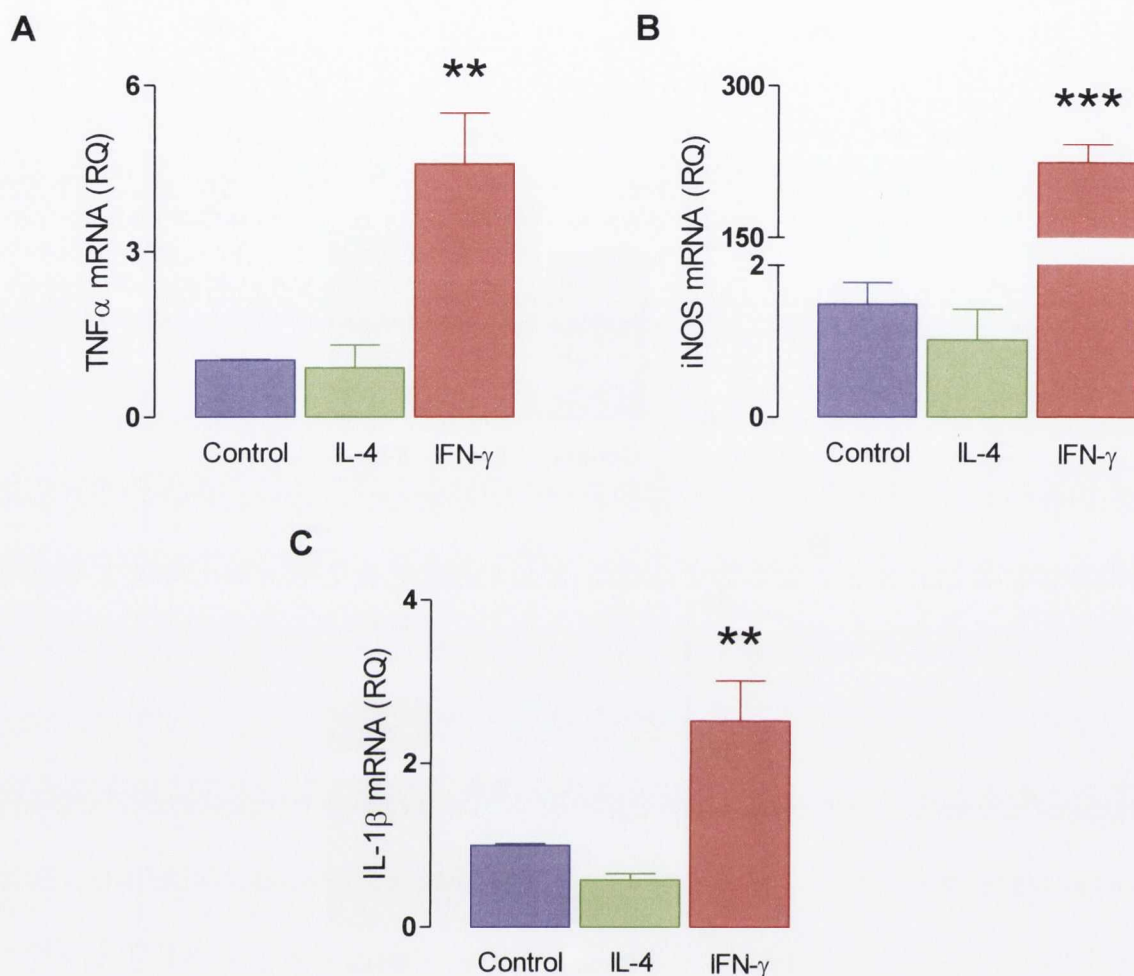


Figure 4.2 IFN- γ increased the mRNA expression of markers of the M1 activation state in microglia

Microglia were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA expression of M1 activation state markers. The data show that IFN- γ significantly increased the mRNA expression of TNF α (A), iNOS (B), and IL-1 β (C). ** p <0.01, *** p <0.001; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=3-5).

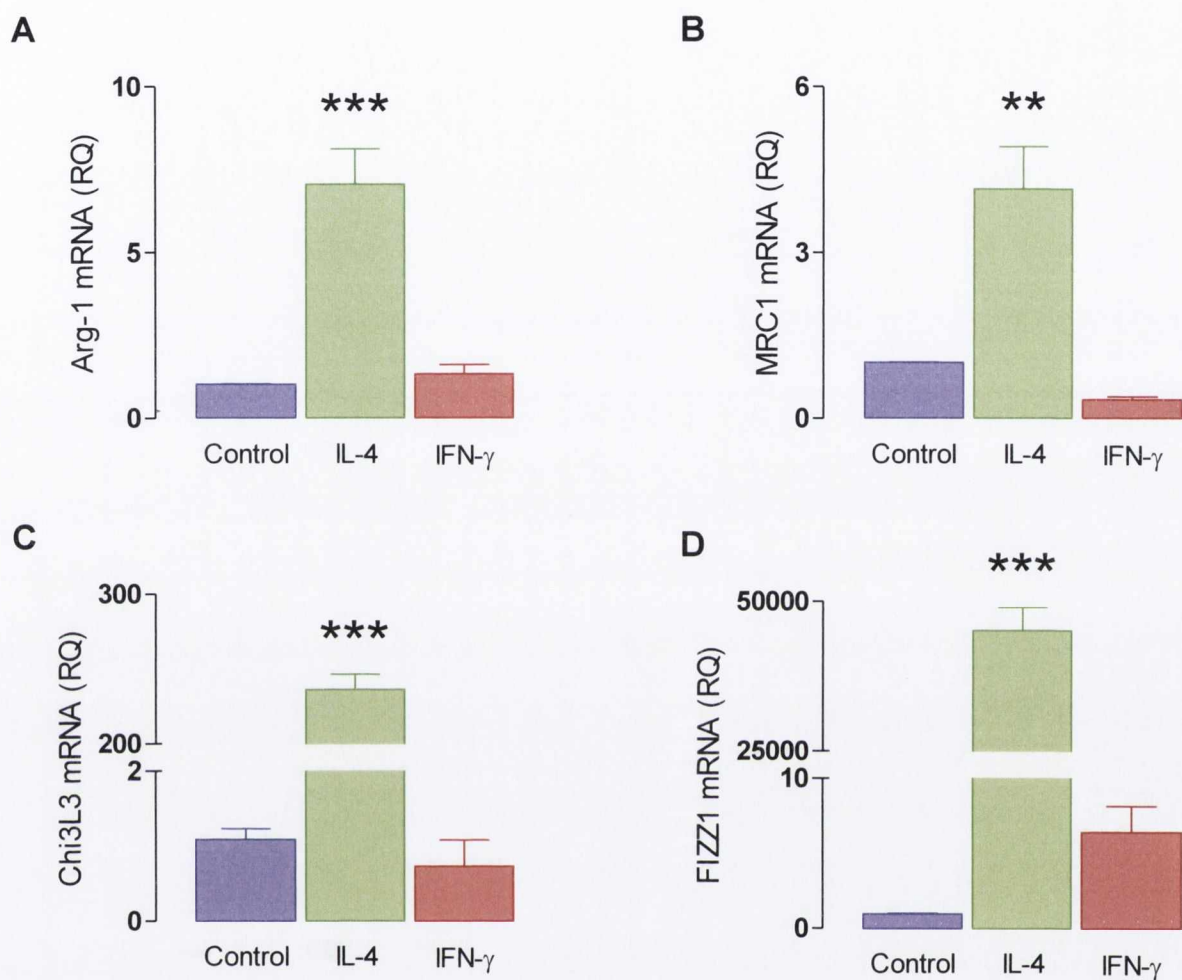


Figure 4.3 IL-4 increased the mRNA expression of markers of the M2 activation state in microglia

Microglia were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA expression of M2 activation state markers. The data show that IL-4 significantly increased the mRNA expression of Arg-1 (A), MRC1 (B), Chi3L3 (C), and FIZZ1 (D). ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=3-5).

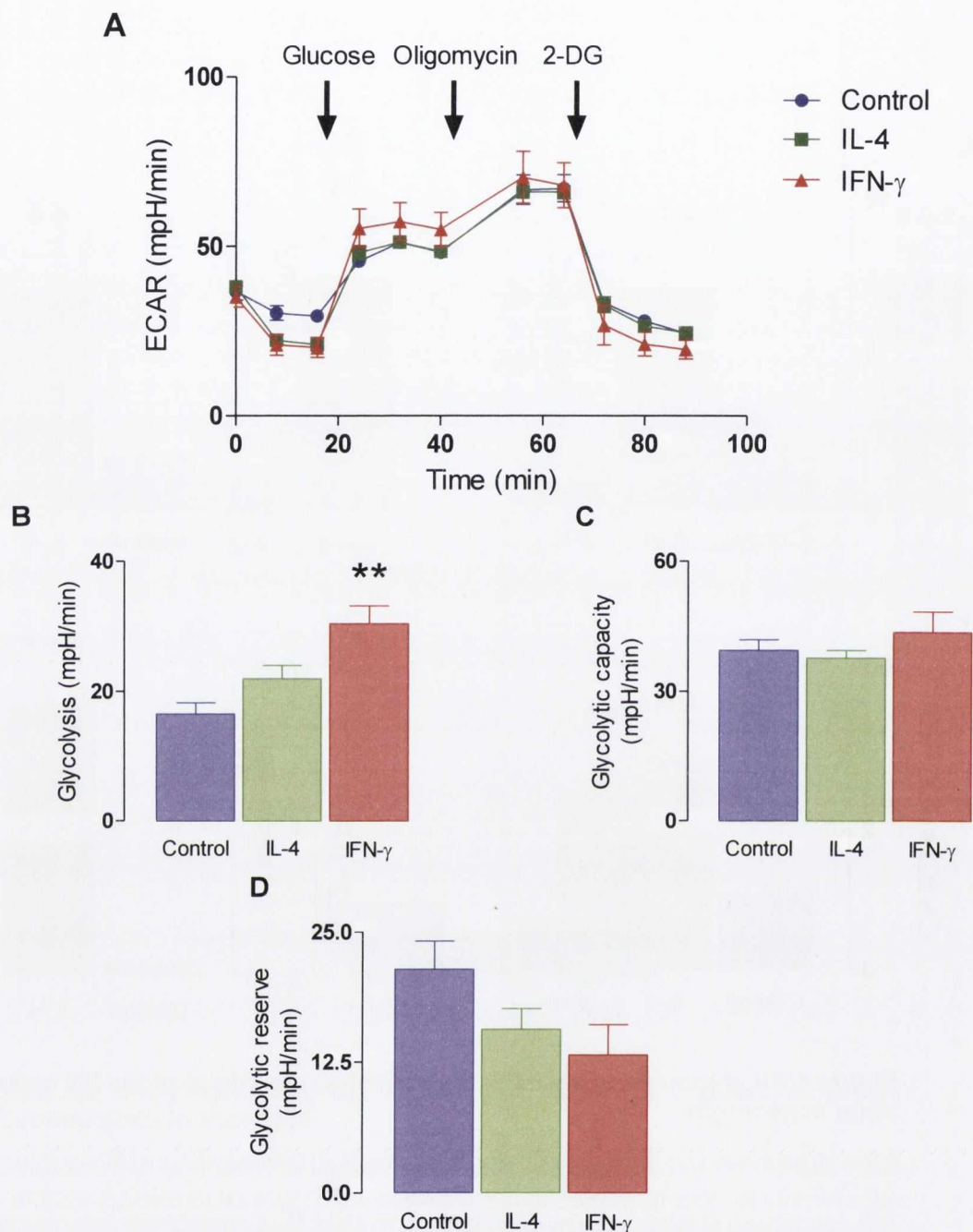


Figure 4.4 IFN- γ significantly increased glycolysis in microglia

Microglia were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and a glycolytic flux assay was carried out to assess the metabolic signature of the cells. The SeaHorse analyser was used to measure ECAR (A) and the data show that IFN- γ significantly increased glycolysis (B) but not glycolytic capacity (C) or glycolytic reserve (D). ** $p < 0.01$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed as mean $\pm$ SEM (n=6).

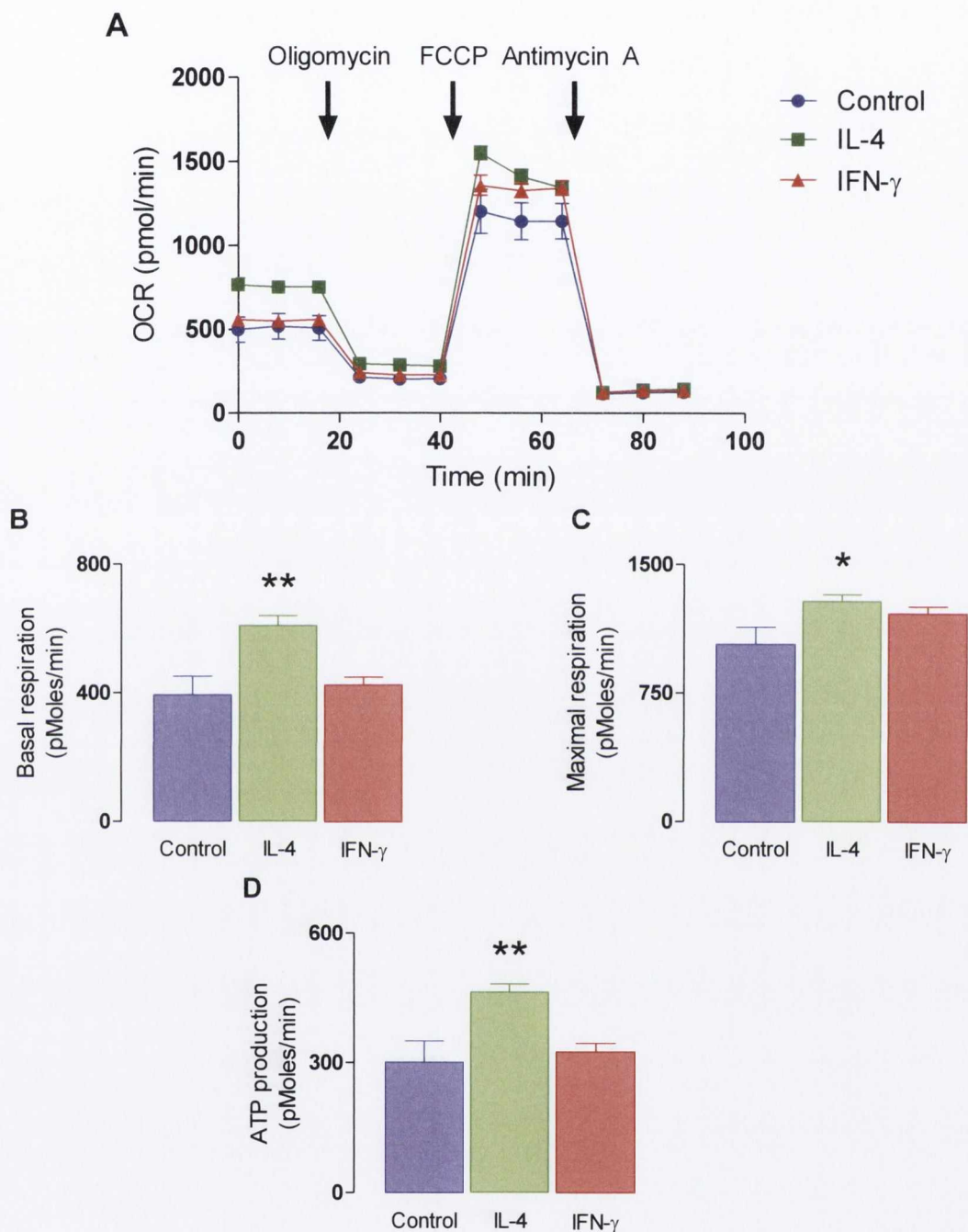


Figure 4.5 IL-4 significantly increased mitochondrial respiration in microglia

Microglia were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and a mitochondrial stress test was carried out to assess the metabolic signature of the cells. The Seahorse analyser was used to measure OCR (A) and IL-4 significantly increased basal respiration of cells (B). IL-4 also increased the maximal respiration (C) and ATP production (D) of microglia. * $p < 0.05$, ** $p < 0.01$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed as mean $\pm$ SEM (n=6).

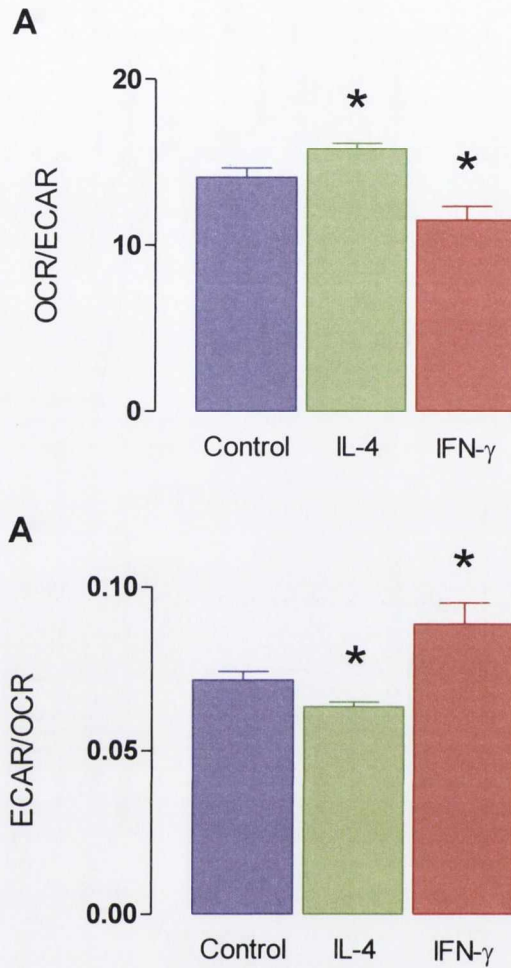


Figure 4.6 IFN- γ decreased OCR/ECAR and increased ECAR/OCR ratio in microglia, while IL-4 had the opposite effect

Basal ECAR and OCR values from the mitochondrial stress test were used to calculate ECAR/OCR (A) and OCR/ECAR (B) in microglia. The data show that IFN- γ and IL-4 had opposite effects on the induction of a metabolic signature in microglia. * $p < 0.05$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed as mean $\pm$ SEM (n=5).

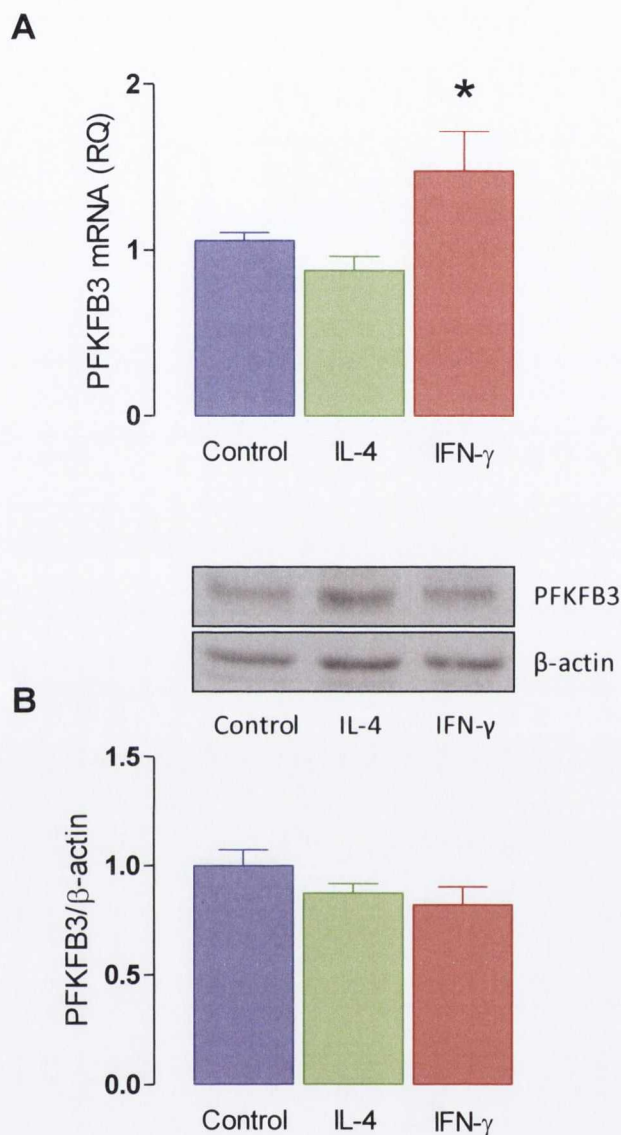


Figure 4.7 IFN- γ significantly increased PFKFB3 mRNA in microglia

Microglia were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA and protein expression of PFKFB3. The data show that the IFN- γ significantly increased the mRNA expression of PFKFB3 (A), however no change was observed at the protein level (B). Western immunoblotting data were normalised to β -actin and inserts illustrate representative blots for target proteins. * $p < 0.05$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=6-9).

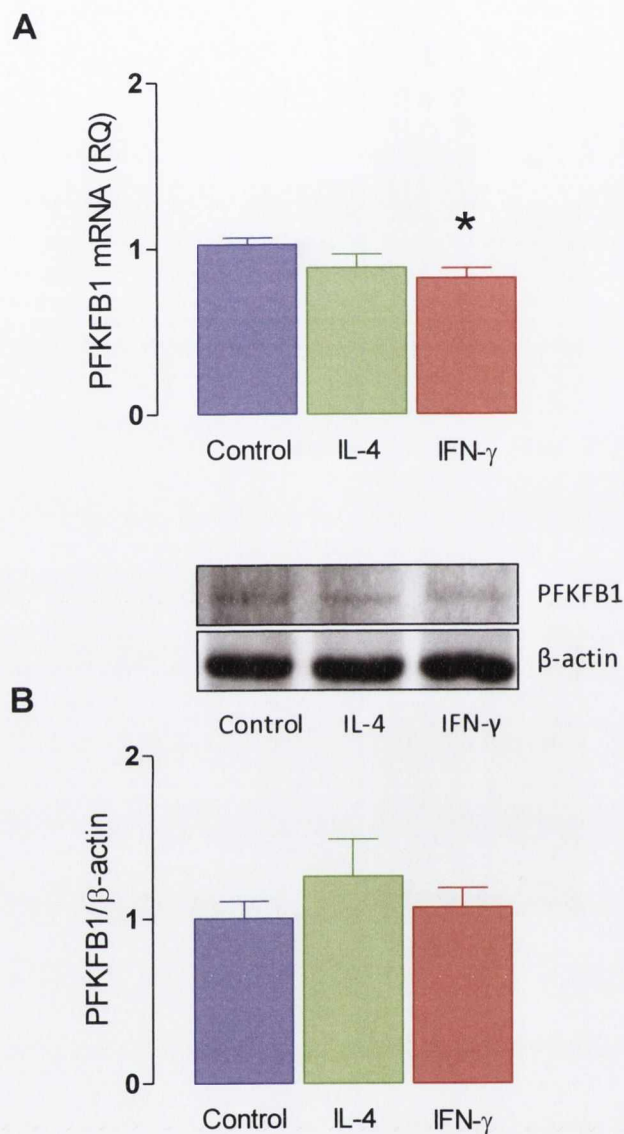


Figure 4.8 IFN- γ significantly decreased the PFKFB1 mRNA in microglia

Microglia were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA and protein expression of PFKFB1. The data show that the IFN- γ significantly decreased the mRNA expression of PFKFB1 (A), however no change was observed at the protein level (B). Western immunoblotting data were normalised to β -actin and inserts illustrate representative blots for target proteins. * $p < 0.05$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed as mean $\pm$ SEM (n=6-9).

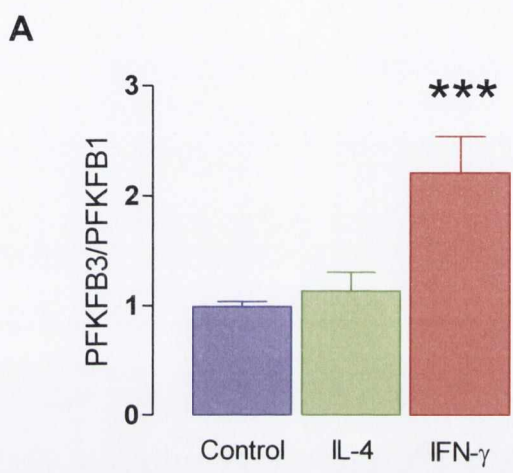


Figure 4.9 IFN-γ significantly increased PFKFB3/PFKFB1 in microglia

Microglia were incubated with media alone, IL-4 (20ng/mL), or IFN-γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA and protein expression of PFKFB3. The data show that the IFN-γ significantly increased the mRNA expression of PFKFB3/PFKFB1, while IL-4 had no effect (A). *** $p < 0.001$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed relative to control as mean ± SEM (n=9).

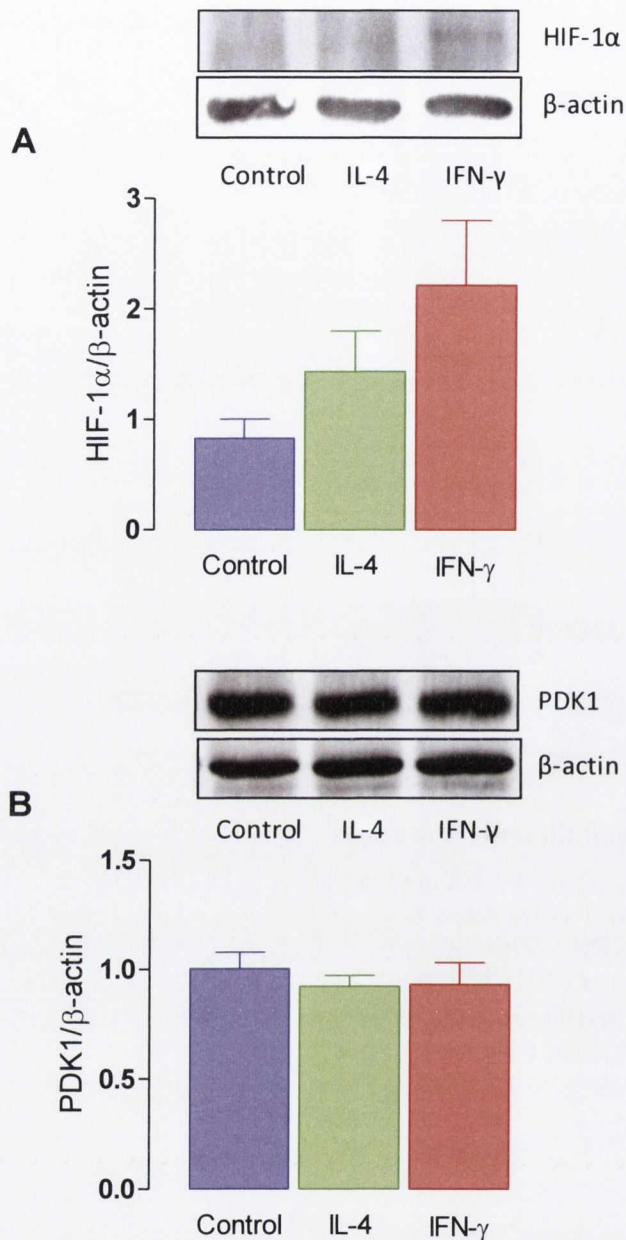


Figure 4.10 HIF-1α expression was induced by IFN-γ, but this had no effect on PDK1 expression in microglia

Microglia were incubated with media alone, IL-4 (20ng/mL), or IFN-γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of protein expression of HIF-1α and PDK1. The data show that the IFN-γ increase in HIF-1α expression did not reach significance (A), and there was no change in the PDK1 expression (B). Western immunoblotting data were normalised to β-actin and inserts illustrate representative blots for target proteins. Data are expressed relative to control as mean ± SEM (n=6).

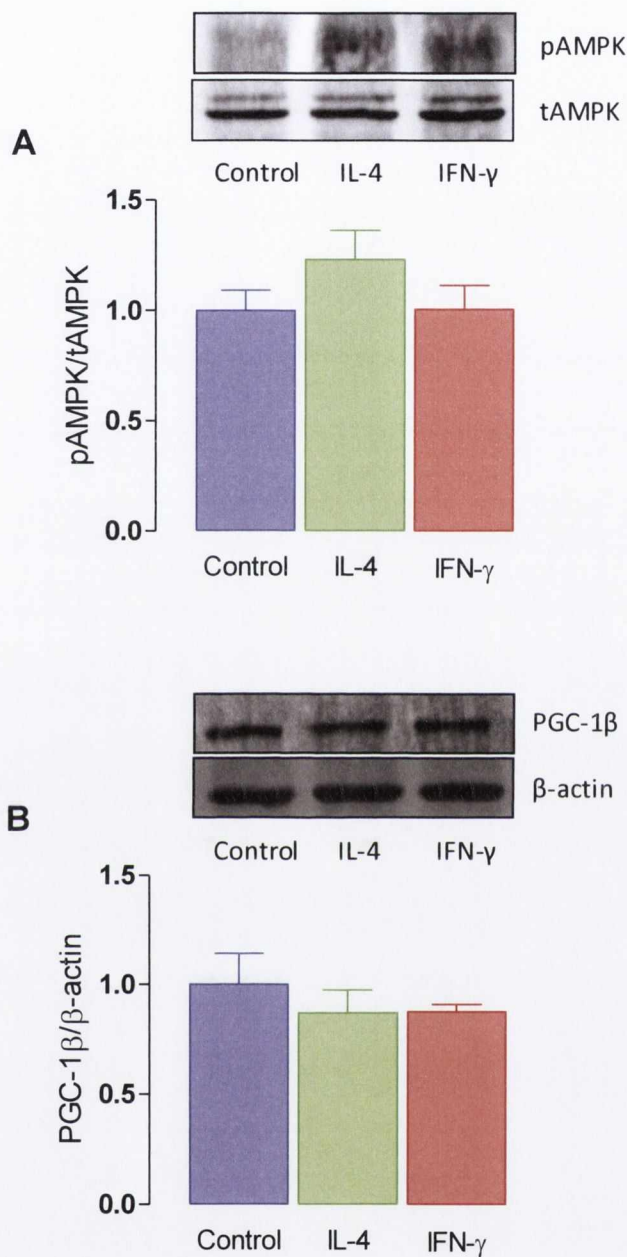


Figure 4.11 Polarised microglia did not alter pAMPK & PGC1 β expression

Microglia were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of protein expression of pAMPK and PGC-1 β . The data show that the phosphorylation of AMPK did not change following stimulation with IL-4 or IFN- γ (A), and the expression of PGC-1 β did not change in either activation state (B). Western immunoblotting data were normalised to β -actin and inserts illustrate representative blots for target proteins. Data are expressed relative to control as mean $\pm$ SEM (n=6).

).

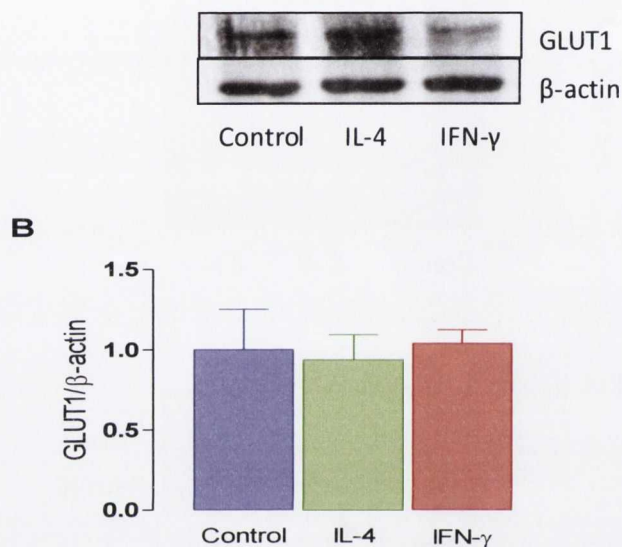


Figure 4.12 Polarised microglia did not alter GLUT1 expression in microglia

Microglia were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA and protein expression of GLUT1. The data show that neither IL-4 nor IFN- γ affected GLUT1 expression (A) Western immunoblotting data were normalised to β -actin and inserts illustrate representative blots for target proteins. Data are expressed relative to control as mean $\pm$ SEM (n=6).

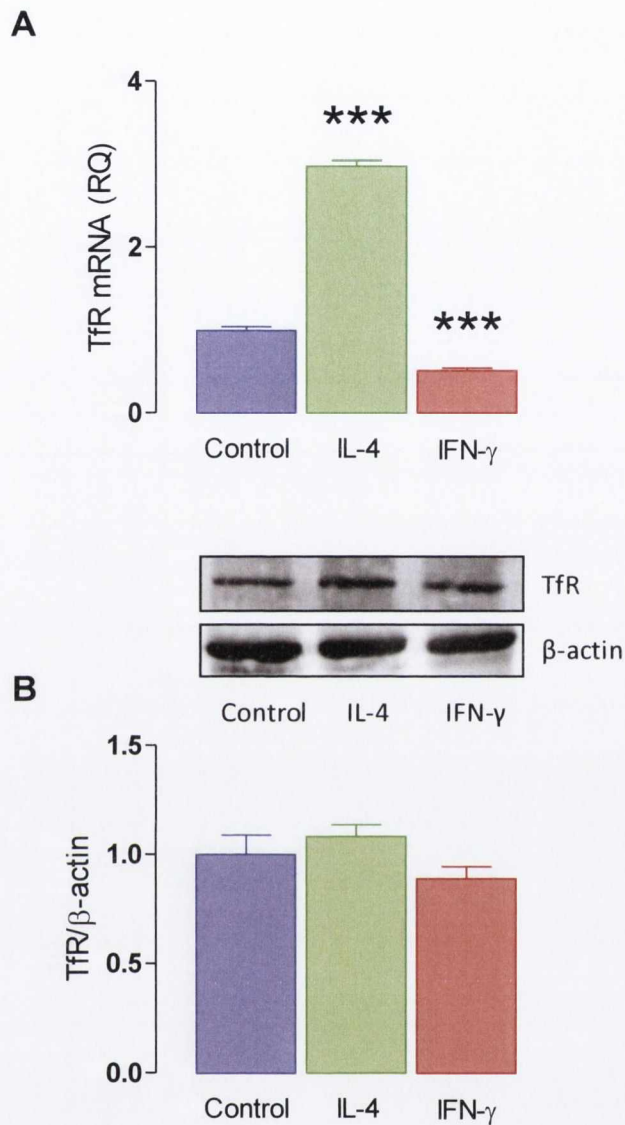


Figure 4.13 IL-4 and IFN-γ had opposite effects on TfR mRNA expression in microglia

Microglia were incubated with media alone, IL-4 (20ng/mL), or IFN-γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA and protein expression of TfR. The data show that TfR mRNA was significantly increased by IL-4, and significantly decreased by IFN-γ (A), however no change was observed at the protein level (B). Western immunoblotting data were normalised to β-actin and inserts illustrate representative blots for target proteins. *** $p < 0.001$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=3-9).

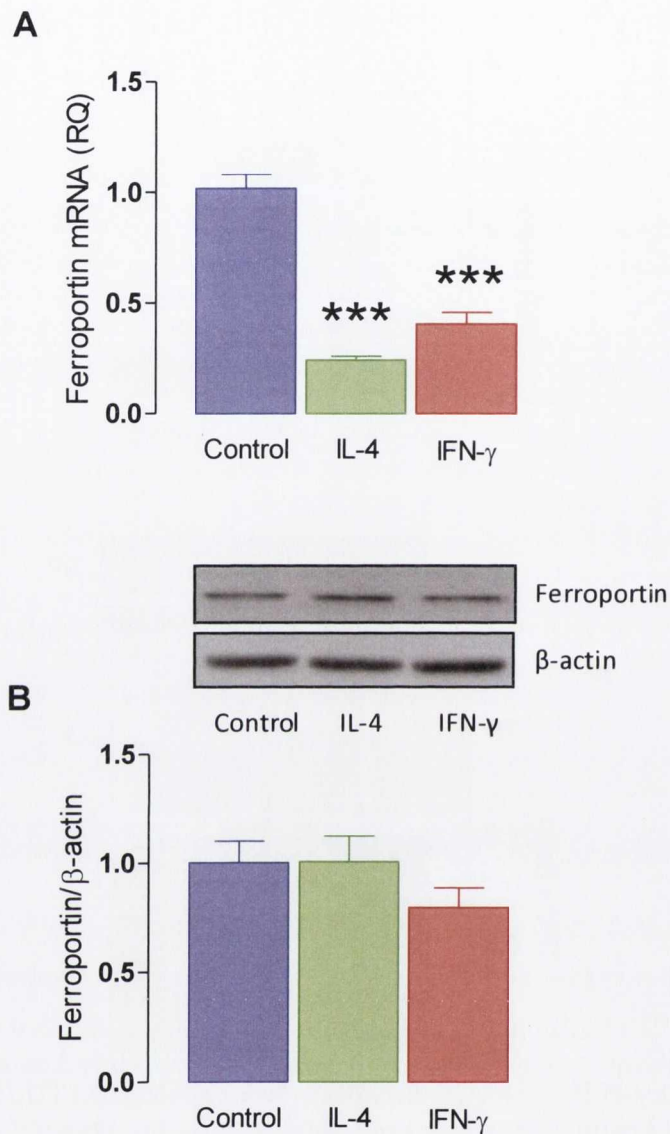


Figure 4.14 IL-4 and IFN- γ decreased the mRNA expression of ferroportin in microglia

Microglia were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA and protein expression of ferroportin. The data show that the mRNA expression of ferroportin was significantly decreased by both IL-4 and IFN- γ (A), yet the protein expression was not significantly altered by either stimulus (B). Western immunoblotting data were normalised to β -actin and inserts illustrate representative blots for target proteins. *** $p < 0.001$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=4-9).

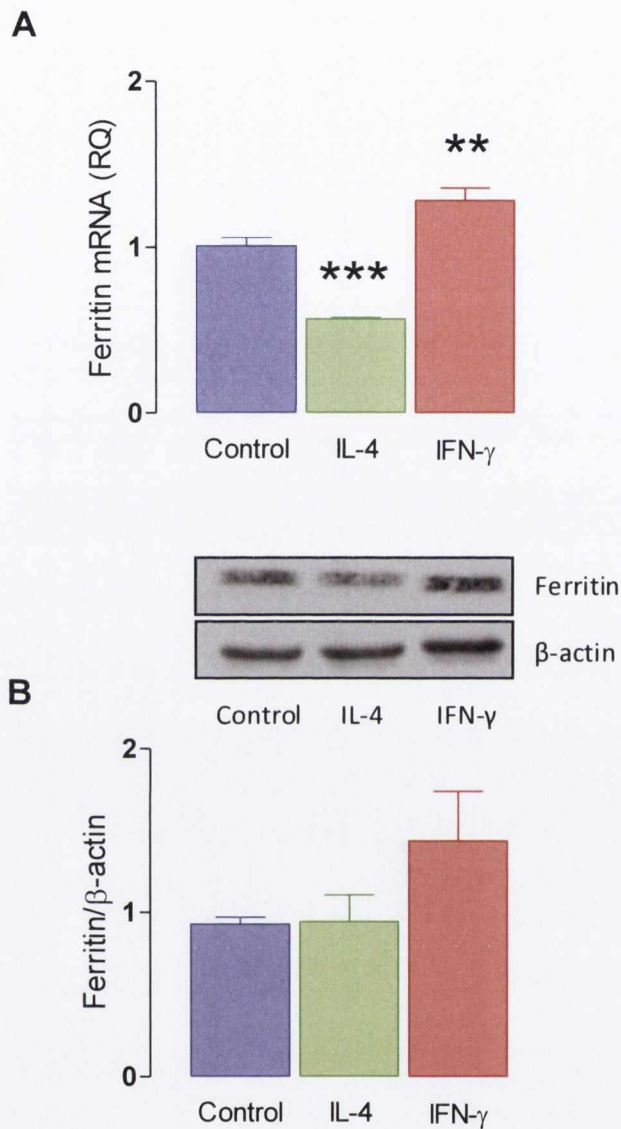


Figure 4.15 IL-4 and IFN- γ had contrasting effects on ferritin mRNA expression in microglia

Microglia were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA and protein expression of ferritin. The data show that the mRNA expression of ferritin was significantly decreased by IL-4 and increased by IFN- γ (A). There was a trend towards an IFN- γ -induced increase in ferritin protein expression, but this effect is not statistically significant (B). Western immunoblotting data were normalised to β -actin and inserts illustrate representative blots for target proteins. ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=4-7).

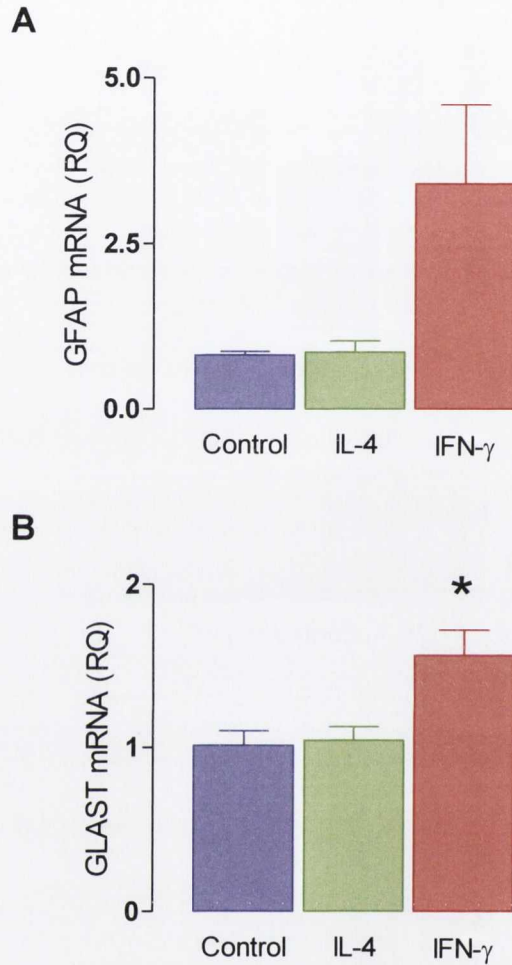


Figure 4.16 IFN- γ increased the mRNA expression of GLAST in astrocytes

Astrocytes were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA expression of markers of activation. The data show that IFN- γ increased the mRNA expression of GFAP, although the effect was not statistically significant (A). There was a statistically significant IFN- γ -induced increase in the mRNA expression of GLAST (B). * $p < 0.05$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=4).

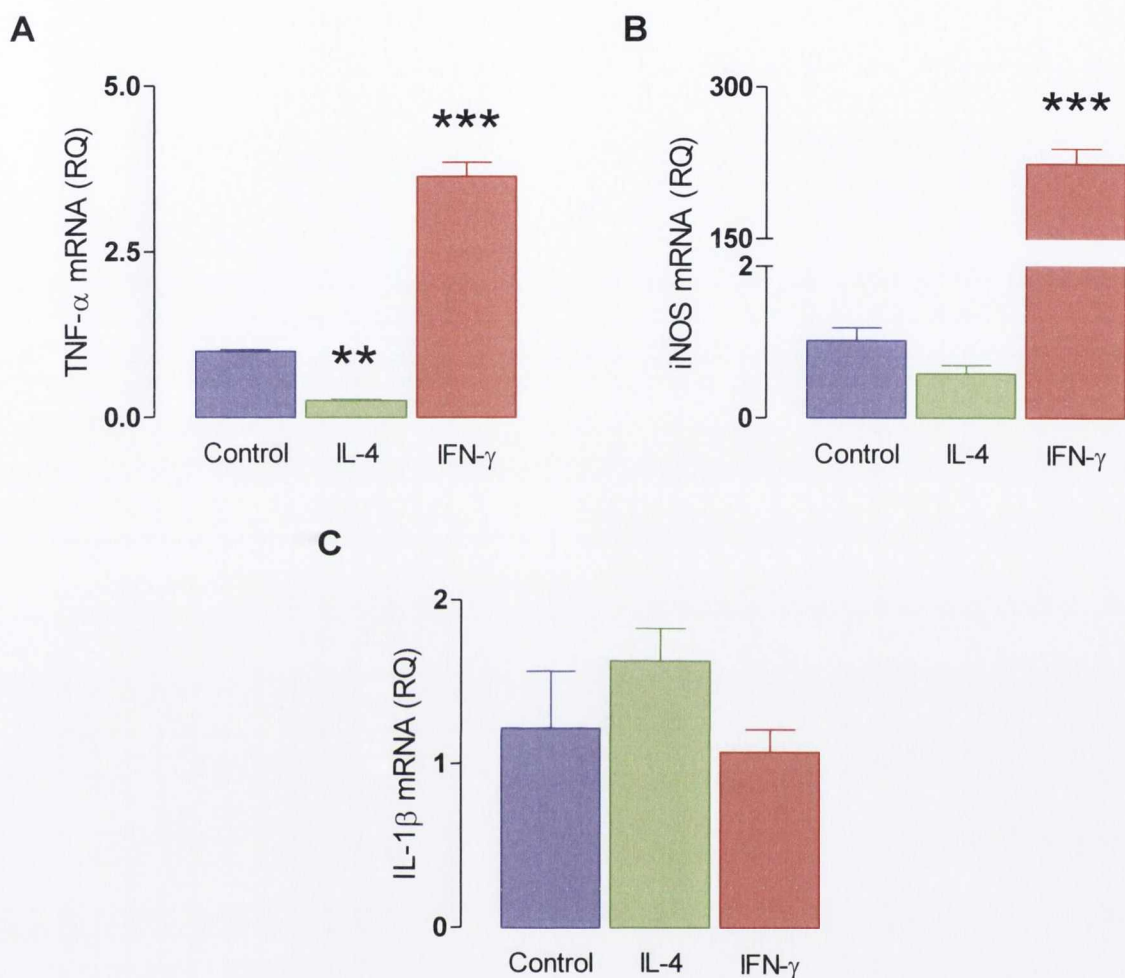


Figure 4.17 IFN-γ increased the mRNA expression of markers of the M1 activation state in astrocytes

Astrocytes were incubated with media alone, IL-4 (20ng/mL), or IFN-γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA expression of M1 activation state markers. The data show that IFN-γ significantly increased the mRNA expression of TNFα (A), iNOS (B), but not IL-1β (C). ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=4).

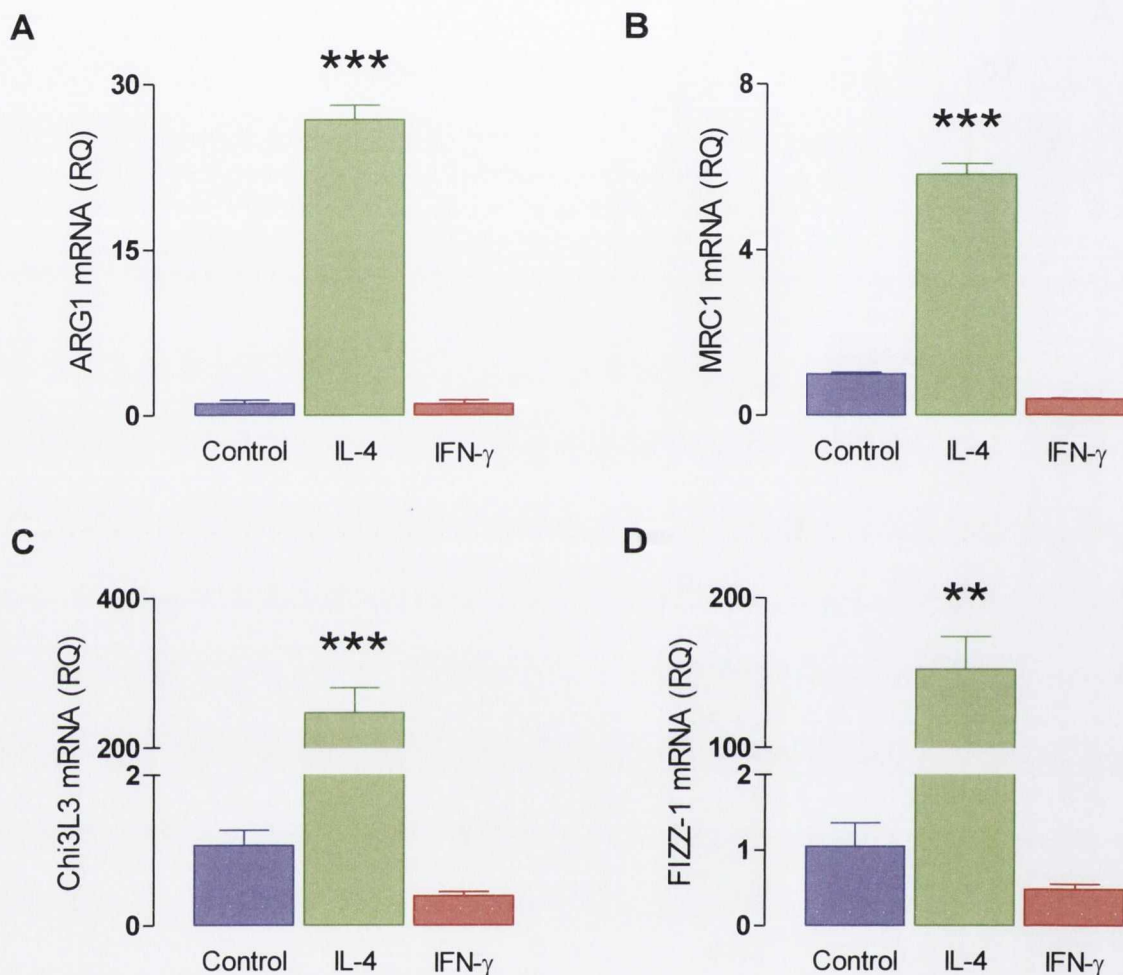


Figure 4.18 IL-4 increased M2 activation state markers in astrocytes

Astrocytes were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and cells were harvested for assessment of mRNA expression of M2 activation state markers. The data show that IL-4 significantly increased the mRNA expression of Arg-1 (A), MRC1 (B), Chi3L3 (C), and FIZZ1 (D). ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=4).

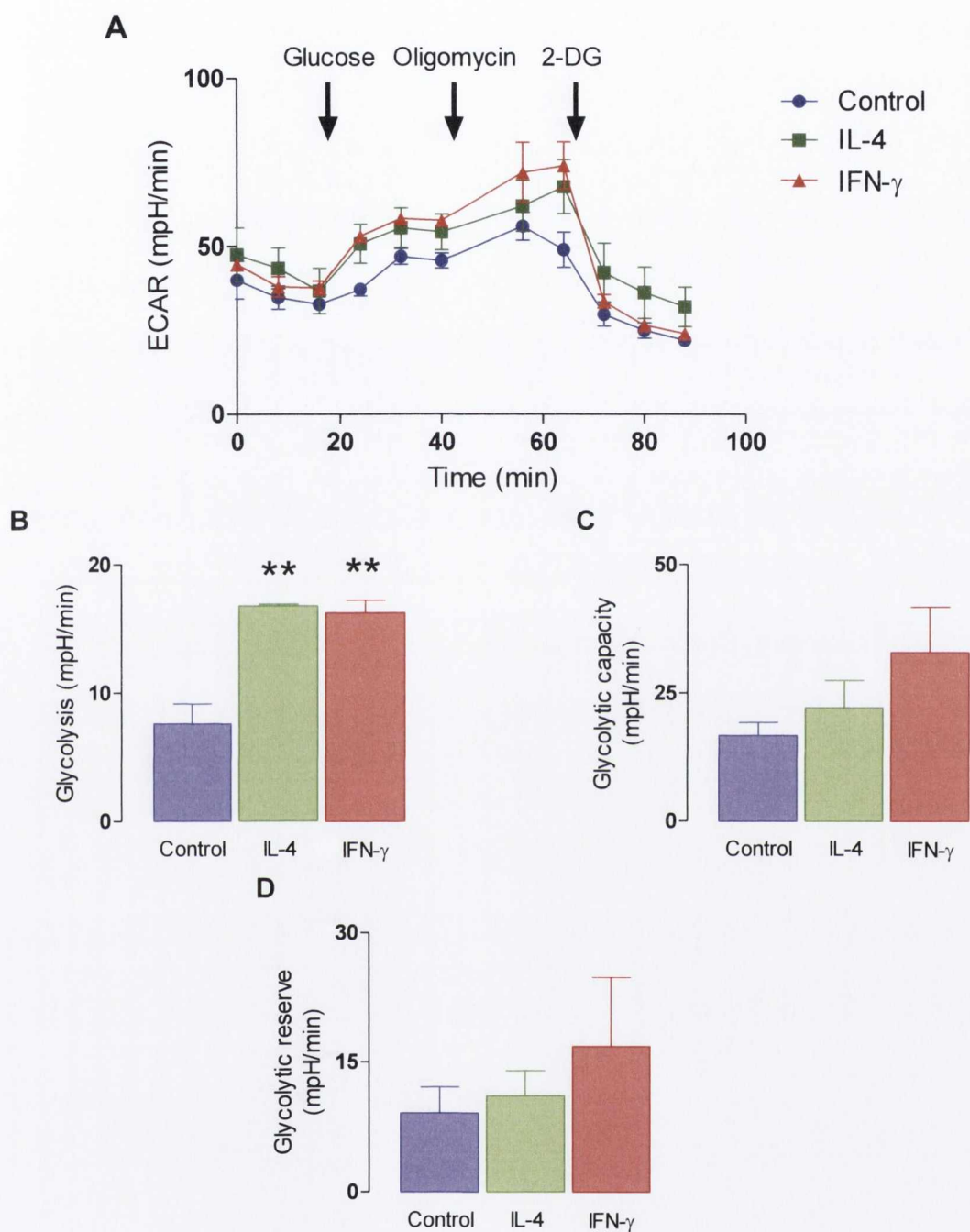


Figure 4.19 IL-4 and IFN- γ significantly increase the glycolytic rate in astrocytes

Astrocytes were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and a glycolytic flux assay was carried out to assess the metabolic signature of the cells. The Seahorse analyser was used to measure ECAR (A) and the data show that IL-4 and IFN- γ significantly increased the glycolytic rate of cells (B), but did not alter glycolytic capacity (C) or glycolytic reserve (D). ** $p < 0.01$; one-way ANOVA with Dunnett's post hoc analysis. Data are expressed as mean $\pm$ SEM (n=4).

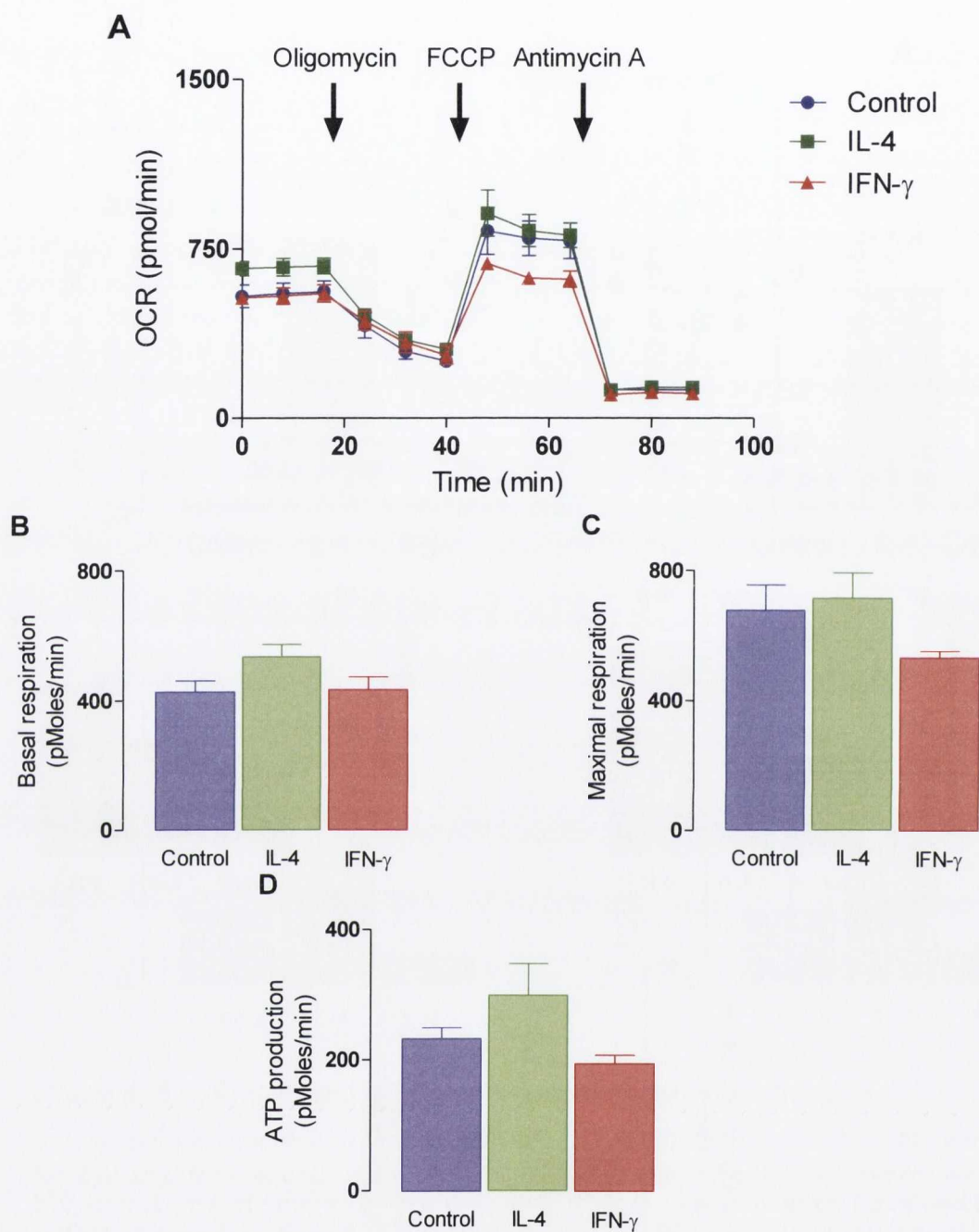


Figure 4.20 IL-4 or IFN- γ did not affect oxidative metabolism in astrocytes

Astrocytes were incubated with media alone, IL-4 (20ng/mL), or IFN- γ (50ng/mL) for 24h *in vitro* and a mitochondrial stress test was carried out to assess the metabolic signature of the cells. The Seahorse analyser was used to measure OCR (A) and the data show that there was no change in basal respiration (B), maximal respiration (C), or ATP production of cells (D). Data are expressed as mean $\pm$ SEM (n=3).

4.4 Discussion

The aim of this study was to determine whether changes in metabolism in microglia accompanied the changes in phenotypic markers induced by IFN- γ and IL-4, and to establish whether these stimuli induced similar changes in astrocytes. The most significant findings are that IFN- γ , which predictably increased mRNA expression of TNF α and iNOS, triggered an increase in glycolysis and expression of PFKFB3 and increased iron retention reflected by an increase in ferritin and decreased ferroportin expression. Interestingly IFN- γ exerted largely similar effects in astrocytes.

The data presented in Chapter 3 and the findings of several researchers (Nathan et al., 1983, Stein et al., 1992, Martinez and Gordon, 2014) have indicated that IFN- γ and IL-4 trigger changes in macrophages that typify M1 and M2 phenotypes. The present data show that similar changes occur in microglia with IFN- γ -induced increases in TNF α , iNOS and IL-1 β , and IL-4 increasing Arg-1, MRC1, Chi3L3, and FIZZ1. These data support the findings of other groups that reported similar IFN- γ - and IL-4-induced changes in cultured microglia prepared from neonates (Chhor et al., 2013b, Colton and Wilcock, 2010). These effects have been mirrored in microglia prepared from adult brains, which responded in the same way to IFN- γ with increased expression of TNF α and iNOS (Jones et al., 2015).

An important finding presented here is that IFN- γ induced a metabolic shift in microglia and increased glycolysis, whereas IL-4 increased oxidative metabolism. The ECAR/OCR and OCR/ECAR data make clear the fact that microglia preferentially utilise different metabolic pathways in response to polarising stimuli, as IFN- γ increased ECAR/OCR, and IL-4 increased OCR/ECAR. These changes demonstrate that microglia differentially adopt metabolic signatures in a similar manner to macrophages. As described in Chapter 3, pro-inflammatory macrophages preferentially use glycolysis to rapidly generate ATP to meet the demands of activation, generating

metabolic resources that drive inflammation in the process, such as ROS and TCA cycle intermediates (Tannahill et al., 2013). In contrast, increased oxidative metabolism provides alternatively activated macrophages with an abundant, sustainable source of ATP.

Although reports on the metabolic reprogramming of microglia are lacking, studies examining the metabolism of BV2 microglial cells demonstrate that LPS increases glycolysis, as indicated by increased ECAR in these cells, suggesting metabolic reprogramming takes place (Voloboueva et al., 2013, Orihuela et al., 2016). While BV2 cells are poor representatives of primary microglia (Stansley et al., 2012), increased glycolysis has also been shown in microglia following LPS-stimulation (Orihuela et al., 2016). The present study is consistent with this report, with IFN- γ increasing glycolysis in microglial cultures. The present study also revealed an IL-4-induced increase in oxidative metabolism which previous reports have failed to observe (Orihuela et al., 2016). This may be because microglia from the CD1 mice used by Orihuela et al. exhibit greater inflammatory responses than microglia isolated from the C57/BL6 mice used here (Nikodemova and Watters, 2011) and so, the effects of anti-inflammatory cytokines may not be as robust.

The underlying mechanisms that facilitate this metabolic switch in microglia were investigated to determine whether microglia utilised similar processes to macrophages. In the present study, IFN- γ increased mRNA expression of PFKFB3 and decreased PFKFB1 in microglia and the IFN- γ -induced increase in PFKFB3/PFKFB1 reflects the increased glycolytic rate of these cells, and suggests that the differential expression of these enzymes can be used as a proxy marker to identify the metabolic signature of M1-like microglia, as well as M1 macrophages. These changes have not yet been reported in microglia, but are consistent with findings in M1 polarised macrophages. As described in Chapter 3, classical activation of macrophages involves a switch in expression of PFKFB1 to the more active PFKFB3 isoform, which

accommodates increased glycolytic flux. Changes in protein expression of these enzymes would be more informative than mRNA expression and has been observed in macrophages stimulated with LPS (Rodriguez-Prados et al., 2010), but not in microglia. The absence of changes in protein expression may be because changes are more modest in microglia than macrophages and are difficult to detect, or because IFN- γ may not be as potent a stimulus as LPS and therefore may not induce as robust an effect.

The link between PFKFB3 and glycolysis identifies the enzyme as a potential therapeutic target for neuroinflammatory disorders, which are characterised by increased populations of pro-inflammatory, glycolytic microglia. There are no reports examining the effects of PFKFB3 inhibition in microglia, but numerous studies have investigated PFKFB3 inhibition as a potential cancer treatment and have shown that it is possible to attenuate the Warburg effect in cancer cells. For example, siRNA inhibits glycolysis and cell proliferation in HeLa cells (Calvo et al., 2006) and small molecule inhibitors of PFKFB3, 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) and 1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one (PFK15), inhibit the proliferation of *ras*-transformed tumour cells and induce apoptosis in transformed cells respectively (Clem et al., 2013, Clem et al., 2008). Since the Warburg effect, or aerobic glycolysis, can be attenuated by PFKFB3 inhibition, it may be possible to dampen pro-inflammatory immune responses in a similar manner.

HIF-1 α was investigated in microglia following IFN- γ and IL-4 stimulation, as it induces expression of *pfkfb3* (Obach et al., 2004) and has been shown to drive the metabolic reprogramming of M1 macrophages (Kelly and O'Neill, 2015) by increasing glycolysis (Obach et al., 2004, Freerman et al., 2014) and inhibiting oxidative metabolism (Kim et al., 2006a), the expression of this transcription factor. IFN- γ induced a 2.5 fold increase in HIF-1 α expression in microglia and although HIF-1 α expression in polarised microglia has not been reported, the apparent increase observed in the present study is

consistent with changes observed in LPS treated BV2 cells (Oh et al., 2008) and macrophages (Tannahill et al., 2013, Blouin et al., 2004).

In the absence of a significant increase in HIF-1 α expression in M1-like microglia, PDK-1 expression was predictably unchanged in these cells, which was highlighted by a functional TCA cycle in these cells as indicated by an absence of a decrease in OCR. PDK-1 expression is induced by HIF-1 α and plays a role in the induction of the metabolic signature of M1 macrophages by inhibiting the conversion of pyruvate to acetyl-CoA, thus starving the TCA cycle of substrate (Kim et al., 2006a). HIF-1 α mediated metabolic reprogramming was initially described in cells under hypoxic conditions, but also occurs in the presence of oxygen following activation of inflammatory cells (Palsson-McDermott et al., 2015). The HIF-1 α -PDK1 axis plays an essential role in the hypoxia induced activation of peritoneal macrophages, and HIF-1 α knockout or siRNA inhibition of PDK1 inhibits the metabolic switching of these cells (Semba et al., 2016). The present study suggests that HIF-1 α may not play a crucial role in the metabolic reprogramming of microglia, like it does in macrophages. Indeed, increases in PFKFB3 expression have been reported in LPS-stimulated peritoneal macrophages prior to increases in HIF-1 α being observed, and transcription can occur independently of HIF-1 α (Rodriguez-Prados et al., 2010). This suggests the presence of other yet unidentified factors responsible for mediating metabolic reprogramming of M1 microglia. For example, the Sp1 transcription factor induces *pfkfb3* transcription following the synergistic stimulation of macrophages with adenosine and LPS (Ruiz-Garcia et al., 2011).

In the present study, there was an increase in AMPK activation, albeit insignificant, observed in IL-4-stimulated microglia, while IFN- γ had no effect. This is consistent with the literature, AMPK is associated with the metabolic profile of M2 activated macrophages, as it increases the expression of proteins involved in oxidative phosphorylation (Vats et al., 2006), fatty acid metabolism and mitochondrial enzymes (Winder et al., 2000), while also

inhibiting the metabolic reprogramming of M1 cells (Hardie, 2007). In macrophages, AMPK is associated with anti-inflammatory phenotype, antagonising LPS signalling through NF- κ B inhibition (Ak and Levine, 2010), and conversely expression of AMPK is decreased in macrophages stimulated with LPS (Sag et al., 2008). Furthermore, increased experimental autoimmune encephalomyelitis (EAE) disease severity is observed in *AMPK- α 1^{-/-}* mice, accompanied by increased IFN- γ secretion (Nath et al., 2009), suggesting that AMPK plays a role in modulating the inflammatory response in the CNS.

PGC-1 β , which is downstream of AMPK and also STAT6, induces mitochondrial respiration and biogenesis (St-Pierre et al., 2003, Shao et al., 2010), thus driving oxidative metabolism. However in the present study, there was no increase in PGC-1 β expression observed following IL-4 stimulation. Reports have demonstrated increased PGC-1 β in macrophages in response to IL-4 is essential for M2 activation and PGC-1 β expression attenuates inflammation (Vats et al., 2006). PPAR γ is activated by PGC-1 β and plays a role in modulation of microglial inflammation (Bernardo et al., 2000), which suggests that PGC-1 β expression should be also be increased in M2-like macrophages. This suggests that the IL-4-induced increase in respiratory rate in microglia is be mediated by other yet unknown factors.

Iron deposits in the brain are associated with ageing and neurodegenerative disorders (Andersen et al., 2014, Connor et al., 1995), highlighting the importance of iron homeostasis in the CNS. Macrophages alter their iron handling properties in response to polarisation, as described in Chapter 3, but the iron handling capabilities polarised microglia is less well defined. Here, IFN- γ -stimulated microglia expressed low levels of TfR mRNA, high levels of ferritin mRNA, and low levels of ferroportin mRNA. These data suggest that iron metabolism in pro-inflammatory microglia is similar to that of M1 macrophages, displaying low iron uptake and efflux, but with high intracellular iron concentrations This is is consistent with reports showing

iron and ferritin accumulation within microglia in response to neuroinflammation (Thomsen et al., 2015). There were corresponding changes in protein expression, but perhaps a more potent stimulus, such as LPS, is required for these changes to reach significance.

Iron handling proteins in IL-4-stimulated microglia were assessed and these cells demonstrated high levels of TfR, low levels of ferritin, and low levels of ferroportin. In contrast to the apparent changes in protein expression observed in M1-like microglia, IL-4 stimulation did not alter the protein expression of any of the markers assessed. Perhaps a combination of IL-4 and IL-13, which is commonly used in the literature to induce an M2-like phenotype, would be capable of inducing such changes. The changes observed in TfR and ferritin were predictable, as the M2 phenotype in macrophages is associated with increased levels of iron recycling and decreased iron retention to facilitate the matrix remodelling and anti-inflammatory properties of the cell (Cairo et al., 2011). However, the decreased ferroportin expression was unexpected, but mirrors changes observed in IL-4 stimulated BMDMs shown in Chapter 3, suggesting that iron efflux from M2-like cells is more complex than previously believed. As described in Chapter 3, other proteins involved in iron handling have been associated with pro- and anti-inflammatory phenotypes in macrophages, such as hepcidin and DMT1, and perhaps examine the expression of these would provide a clearer indication of the iron handling properties of polarised microglia.

One of the more novel aspects of the present finding is that astrocytes respond to IFN- γ and IL-4 in a manner similar to microglia. Specifically, IFN- γ increased expression of both TNF α and iNOS and IL-4 expression increased expression of Arg-1, MRC1, Chi3L3 and FIZZ1 to approximately the same extent in both cell types. This is consistent with a recent report showing increased Arg-1 and MRC1 expression in IL-4 stimulated astrocytes, and increased iNOS and TNF α in cells treated with a combination of LPS and IFN- γ (Jang et al., 2013). The present study expands this by demonstrating

IL-4-induced increases in *Chi3L3* and *FIZZ1* and also reveals that IFN- γ alone stimulates polarisation of astrocytes to an M1-like phenotype. In addition, unpublished data from this laboratory confirmed the ability of astrocytes to adopt M1- and M2-like phenotypes. The responses of microglia and astrocytes were not identical however; whereas IFN- γ increased IL-18 mRNA in microglia, no change was observed in astrocytes.

Astrocytes were assessed to determine if they undergo the metabolic reprogramming observed in BMDMs and microglia in response to IFN- γ and IL-4. Both stimuli significantly increased glycolysis in astrocytes and no changes in the glycolytic capacity or glycolytic reserve were observed. Increased glycolysis in astrocytes has been reported to occur in astrocytes following stimulation with IFN- γ and LPS, through AMPK and PFKFB3 expression (Almeida et al., 2004). Oxidative metabolism in astrocytes did not change with either stimulus. These data suggest that differential metabolic reprogramming in polarised astrocytes does not occur, as cells respond to stimuli in the same way, namely by increasing glycolytic flux. This may be because immune activity is a secondary function of astrocytes and reprogramming occurs in specialised cells of the immune system.

The aim of this study was to characterise the metabolic profile of polarised microglia to assess whether changes occur in these cells upon activation. Like macrophages, microglia can adopt M1-like and M2-like phenotypes in response to stimuli in the microenvironment. Chronic pro-inflammatory activation of microglia is associated with most neuroinflammatory diseases (Tang and Le, 2016) and altering the activation state of these cells to an anti-inflammatory M2-like phenotype can alleviate the neurodegeneration that accompanies these disorders. Increasing evidence suggests a role of metabolic reprogramming in the regulation of the innate and adaptive immune response (Chang and Pearce, 2016, Mills and O'Neill, 2016) and the function of pro-inflammatory immune cells can be modulated by targeting metabolic pathways. The present study demonstrates that metabolic reprogramming

occurs in polarised microglia, which leads to the possibility for therapeutic interventions for neurodegenerative disorders that target metabolism of inflammatory cells.

Chapter 5: Functional consequences of glial activation

5.1 Introduction

As described in Chapter 4, microglia adopt M1-like or M2-like activation states upon detection of specific stimuli. While the chronic activation of the M1 phenotype induces a pro-inflammatory environment associated with neurodegeneration, the M2 phenotype is considered to be anti-inflammatory and is important in the resolution of inflammation (Lynch, 2009). These phenotypes are characterised by the expression of particular markers designated as markers of activation (Chhor et al., 2013b) but this paradigm fails when extrapolated to an *in vivo* setting as microglia seem to have near-infinite plasticity that lends itself ideally to dealing with an endless variety of environmental challenges (Martinez and Gordon, 2014). Establishing functional characteristics of activated microglia provides greater insights into the consequences of microglial activation. By understanding induction of functional differences, it may be possible to understand why microglia contribute to neuroinflammatory disorders.

According to the amyloid hypothesis of AD, A β deposition due to aberrant phagocytic function of microglia is central to disease pathology (Hardy and Selkoe, 2002). While pro-inflammatory M1-like microglia are co-localised with A β depositions in the AD brain, it is believed that phagocytic function is impaired and A β clearance is diminished (Krabbe et al., 2013). However, M2 macrophage activation has also been shown to decrease phagocytic capability (Varin et al., 2010) and it is unclear how phenotype affects microglial phagocytosis. Following the phagocytosis of an insulting agent, antigen presentation primes T cells to generate an adaptive immune response. The infiltration of T cells has been associated with disease pathology in AD and MS (Bryson and Lynch, 2016, Sawcer et al., 2011), and while M1 macrophages can induce T_h1 cell differentiation (Arnold et al., 2015), the antigen presenting capabilities of microglia are less well defined.

The recruitment of other immune cells by polarised microglia is an important aspect of the immune response. Pro-inflammatory microglia secrete a number

of chemotactic proteins that induce the migration of peripheral immune cells through the BBB (D'Aversa et al., 2002). The recruited cells exacerbate the inflammatory conditions experienced in the CNS and infiltrating peripheral immune cells have been implicated in a number of neurodegenerative disorders such as MS and AD (Benveniste, 1997, Fiala et al., 2002). Microglia are also capable of secreting a number of growth factors that impact surrounding neurons. Microglia-specific BDNF plays an important role in learning and memory (Parkhurst et al., 2013), and decreased expression of IGF1 has been associated with pro-inflammatory microglia in AD (Steen et al., 2005). By assessing secretion profile of polarised microglia, we can further our understanding the functional consequences of microglial polarisation.

The aims of this study are to investigate the functional consequences of microglial polarisation in terms of their:

1. Phagocytosis of latex beads and A β
2. Antigen presentation and induction of T cell responses
3. Secretion of chemokines and growth factors

5.2 Methods

Primary microglia were procured from neonatal mouse pups and polarised to M1-like and M2-like activation states respectively. Cells were harvested for assessment of the mRNA expression of markers associated with phagocytosis, antigen presentation and growth factors in polarised microglia using RT-PCR and supernatants from these cultures were analysed using ELISA to investigate the secretion of chemokines.

Primary mixed glia were procured from neonatal mouse and incubated with fluorescently-labelled latex beads or FITC-conjugated A β for 2h *in vitro*. Cells were examined using ICC to confirm bead or A β uptake. Mixed glial cultures were stimulated with IFN- γ or IL-4 for 24h *in vitro* prior to 2h incubation with fluorescent particles and FACS analysis was used to assess the differences in phagocytic capabilities of the M1-like or M2-like phenotypes.

Primary microglia were procured from neonatal mouse pups and polarised to M1-like and M2-like activation states respectively. Cells were then irradiated and co-cultured with CTV-stained T cells isolated from the spleens of 9 month-old GFP mice for 7d. T cells were assessed for proliferation and differentiation by FACS and supernatants were assessed by ELISA for the secretion of cytokines.

5.3 Results

Primary glial cultures were generated from neonatal mouse pups and incubated for 2h in the presence of fluorescently-labelled latex beads. Cells were stained and assessed by ICC and confocal microscopy to visualise uptake of fluorescent particles. Bead uptake in primary microglial cultures was demonstrated by ICC showing the co-localisation of fluorescent beads with the Iba1⁺ microglial cells (Figure 5.1A). Staining of primary mixed glial cultures with GFAP revealed that fluorescently-labelled latex beads were not phagocytosed by astrocytes (Figure 5.1B). Bead internalisation was confirmed using confocal microscopy, which demonstrated the absence of beads in GFAP⁺ astrocytes, but the accumulation of the fluorescent particles inside microglial cells, identified by DAPI stained nuclei (Figure 5.1C).

Primary microglia and mixed glia generated from neonatal mouse pups were incubated for 2h in the presence of FITC-conjugated A β , and assessed for phagocytosis of peptide by ICC. Staining of microglia with anti-Iba1 revealed the co-localisation of FITC-A β with Iba1⁺ microglia cells, indicative of phagocytosis (Figure 5.2A). Mixed glial cultures stained with anti-GFAP reveal that GFAP⁺ astrocytes do not phagocytose FITC-A β , but fluorescent peptide accumulates within GFAP⁻ microglia, identified by DAPI stained nuclei (Figure 5.2B).

Mixed glial cultures were stimulated with IFN- γ or IL-4 for 24h *in vitro*, and incubated for 2h with fluorescently-labelled latex beads or FITC-conjugated A β to determine whether microglial polarisation is accompanied with a change in microglial phagocytic function. Phagocytosis by microglia was assessed by FACS and analysis of the mean data revealed that neither IL-4 nor IFN- γ altered the phagocytosis of fluorescently-labelled latex beads or FITC-conjugated A β when compared with unstimulated microglia (Figure 5.3A, B). There was an apparent decrease in FITC-A β phagocytosis by IFN- γ stimulated cells, but this decrease did not reach statistical significance.

Polarised microglia were assessed for the mRNA expression of markers associated with phagocytosis by RT-PCR. IL-4 significantly increased CD68 mRNA expression ($**p<0.01$; one-way ANOVA with Dunnett's post hoc analysis; Figure 5.4A) and both IL-4 and IFN- γ decreased CD33 expression ($*p<0.05$, $**p<0.01$; one-way ANOVA with Dunnett's post hoc analysis; Figure 5.4C), but no change was observed in TREM2 (Figure 5.4B).

To assess the antigen presenting functions of polarised microglia, the mRNA expression of proteins involved in the presentation of antigen to T cells was assessed by RT-PCR. MHC II expression was significantly increased by IFN- γ ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 5.5A), while IL-4 had no effect on its expression (Figure 5.5A). Neither stimulus affected the expression of CD80, although there was an apparent increase in IFN- γ -stimulated microglia which did not reach statistical significance (Figure 5.5B). IFN- γ significantly increased CD86 ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 5.5C), while IL-4 has no effect on its expression (Figure 5.5C). The ability of polarised microglia to induce the proliferation of T cells was assessed using a CTV proliferation assay, which revealed that neither activation of the M1-like nor M2-like phenotypes affected T cell proliferation (Figure 5.6A). The differentiation of T cell subtypes was examined to investigate which populations were induced by co-culture with M1-like or M2-like polarised microglia.

The expression of IFN- γ , an indicator of T_H1 cell differentiation, was increased in T cells co-cultured with IFN- γ - and IL-4-stimulated microglia when compared to T cells co-cultured with unstimulated cells ($*p<0.05$, $**p<0.01$; one-way ANOVA with Dunnett's post hoc analysis; Figure 5.7A). However, increased secretion of the cytokine was only observed in T cells co-cultured with IFN- γ -treated microglia ($*p<0.05$; one-way ANOVA with Dunnett's post hoc analysis; Figure 5.7B). Differentiation of T cells to a T_H17 subtype was assessed by examining IL-17 expression. While neither stimulus affected the expression of IL-17 in T cells (Figure 5.7C), secretion of the

cytokine was significantly decreased from T cells when co-cultured with IL-4-treated microglia ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 5.7D). T cell expression of IL-4, an indicator of T_H2 cell differentiation, was not affected by co-culture with microglia of either phenotype (Figure 5.7E), although an apparent decrease in IL-4 expression was observed in T cells co-cultured with M1-like microglia.

Microglial secretion of chemokines plays an important role in the generation of an immune response and the release of chemokines from polarised microglia was examined by ELISA. CCL2 secretion was significantly increased by both IFN- γ and IL-4 stimulation of microglia ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 5.8A). Microglial CCL3 and CCL5 secretion was also induced by IFN- γ ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 5.8B, C), but not by IL-4 (Figure 5.8B, C).

Growth factors in the CNS can help modulate neuroinflammation and promote neuronal health and expression of growth factors was assessed in IFN- γ and IL-4-stimulated microglia by RT-PCR. Neither stimulus altered NGF expression (Figure 5.9A). IL-4 significantly increased BDNF expression ($*p<0.05$; one-way ANOVA with Dunnett's post hoc analysis; Figure 5.9B), but unchanged in IFN- γ -treated cells. IL-4 also induced a significant increase in IGF1 expression ($***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 5.9C), while in contrast, a significant decrease was observed with IFN- γ stimulation ($*p<0.05$, $***p<0.001$; one-way ANOVA with Dunnett's post hoc analysis; Figure 5.9C).

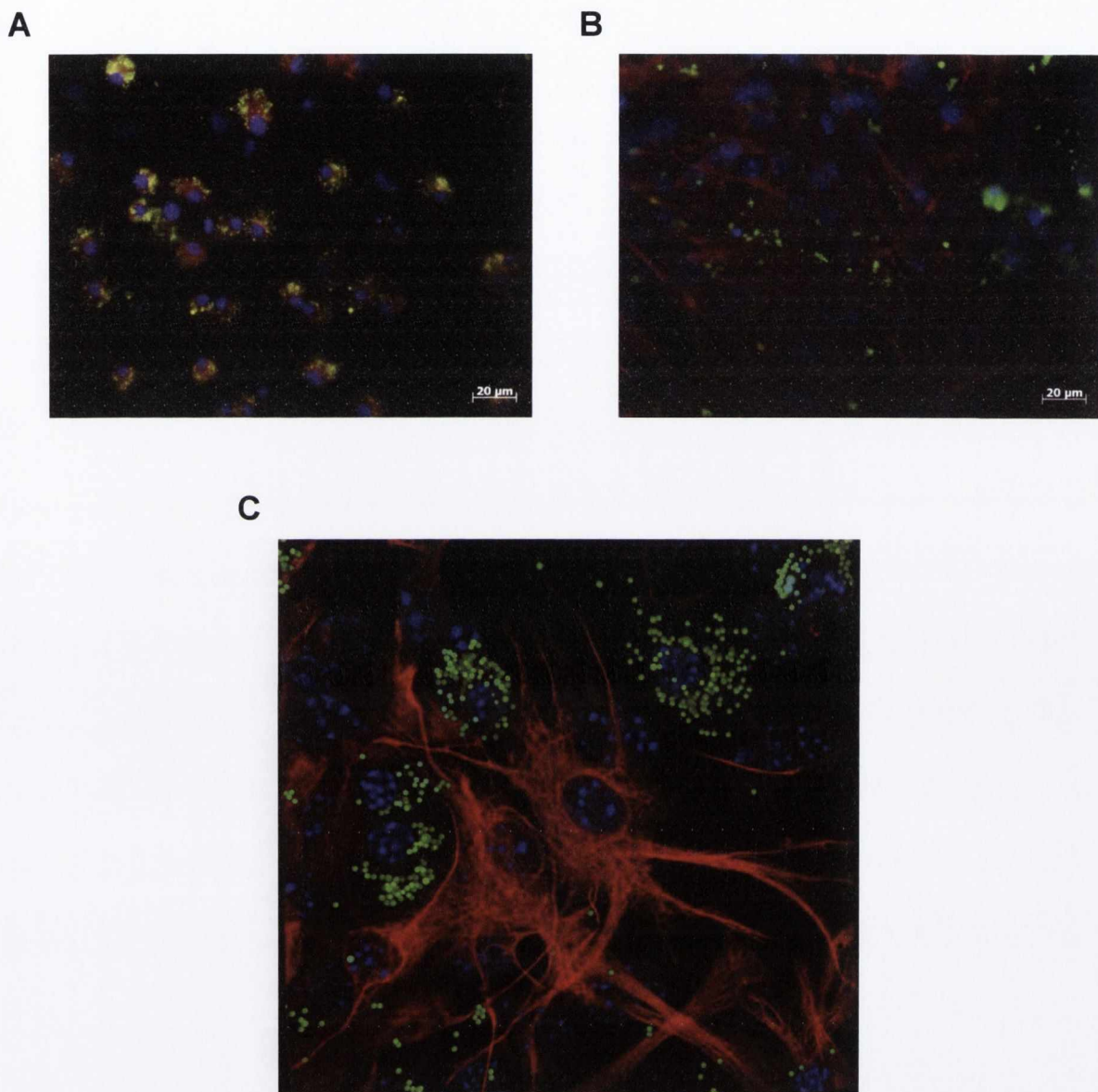


Figure 5.1 Fluorescently-labelled latex beads were taken up by microglia, but not astrocytes *in vitro*

Primary microglia or mixed glia were incubated with fluorescently-labelled beads (green) for 2h. (A) Microglia were stained with anti-Iba1 (red) and counterstained with DAPI (blue) to identify nuclei and fluorescent beads were present in Iba1⁺ cells. (B) Cells were stained with anti-GFAP (red) to identify astrocytes and counterstained with DAPI (blue) and there was no evidence of bead uptake in GFAP⁺ cells. (C) Confocal microscopy confirmed the intracellular localisation of beads in GFAP⁻ cells.

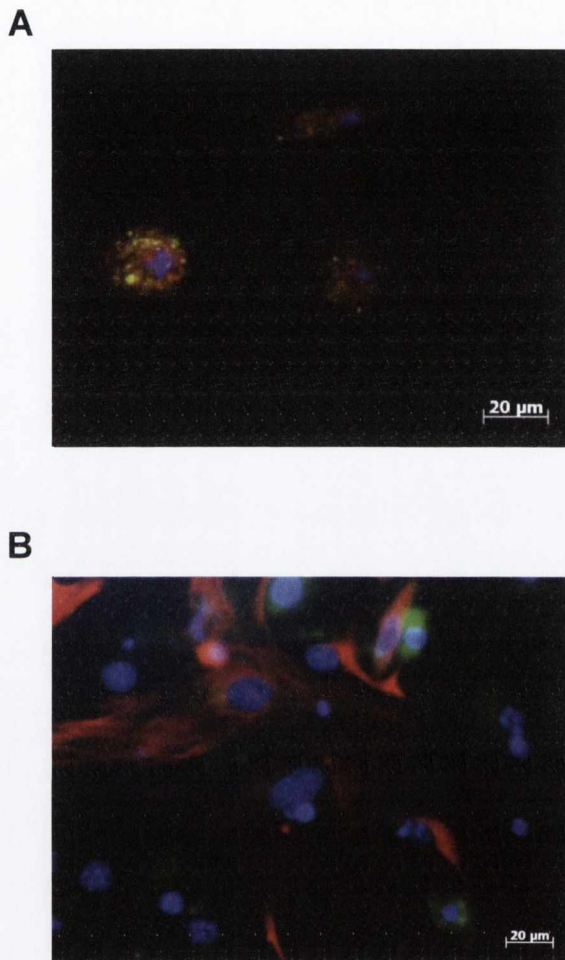


Figure 5.2 Microglia were shown to engulf FITC-conjugated A β

Primary microglia or mixed glia were incubated with FITC-conjugated A β (green) for 2h. (A) Microglial cultures were stained with anti-Iba1 (red) to identify cells and counterstained with DAPI (blue) to identify nuclei and FITC-conjugated A β was found to accumulate within Iba1⁺ microglia. (B) Mixed glial cultures were stained with anti-GFAP (red) to identify astrocytes and counterstained with DAPI (blue) and fluorescent peptide was shown to be engulfed by GFAP⁺ microglia.

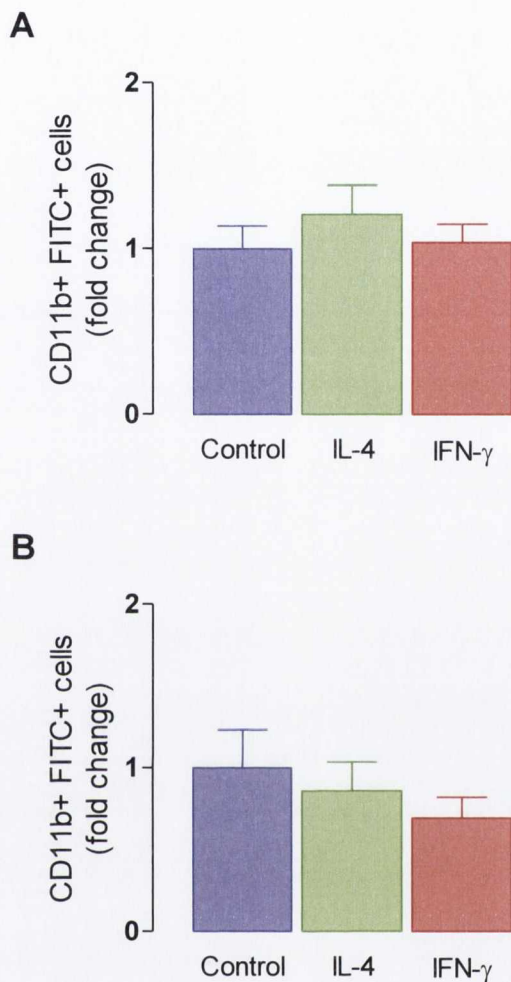


Figure 5.3 Neither IL-4 nor IFN-γ affected phagocytosis of fluorescently-labelled latex beads

Mixed glia were incubated in the presence or absence of IL-4 (20ng/mL) or IFN-γ (50ng/mL) for 24h *in vitro*, followed by a 2h incubation with fluorescently-labelled latex beads (A) or FITC-conjugated Aβ (B). Phagocytosis was assessed by flow cytometry and the data show that neither IL-4 or IFN-γ affected the microglial phagocytosis of beads (A) or FITC-Aβ (B). Data are expressed relative to control as mean ± SEM (n=8).

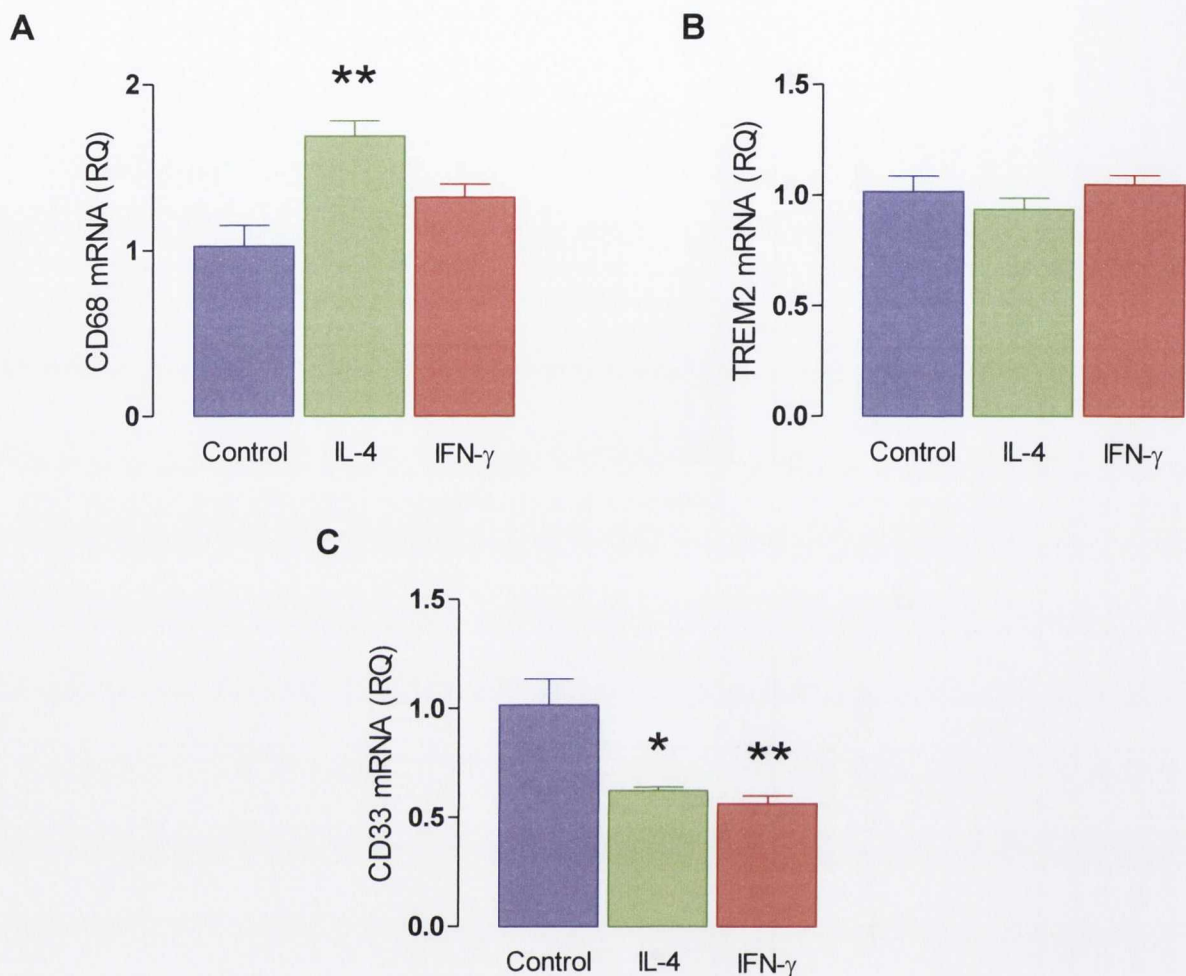


Figure 5.4 IL-4 increased the mRNA expression of CD68 and both IL-4 and IFN- γ decreased the expression of CD33 in microglia

Microglia were incubated in the presence or absence of IL-4 (20ng/mL) or IFN- γ (50ng/mL) for 24h and cells were harvested for assessment of mRNA expression. IL-4 significantly increased CD68 (A). Neither stimulus affected TREM2 expression (B), but both significantly decreased CD33 expression (C). * $p < 0.05$, ** $p < 0.01$; one-way ANOVA with Dunnett's post-hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=3-7).

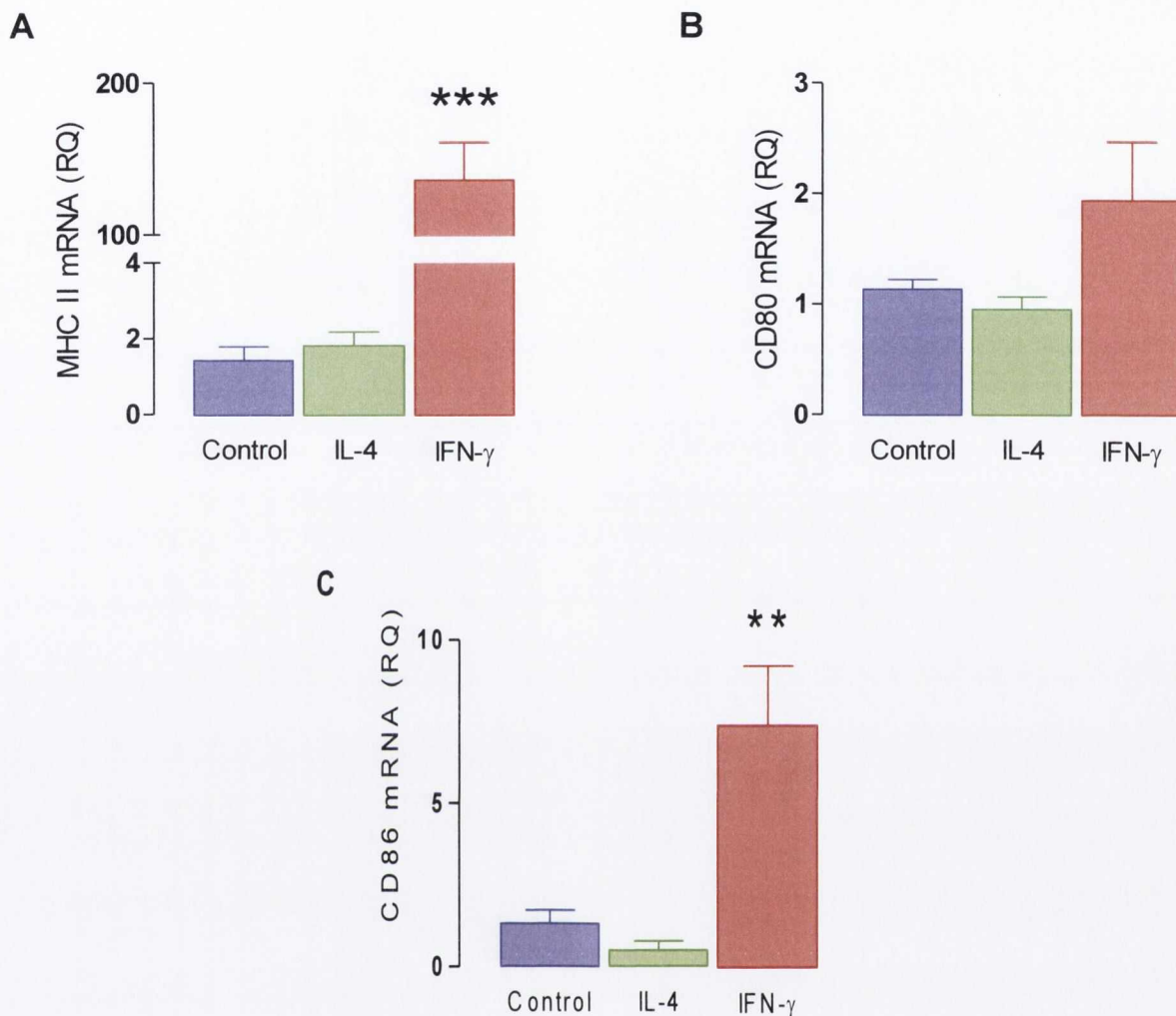


Figure 5.5 IFN- γ increased the mRNA expression of MHC II and CD86 in microglia

Microglia were incubated in the presence or absence of IL-4 (20ng/mL) or IFN- γ (50ng/mL) for 24h and cells were harvested for assessment of mRNA expression. IFN- γ significantly increased MHC II (A) and while CD80 was unchanged by either stimulus (B), CD86 is significantly increased by IFN- γ and unchanged by IL-4 (C). ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA with Dunnett's post-hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=3).

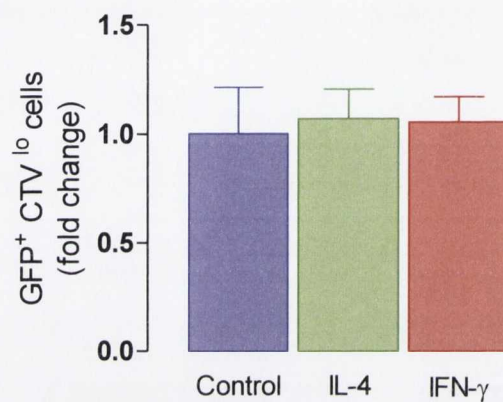


Figure 5.6 Neither IL-4 or IFN-γ affected microglial capacity to induce T cell proliferation

T cells prepared from GFP mice were stained with CTV and cultured for 7 days with irradiated microglia which had been incubated in the presence or absence of IL-4 (20ng/mL) or IFN-γ (50ng/mL) for 24h *in vitro*. T cells were harvested for assessment of proliferation by flow cytometry and the data show that stimulation of microglia with IL-4 or IFN-γ had no effect on their ability to induce T cell proliferation. Data are expressed relative to media alone control as mean $\pm$ SEM (n=3).

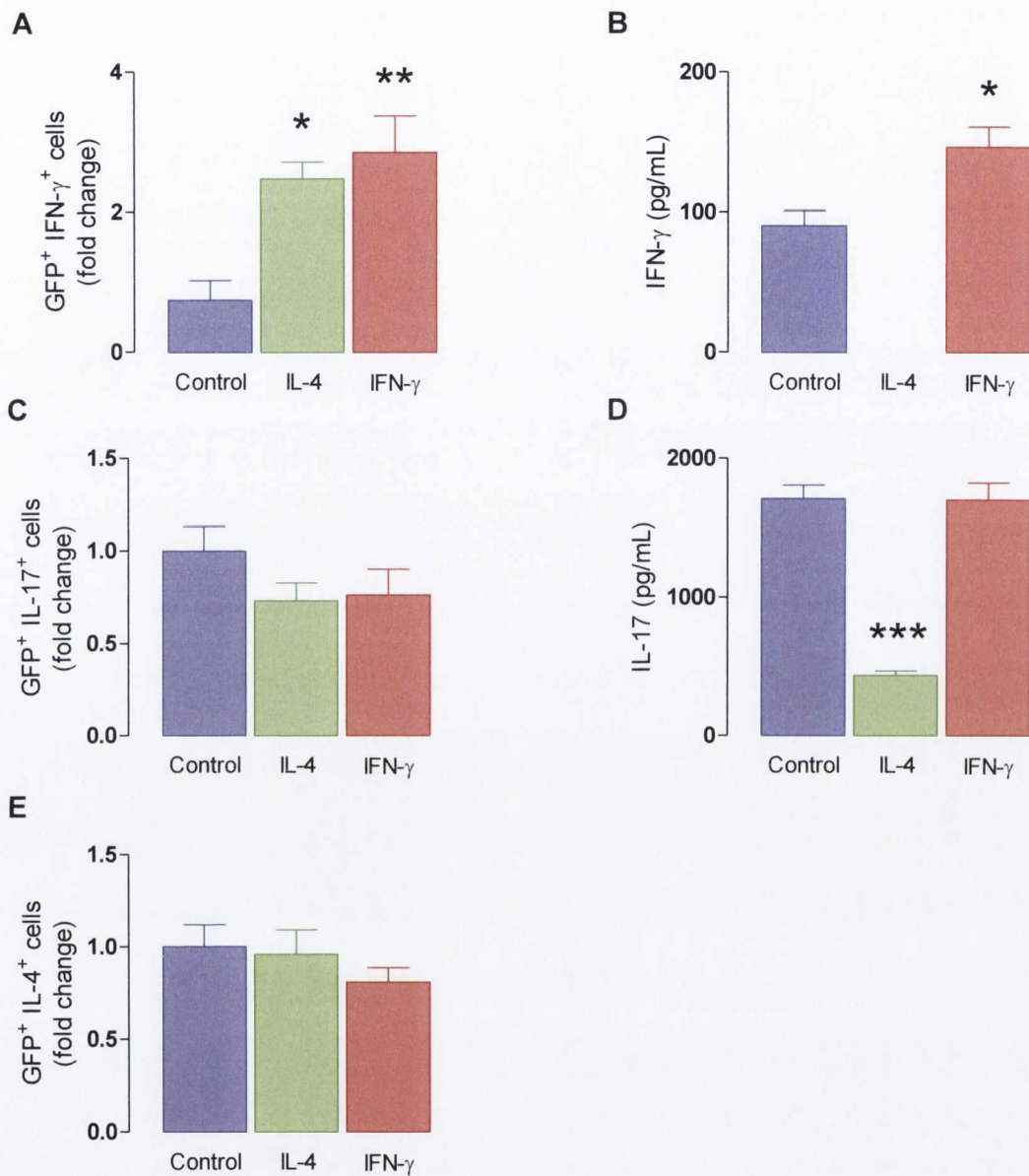


Figure 5.7 IFN-γ and IL-4 stimulated microglia induced the proliferation of IFN-γ⁺ T cells

T cells from the CTV assay were analysed for cytokine expression by flow cytometry and cytokine release by ELISA. The data show that pre-treatment of microglia with IL-4 and IFN-γ prior to co-culture significantly increased IFN-γ expression in T cells (A), although only IFN-γ treated microglia induced a significant increase in IFN-γ secretion (B). Neither stimulus affected IL-17 expression in T cells (C), but IL-4 treated microglia significantly decreased its release (D). Neither IL-4 nor IFN-γ-treated microglia altered IL-4 expression in T cells (E). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA with Dunnett's post-hoc analysis. Data are expressed relative to control as mean $\pm$ SEM ($n=3$).

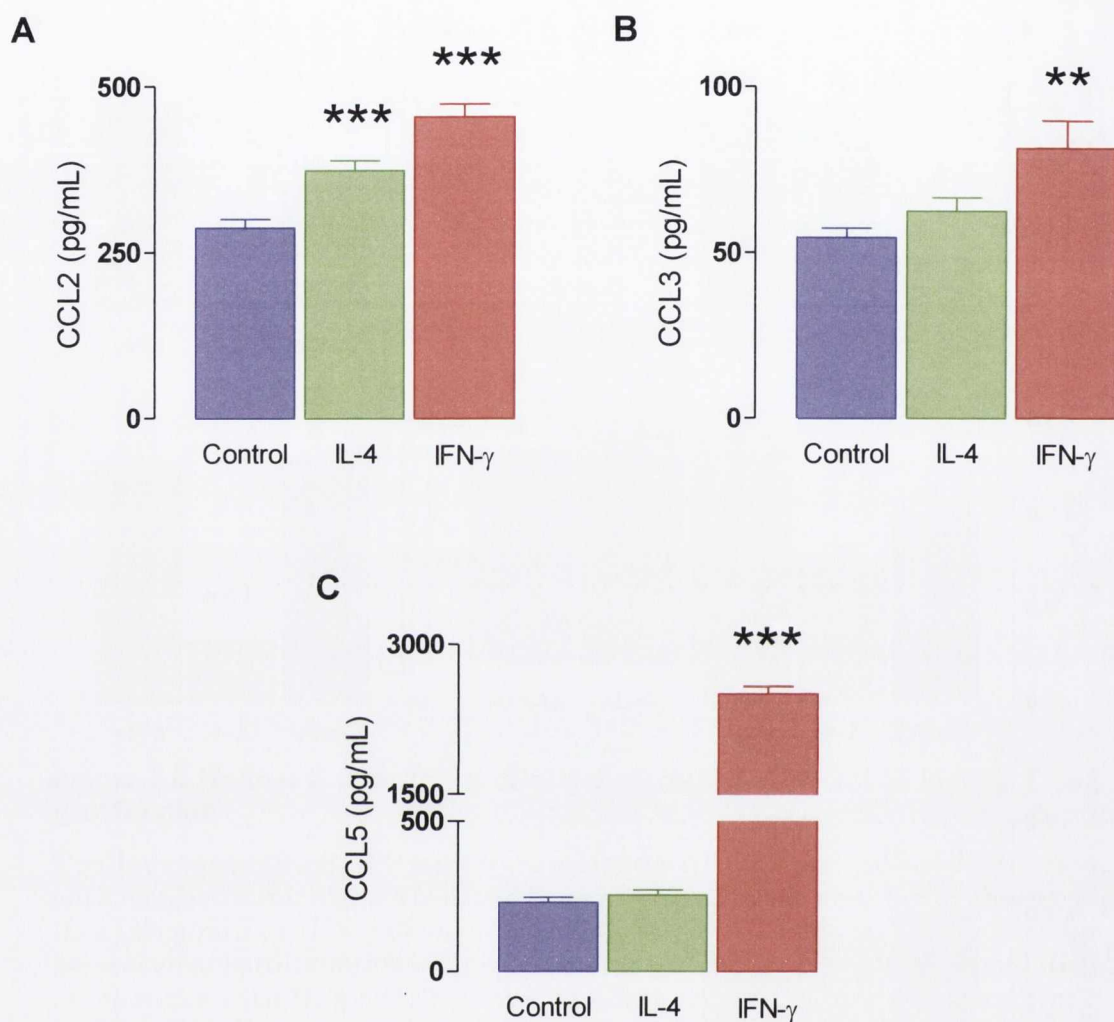


Figure 5.8 IFN- γ significantly increased microglial secretion of chemokines

Microglia were incubated in the presence or absence of IL-4 (20ng/mL) or IFN- γ (50ng/mL) for 24h and supernatants were harvested for assessment chemokine secretion. The data show that IFN- γ and IL-4 significantly increased the expression of CCL2 (A), while IFN- γ also increased the expression of CCL3 (B) and CCL5 (C). ** p < 0.01, *** p < 0.001; one-way ANOVA with Dunnett's post-hoc analysis. Data are expressed relative to control as mean $\pm$ SEM (n=3).

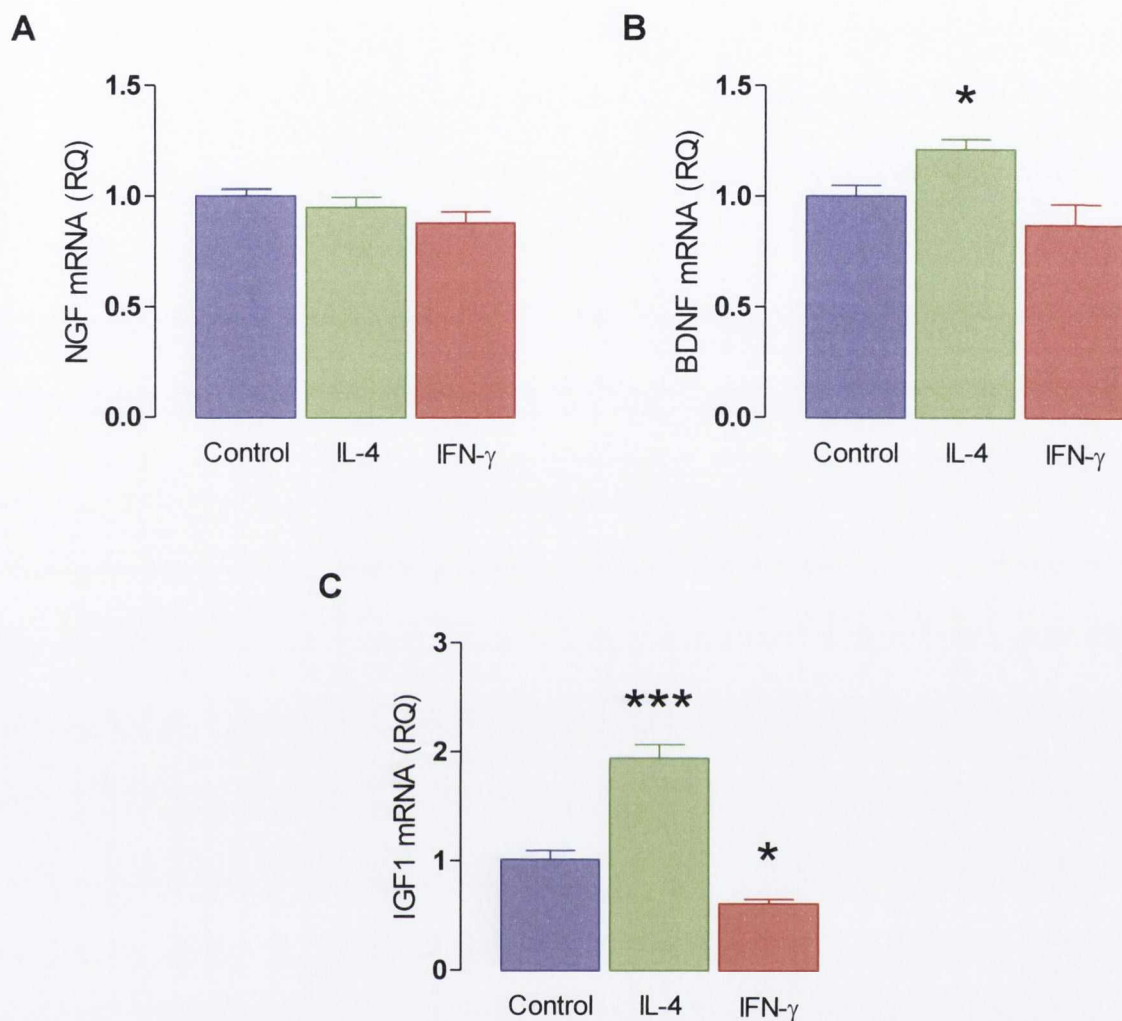


Figure 5.9 IL-4 induced microglial secretion of BDNF and IGF1

Microglia were incubated in the presence or absence of IL-4 (20ng/mL) or IFN-γ (50ng/mL) for 24h and cells were harvested for assessment of mRNA expression. The data show that neither IFN-γ nor IL-4 affected the mRNA expression of NGF (A). IL-4 significantly increased the mRNA expression of BDNF (B) and IGF1 (C), while IFN-γ significantly decreased the mRNA expression of IGF1. * $p < 0.05$, *** $p < 0.001$; one-way ANOVA with Dunnett's post-hoc analysis. Data are expressed relative to control as mean ± SEM (n=3).

5.4 Discussion

The aim of this study was to investigate the functional consequences of microglial polarisation. The most interesting findings were that IFN- γ induced a modest decrease in the phagocytosis of FITC-A β , but neither IFN- γ - nor IL-4-stimulated microglia induced an increase in T cell proliferation. IFN- γ also increased the secretion of chemokines CCL2, CCL3, and CCL5 and decreased expression of IGF1. In contrast, IL-4 increased the expression of IGF1, as well as BDNF.

In the present study, fluorescently-labelled latex beads and FITC-labelled A β were used to assess microglial phagocytosis. Internalisation of these particles by microglia was confirmed using immunocytochemistry and confocal microscopy, which demonstrated the presence of fluorescently-labelled beads and FITC-labelled A β in microglia, but not astrocytes. This is consistent with reports demonstrating the phagocytosis of FITC-labelled A β by microglia (Hickman et al., 2008), but previous studies have also demonstrated the phagocytosis of FITC-labelled A β by astrocytes (Jones et al., 2013). Internalisation of FITC-labelled A β by astrocytes has been shown to occur heterogeneously, with some populations taking up the same quantities of peptide as microglia, whilst other populations take up no peptide at all (Mandrekar et al., 2009), which may explain the discrepancies between reports on astrocyte phagocytosis. Furthermore, mixed glial cultures were assessed in the present study rather than purified astrocytes, and decreased phagocytosis may be due to the presence of phagocytic microglia. Although astrocytes may be capable of the phagocytosis of A β under certain circumstances, as demonstrated by the expression of TLR2, TLR4 and RAGE (Casula et al., 2011), their proficiency as phagocytes does not appear to be as great as microglia.

Since it has been hypothesised that activated microglia have altered phagocytic function, IFN- γ - and IL-4-stimulated microglia were assessed for

changes in phagocytosis. Both stimuli induced the same differential expression profile for markers of phagocytosis; significantly increased CD68 expression and significantly decreased CD33 expression, while TREM2 expression remained unchanged. CD68 and TREM2 are associated with increased microglial phagocytosis (Hsieh et al., 2009, Taylor et al., 2005, Zotova et al., 2013), while CD33 modulates TREM2 activity (Chan et al., 2015). Although TREM2 expression is not directly affected, the decrease in CD33 indicates increased TREM2 activity, which together with the increased CD68 expression, suggests increased phagocytic capabilities in both phenotypes.

To determine whether these changes in expression corresponded with altered phagocytic ability, the uptake of fluorescently-labelled latex beads and FITC-labelled A β by polarised microglia was assessed by FACS. The data suggest that priming microglia to an M1-like or M2-like phenotype has no effect of the phagocytosis of fluorescently-labelled latex beads, but there was an apparent decrease in the phagocytosis of FITC-labelled A β by M1-like cells compared with control cells. This decrease did not reach statistical significance, but could indicate that pro-inflammatory microglia are not as proficient at phagocytosing FITC-labelled A β .

There are conflicting reports of the effects of IL-4 and IFN- γ on phagocytosis, which differ depending on cell type and the particle being phagocytosed. Although IL-4 has been reported to decrease phagocytosis of pathogens by macrophages (Varin et al., 2010), Arg-1 positive microglia reduce A β deposition in AD suggesting that M2-like microglia have increased phagocytic capability (Cherry et al., 2015). There are also conflicting reports of the effects of IFN- γ on phagocytosis in macrophages (Marodi et al., 1993, Frausto-Del-Rio et al., 2012).

In AD, microglia are responsible for the phagocytosis and degradation of A β (Lee and Landreth, 2010) and impaired phagocytic function leads to chronic inflammation and neurodegeneration associated with disease progression (El

Khoury and Luster, 2008). The deposition of A β has been attributed to impaired phagocytic function of pro-inflammatory microglia (Krabbe et al., 2013) and microglia in the APP/PS1 mouse model of AD express decreased levels of the A β -binding scavenger receptors SRA, CD36, and RAGE, and are conversely associated with increased expression of IL-1 β and TNF α (Hickman et al., 2008). Even though markers of phagocytosis are increased in M1-like microglia in the present study, phagocytosis of A β is decreased. This suggests that pro-inflammatory microglia are primed for phagocytosis, but A β induces changes that impair phagocytic function, leading to increased plaque deposition. It may be that decreased phagocytic function observed in aged microglia (Njie et al., 2012a) leads to A β deposition, which accelerates AD pathology and plaque burden (Streit et al., 2009).

Although the present study did not reveal any increases in the phagocytosis of fluorescently labelled latex beads or the FITC-labelled A β by M2-like microglia, this may be due to the activation of these cells to a pro-inflammatory state by these molecules. A β is widely reported to induce a pro-inflammatory M1-like phenotype in microglia (Saijo and Glass, 2011b, Floden and Combs, 2006) and may have induced this activation state in the previously unstimulated cells, causing them to increase phagocytosis. A phenotypic switch subsequent to the adoption of the M2-like state may also have occurred due to the plastic nature of these cells. Microglial bead saturation could also account for the absence of differences in phagocytosis between phenotypes. As demonstrated by microscopy, there was robust uptake of fluorescent particles by microglia, with all cells positively staining for substrate, making differences in uptake difficult to differentiate by FACS.

Infiltrating T cells are the primary inflammatory aggressors in MS (Lassmann et al., 2001) and have also been associated with AD pathology (Bryson and Lynch, 2016). The ability of polarised microglia to generate an adaptive immune response was investigated and, although IFN- γ increased the expression of markers associated with antigen presentation, neither IFN-

γ nor IL-4 had any effect on the induction of T cell proliferation. T cell differentiation was assessed by ELISA and IFN- γ secretion was significantly increased from T cells co-cultured with M1-like microglia, suggesting that pro-inflammatory microglia promote a positive feedback loop to amplify the inflammatory response. In contrast to this, M2-like microglia decreased T cell secretion of IL-17, demonstrating the anti-inflammatory capacity of this phenotype.

The proliferation and differentiation of T cells by microglia has been demonstrated (Aloisi et al., 2000, Zhang et al., 2002, Aloisi et al., 1999), and these findings are in contrast to previous reports showing that IFN- γ stimulated microglia, which had been irradiated and co-cultured with T cells, increased T cell proliferation (Li et al., 2007). However, this study used a different T cell stain to assess proliferation, and also co-cultured cells for 3 days rather than the 7 day incubation period used in the present study. The discrepancy may be due to the irradiated microglia disintegrating over time and releasing cytosolic molecules that boost the proliferation of T cells, bringing all samples to an equal level of proliferation. Alternatively, T cell proliferation may slow over time, and the numbers of proliferated cells may reach a maximal capacity for the size of the culture plate well.

During CNS inflammation, microglia produce chemokines which recruit infiltrating peripheral immune cells (Aloisi, 2001), and chemokines and chemokine receptor expression is upregulated in neurodegenerative disorders (Cartier et al., 2005). IFN-induced increased secretion of CCL2, CCL3, and CCL5 from microglia and although IL-4 increased CCL2 secretion, no changes in CCL3 or CCL5 were observed. These data suggest that M1-like microglia enhance inflammation by inducing the infiltration of peripheral immune cells, while M2-like cells induce a less severe level of inflammation. These findings complement the literature, which reports that activation of microglia to an M1-like phenotype is associated with increased chemokine

expression in neuroinflammatory disorders (Krabbe et al., 2013, Streit et al., 2009).

The expression of growth factors were assessed in IFN- γ - and IL-4-stimulated microglia, and the data show that IFN- γ decreases IGF1 expression, while IL-4 increases BDNF and IGF1. These results consistent with the literature, as M1-like microglia and decreased levels of IGF1 and BDNF are associated with the pro-inflammatory environment seen in AD (Mattson, 2008, Westwood et al., 2014, Gasparini et al., 2002). The data suggest that skewing microglial phenotype to the M2-like in such disorders may boost growth factor levels and help alleviate the inflammation in this disorder.

The findings of this study assist in the characterisation of the functional ability of inflammatory microglia associated with neuroinflammatory disorders. M1-like cells display increased levels of phagocytic markers, but this is not associated with increased A β phagocytosis. These cells can also induce the differentiation of T_h1 cells and recruit other immune cells to the site of inflammation through the secretion of chemokines. In contrast, M2-like microglia do not secrete the same quantities of chemokines and enhance neuronal health through expression of growth factors.

Chapter 6 Characterisation of age- and genotype-related effects on microglial activation

6.1 Introduction

Several studies have demonstrated that glial activation occurs with age and increased expression of markers of both activated microglia and astrocytes have been reported (Lynch et al., 2010). In the case of microglia, some researchers have interpreted this as an indication that microglia become primed with age and are therefore more susceptible to inflammatory stimuli than microglia from the brain of young animals (von Bernhardi et al., 2010). Consistent with this, it has been shown that systemic or central administration of LPS to aged animals increases hippocampal expression of TNF α , IL-1 β and IL-6, markers of microglial activation, to a greater extent than young animals (Hoogland et al., 2015). More recently, a similar increased responsiveness to LPS has been reported in isolated microglia prepared from the brain of aged, compared with young, mice (Sierra et al., 2007, Wynne et al., 2010). This age-related change in microglial plasticity may, at least in part, explain the synaptic and neuronal deficits that progress with age (Loane et al., 2009).

Microglial activation, and indeed astrocytic activation, are also characteristics of Alzheimer's disease (AD) and therefore the disease, like most neurodegenerative diseases, is characterized by neuroinflammatory changes (Heneka et al., 2015). Similar changes have been described in animal models of several neurodegenerative diseases including APP/PS1 mice (Colton and Wilcock, 2010). There is compelling evidence demonstrating that mRNA expression of TNF α and iNOS is increased in the hippocampus of APP/PS1 mice and this suggests that the microglia adopt an M1-like state (Minogue et al., 2014a). Furthermore, stimulation of microglia prepared from APP/PS1 mice with IFN γ induces a greater increase in TNF α and iNOS mRNA than it induces in microglia from WT mice (Jones et al., 2015).

The data presented in Chapter 4 indicated that the inflammatory changes induced by IFN- γ in microglia prepared from neonatal mice were accompanied by a metabolic switch to glycolysis, mirroring the evidence

obtained from BMDMs in Chapter 3, and consistent with the literature (Galvan-Pena and O'Neill, 2014). This was associated with changes in PFKFB3 and PFKFB1, which are modulators of glycolysis (Ruiz-Garcia et al., 2011), and with a shift in iron handling by cells. Here the objective was to determine whether a similar switch was observed in microglia prepared from APP/PS1, compared with WT mice and to establish whether there was evidence of an age-related change.

The aims of this study are:

1. To determine whether expression of markers of the M1 phenotype is greater in microglia from aged and/or APP/PS1 mice compared with young WT mice.
2. To establish whether there is an age- or genotype-related change in metabolic signature and iron handling function in microglia.

6.2 Methods

Groups of 9 month and 17 month old APP/PS1 mice and their littermate WT controls were used in this study. The young WT mice consisted of 4 females and 3 males, and APP/PS1 mice of 4 females and 2 males. Aged WT mice consisted of 6 females and 1 male, and APP/PS1 mice of 4 females and 2 males. Mice were anaesthetized using urethane (1.5 g/kg) and cardiac perfusion was carried out. Brains were dissected free and the cerebellum removed. Hippocampal tissue was snap frozen in liquid nitrogen and stored at -80°C for mRNA analysis and microglia were isolated from the remaining tissue as described in section 2.2 for analysis by SeaHorse, as described below, and RT-PCR, as described in section 2.8.

6.2.1 Modified SeaHorse mitochondrial stress test for isolated microglia

Preliminary experiments were carried out on cells isolated from the brains of 9 month-old WT mice in order to optimize the conditions required for assessment of metabolism. It was found that isolated microglia from adult mice were less viable than the primary microglial cultures procured from neonatal mice, and did not survive when subjected to the same stresses. Therefore, modifications were made to the experimental protocol.

Firstly, poly-D-lysine was used to coat the plates to ensure adherence of the cells. Plates were incubated overnight in the flow hood in the presence of poly-D-lysine (50ng/mL; 200µL/well), and were protected from ultraviolet light using tinfoil. The following day, the poly-D-lysine was removed and the wells were washed twice with sterile H₂O.

Preliminary experiments were carried out to assess the impact of time on cell viability. Cell death occurred when cells were left overnight to adhere to the cell culture plates, and cell respiration was inversely related to time in culture. To overcome this problem, cells were seeded in SeaHorse assay media (100µL/well) and incubated for 1h prior to being topped up to 475µL/well with assay media. At this point, the plate was placed in a no-CO₂ incubator for 1h prior to reading. Thus, preparation time was reduced to 2h.

The mitochondrial stress test was modified for use in cells prepared from adult mice. Preliminary experiments showed that the response to FCCP was minimal when cells had previously been incubated with oligomycin and therefore this step was removed. The reason for the poor FCCP response in oligomycin-treated cells is not clear, but may be due to cell death following 24min without the production of ATP from the electron transport chain. Therefore, in the modified procedure, no oligomycin was added during the assay, but FCCP and antimycin A were added to ports A and B of the sensor cartridge.

The mitochondrial stress test was carried out in preference to the glycolytic stress test due because the cells failed to recover following the incubation in glucose-free media.

6.3 Results

Expression of M1 activation state markers was investigated in hippocampal tissue from 9 month-old and 17 month-old WT and APP/PS1 mice. A genotype-related increase in TNF α mRNA was observed ($F_{(1,28)}=6.34$, $p=0.018$, two-way ANOVA; Figure 6.1A) but there was no age-related change. A significant age x genotype interaction effect was also observed ($F_{(1,28)}=5.49$, $p=0.026$; two-way ANOVA; Figure 6.1A). iNOS mRNA remained unchanged by age or genotype (Figure 6.1B).

Microglia, isolated from the brains of these mice, were also assessed for mRNA expression of TNF α and iNOS. The data indicate that there was an age-related increase in TNF α mRNA ($F_{(1,25)}=8.37$, $p=0.008$; two-way ANOVA; Figure 6.2A), but no genotype-related change. A significant age x genotype interaction effect was also observed ($F_{(1,25)}=4.68$, $p=0.041$; two-way ANOVA; Figure 6.2A). iNOS mRNA remained unchanged by age or genotype (Figure 6.2B).

As indicated in Chapter 3, M1 macrophages preferentially utilize glycolysis as their energy source and M2 macrophages preferentially utilize oxidative phosphorylation. Here the possibility that age and/or phenotype modulated microglial metabolism was examined using SeaHorse technology. First, the data from analysis of the effect of age on metabolic signature of microglia are presented. A small age-related increase in basal OCR was observed (Figure 6.3A); analysis of the mean data indicated that this increase did not reach statistical significance (Figure 6.3B). Addition of the uncoupling agent, FCCP, and the electron transport chain inhibitor, antimycin A allows analysis of maximal respiration. The data show that FCCP increased OCR to a greater extent in microglia from 9 month-old, compared with 17 month-old, mice (Figure 6.3A) and addition of antimycin A reduced OCR to the same extent in both preparations. Maximal respiration was significantly decreased in microglia from aged mice compared with young mice (** $p<0.01$; one-way ANOVA with Dunnett's post hoc analysis; Figure 6.3B).

Microglia isolated from the brains of 9 month-old WT and APP/PS1 mice were assessed for OCR. The data show that there was a small genotype-related decrease in respiration (Figure 6.4A) but analysis of the mean data indicated that this decrease did not reach statistical significance (Figure 6.4B). There was no genotype-related difference observed in the maximal respiration of these cells (Figure 6.4C).

Next, the effect of age on the metabolic signature of microglia isolated from 9 month-old was compared with microglia from 17 month-old APP/PS1 mice (Figure 6.5A). The data revealed an age-associated increase in basal respiration (** $p < 0.01$; one-way ANOVA with Dunnett's post hoc analysis; Figure 6.5B). Microglia from the brains of aged mice also showed a statistically significant increase in maximal respiration (** $p < 0.01$; one-way ANOVA with Dunnett's post hoc analysis; Figure 6.5C).

The effect of genotype on the metabolic signature of microglia isolated from 17 month-old mice was assessed. Basal respiration was similar in cells from WT and APP/PS1 mice, but the addition of FCCP had a greater effect on cells from APP/PS1 mice (Figure 6.6A). While mean basal respiration was unchanged with genotype (Figure 6.6B), maximal respiration was significantly greater in microglia from APP/PS1 mice (** $p < 0.01$; one-way ANOVA with Dunnett's post hoc analysis; Figure 6.6C).

Analysis of age- and genotype-related changes together showed that microglia isolated from the brains of 17 month-old APP/PS1 mice exhibited the highest rate of oxygen consumption, while the lowest level of respiration was observed in the microglia isolated from the brains of 9 month-old APP/PS1 mice (Figure 6.7A). The data indicate that there was an overall age-related increase in microglial basal respiration ($F_{(1,16)}=11.97$, $p=0.0032$; two-way ANOVA; Figure 6.7B) but there was no genotype-related change. In contrast to this, a genotype-related change in maximal respiration of microglia was observed ($F_{(1,16)}=10.48$, $p=0.0052$; two-way ANOVA; Figure 6.7C), however this manifested in contrasting effects depending on the age

groups, as indicated by the statistically significant interaction ($F_{(1,16)}=34.46$, $p=0.0001$; two-way ANOVA; Figure 6.7C). The data suggest that there was a genotype-related decrease in maximal respiration in the 9 month-old group, and a genotype-related increase in maximal respiration in the 17 month-old group. There were no age-associated effects observed on the maximal respiration of cells.

The data presented in Chapter 4 suggested that PFKFB3:PFKFB1 may be correlated with glycolysis and therefore expression of these enzymes in microglia from 9 month-old and 17 month-old WT and APP/PS1 mice was assessed. While neither significant age- nor genotype-related changes in expression of either enzyme was observed (Figure 6.8A, B), the PFKFB3:PFKFB1 ratio revealed a significant age-related increase ($F_{(1,33)}=5.47$, $p=0.026$, two-way ANOVA; Figure 6.8C).

The data presented in Chapter 4 suggested that an inflammatory phenotype in microglia was correlated with increased iron storage and therefore the expression of markers of iron handling was assessed in microglia isolated from the mice. Modest age- and genotype-related increases in ferritin expression (Figure 6.9A), but no statistically significant changes, were observed. Ferroportin expression was significantly decreased by both age ($F_{(1,43)}=21.38$, $p<0.0001$; two-way ANOVA; Figure 6.9B) and genotype ($F_{(1,43)}=16.48$, $p=0.0002$; two-way ANOVA; Figure 6.9B) and there was a significant age x genotype interaction was observed ($F_{(1,43)}=16.73$, $p=0.0002$; two-way ANOVA; Figure 6.9B).

Analysis of the mRNA expression of PFKFB1 and PFKFB3 in hippocampal tissue from the 9 month-old and 17 month-old WT and APP/PS1 mice revealed neither an age-, nor a genotype-, related change (Table 6.10A, B). A significant interaction effect was observed in the expression of PFKFB3, with macroglia from younger animals displaying elevated levels of the enzyme. The expression of ferritin and ferroportin was also assessed in this tissue. The data suggest that there was an age-related decrease in ferritin

($F_{(1,25)}=4.84$, $p=0.0373$; two-way ANOVA; Table 6.10C) and no genotype-related changes. A genotype-related increase in ferroportin was observed ($F_{(1,26)}=6.551$, $p=0.0166$; two-way ANOVA; Table 6.10D), but no age-related changes were identified.

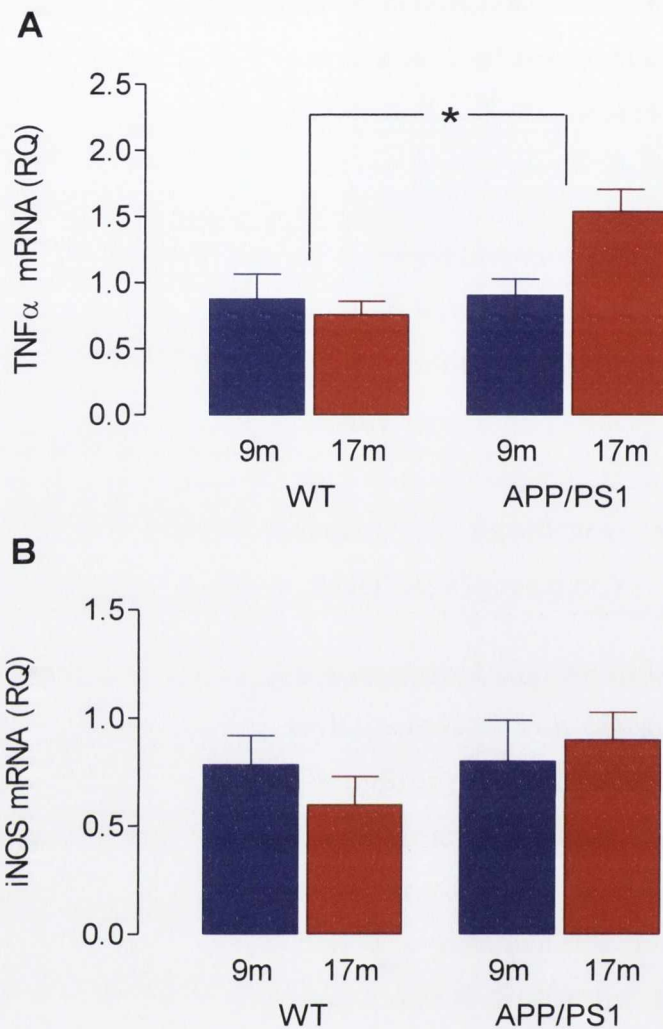


Figure 6.1 Increased TNF- α mRNA was observed in hippocampus of APP/PS1 mice

Hippocampal tissue prepared from groups of 9 month-old and 17 month-old WT and APP/PS1 mice was assessed for the mRNA expression of TNF α and iNOS using RT-PCR. The data show a significant genotype x age interaction in TNF α mRNA (A). No effect of age or genotype was observed in iNOS mRNA (B). Data are expressed relative to young WT values as mean $\pm$ SEM (n=6-12).

A: Genotype_{effect} [$F_{(1,28)}=6.34, p=0.018$], age_{effect} [$F_{(1,28)}=2.61, p=0.117$], interaction_{effect} [$F_{(1,28)}=5.49, *p=0.026$]

B: Genotype_{effect} [$F_{(1,27)}=0.79, p=0.379$], age_{effect} [$F_{(1,27)}=0.06, p=0.808$], interaction_{effect} [$F_{(1,27)}=0.63, p=0.432$]

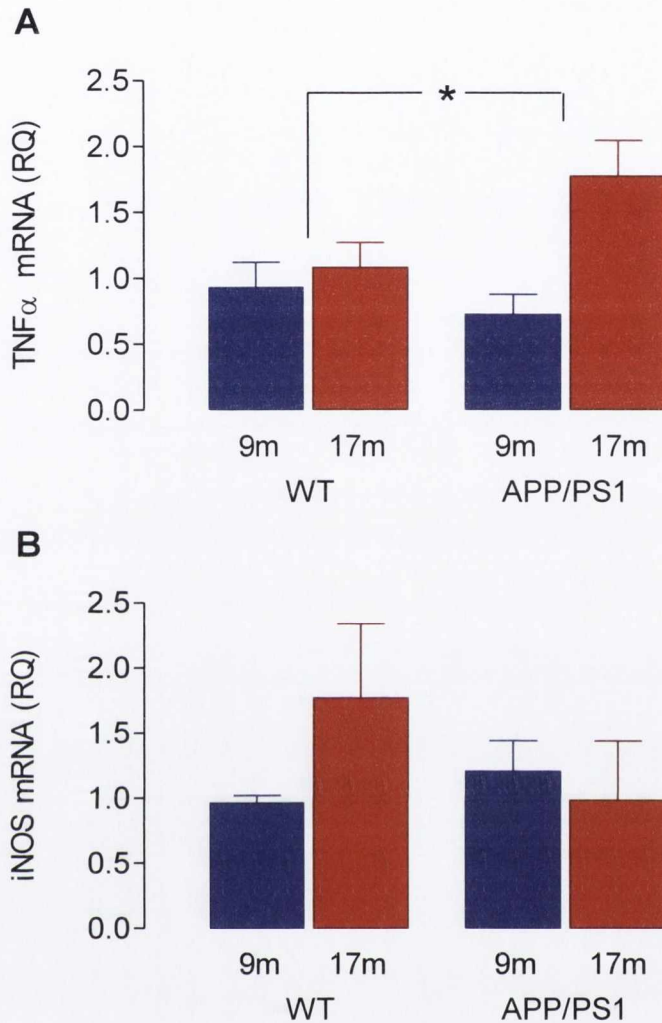


Figure 6.2. Microglial expression of TNF α mRNA was affected by age and genotype

Isolated microglia were prepared from groups of 9 month-old and 17 month-old WT and APP/PS1 mice and assessed for mRNA expression of TNF α and iNOS using RT-PCR. The data show a significant genotype x age interaction in TNF α mRNA (A). No effect of age or genotype was observed in iNOS mRNA (B). Data are expressed relative to young WT values as mean $\pm$ SEM (n=6-10).

A: Genotype_{effect} [$F_{(1,25)}=1.38$, $p=0.251$], age_{effect} [$F_{(1,25)}=8.37$, $p=0.008$], interaction_{effect} [$F_{(1,25)}=4.68$, $*p=0.041$]

B: Genotype_{effect} [$F_{(1,27)}=0.54$, $p=0.77$], age_{effect} [$F_{(1,27)}=0.62$, $p=0.435$], interaction_{effect} [$F_{(1,27)}=1.95$, $p=0.174$]

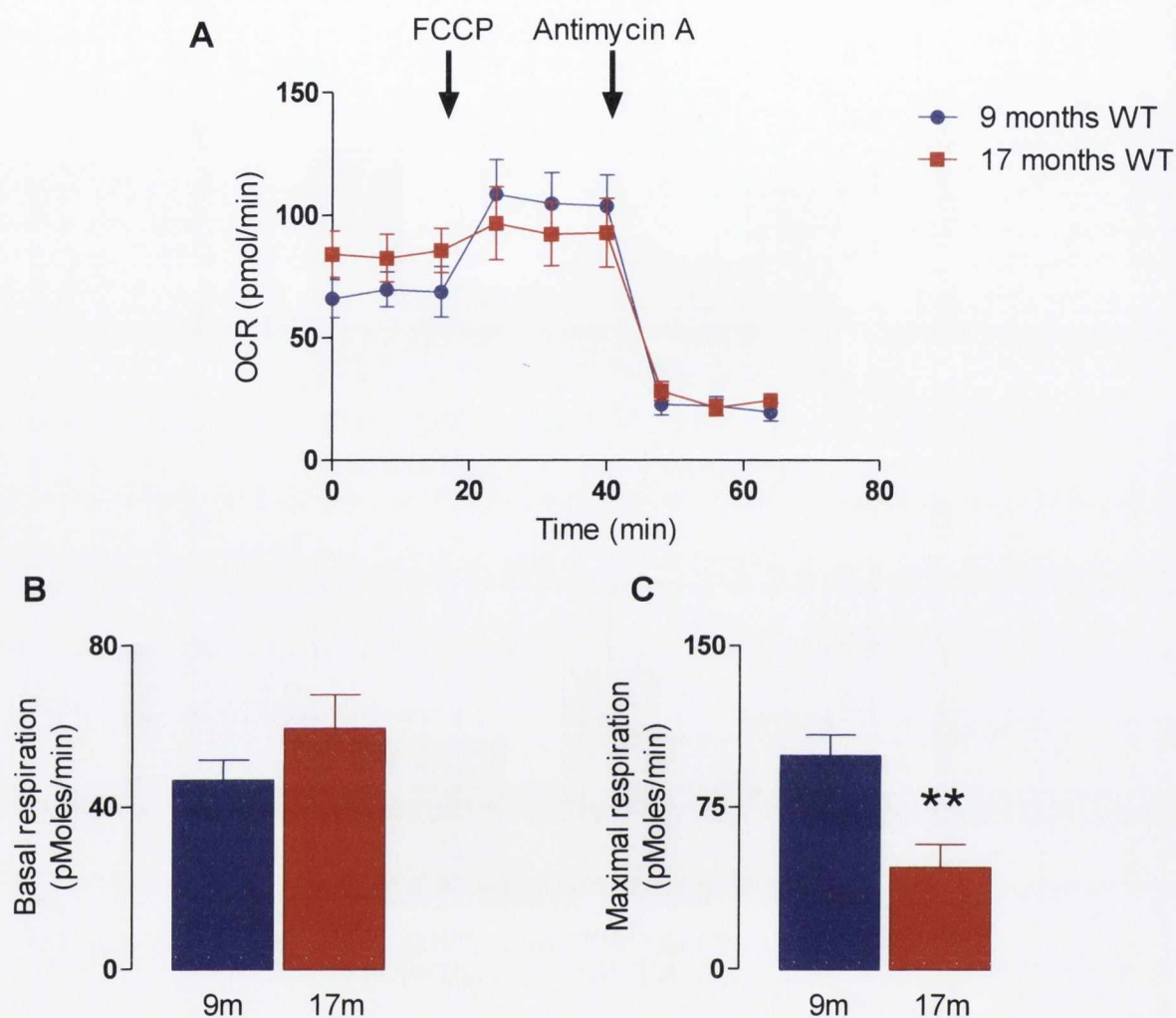


Figure 6.3 Maximal respiration was decreased in aged WT mice compared with younger controls

Microglia were isolated from the brains of 9 month-old and 17 month-old WT mice and a mitochondrial stress test was carried out to assess the metabolism of the cells. The SeaHorse analyser was used to measure OCR (A) and the data show that there were no significant change in the basal respiration of cells with age (B). However, there was a significant decrease observed in the maximal respiration of cells from the older animals (C). ** $p < 0.01$; student's t-test for independent means. Data are expressed as mean $\pm$ SEM ($n=5$).

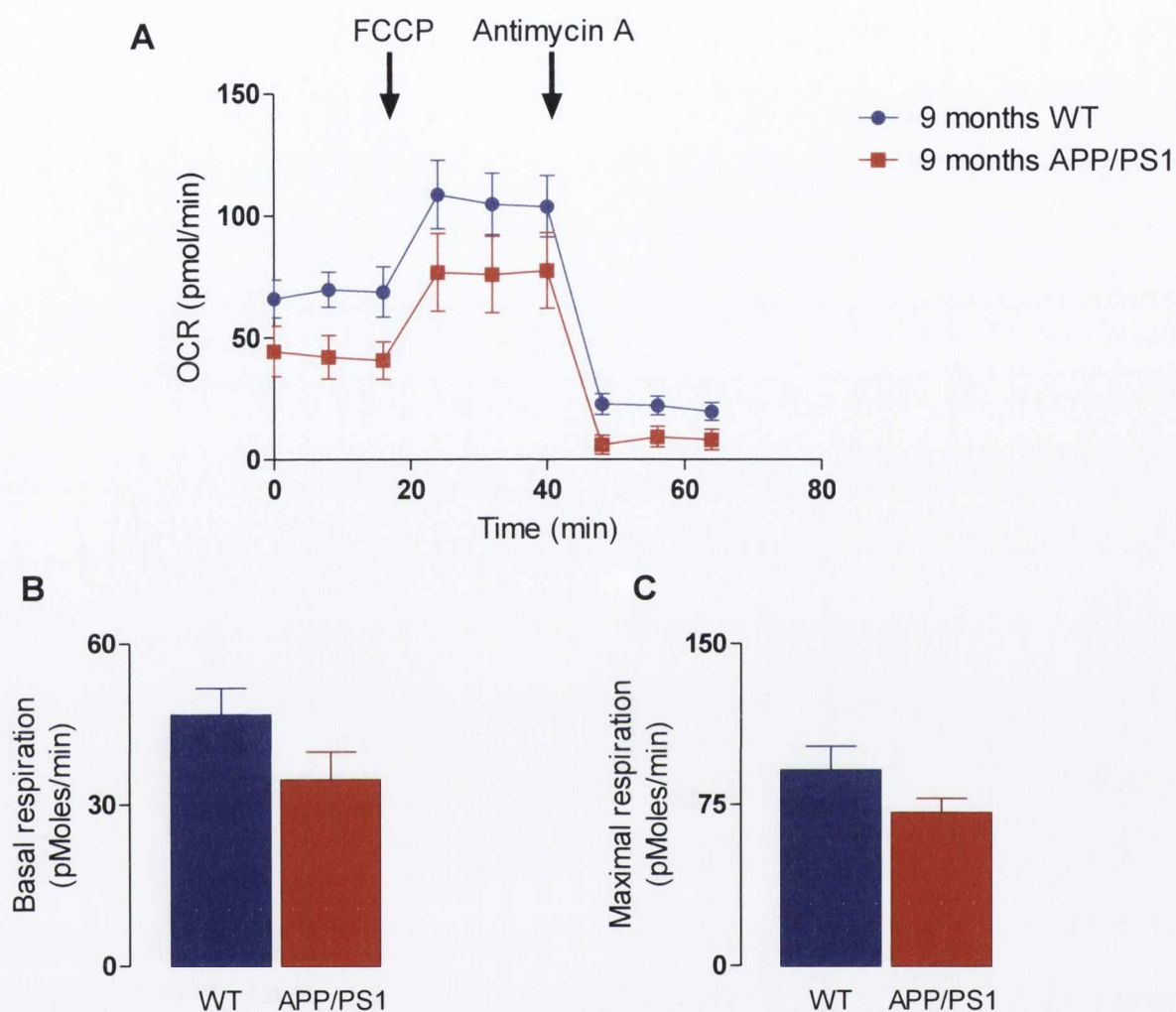


Figure 6.4 There was no change in respiration in 9 month-old APP/PS1 mice compared with WT controls

Microglia were isolated from the brains of 9 month-old WT and APP/PS1 mice and a mitochondrial stress test was carried out to assess the metabolism of the cells. The SeaHorse analyser was used to measure OCR (A) and the data show that there were decreases in both the basal respiration of cells (B) and the maximal respiration of cells (C) in microglia from APP/PS1 mice, but no statistically significant changes in either measure. Data are expressed as mean $\pm$ SEM (n=5).

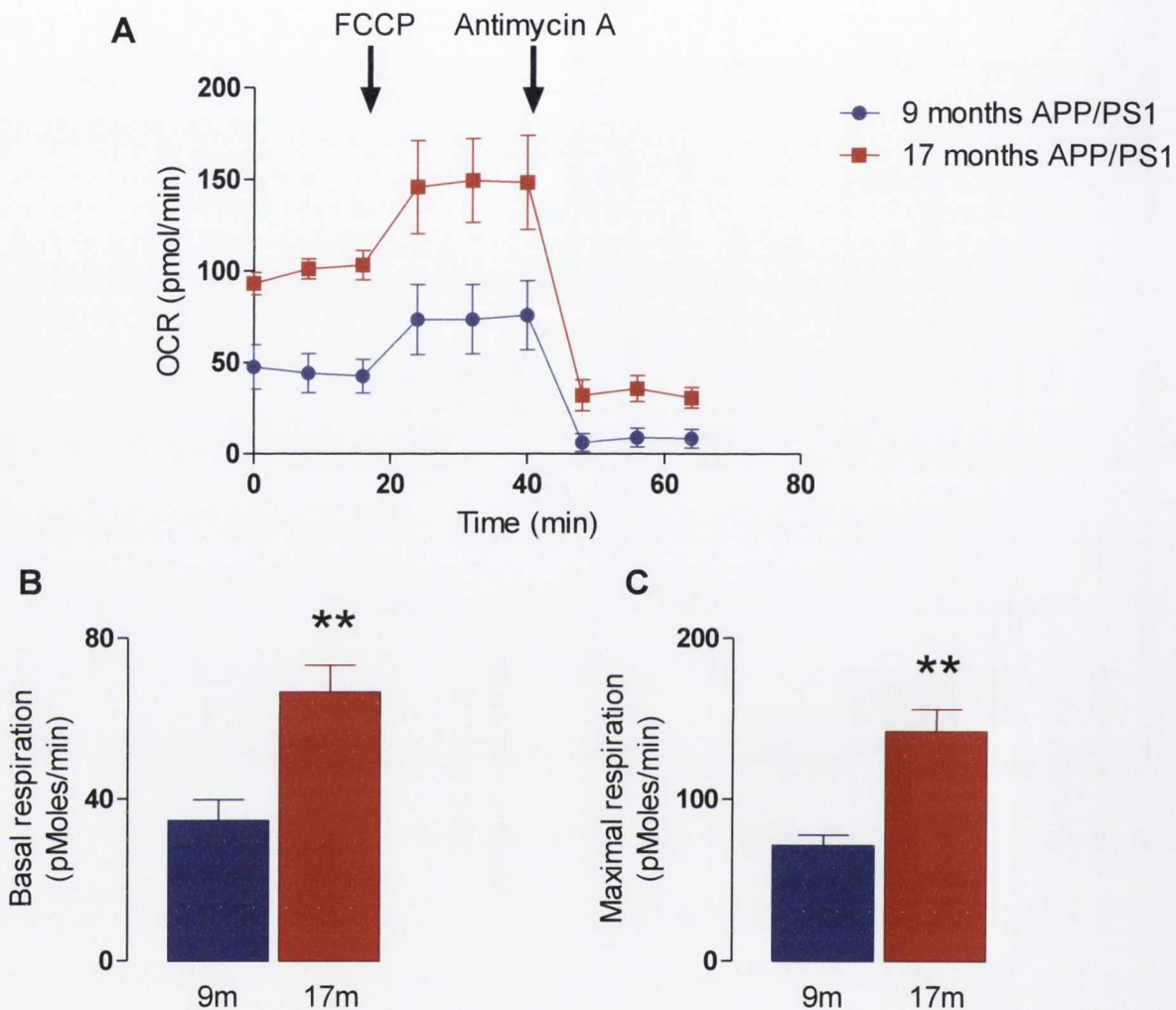


Figure 6.5 Respiration was higher in aged APP/PS1 mice compared with younger controls

Microglia were isolated from the brains of 9 month-old and 17 month-old APP/PS1 mice and a mitochondrial stress test was carried out to assess the metabolism of the cells. The SeaHorse analyser was used to measure OCR (A) and the data show that cells from the older mice exhibited significant increases in both the basal respiration (B) and maximal respiration (C). ** $p < 0.01$; student's t-test for independent means. Data are expressed as mean $\pm$ SEM ($n = 5$).

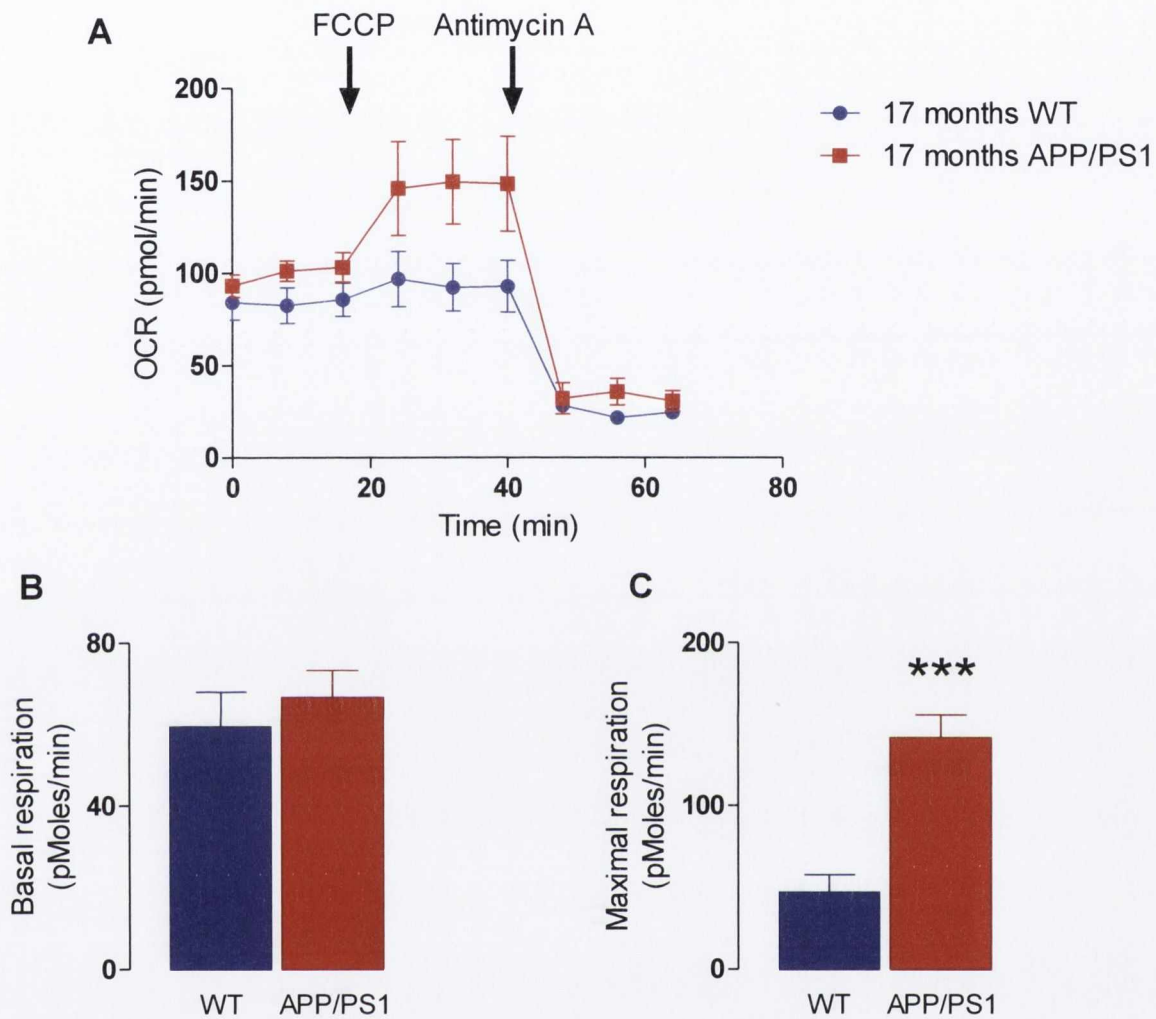


Figure 6.6 Maximal respiration was increased in aged APP/PS1 microglia

Microglia were isolated from the brains of 17 month-old WT and APP/PS1 mice and a mitochondrial stress test was carried out to assess the metabolism of the cells. The SeaHorse analyser was used to measure OCR (A) and the data show that there no changes in basal respiration (B), but that there was a significant increase in the maximal respiration of cells from the APP/PS1 mice (C). *** $p < 0.001$; student's t-test for independent means. Data are expressed as mean $\pm$ SEM (n=5).

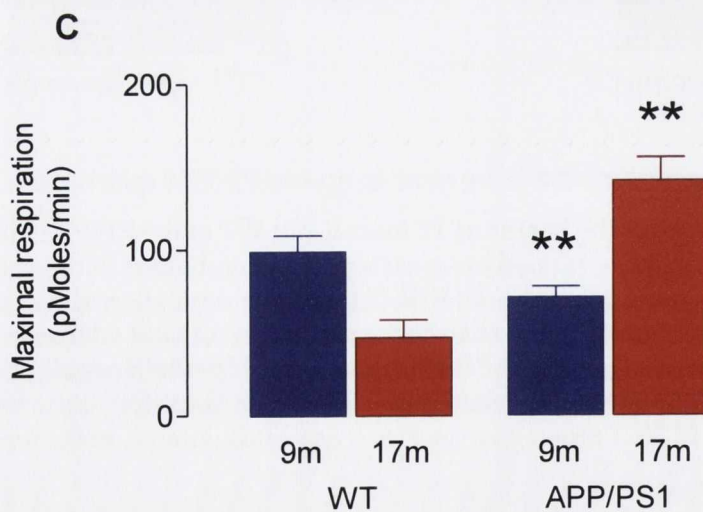
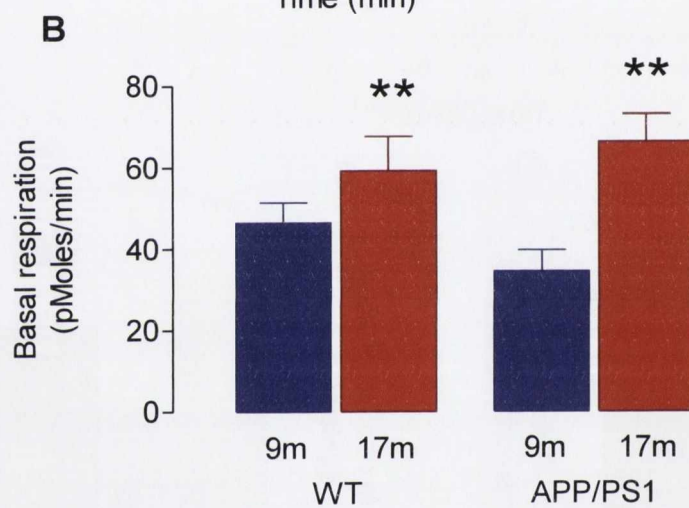
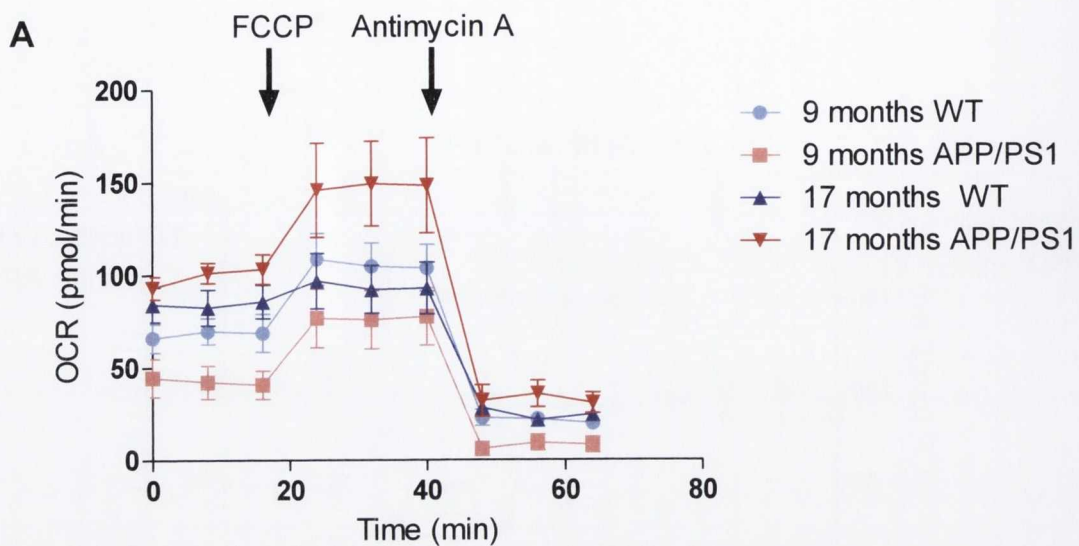


Figure 6.7 Age- and genotype-effects on microglial respiration

Microglia were isolated from the brains of 9 and 17 month-old WT and APP/PS1 mice and a mitochondrial stress test was carried out to assess the metabolic signature of the cells. The SeaHorse analyser was used to measure OCR (A) and the data show that there was a significant age-associated increase in the basal respiration of cells (B). Significant genotype-related changes were observed in the maximal respiration from these cells, although with contrasting results (C). ** $p < 0.01$; Two-way ANOVA followed by Bonferroni *post-hoc* analysis. Data are expressed relative to WT controls as mean $\pm$ SEM (n=4-6).

B: Genotype_{effect} [$F_{(1,16)}=0.1528$, $p=0.701$], age_{effect} [$F_{(1,16)}=11.97$, $p=0.0032$], interaction_{effect} [$F_{(1,16)}=2.2$, $p=0.1575$]

C: Genotype_{effect} [$F_{(1,16)}=10.48$, $p=0.0052$], age_{effect} [$F_{(1,16)}=0.8363$, $p=0.374$], interaction_{effect} [$F_{(1,16)}=34.46$, $p=0.0001$]

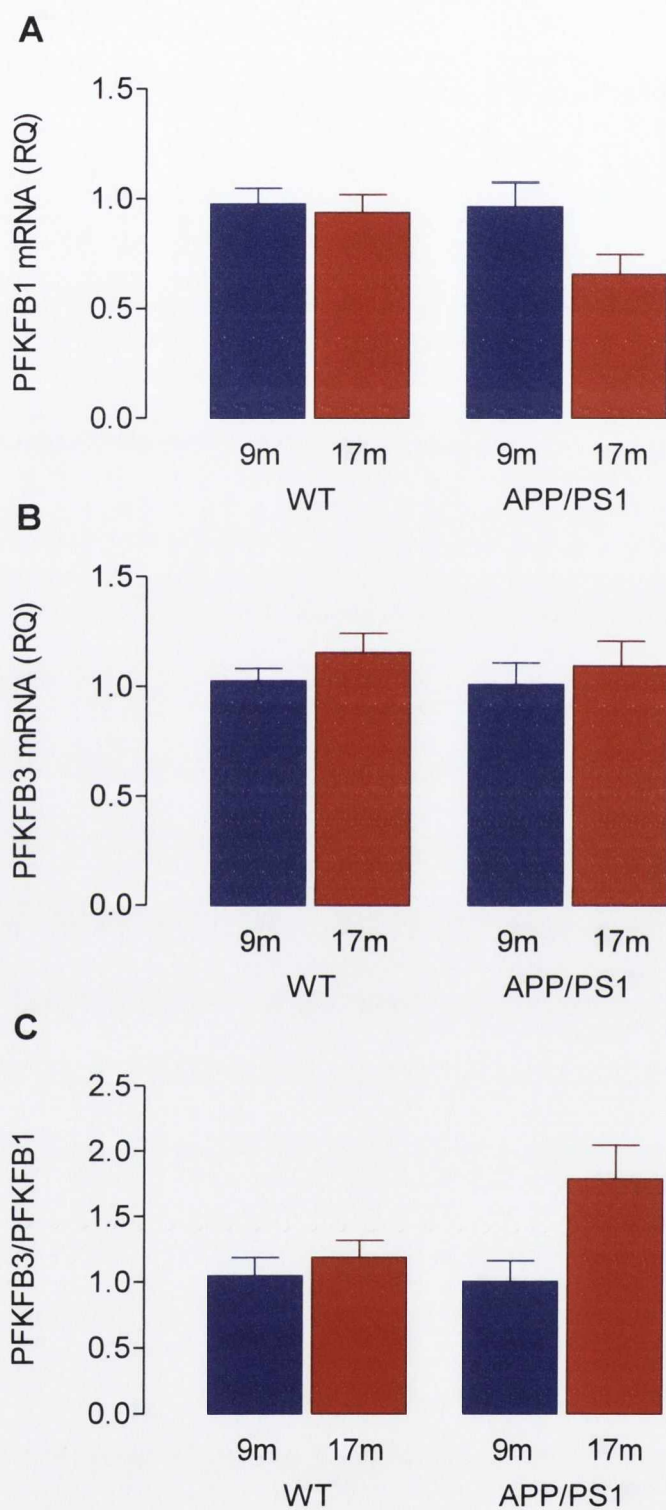


Figure 6.8 PFKBF3:PFKFB1 was increased in an age-dependent manner

Isolated microglia were prepared from groups of 9 month-old and 16 month-old WT and APP/PS1 mice and assessed for mRNA expression of PFKFB1 and PFKFB3 using RT-PCR. Although there was no age- or genotype-effect on the expression of either enzyme (A,B), there was a significant effect of age-associated increase in PFKFB3:PFKFB1 (C). Data are expressed as means $\pm$ SEM (n=6-12).

A: Genotype_{effect} [$F_{(1,35)}=2.52$, $p=0.121$], age_{effect} [$F_{(1,35)}=3.49$, $p=0.071$], interaction_{effect} [$F_{(1,35)}=2.04$, $p=0.161$]

B: Genotype_{effect} [$F_{(1,44)}=0.167$, $p=0.685$], age_{effect} [$F_{(1,44)}=1.27$, $p=0.264$], interaction_{effect} [$F_{(1,44)}=0.055$, $p=0.815$]

C: Genotype_{effect} [$F_{(1,33)}=0.205$, $p=0.161$], age_{effect} [$F_{(1,33)}=5.47$, $p=0.026$], interaction_{effect} [$F_{(1,33)}=2.68$, $p=0.111$]

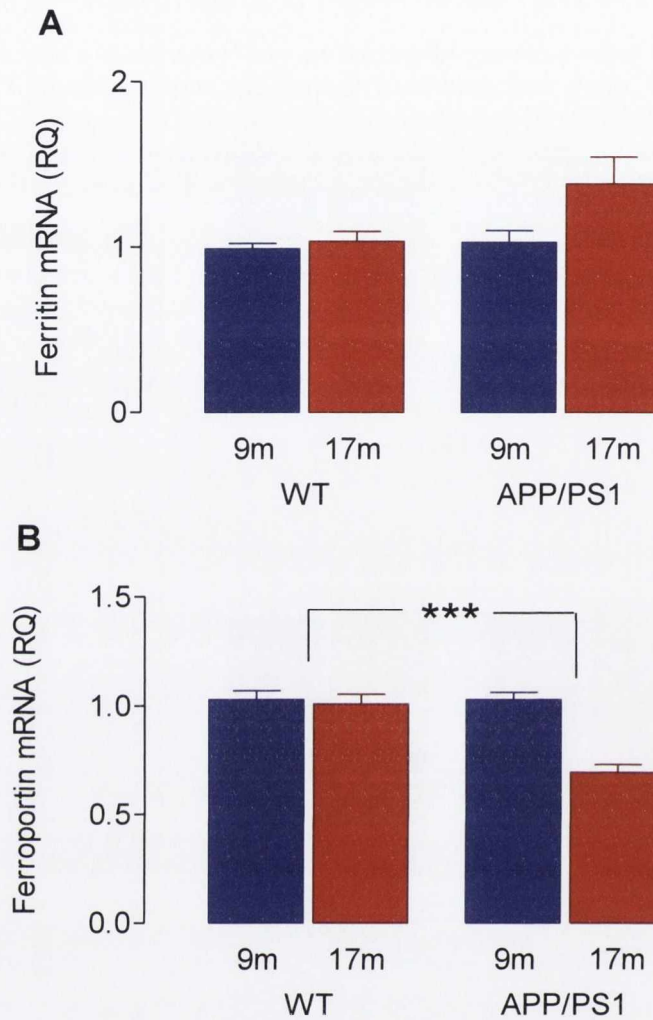


Figure 6.9 Ferroportin mRNA expression was decreased in microglia from aged APP/PS1 mice

Isolated microglia were prepared from groups of 9 and 17 month-old WT and APP/PS1 mice and assessed for mRNA expression of ferritin and ferroportin using RT-PCR. There was no significant age- or genotype-effect in ferritin expression (A), but significant age- and genotype-effects were observed in ferroportin mRNA. Data are expressed as means $\pm$ SEM (n=6-15).

A: Genotype_{effect} [$F_{(1,43)}=3.61$, $p=0.064$], age_{effect} [$F_{(1,43)}=3.43$, $p=0.071$], interaction_{effect} [$F_{(1,43)}=2.17$, $p=0.148$]

B: Genotype_{effect} [$F_{(1,43)}=16.48$, $p=0.0002$], age_{effect} [$F_{(1,43)}=21.38$, $p<0.0001$], interaction_{effect} [$F_{(1,43)}=16.73$, $p=0.0002$]

Target	Source of variation	df	SS	MS	F	P value
A) PFKFB1						
	Interaction	1	0.01271	0.01271	0.07137	0.7922
	Genotype	1	0.3653	0.3653	2.050	0.1684
	Age	1	0.007502	0.007502	0.04211	0.8396
	Residual	19	3.385	0.1781		
B) PFKFB3						
	Interaction	1	0.8951	0.8951	4.304	0.0499
	Genotype	1	0.05967	0.05967	0.2869	0.5976
	Age	1	0.4586	0.4586	2.205	0.1517
	Residual	22	4.576	0.2080		
C) Ferritin						
	Interaction	1	0.1088	0.1088	0.8531	0.3645
	Genotype	1	0.09742	0.09742	0.7640	0.3904
	Age	1	0.6172	0.6172	4.840	0.0373
	Residual	25	3.188	0.1275		
D) Ferroportin						
	Interaction	1	1.649	1.649	1.548	0.2246
	Genotype	1	6.978	6.978	6.551	0.0166
	Age	1	0.1266	0.1266	0.1188	0.7331
	Residual	26	27.70	1.065		

Table 6.1 Neither age nor genotype affected the mRNA expression of PFKFB isozymes in hippocampal tissue, but age and genotype affected ferritin and ferroportin expression respectively

Groups of 9 and 17 month-old WT and APP/PS1 mice were sacrificed and hippocampal tissue was assessed for the mRNA expression of markers of metabolic profile and iron handling using RT-PCR. There were no age- or genotype-related effects on the mRNA expression of PFKFB1 (A), or PFKFB3 (B). Ferritin mRNA expression decreased in an age-associated manner (C) and a genotype-associated decrease in ferroportin mRNA expression was observed (D). Data are expressed relative to young WT values as mean $\pm$ SEM (n=4-8).

6.4 Discussion

The primary aim of this study was to assess changes in expression of activation state markers associated with an M1 phenotype that occur with age and/or an APP/PS1 genotype and to determine whether these changes coincide with changes in metabolism and iron handling by cells. Previous studies indicated a detrimental role of M1-like microglia in neurodegenerative diseases and ageing (Lynch et al., 2010, Lynch, 2014). Therefore, the hypothesis here was that increased expression of M1 activation state markers would be observed in hippocampal tissue, and in microglia, isolated from the brains of aged and/or APP/PS1 mice, and that altered metabolic and iron handling profiles, similar to those observed in IFN- γ -stimulated microglia *in vitro* described in Chapter 4, would be observed.

The data revealed significant age- and genotype-associated increases in TNF α expression in hippocampal tissue and microglia respectively. Microglia isolated from aged APP/PS1 mice demonstrated increased oxygen consumption, indicative of a higher metabolic rate. This was accompanied by an increase in PFKFB3:PFKFB1, which, as demonstrated in Chapters 3 and 4, is indicative of increased glycolysis and an M1 phenotype. Furthermore, microglia isolated from these mice also exhibited an expression profile indicative of increased iron retention. This provides evidence for the first time, that metabolic reprogramming may occur in microglia with neuroinflammation.

The increased expression of TNF α observed in hippocampal tissue from aged mice supports evidence suggesting that microglia become primed to adopt a pro-inflammatory phenotype with age (Lynch et al., 2010). Microarray analysis on brain tissue from young and aged animals has demonstrated the upregulation of genes encoding for inflammatory cytokines (Prolla, 2002a) and chemokines (Lee et al., 2000) in the aged brain and several studies

explicitly report increases in TNF α expression with age (Sierra et al., 2007, Njie et al., 2012b).

Although there was no overall genotype-associated increase observed in hippocampal TNF α expression, there was a marked increase in expression in 17 month-old mice compared with age matched WT controls, suggesting that an APP/PS1 genotype is associated with increased TNF α at an advanced age. Increased TNF α expression has been reported to occur in the hippocampus of APP/PS1 mice at 14 and 24 months of age (Minogue et al., 2014a), but perhaps disease pathology has not progressed sufficiently for the detection of changes at 9 months.

When microglia isolated from the brains of these animals were assessed for expression of these M1-like activation state markers, similar changes in TNF α expression were observed. This is consistent with the idea that classically-activated microglia are associated with ageing (Lynch et al., 2010) and neurodegeneration (Heneka et al., 2015). Chronically activated pro-inflammatory microglia induce neuronal cell death associated with neurodegenerative disorders (Tang and Le, 2016), and treatment of microglia with A β induces an M1-like phenotype, demonstrated by increased expression of TNF α , IL-1 β , and iNOS (Jones et al., 2015). iNOS expression has been shown to increase in microglial-enriched population isolated from brain tissue from APP/PS1 compared with WT control at 14 months (Jones et al., 2015), but this change was not observed in the present study. The differential expression of TNF α and iNOS in isolated microglia demonstrates the complexities and shortcomings of the M1/M2 paradigm. Although *in vitro* evidence suggests that these markers are co-expressed in response to pro-inflammatory stimuli, this is too simplistic to be considered relevant in the *in vivo* setting.

The most novel finding of this study was the characterisation of the metabolic profiles of microglia isolated from aged and APP/PS1 mice. The basal metabolic rate was significantly increased in microglia in an age-dependent

manner. This increase suggests that microglia in the aged brain are metabolically active, consistent with the hypothesis that microglia of the aged brain are primed or reactive, and respond more readily to inflammatory stimuli (Norden and Godbout, 2013). Microglia in the aged brain may require more oxygen to carry out their actions due to impaired function and senescence, which has also been reported to occur with age (Streit and Xue, 2009). The age-associated increase in metabolism is perhaps consistent with the absence of an age-related increase in iNOS. NO is associated with decreased oxidative metabolism, as it has been shown to inhibit complexes I and II in the respiratory complex, inhibit ATP synthase, and damage mitochondrial DNA and mitochondrial membranes (Brown, 2001, Borutaite et al., 2000). However, the data is at variance with reports showing that ageing downregulates genes associated with mitochondrial electron transport and oxidative phosphorylation (Kayo et al., 2001), although this was observed in skeletal muscle of rhesus monkeys and may be very different to microglia. Interestingly, microglia isolated from APP/PS1 mice, which have previously been shown to become senescent in AD (Streit et al., 2009), exhibited no changes in basal metabolism compared with age-matched controls.

The change in OCR in response to FCCP is an indicator of the maximal respiratory capacity of cells and here an age-related decrease in microglia isolated from WT mice was observed. These data suggest that the responsiveness of cells to the increased energy demands associated with age-related activation is decreased. However, the maximal respiratory capacity of microglia from 17 month-old APP/PS1 mice was significantly higher than isolated microglia from all other groups. Enhanced oxidative metabolism is associated with long-term activation of immune cells, such as memory T cells and perhaps increased oxidative metabolism is necessary to facilitate chronic activation of microglia.

The finding that microglia from aged APP/PS1 mice have increased aerobic basal respiration and a heightened maximal respiratory capacity was

unexpected, as these changes are similar to the metabolic reprogramming observed in IL-4-stimulated microglia in Chapter 4. A possible explanation is that, although acute activation of a pro-inflammatory microglial phenotype is accompanied by increased glycolysis, another switch occurs with chronic activation to sustain the inflammatory activity of the cell.

As well as showing an age-associated increase in oxidative metabolism in microglia, the data revealed a significant age-related increase in PFKFB3:PFKFB1, with microglia from 17 month-old APP/PS1 mice demonstrating the highest PFKFB3:PFKFB1 ratio. As demonstrated in Chapters 3 and 4, this is indicative of an increase in glycolysis. A number of glycolytic enzymes have previously been shown to be increased in CHME-5 cells, a human microglial cell line, in response to treatment with plasma from AD patients (Jayasena et al., 2015), suggesting increased microglial glycolysis could be a hallmark of the condition. The data in the present study suggest that microglia from aged APP/PS1 have both increased levels of oxidative metabolism as well as an increased rate of glycolysis, consistent with a number of reports that implicate increased and chronic microglial activity in neurodegeneration (Lynch et al., 2010, Heneka et al., 2015).

The effect of age and an APP/PS1 genotype on the iron handling properties of microglia were assessed by examining the expression of ferritin and ferroportin. Microglia isolated from aged APP/PS1 mice were shown to increase and decrease ferritin and ferroportin expression respectively. These cells display characteristics of the M1-like phenotype (Minogue et al., 2014a), and the findings are consistent with the observation that IFN- γ -stimulation of microglia increased ferritin and decreased ferroportin (Chapter 4). In short, the results suggest that an inflammatory microglial phenotype is associated with increased iron retention. Interestingly ferritin-mediated iron accumulation is a feature of AD, PD and ALS (Oshiro et al., 2011), and impaired ferroportin-mediated iron export has been suggested to contribute to neurotoxicity in these diseases (Raha et al., 2013).

The most interesting findings of this study were that microglia from aged APP/PS1 mice demonstrate a significantly increased basal level of oxidative metabolism and PFKFB3:PFKFB1, indicative of highly active cells that utilise both glycolysis and oxidative phosphorylation to generate ATP. These cells also display similar iron handling properties to M1-like microglia *in vitro*, which are indicative of increased iron retention.

Chapter 7 General discussion

7.1 General discussion

The overall aim of this study was to assess whether novel markers of microglial activation could be identified and, specifically, to determine whether IFN- γ -activated and IL-4-activated microglia could be identified on the basis of metabolic changes and iron handling capabilities. The primary findings were that microglia adopted different metabolic and iron handling profiles upon polarisation with these cytokines, and that this switch was accompanied by changes in the ratio between expression of PFKFB3 and PFKFB1. IFN- γ increased glycolytic rate and PFKFB3:PFKFB1 expression in microglia *in vitro*, while IL-4 stimulation increased mitochondrial respiration. An important objective of this study was to determine whether changes might be observed in microglia from aged mice or APP/PS1 mice compared with young and WT mice respectively. The data show that microglia isolated from the brains of 17 month-old APP/PS1 mice displayed increased oxidative metabolism as well as increased PFKFB3:PFKFB1, suggestive of increased activity, and an iron retentive profile. These findings demonstrate a means for characterisation of microglial activation based on metabolism and iron handling that is novel and potentially valuable in the context of understanding more fully changes that develop with age.

	BMDMs	Primary microglia	Primary astrocytes	Microglia isolated from adult mice
M1 activation state markers	Increased with IFN- γ	Increased with IFN- γ	Increased with IFN- γ	Increased with age
M2 activation state markers	Increased with IL-4	Increased with IL-4	Increased with IL-4	N/A
Glycolytic rate	Increased with IFN- γ	Increased with IFN- γ	Increased with IFN- γ & IL-4	N/A
Basal respiration	Increased with IL-4	Increased with IL-4	Unchanged	Increased with age
PFKFB3:PFKFB1	Increased with IFN- γ	Increased with IFN- γ	N/A	Increased with age
Iron retention	Increased with IFN- γ	Increased with IFN- γ	N/A	Increased with age

Table 7.1 Summary of changes in metabolism and iron handling profile

The intrinsic link between metabolism and function in immune cells is a relatively recent discovery (Olefsky and Glass, 2010a, Rodriguez-Prados et al., 2010, Michalek et al., 2011). Pro-inflammatory M1 macrophages and effector T cells have been shown to increase glycolysis in order to meet the energy demands associated with activation (Rodriguez-Prados et al., 2010, Buck et al., 2015). Glycolysis is not as efficient as oxidative metabolism, but the switch enables cells to rapidly produce ATP to deal with immediate threats, provided there is sufficient glucose. These cells also display impaired mitochondrial respiration, which mediates the generation of inflammatory mediators, such as succinate and citrate (Galvan-Pena and O'Neill, 2014, Tannahill et al., 2013). In contrast, the activation of macrophages by IL-4 to induce an M2-like phenotype is associated with increased mitochondrial respiration, which is also observed in T_{reg} and memory T cells (Nomura et al., 2016, Michalek et al., 2011). This metabolic change is reflective of the fact that these cells are involved in long-term activities that require sustainable production of ATP.

Metabolic reprogramming has been studied in macrophages for the past few years, but reports investigating this phenomenon in microglia are very limited. However, increased glucose uptake, PFK1 activity and ECAR in BV2 cells following LPS stimulation has been described and this is accompanied by decreased OCR (Gimeno-Bayón et al., 2014, Voloboueva et al., 2013, Orihuela et al., 2016). Although this is consistent with findings in macrophages, BV2 cells fail to replicate at least some functions of microglia and it is questionable whether they can be considered as a suitable model for primary microglia (Stansley et al., 2012). Oriheula and colleagues have shown that primary microglia undergo metabolic reprogramming in a similar manner to macrophages, with increased glycolysis and decreased oxidative respiration following LPS stimulation (Orihuela et al., 2016). However, they failed to observe any changes in metabolism in microglia treated with a combination of IL-4 and IL-13. The present study is the first to demonstrate that IFN- γ -induced expression of M1 activation state markers TNF α , iNOS

and IL-1 β in primary microglia is accompanied by increased glycolysis, and that IL-4-induced increases in M2 activation state markers Arg-1, MRC1, Chi3L3 and FIZZ1 are accompanied by increased oxidative metabolism. This novel way of characterising microglial phenotype provides a greater insight into the functional capacity of cells than characterisation through the expression of specific activation state markers, which is now considered outdated (Martinez and Gordon, 2014, Murray et al., 2014).

This study demonstrates that the ratio between expression of PFKFB3 and PFKFB1 can be used as a marker for the metabolic profile and activation state of macrophages and microglia. PFKFB3 has been shown to play an important role in mediating the increased glycolytic rate associated with M1 macrophages. The data presented here demonstrate that PFKFB3 is also more active in M1-like microglia. Interestingly PFKFB3, unlike other members of that family of enzymes, has a predominantly kinase activity which drives glycolytic flux and mediates increased glycolytic rate.

Whereas macrophages and microglia share many properties, there are several distinctions that separate the cells. One difference that is relevant to the present findings is that HIF-1 α expression is not robustly increased in microglia following IFN- γ stimulation. PFKFB3 expression is induced by HIF-1 α and this transcription factor is believed to be a key mediator in enabling the aerobic glycolysis that occurs in M1 macrophages. Although modest increases in HIF-1 α were observed in IFN- γ -stimulated microglia, the expression of downstream proteins, such as PDK1 and GLUT1, were unchanged. Failure to detect changes in the expression of these proteins suggests that metabolic reprogramming in microglia is mediated by mechanisms that are different in macrophages. Other transcription factors have been associated with mediating increased glycolysis and glucose uptake, as c-Myc has been shown to do in transformed rat fibroblasts (Osthus et al., 2000). Perhaps a combination of HIF-1 α and other, yet unidentified factors, mediate the metabolic changes observed in M1-like microglia.

Decreased mitochondrial respiration has been shown to occur in M1 macrophages is believed to be mediated by PDK1. However, in the present study, IFN- γ stimulation of microglia did not alter OCR or PDK1 expression, suggesting that M1-like microglia do not inhibit oxidative metabolism like macrophages.

A possible explanation for the absence of changes in OCR and the expression of proteins downstream of HIF-1 α may be the stimuli used. IFN- γ was used in the present study to induce the M1 activation state and, while increases in archetypal activation state markers were observed, this may not be as potent an activator as LPS alone, or a combination of LPS and IFN- γ , which have been shown to increase HIF-1 α expression or decrease OCR in macrophages. Perhaps further investigation into the mechanisms mediating the metabolic reprogramming of microglia is required using LPS to induce more robust changes.

Similarly, a combination of IL-4 and IL-13 could be used to induce a greater response in microglia for the adoption of the M2 activation state. In the present study, IL-4 increased expression of PGC-1 β and pAMPK were not observed. Although AMPK activity is associated with an anti-inflammatory M2 phenotype, STAT6^{-/-} mice do not show attenuated expression of the enzyme, suggesting that IL-4 stimulation does not affect its activity (Vats et al., 2006), and it may therefore require dual stimulation with IL-4 and IL-13 to induce changes.

Astrocytes have been shown to play a role in the immune response in the CNS, and secrete cytokines and chemokines in response to inflammatory mediators (Lau and Yu, 2001, Smits et al., 2002). In the present study, IFN- γ and IL-4 upregulated the expression of M1 and M2 activation state markers respectively in astrocytes, but this was not accompanied by the changes in ECAR and OCR observed in macrophages and microglia. This suggests that metabolic reprogramming may be a phenomenon reserved for cells of myeloid lineage and also, as shown, in lymphocytes. The role of astrocytes in the

immune response in the CNS may be an auxiliary one, as they failed to phagocytose fluorescently-labelled latex beads or FITC-A β in mixed glial cultures, while microglia demonstrated robust uptake of both. Although astrocytes have previously been shown to be capable of phagocytosis, it may be that they do not do so in the presence of the more phagocytic microglia.

This study is the first to examine the metabolic profile of microglia isolated from the brains of adult mice. The increase in basal oxygen consumption observed in microglia isolated from older animals suggests increased activity in these cells. It has been hypothesised that microglia become senescent with age (Streit, 2006) and it might have been predicted that these cells may be metabolically compromised. On the face of it, the increased oxygen consumption is inconsistent with this. However, an alternative explanation may be that dysfunctional microglia fail to modulate their metabolic demands for normal functions and therefore require more oxygen. This may be consistent with their increased secretory activity, as they are known to increase their production and release of inflammatory mediators. Indeed, microglia become over active with age, which could explain the increased responsiveness of cells from aged animals to inflammatory stimuli (Minogue et al., 2014b, Perry and Teeling, 2013). Although basal OCR was increased with age, so too was PFKFB3:PFKFB1, which was shown to be associated with increased glycolysis and a pro-inflammatory phenotype in macrophages and microglia *in vitro*. This suggests that cells increase both glycolysis and oxidative metabolism and provides evidence that microglia become inappropriately activated with age.

The increased maximal respiratory capacity observed in microglia isolated from the brains of APP/PS1 mice was an interesting finding; it might suggest that the persistent presence of A β causes cells to become more responsive to inflammatory stimuli like IFN- γ , consistent with the synergy that has been shown to exist (Jones et al., 2015). There was no genotype-related change in basal OCR, but an increase in PFKFB3:PFKFB1 in microglia from 17 month-

old APP/PS1 mice indicative of a glycolytic phenotype that may prevail in these cells, which is associated with the M1 activation state *in vitro*. This is consistent with the literature (Minogue et al., 2014b) and the increased TNF α expression observed in these cells and suggests that microglial activation can be characterised by metabolic profile.

It is clear that further studies are required to investigate metabolic reprogramming with age and APP/PS1 genotype. PFKFB3:PFKFB1 can be used as a proxy marker for metabolic profile, but more direct measures are necessary. Glycolytic stress tests were found to be problematic in cells prepared from adult mice; specifically, cells failed to recover from the glucose deprivation required for the assay. Glucose uptake and lactate production could be assessed as alternate measures to investigate the glycolytic nature of these cells.

Similar changes in the expression of iron handling proteins in both macrophages and microglia were observed *in vitro*. Consistent with the current understanding of how iron is handled in polarised macrophages (Recalcati et al., 2010, Cairo et al., 2011); IFN- γ induced an iron-retentive phenotype as indicated by increased ferritin expression, whereas IL-4 increased the expression of TfR and decreased the expression of ferritin, indicative of iron recycling in cells. This suggests that the expression of these proteins may be used to further characterise an inflammatory phenotype in microglia. Microglia isolated from the brains of aged and APP/PS1 mice showed increased expression of ferritin and decreased expression of ferroportin, paralleling the profile observed in IFN- γ stimulated microglia *in vitro*. This is consistent with reports demonstrating that microglia in the CNS of APP/PS1 mice adopt an M1-like phenotype (Jones et al., 2015) and that pro-inflammatory ferritin-mediated iron retention is associated with neuroinflammatory disorders, such as AD (Ayton et al., 2015, Thomsen et al., 2015). It has been suggested that iron accumulation in these conditions is a due to the inflammatory environment (Urrutia et al., 2013), but iron

accumulation also exacerbates and promotes an inflammatory phenotype (Siegert et al., 2015). It remains to be determined whether iron accumulation drives or is a result of inflammation.

It is important to note that there are other proteins involved in iron homeostasis that were not assessed here, such as DMT1 and hepcidin, that are associated with inflammatory phenotype in macrophages (Urrutia et al., 2013). DMT1 is involved in the transport of iron into the cell, while hepcidin induces the internalisation of ferroportin and promotes iron retention. Therefore the present data represent an incomplete portrayal of the iron handling properties of polarised cells. Assessment of the uptake of iron nanoparticles by cells, perhaps fluorescently labelled for flow cytometric analysis would provide a more thorough representation of how these different stimuli alter iron homeostasis in microglia.

IFN- γ - and IL-4-stimulated microglia were assessed for their phagocytic, antigen presentation, and secretory capabilities, in order to provide a further insight into the functional consequences of polarisation. There are conflicting reports on the effects of polarisation on microglial phagocytosis, but the present data suggest that neither IFN- γ nor IL-4 stimulation affected phagocytosis of latex beads, or A β , suggesting that there is no phenotypic modulation of phagocytic function. It must be noted that robust phagocytosis was observed in unstimulated microglia when examined by microscopy, indicating that a ceiling effect may have prevented any modulation by IFN- γ or IL-4. Although neither stimulus affected phagocytosis or the microglial-induced proliferation of T cells, there were distinct profiles observed between IFN- γ and IL-4 polarised microglia in terms of their expression of secretory proteins. IFN- γ increased the expression of chemokines which promote a heightened immune response and are associated with neurodegeneration; these included CCL2, CCL3 and CCL5 (Cartier et al., 2005). M2-like cells were associated with increased expression of growth factors, which serve to

protect and promote neuronal health. These findings support several previous reports indicating that M2-like cells are associated with repair.

This study demonstrates that microglial activation states can be characterised by their metabolic and iron handling profiles. It would be beneficial for future research into this area to elucidate the mechanisms that mediate metabolic reprogramming in microglia, as this could identify possible therapeutic targets for modulating microglial activation. Chronic activation of pro-inflammatory M1-like microglia is associated with most neurodegenerative disorders, and much research has attempted to quell the inflammatory phenotype observed in these cells. By targeting the metabolism of activated cells, it may be possible to induce a switch in microglial phenotype. Disrupting metabolic pathways has been shown to alter functional outcome of activation in macrophages (Mills and O'Neill, 2016). For example, inhibition of HIF-1 α activity attenuates the Warburg effect in LPS-stimulated macrophages, which is accompanied by decreased IL-1 β secretion (Palsson-McDermott et al., 2015). Similarly, increasing AMPK activity can skew macrophages to an M2 phenotype (Mounier et al., 2013). T cell function can also be modulated by targeting the metabolic reprogramming associated with activation (Chang and Pearce, 2016). For example, inhibition of PDK1 diminishes T_h17 cell-mediated inflammation in models of inflammatory bowel disease and EAE (Gerriets et al., 2015). With further research, it may be possible to abrogate microglial-mediated inflammation through pharmacological blockade of metabolism as a therapeutic possibility in neurodegenerative disorders.

Chapter 8: References

- AJAMI, B., BENNETT, J. L., KRIEGER, C., TETZLAFF, W. & ROSSI, F. M. 2007. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nature neuroscience*, 10, 1538-1543.
- AK, P. & LEVINE, A. J. 2010. p53 and NF- κ B: different strategies for responding to stress lead to a functional antagonism. *FASEB J*, 24, 3643-52.
- AKIRA, S. & TAKEDA, K. 2004. Toll-like receptor signalling. *Nature reviews immunology*, 4, 499-511.
- ALEXOPOULOU, L., HOLT, A. C., MEDZHITOV, R. & FLAVELL, R. A. 2001. Recognition of double-stranded RNA and activation of NF- κ B by Toll-like receptor 3. *Nature*, 413, 732-738.
- ALMEIDA, A., MONCADA, S. & BOLANOS, J. P. 2004. Nitric oxide switches on glycolysis through the AMP protein kinase and 6-phosphofructo-2-kinase pathway. *Nat Cell Biol*, 6, 45-51.
- ALOISI, F. 2001. Immune function of microglia. *Glia*, 36, 165-179.
- ALOISI, F., PENNA, G., POLAZZI, E., MINGHETTI, L. & ADORINI, L. 1999. CD40-CD154 interaction and IFN- γ are required for IL-12 but not prostaglandin E2 secretion by microglia during antigen presentation to Th1 cells. *Journal of Immunology*, 162, 1384-1391.
- ALOISI, F., RIA, F. & ADORINI, L. 2000. Regulation of T-cell responses by CNS antigen-presenting cells: different roles for microglia and astrocytes. *Immunology Today*, 21, 141-147.
- ANDERSEN, H. H., JOHNSEN, K. B. & MOOS, T. 2014. Iron deposits in the chronically inflamed central nervous system and contributes to neurodegeneration. *Cellular and Molecular Life Sciences*, 71, 1607-1622.
- ANDREWS, A. L., HOLLOWAY, J. W., HOLGATE, S. T. & DAVIES, D. E. 2006. IL-4 receptor alpha is an important modulator of IL-4 and IL-13 receptor binding: implications for the development of therapeutic targets. *J Immunol*, 176, 7456-61.
- ARNOLD, C. E., GORDON, P., BARKER, R. N. & WILSON, H. M. 2015. The activation status of human macrophages presenting antigen determines the efficiency of Th17 responses. *Immunobiology*, 220, 10-19.
- ASHE, K. H. 2001. Learning and memory in transgenic mice modeling Alzheimer's disease. *Learning & Memory*, 8, 301-308.
- ATSUMI, T., NISHIO, T., NIWA, H., TAKEUCHI, J., BANDO, H., SHIMIZU, C., YOSHIOKA, N., BUCALA, R. & KOIKE, T. 2005. Expression of inducible 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase/PFKFB3 isoforms in adipocytes and their potential role in glycolytic regulation. *Diabetes*, 54, 3349-57.
- AYTON, S., FAUX, N. G., BUSH, A. I. & ALZHEIMER'S DISEASE NEUROIMAGING, I. 2015. Ferritin levels in the cerebrospinal fluid predict Alzheimer's disease outcomes and are regulated by APOE. *Nat Commun*, 6.
- BABES, V. 1892. Sur certains caractères des lésions histologiques de la rage. *Ann Inst Pasteur Lille*, 6, 209-223.

- BAMBERGER, M. E., HARRIS, M. E., MCDONALD, D. R., HUSEMANN, J. & LANDRETH, G. E. 2003. A cell surface receptor complex for fibrillar beta-amyloid mediates microglial activation. *J Neurosci*, 23, 2665-74.
- BAMBERGER, M. E. & LANDRETH, G. E. 2001. Microglial interaction with β -amyloid: Implications for the pathogenesis of Alzheimer's disease. *Microscopy research and technique*, 54, 59-70.
- BAUER, S., KIRSCHNING, C. J., HÄCKER, H., REDECKE, V., HAUSMANN, S., AKIRA, S., WAGNER, H. & LIPFORD, G. B. 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proceedings of the National Academy of Sciences of the United States of America*, 98, 9237-9242.
- BEKRIS, L. M., YU, C.-E., BIRD, T. D. & TSUANG, D. W. 2010. Genetics of Alzheimer Disease. *Journal of geriatric psychiatry and neurology*, 23, 213-227.
- BENVENISTE, E. N. 1998. Cytokine actions in the central nervous system. *Cytokine & growth factor reviews*, 9, 259-275.
- BENVENISTE, N. E. 1997. Role of macrophages/microglia in multiple sclerosis and experimental allergic encephalomyelitis. *Journal of Molecular Medicine*, 75, 165-173.
- BERNARDO, A., LEVI, G. & MINGHETTI, L. 2000. Role of the peroxisome proliferator-activated receptor-gamma (PPAR-gamma) and its natural ligand 15-deoxy-Delta12, 14-prostaglandin J2 in the regulation of microglial functions. *Eur J Neurosci*, 12, 2215-23.
- BHATIA, S., EDIDIN, M., ALMO, S. C. & NATHENSON, S. G. 2006. B7-1 and B7-2: similar costimulatory ligands with different biochemical, oligomeric and signaling properties. *Immunol Lett*, 104, 70-5.
- BIBER, K., NEUMANN, H., INOUE, K. & BODDEKE, H. W. 2007. Neuronal 'On' and 'Off' signals control microglia. *Trends Neurosci*, 30, 596-602.
- BISWAS, SUBHRA K. & MANTOVANI, A. 2012. Orchestration of Metabolism by Macrophages. *Cell Metabolism*, 15, 432-437.
- BLACK, R. A., RAUCH, C. T., KOZLOSKY, C. J., PESCHON, J. J., SLACK, J. L., WOLFSON, M. F., CASTNER, B. J., STOCKING, K. L., REDDY, P., SRINIVASAN, S., NELSON, N., BOIANI, N., SCHOOLEY, K. A., GERHART, M., DAVIS, R., FITZNER, J. N., JOHNSON, R. S., PAXTON, R. J., MARCH, C. J. & CERRETTI, D. P. 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature*, 385, 729-33.
- BLOCK, M. L. & HONG, J.-S. 2005. Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. *Progress in neurobiology*, 76, 77-98.
- BLOUIN, C. C., PAGE, E. L., SOUCY, G. M. & RICHARD, D. E. 2004. Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor 1alpha. *Blood*, 103, 1124-30.
- BOBARYKINA, A. Y., MINCHENKO, D. O., OPENTANOVA, I. L., MOENNER, M., CARO, J., ESUMI, H. & MINCHENKO, O. H. 2006. Hypoxic regulation of PFKFB-3 and PFKFB-4 gene expression

in gastric and pancreatic cancer cell lines and expression of PFKFB genes in gastric cancers. *ACTA BIOCHIMICA POLONICA-ENGLISH EDITION*-, 53, 789.

- BOGDAN, C. 2001. Nitric oxide and the immune response. *Nature immunology*, 2, 907-916.
- BORNEMANN, K. D., WIEDERHOLD, K.-H., PAULI, C., ERMINI, F., STALDER, M., SCHNELL, L., SOMMER, B., JUCKER, M. & STAUFENBIEL, M. 2001. A β -induced inflammatory processes in microglia cells of APP23 transgenic mice. *The American journal of pathology*, 158, 63-73.
- BORUTAITE, V., BUDRIUNAITE, A. & BROWN, G. C. 2000. Reversal of nitric oxide-, peroxynitrite- and S-nitrosothiol-induced inhibition of mitochondrial respiration or complex I activity by light and thiols. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1459, 405-412.
- BRAND, K. 1985. Glutamine and glucose metabolism during thymocyte proliferation. Pathways of glutamine and glutamate metabolism. *Biochemical Journal*, 228, 353-361.
- BROWN, G. C. 2001. Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1504, 46-57.
- BRUICK, R. K. & MCKNIGHT, S. L. 2001. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science*, 294, 1337-40.
- BRUNELLI, S. & ROVERE-QUERINI, P. 2008. The immune system and the repair of skeletal muscle. *Pharmacol Res*, 58, 117-21.
- BRYSON, K. J. & LYNCH, M. A. 2016. Linking T cells to Alzheimer's disease: from neurodegeneration to neurorepair. *Curr Opin Pharmacol*, 26, 67-73.
- BUCK, M. D., O'SULLIVAN, D. & PEARCE, E. L. 2015. T cell metabolism drives immunity. *J Exp Med*, 212, 1345-60.
- BUTTINI, M., LIMONTA, S. & BODDEKE, H. W. 1996. Peripheral administration of lipopolysaccharide induces activation of microglial cells in rat brain. *Neurochem Int*, 29, 25-35.
- BYRD, T. F. & HORWITZ, M. A. 1989. Interferon gamma-activated human monocytes downregulate transferrin receptors and inhibit the intracellular multiplication of *Legionella pneumophila* by limiting the availability of iron. *J Clin Invest*, 83, 1457-65.
- BYRD, T. F. & HORWITZ, M. A. 1993. Regulation of transferrin receptor expression and ferritin content in human mononuclear phagocytes. Coordinate upregulation by iron transferrin and downregulation by interferon gamma. *J Clin Invest*, 91, 969-76.
- CAIRO, G., RECALCATI, S., MANTOVANI, A. & LOCATI, M. 2011. Iron trafficking and metabolism in macrophages: contribution to the polarized phenotype. *Trends in immunology*, 32, 241-247.
- CALVO, M. N., BARTRONS, R., CASTAÑO, E., PERALES, J. C., NAVARRO-SABATÉ, A. & MANZANO, A. 2006. PFKFB3 gene silencing decreases glycolysis, induces cell-cycle delay and inhibits anchorage-independent growth in HeLa cells. *FEBS Letters*, 580, 3308-3314.

- CAMERON, B. & LANDRETH, G. E. 2010. Inflammation, Microglia and Alzheimer's Disease. *Neurobiology of disease*, 37, 503-509.
- CAO, D., LU, H., LEWIS, T. L. & LI, L. 2007. Intake of sucrose-sweetened water induces insulin resistance and exacerbates memory deficits and amyloidosis in a transgenic mouse model of Alzheimer disease. *Journal of Biological Chemistry*, 282, 36275-36282.
- CARDONA, A. E., PIORO, E. P., SASSE, M. E., KOSTENKO, V., CARDONA, S. M., DIJKSTRA, I. M., HUANG, D., KIDD, G., DOMBROWSKI, S. & DUTTA, R. 2006. Control of microglial neurotoxicity by the fractalkine receptor. *Nature neuroscience*, 9, 917-924.
- CARPENTIER, P. A., BEGOLKA, W. S., OLSON, J. K., ELHOFY, A., KARPUS, W. J. & MILLER, S. D. 2005. Differential activation of astrocytes by innate and adaptive immune stimuli. *Glia*, 49, 360-74.
- CARROLL, K. C., VIOLLET, B. & SUTTLES, J. 2013. AMPK α 1 deficiency amplifies proinflammatory myeloid APC activity and CD40 signaling. *J Leukoc Biol*, 94, 1113-21.
- CARTIER, L., HARTLEY, O., DUBOIS-DAUPHIN, M. & KRAUSE, K. H. 2005. Chemokine receptors in the central nervous system: role in brain inflammation and neurodegenerative diseases. *Brain Res Brain Res Rev*, 48, 16-42.
- CASULA, M., IYER, A. M., SPLIT, W. G., ANINK, J. J., STEENTJES, K., STA, M., TROOST, D. & ARONICA, E. 2011. Toll-like receptor signaling in amyotrophic lateral sclerosis spinal cord tissue. *Neuroscience*, 179, 233-43.
- CAVAILLON, J.-M. 2011. The historical milestones in the understanding of leukocyte biology initiated by Elie Metchnikoff. *Journal of leukocyte biology*, 90, 413-424.
- CHAN, G., WHITE, C. C., WINN, P. A., CIMPEAN, M., REPLOGLE, J. M., GLICK, L. R., CUERDON, N. E., RYAN, K. J., JOHNSON, K. A., SCHNEIDER, J. A., BENNETT, D. A., CHIBNIK, L. B., SPERLING, R. A., BRADSHAW, E. M. & DE JAGER, P. L. 2015. CD33 modulates TREM2: convergence of Alzheimer loci. *Nat Neurosci*, 18, 1556-8.
- CHANG, C.-H. & PEARCE, E. L. 2016. Emerging concepts of T cell metabolism as a target of immunotherapy. *Nature immunology*, 17, 364-368.
- CHEN, G. & GOEDDEL, D. V. 2002. TNF-R1 signaling: a beautiful pathway. *Science*, 296, 1634-1635.
- CHEN, G., SHAW, M. H., KIM, Y.-G. & NUÑEZ, G. 2009. NOD-like receptors: role in innate immunity and inflammatory disease. *Annual Review of Pathological Mechanical Disease*, 4, 365-398.
- CHERRY, J. D., OLSCHOWKA, J. A. & O'BANION, M. K. 2015. Arginase 1+ microglia reduce A β plaque deposition during IL-1 β -dependent neuroinflammation. *J Neuroinflammation*, 12, 203.
- CHESNEY, J., MITCHELL, R., BENIGNI, F., BACHER, M., SPIEGEL, L., AL-ABED, Y., HAN, J. H., METZ, C. & BUCALA, R. 1999. An inducible gene product for 6-phosphofructo-2-kinase with an AU-rich instability element: role in tumor cell glycolysis and the Warburg effect. *Proc Natl Acad Sci U S A*, 96, 3047-52.

- CHHOR, V., LE CHARPENTIER, T., LEBON, S., ORÉ, M.-V., CELADOR, I. L., JOSSEAND, J., DEGOS, V., JACOTOT, E., HAGBERG, H. & SÄVMAN, K. 2013a. Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro. *Brain, behavior, and immunity*, 32, 70-85.
- CHHOR, V., LE CHARPENTIER, T., LEBON, S., ORÉ, M.-V., CELADOR, I. L., JOSSEAND, J., DEGOS, V., JACOTOT, E., HAGBERG, H., SÄVMAN, K., MALLARD, C., GRESENS, P. & FLEISS, B. 2013b. Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro. *Brain, Behavior, and Immunity*, 32, 70-85.
- CHLOSTA, S., FISHMAN, D. S., HARRINGTON, L., JOHNSON, E. E., KNUTSON, M. D., WESSLING-RESNICK, M. & CHERAYIL, B. J. 2006. The Iron Efflux Protein Ferroportin Regulates the Intracellular Growth of *Salmonella enterica*. *Infection and Immunity*, 74, 3065-3067.
- CHOI, S. S., LEE, H. J., LIM, I., SATOH, J. & KIM, S. U. 2014. Human astrocytes: secretome profiles of cytokines and chemokines. *PLoS One*, 9, e92325.
- CHURCH, L. D., COOK, G. P. & MCDERMOTT, M. F. 2008. Primer: inflammasomes and interleukin 1 β in inflammatory disorders. *Nature Clinical Practice Rheumatology*, 4, 34-42.
- CITRON, M., OLTERS DORF, T., HAASS, C., MCCONLOGUE, L., HUNG, A. Y., SEUBERT, P., VIGO-PELFREY, C., LIEBERBURG, I. & SELKOE, D. J. 1992. Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. *Nature*, 360, 672-4.
- CLARKE, R. M., O'CONNELL, F., LYONS, A. & LYNCH, M. A. 2007. The HMG-CoA reductase inhibitor, atorvastatin, attenuates the effects of acute administration of amyloid- β 1-42 in the rat hippocampus in vivo. *Neuropharmacology*, 52, 136-145.
- CLEM, B., TELANG, S., CLEM, A., YALCIN, A., MEIER, J., SIMMONS, A., RASKU, M. A., ARUMUGAM, S., DEAN, W. L., EATON, J., LANE, A., TRENT, J. O. & CHESNEY, J. 2008. Small-molecule inhibition of 6-phosphofructo-2-kinase activity suppresses glycolytic flux and tumor growth. *Mol Cancer Ther*, 7, 110-20.
- CLEM, B. F., O'NEAL, J., TAPOLSKY, G., CLEM, A. L., IMBERT-FERNANDEZ, Y., KERR, D. A., KLARER, A. C., REDMAN, R., MILLER, D. M., TRENT, J. O., TELANG, S. & CHESNEY, J. 2013. Targeting 6-Phosphofructo-2-Kinase (PFKFB3) as a Therapeutic Strategy against Cancer. *Molecular cancer therapeutics*, 12, 1461-1470.
- COLTON, C. A. 2009. Heterogeneity of microglial activation in the innate immune response in the brain. *J Neuroimmune Pharmacol*, 4, 399-418.
- COLTON, C. A. & WILCOCK, D. M. 2010. Assessing activation states in microglia. *CNS & Neurological Disorders-Drug Targets (Formerly Current Drug Targets-CNS & Neurological Disorders)*, 9, 174-191.
- CONNOR, J. R., SNYDER, B. S., AROSIO, P., LOEFFLER, D. A. & LEWITT, P. 1995. A quantitative analysis of isoferitins in select regions of aged, parkinsonian, and Alzheimer's diseased brains. *J Neurochem*, 65, 717-24.
- CORNA, G., CAMPANA, L., PIGNATTI, E., CASTIGLIONI, A., TAGLIAFICO, E., BOSURGI, L., CAMPANELLA, A., BRUNELLI, S., MANFREDI, A. A. & APOSTOLI, P. 2010. Polarization

- dictates iron handling by inflammatory and alternatively activated macrophages. *haematologica*, 95, 1814-1822.
- CRAMER, T., YAMANISHI, Y., CLAUSEN, B. E., FORSTER, I., PAWLINSKI, R., MACKMAN, N., HAASE, V. H., JAENISCH, R., CORR, M., NIZET, V., FIRESTEIN, G. S., GERBER, H. P., FERRARA, N. & JOHNSON, R. S. 2003. HIF-1 α is essential for myeloid cell-mediated inflammation. *Cell*, 112, 645-57.
- CUMANO, A. & GODIN, I. 2007. Ontogeny of the hematopoietic system. *Annu. Rev. Immunol.*, 25, 745-785.
- CURTSINGER, J. M., SCHMIDT, C. S., MONDINO, A., LINS, D. C., KEDL, R. M., JENKINS, M. K. & MESCHER, M. F. 1999. Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *J Immunol*, 162, 3256-62.
- D'AVERSA, T. G., WEIDENHEIM, K. M. & BERMAN, J. W. 2002. CD40-CD40L interactions induce chemokine expression by human microglia: implications for human immunodeficiency virus encephalitis and multiple sclerosis. *Am J Pathol*, 160, 559-67.
- DASHTY, M. 2013. A quick look at biochemistry: carbohydrate metabolism. *Clinical biochemistry*, 46, 1339-1352.
- DATTA, G., KRAMER, P. A., JOHNSON, M. S., SAWADA, H., SMYTHIES, L. E., CROSSMAN, D. K., CHACKO, B., BALLINGER, S. W., WESTBROOK, D. G., MAYAKONDA, P., ANANTHARAMAIAH, G. M., DARLEY-USMAR, V. M. & WHITE, C. R. 2015. Bioenergetic programming of macrophages by the apolipoprotein A-I mimetic peptide 4F. *Biochem J*, 467, 517-27.
- DELGOFFE, G. M., KOLE, T. P., ZHENG, Y., ZAREK, P. E., MATTHEWS, K. L., XIAO, B., WORLEY, P. F., KOZMA, S. C. & POWELL, J. D. 2009. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity*, 30, 832-44.
- DELGOFFE, G. M., POLLIZZI, K. N., WAICKMAN, A. T., HEIKAMP, E., MEYERS, D. J., HORTON, M. R., XIAO, B., WORLEY, P. F. & POWELL, J. D. 2011. The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2. *Nat Immunol*, 12, 295-303.
- DONG, Y. & BENVENISTE, E. N. 2001. Immune function of astrocytes. *Glia*, 36, 180-190.
- DONOVAN, A., BROWNLIE, A., ZHOU, Y., SHEPARD, J., PRATT, S. J., MOYNIHAN, J., PAW, B. H., DREJER, A., BARUT, B., ZAPATA, A., LAW, T. C., BRUGNARA, C., LUX, S. E., PINKUS, G. S., PINKUS, J. L., KINGSLEY, P. D., PALIS, J., FLEMING, M. D., ANDREWS, N. C. & ZON, L. I. 2000. Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. *Nature*, 403, 776-81.
- DOYLE, S. E., O'CONNELL, R. M., MIRANDA, G. A., VAIDYA, S. A., CHOW, E. K., LIU, P. T., SUZUKI, S., SUZUKI, N., MODLIN, R. L., YE, W. C., LANE, T. F. & CHENG, G. 2004. Toll-like receptors induce a phagocytic gene program through p38. *J Exp Med*, 199, 81-90.
- DUFF, K., ECKMAN, C., ZEHR, C., YU, X., PRADA, C. M., PEREZ-TUR, J., HUTTON, M., BUEE, L., HARIGAYA, Y., YAGER, D., MORGAN, D., GORDON, M. N., HOLCOMB, L., REFOLO, L.,

- ZENK, B., HARDY, J. & YOUNKIN, S. 1996. Increased amyloid-beta42(43) in brains of mice expressing mutant presenilin 1. *Nature*, 383, 710-3.
- EL KASMI, K. C. & STENMARK, K. R. 2015. Contribution of metabolic reprogramming to macrophage plasticity and function. *Semin Immunol*, 27, 267-75.
- EL KHOURY, J. & LUSTER, A. D. 2008. Mechanisms of microglia accumulation in Alzheimer's disease: therapeutic implications. *Trends Pharmacol Sci*, 29, 626-32.
- EPELMAN, S., LAVINE, K. J., BEAUDIN, A. E., SOJKA, D. K., CARRERO, J. A., CALDERON, B., BRIJA, T., GAUTIER, E. L., IVANOV, S. & SATPATHY, A. T. 2014. Embryonic and adult-derived resident cardiac macrophages are maintained through distinct mechanisms at steady state and during inflammation. *Immunity*, 40, 91-104.
- ERWIG, L. P. & HENSON, P. M. 2007. Immunological consequences of apoptotic cell phagocytosis. *Am J Pathol*, 171, 2-8.
- EVERTS, B., AMIEL, E., HUANG, S. C.-C., SMITH, A. M., CHANG, C.-H., LAM, W. Y., REDMANN, V., FREITAS, T. C., BLAGIH, J., VAN DER WINDT, G. J. W., ARTYOMOV, M. N., JONES, R. G., PEARCE, E. L. & PEARCE, E. J. 2014. TLR-driven early glycolytic reprogramming via the kinases TBK1- $\text{IKK}[\epsilon]$ supports the anabolic demands of dendritic cell activation. *Nat Immunol*, 15, 323-332.
- FARINA, C., ALOISI, F. & MEINL, E. 2007. Astrocytes are active players in cerebral innate immunity. *Trends in immunology*, 28, 138-145.
- FELDMANN, M. 2002. Development of anti-TNF therapy for rheumatoid arthritis. *Nat Rev Immunol*, 2, 364-71.
- FIALA, M., LIU, Q. N., SAYRE, J., POP, V., BRAHMANDAM, V., GRAVES, M. C. & VINTERS, H. V. 2002. Cyclooxygenase-2-positive macrophages infiltrate the Alzheimer's disease brain and damage the blood-brain barrier. *Eur J Clin Invest*, 32, 360-71.
- FLODEN, A. M. & COMBS, C. K. 2006. β -Amyloid Stimulates Murine Postnatal and Adult Microglia Cultures in a Unique Manner. *The Journal of Neuroscience*, 26, 4644-4648.
- FÖRSTERMANN, U. & SESSA, W. C. 2012. Nitric oxide synthases: regulation and function. *European Heart Journal*, 33, 829-837.
- FRANCESCHINI, A., CAPECE, M., CHIOZZI, P., FALZONI, S., SANZ, J. M., SARTI, A. C., BONORA, M., PINTON, P. & DI VIRGILIO, F. 2015. The P2X7 receptor directly interacts with the NLRP3 inflammasome scaffold protein. *The FASEB Journal*, 29, 2450-2461.
- FRAUSTO-DEL-RIO, D., SOTO-CRUZ, I., GARAY-CANALES, C., AMBRIZ, X., SOLDEVILA, G., CARRETERO-ORTEGA, J., VAZQUEZ-PRADO, J. & ORTEGA, E. 2012. Interferon gamma induces actin polymerization, Rac1 activation and down regulates phagocytosis in human monocytic cells. *Cytokine*, 57, 158-68.
- FREEMERMAN, A. J., JOHNSON, A. R., SACKS, G. N., MILNER, J. J., KIRK, E. L., TROESTER, M. A., MACINTYRE, A. N., GORAKSHA-HICKS, P., RATHMELL, J. C. & MAKOWSKI, L. 2014. Metabolic reprogramming of macrophages: glucose transporter 1 (GLUT1)-mediated glucose metabolism drives a proinflammatory phenotype. *J Biol Chem*, 289, 7884-96.

- GALIC, S., FULLERTON, M. D., SCHERTZER, J. D., SIKKEMA, S., MARCINKO, K., WALKLEY, C. R., IZON, D., HONEYMAN, J., CHEN, Z. P., VAN DENDEREN, B. J., KEMP, B. E. & STEINBERG, G. R. 2011. Hematopoietic AMPK beta1 reduces mouse adipose tissue macrophage inflammation and insulin resistance in obesity. *J Clin Invest*, 121, 4903-15.
- GALVAN-PENA, S. & O'NEILL, L. A. 2014. Metabolic reprogramming in macrophage polarization. *Front Immunol*, 5, 420.
- GAMES, D., ADAMS, D., ALESSANDRINI, R., BARBOUR, R., BERTHELETTE, P., BLACKWELL, C., CARR, T., CLEMENS, J., DONALDSON, T., GILLESPIE, F. & ET AL. 1995. Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. *Nature*, 373, 523-7.
- GANZ, T. 2007. Molecular control of iron transport. *J Am Soc Nephrol*, 18, 394-400.
- GANZ, T. 2009. Iron in innate immunity: starve the invaders. *Current Opinion in Immunology*, 21, 63-67.
- GARCIA-ALLOZA, M., ROBBINS, E. M., ZHANG-NUNES, S. X., PURCELL, S. M., BETENSKY, R. A., RAJU, S., PRADA, C., GREENBERG, S. M., BACSKAI, B. J. & FROSCH, M. P. 2006. Characterization of amyloid deposition in the APPswe/PS1dE9 mouse model of Alzheimer disease. *Neurobiology of disease*, 24, 516-524.
- GASPARINI, L., NETZER, W. J., GREENGARD, P. & XU, H. 2002. Does insulin dysfunction play a role in Alzheimer's disease? *Trends Pharmacol Sci*, 23, 288-93.
- GERRIETS, V. A., KISHTON, R. J., NICHOLS, A. G., MACINTYRE, A. N., INOUE, M., ILKAYEVA, O., WINTER, P. S., LIU, X., PRIYADHARSHINI, B., SLAWINSKA, M. E., HAEBERLI, L., HUCK, C., TURKA, L. A., WOOD, K. C., HALE, L. P., SMITH, P. A., SCHNEIDER, M. A., MACIVER, N. J., LOCASALE, J. W., NEWGARD, C. B., SHINOHARA, M. L. & RATHMELL, J. C. 2015. Metabolic programming and PDHK1 control CD4+ T cell subsets and inflammation. *J Clin Invest*, 125, 194-207.
- GIMENO-BAYÓN, J., LÓPEZ-LÓPEZ, A., RODRÍGUEZ, M. & MAHY, N. 2014. Glucose pathways adaptation supports acquisition of activated microglia phenotype. *Journal of neuroscience research*, 92, 723-731.
- GINHOUX, F., GRETER, M., LEBOEUF, M., NANDI, S., SEE, P., GOKHAN, S., MEHLER, M. F., CONWAY, S. J., NG, L. G. & STANLEY, E. R. 2010. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science*, 330, 841-845.
- GINHOUX, F. & JUNG, S. 2014. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nature Reviews Immunology*, 14, 392-404.
- GINHOUX, F., LIM, S., HOEFFEL, G., LOW, D. & HUBER, T. 2013. Origin and differentiation of microglia. *Frontiers in Cellular Neuroscience*, 7, 45.
- GORDON, S. 2003a. Alternative activation of macrophages. *Nat Rev Immunol*, 3, 23-35.
- GORDON, S. 2003b. Alternative activation of macrophages. *Nature reviews immunology*, 3, 23-35.

- GORDON, S. & TAYLOR, P. R. 2005. Monocyte and macrophage heterogeneity. *Nat Rev Immunol*, 5, 953-964.
- GOZZELINO, R., JENEY, V. & SOARES, M. P. 2010. Mechanisms of cell protection by heme oxygenase-1. *Annual review of pharmacology and toxicology*, 50, 323-354.
- GRIFFIN, R., NALLY, R., NOLAN, Y., MCCARTNEY, Y., LINDEN, J. & LYNCH, M. A. 2006. The age-related attenuation in long-term potentiation is associated with microglial activation. *J Neurochem*, 99, 1263-72.
- GUILLIAMS, M., DE KLEER, I., HENRI, S., POST, S., VANHOUTTE, L., DE PRIJCK, S., DESWARTE, K., MALISSEN, B., HAMMAD, H. & LAMBRECHT, B. N. 2013. Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. *The Journal of experimental medicine*, 210, 1977-1992.
- HAASS, C., LEMERE, C. A., CAPELL, A., CITRON, M., SEUBERT, P., SCHENK, D., LANNFELT, L. & SELKOE, D. J. 1995. The Swedish mutation causes early-onset Alzheimer's disease by beta-secretase cleavage within the secretory pathway. *Nat Med*, 1, 1291-6.
- HANISCH, U. K. 2002. Microglia as a source and target of cytokines. *Glia*, 40, 140-55.
- HARDIE, D. G. 2007. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol*, 8, 774-85.
- HARDY, J. & SELKOE, D. J. 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science*, 297, 353-6.
- HARRY, G. J. & KRAFT, A. D. 2012. Microglia in the developing brain: a potential target with lifetime effects. *Neurotoxicology*, 33, 191-206.
- HASCHEMI, A., KOSMA, P., GILLE, L., EVANS, C. R., BURANT, C. F., STARKL, P., KNAPP, B., HAAS, R., SCHMID, J. A., JANDL, C., AMIR, S., LUBEC, G., PARK, J., ESTERBAUER, H., BILBAN, M., BRIZUELA, L., POSPISILIK, J. A., OTTERBEIN, L. E. & WAGNER, O. 2012a. The sedoheptulose kinase CARKL directs macrophage polarization through control of glucose metabolism. *Cell Metab*, 15, 813-26.
- HASCHEMI, A., KOSMA, P., GILLE, L., EVANS, CHARLES R., BURANT, CHARLES F., STARKL, P., KNAPP, B., HAAS, R., SCHMID, JOHANNES A., JANDL, C., AMIR, S., LUBEC, G., PARK, J., ESTERBAUER, H., BILBAN, M., BRIZUELA, L., POSPISILIK, J. A., OTTERBEIN, LEO E. & WAGNER, O. 2012b. The Sedoheptulose Kinase CARKL Directs Macrophage Polarization through Control of Glucose Metabolism. *Cell Metabolism*, 15, 813-826.
- HEDESKOV, C. J. 1968. Early effects of phytohaemagglutinin on glucose metabolism of normal human lymphocytes. *Biochem J*, 110, 373-80.
- HENEKA, M. T., CARSON, M. J., EL KHOURY, J., LANDRETH, G. E., BROSSERON, F., FEINSTEIN, D. L., JACOBS, A. H., WYSS-CORAY, T., VITORICA, J., RANSOHOFF, R. M., HERRUP, K., FRAUTSCHY, S. A., FINSEN, B., BROWN, G. C., VERKHRATSKY, A., YAMANAKA, K., KOISTINAHO, J., LATZ, E., HALLE, A., PETZOLD, G. C., TOWN, T., MORGAN, D., SHINOHARA, M. L., PERRY, V. H., HOLMES, C., BAZAN, N. G., BROOKS, D. J., HUNOT, S., JOSEPH, B., DEIGENDESCH, N., GARASCHUK, O., BODDEKE, E., DINARELLO, C. A.,

- BREITNER, J. C., COLE, G. M., GOLENBOCK, D. T. & KUMMER, M. P. 2015. Neuroinflammation in Alzheimer's disease. *Lancet Neurol*, 14, 388-405.
- HENRY, C. J., HUANG, Y., WYNNE, A. M. & GODBOUT, J. P. 2009. Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is associated with exaggerated induction of both pro-inflammatory IL-1 β and anti-inflammatory IL-10 cytokines. *Brain, behavior, and immunity*, 23, 309-317.
- HEPPNER, F. L., RANSOHOFF, R. M. & BECHER, B. 2015. Immune attack: the role of inflammation in Alzheimer disease. *Nat Rev Neurosci*, 16, 358-72.
- HERBER, D. L., MERCER, M., ROTH, L. M., SYMMONDS, K., MALONEY, J., WILSON, N., FREEMAN, M. J., MORGAN, D. & GORDON, M. N. 2007. Microglial activation is required for A β clearance after intracranial injection of lipopolysaccharide in APP transgenic mice. *Journal of Neuroimmune Pharmacology*, 2, 222-231.
- HESSE, M., MODOLELL, M., LA FLAMME, A. C., SCHITO, M., FUENTES, J. M., CHEEVER, A. W., PEARCE, E. J. & WYNN, T. A. 2001. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J Immunol*, 167, 6533-44.
- HICKMAN, S. E., ALLISON, E. K. & EL KHOURY, J. 2008. Microglial dysfunction and defective beta-amyloid clearance pathways in aging Alzheimer's disease mice. *J Neurosci*, 28, 8354-60.
- HIGGINS, G. A. & JACOBSEN, H. 2003. Transgenic mouse models of Alzheimer's disease: phenotype and application. *Behavioural pharmacology*, 14, 419-438.
- HOEFFEL, G., WANG, Y., GRETER, M., SEE, P., TEO, P., MALLERET, B., LEBOEUF, M., LOW, D., OLLER, G. & ALMEIDA, F. 2012. Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages. *The Journal of experimental medicine*, 209, 1167-1181.
- HOEK, R. M., RUULS, S. R., MURPHY, C. A., WRIGHT, G. J., GODDARD, R., ZURAWSKI, S. M., BLOM, B., HOMOLA, M. E., STREIT, W. J. & BROWN, M. H. 2000. Down-regulation of the macrophage lineage through interaction with OX2 (CD200). *Science*, 290, 1768-1771.
- HONTECILLAS, R. & BASSAGANYA-RIERA, J. 2007. Peroxisome proliferator-activated receptor gamma is required for regulatory CD4⁺ T cell-mediated protection against colitis. *J Immunol*, 178, 2940-9.
- HOOGLAND, I. C. M., HOUBOLT, C., VAN WESTERLOO, D. J., VAN GOOL, W. A. & VAN DE BEEK, D. 2015. Systemic inflammation and microglial activation: systematic review of animal experiments. *Journal of Neuroinflammation*, 12, 114.
- HSIAO, K., CHAPMAN, P., NILSEN, S. & ECKMAN, C. 1996. Correlative memory deficits, A beta elevation, and amyloid plaques in transgenic mice. *Science*, 274, 98.
- HSIEH, C. L., KOIKE, M., SPUSTA, S. C., NIEMI, E. C., YENARI, M., NAKAMURA, M. C. & SEAMAN, W. E. 2009. A role for TREM2 ligands in the phagocytosis of apoptotic neuronal cells by microglia. *J Neurochem*, 109, 1144-56.

- HUANG, S. C., EVERTS, B., IVANOVA, Y., O'SULLIVAN, D., NASCIMENTO, M., SMITH, A. M., BEATTY, W., LOVE-GREGORY, L., LAM, W. Y., O'NEILL, C. M., YAN, C., DU, H., ABUMRAD, N. A., URBAN, J. F., JR., ARTYOMOV, M. N., PEARCE, E. L. & PEARCE, E. J. 2014. Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. *Nat Immunol*, 15, 846-55.
- HUME, D. A. 2015. The Many Alternative Faces of Macrophage Activation. *Front Immunol*, 6, 370.
- ISAACS, A. & LINDENMANN, J. 1957. Virus Interference. I. The Interferon. *Proceedings of the Royal Society of London B: Biological Sciences*, 147, 258-267.
- ISHIZUKA, K., KIMURA, T., IGATA-YI, R., KATSURAGI, S., TAKAMATSU, J. & MIYAKAWA, T. 1997. Identification of monocyte chemoattractant protein-1 in senile plaques and reactive microglia of Alzheimer's disease. *Psychiatry Clin Neurosci*, 51, 135-8.
- ITTNER, L. M. & GOTZ, J. 2011. Amyloid-beta and tau--a toxic pas de deux in Alzheimer's disease. *Nat Rev Neurosci*, 12, 65-72.
- IYER, N. V., KOTCH, L. E., AGANI, F., LEUNG, S. W., LAUGHNER, E., WENGER, R. H., GASSMANN, M., GEARHART, J. D., LAWLER, A. M., YU, A. Y. & SEMENZA, G. L. 1998. Cellular and developmental control of O₂ homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev*, 12, 149-62.
- JABLONSKI, K. A., AMICI, S. A., WEBB, L. M., RUIZ-ROSADO JDE, D., POPOVICH, P. G., PARTIDA-SANCHEZ, S. & GUERAU-DE-ARELLANO, M. 2015. Novel Markers to Delineate Murine M1 and M2 Macrophages. *PLoS One*, 10, e0145342.
- JANA, M., PALENCIA, C. A. & PAHAN, K. 2008. Fibrillar amyloid-beta peptides activate microglia via TLR2: implications for Alzheimer's disease. *J Immunol*, 181, 7254-62.
- JANG, E., KIM, J. H., LEE, S., KIM, J. H., SEO, J. W., JIN, M., LEE, M. G., JANG, I. S., LEE, W. H. & SUK, K. 2013. Phenotypic polarization of activated astrocytes: the critical role of lipocalin-2 in the classical inflammatory activation of astrocytes. *J Immunol*, 191, 5204-19.
- JAYASENA, T., POLJAK, A., BRAIDY, N., SMYTHE, G., RAFTERY, M., HILL, M., BRODATY, H., TROLLOR, J., KOCHAN, N. & SACHDEV, P. 2015. Upregulation of glycolytic enzymes, mitochondrial dysfunction and increased cytotoxicity in glial cells treated with Alzheimer's disease plasma. *PLoS One*, 10, e0116092.
- JELLINGER, K., PAULUS, W., GRUNDKE-IQBAL, I., RIEDERER, P. & YODIM, M. 1990. Brain iron and ferritin in Parkinson's and Alzheimer's diseases. *Journal of Neural Transmission-Parkinson's Disease and Dementia Section*, 2, 327-340.
- JONES, R. S., MINOGUE, A. M., CONNOR, T. J. & LYNCH, M. A. 2013. Amyloid-beta-induced astrocytic phagocytosis is mediated by CD36, CD47 and RAGE. *J Neuroimmune Pharmacol*, 8, 301-11.
- JONES, R. S., MINOGUE, A. M., FITZPATRICK, O. & LYNCH, M. A. 2015. Inhibition of JAK2 attenuates the increase in inflammatory markers in microglia from APP/PS1 mice. *Neurobiol Aging*, 36, 2716-24.

- KADOWAKI, H., NISHITOH, H., URANO, F., SADAMITSU, C., MATSUZAWA, A., TAKEDA, K., MASUTANI, H., YODOI, J., URANO, Y., NAGANO, T. & ICHIJO, H. 2005. Amyloid beta induces neuronal cell death through ROS-mediated ASK1 activation. *Cell Death Differ*, 12, 19-24.
- KARRAN, E., MERCKEN, M. & DE STROOPER, B. 2011. The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. *Nat Rev Drug Discov*, 10, 698-712.
- KAWANOKUCHI, J., MIZUNO, T., TAKEUCHI, H., KATO, H., WANG, J., MITSUMA, N. & SUZUMURA, A. 2006. Production of interferon-gamma by microglia. *Mult Scler*, 12, 558-64.
- KAYO, T., ALLISON, D. B., WEINDRUCH, R. & PROLLA, T. A. 2001. Influences of aging and caloric restriction on the transcriptional profile of skeletal muscle from rhesus monkeys. *Proc Natl Acad Sci U S A*, 98, 5093-8.
- KELLY, B. & O'NEILL, L. A. J. 2015. Metabolic reprogramming in macrophages and dendritic cells in innate immunity. *Cell Research*, 25, 771-784.
- KEMNA, E., PICKKERS, P., NEMETH, E., VAN DER HOEVEN, H. & SWINKELS, D. 2005. Time-course analysis of hepcidin, serum iron, and plasma cytokine levels in humans injected with LPS. *Blood*, 106, 1864-6.
- KIELIAN, T. 2006. Toll-like receptors in central nervous system glial inflammation and homeostasis. *Journal of neuroscience research*, 83, 711-730.
- KIM, J.-W., TCHERNYSHYOV, I., SEMENZA, G. L. & DANG, C. V. 2006a. HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. *Cell Metabolism*, 3, 177-185.
- KIM, J. W., TCHERNYSHYOV, I., SEMENZA, G. L. & DANG, C. V. 2006b. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab*, 3, 177-85.
- KIM, S. & PONKA, P. 2000. Effects of interferon-gamma and lipopolysaccharide on macrophage iron metabolism are mediated by nitric oxide-induced degradation of iron regulatory protein 2. *J Biol Chem*, 275, 6220-6.
- KIMELBERG, H. K. & NEDERGAARD, M. 2010. Functions of astrocytes and their potential as therapeutic targets. *Neurotherapeutics*, 7, 338-353.
- KOPF, H., DE LA ROSA, G. M., HOWARD, O. M. & CHEN, X. 2007. Rapamycin inhibits differentiation of Th17 cells and promotes generation of FoxP3+ T regulatory cells. *Int Immunopharmacol*, 7, 1819-24.
- KRABBE, G., HALLE, A., MATYASH, V., RINNENTHAL, J. L., EOM, G. D., BERNHARDT, U., MILLER, K. R., PROKOP, S., KETTENMANN, H. & HEPPNER, F. L. 2013. Functional impairment of microglia coincides with Beta-amyloid deposition in mice with Alzheimer-like pathology. *PLoS One*, 8, e60921.
- KRAWCZYK, C. M., HOLOWKA, T., SUN, J., BLAGIH, J., AMIEL, E., DEBERARDINIS, R. J., CROSS, J. R., JUNG, E., THOMPSON, C. B., JONES, R. G. & PEARCE, E. J. 2010. Toll-like receptor-

- induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood*, 115, 4742-9.
- KREUTZBERG, G. W. 1996. Microglia: a sensor for pathological events in the CNS. *Trends in neurosciences*, 19, 312-318.
- LASSMANN, H., BRUCK, W. & LUCCHINETTI, C. 2001. Heterogeneity of multiple sclerosis pathogenesis: implications for diagnosis and therapy. *Trends Mol Med*, 7, 115-21.
- LASSMANN, H., BRÜCK, W. & LUCCHINETTI, C. Heterogeneity of multiple sclerosis pathogenesis: implications for diagnosis and therapy. *Trends in Molecular Medicine*, 7, 115-121.
- LAU, L. T. & YU, A. C. 2001. Astrocytes produce and release interleukin-1, interleukin-6, tumor necrosis factor alpha and interferon-gamma following traumatic and metabolic injury. *J Neurotrauma*, 18, 351-9.
- LEE, C. K., WEINDRUCH, R. & PROLLA, T. A. 2000. Gene-expression profile of the ageing brain in mice. *Nat Genet*, 25, 294-7.
- LEE, C. Y. & LANDRETH, G. E. 2010. The role of microglia in amyloid clearance from the AD brain. *J Neural Transm (Vienna)*, 117, 949-60.
- LEE, Y. H., LI, Y., UYEDA, K. & HASEMANN, C. A. 2003. Tissue-specific structure/function differentiation of the liver isoform of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *J Biol Chem*, 278, 523-30.
- LEWANDOWSKA, E., WIERZBA-BOBROWICZ, T., KOSNO-KRUSZEWSKA, E., LECHOWICZ, W., SCHMIDT-SIDOR, B., SZPAK, G. M., BERTRAND, E., PASENNIK, E. & GWIAZDA, E. 2004. Ultrastructural evaluation of activated forms of microglia in human brain in selected neurological diseases (SSPE, Wilson's disease and Alzheimer's disease). *Folia Neuropathol*, 42, 81-91.
- LI, J., HSU, H.-C. & MOUNTZ, J. D. 2012. Managing Macrophages in Rheumatoid Arthritis by Reform or Removal. *Current rheumatology reports*, 14, 445-454.
- LI, M., WANG, Y., GUO, R., BAI, Y. & YU, Z. 2007. Glucocorticoids impair microglia ability to induce T cell proliferation and Th1 polarization. *Immunology Letters*, 109, 129-137.
- LIU, B. & HONG, J.-S. 2003. Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. *Journal of Pharmacology and Experimental Therapeutics*, 304, 1-7.
- LIU, L., LU, Y., MARTINEZ, J., BI, Y., LIAN, G., WANG, T., MILASTA, S., WANG, J., YANG, M., LIU, G., GREEN, D. R. & WANG, R. 2016. Proinflammatory signal suppresses proliferation and shifts macrophage metabolism from Myc-dependent to HIF1alpha-dependent. *Proc Natl Acad Sci U S A*, 113, 1564-9.
- LOANE, D. J., DEIGHAN, B. F., CLARKE, R. M., GRIFFIN, R. J., LYNCH, A. M. & LYNCH, M. A. 2009. Interleukin-4 mediates the neuroprotective effects of rosiglitazone in the aged brain. *Neurobiol Aging*, 30, 920-31.

- LOCKSLEY, R. M., KILLEEN, N. & LENARDO, M. J. 2001. The TNF and TNF Receptor Superfamilies: Integrating Mammalian Biology. *Cell*, 104, 487-501.
- LOKE, P., NAIR, M. G., PARKINSON, J., GUILIANO, D., BLAXTER, M. & ALLEN, J. E. 2002. IL-4 dependent alternatively-activated macrophages have a distinctive in vivo gene expression phenotype. *BMC Immunol*, 3, 7.
- LOO, Y.-M. & GALE, M. 2011. Immune signaling by RIG-I-like receptors. *Immunity*, 34, 680-692.
- LOWENSTEIN, C. J. & PADALKO, E. 2004. iNOS (NOS2) at a glance. *J Cell Sci*, 117, 2865-7.
- LUCAS, S. M., ROTHWELL, N. J. & GIBSON, R. M. 2006. The role of inflammation in CNS injury and disease. *British journal of pharmacology*, 147, S232-S240.
- LUE, L. F., RYDEL, R., BRIGHAM, E. F., YANG, L. B., HAMPEL, H., MURPHY, G. M., JR., BRACHOVA, L., YAN, S. D., WALKER, D. G., SHEN, Y. & ROGERS, J. 2001. Inflammatory repertoire of Alzheimer's disease and nondemented elderly microglia in vitro. *Glia*, 35, 72-9.
- LUNT, S. Y. & VANDER HEIDEN, M. G. 2011. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu Rev Cell Dev Biol*, 27, 441-64.
- LYMAN, M., LLOYD, D. G., JI, X., VIZCAYCHIPI, M. P. & MA, D. 2014. Neuroinflammation: the role and consequences. *Neuroscience research*, 79, 1-12.
- LYNCH, A. M., MURPHY, K. J., DEIGHAN, B. F., O'REILLY, J.-A., GUN'KO, Y. K., COWLEY, T. R., GONZALEZ-REYES, R. E. & LYNCH, M. A. 2010. The Impact of Glial Activation in the Aging Brain. *Aging and Disease*, 1, 262-278.
- LYNCH, M. 2009. The Multifaceted Profile of Activated Microglia. *Molecular Neurobiology*, 40, 139-156.
- LYNCH, M. A. 2014. The impact of neuroimmune changes on development of amyloid pathology; relevance to Alzheimer's disease. *Immunology*, 141, 292-301.
- LYONS, A., DOWNER, E. J., CROTTY, S., NOLAN, Y. M., MILLS, K. H. & LYNCH, M. A. 2007a. CD200 ligand–receptor interaction modulates microglial activation in vivo and in vitro: a role for IL-4. *The Journal of neuroscience*, 27, 8309-8313.
- LYONS, A., GRIFFIN, R. J., COSTELLOE, C. E., CLARKE, R. M. & LYNCH, M. A. 2007b. IL-4 attenuates the neuroinflammation induced by amyloid- β in vivo and in vitro. *Journal of neurochemistry*, 101, 771-781.
- MAJUMDAR, A., CRUZ, D., ASAMOAH, N., BUXBAUM, A., SOHAR, I., LOBEL, P. & MAXFIELD, F. R. 2007. Activation of Microglia Acidifies Lysosomes and Leads to Degradation of Alzheimer Amyloid Fibrils. *Molecular Biology of the Cell*, 18, 1490-1496.
- MAN, K. & KALLIES, A. 2015. Synchronizing transcriptional control of T cell metabolism and function. *Nat Rev Immunol*, 15, 574-84.
- MANDREKAR, S., JIANG, Q., LEE, C. Y. D., KOENIGSKNECHT-TALBOO, J., HOLTZMAN, D. M. & LANDRETH, G. E. 2009. Microglia Mediate the Clearance of Soluble A β through Fluid Phase Macropinocytosis. *The Journal of Neuroscience*, 29, 4252-4262.

- MARODI, L., SCHREIBER, S., ANDERSON, D. C., MACDERMOTT, R. P., KORCHAK, H. M. & JOHNSTON, R. B., JR. 1993. Enhancement of macrophage candidacidal activity by interferon-gamma. Increased phagocytosis, killing, and calcium signal mediated by a decreased number of mannose receptors. *J Clin Invest*, 91, 2596-601.
- MARTINEZ, F. O. & GORDON, S. 2014. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Reports*, 6, 13.
- MARTINEZ, F. O., SICA, A., MANTOVANI, A. & LOCATI, M. 2008. Macrophage activation and polarization. *Frontiers in bioscience : a journal and virtual library* [Online], 13. Available: <http://europepmc.org/abstract/MED/17981560>
<http://dx.doi.org/10.2741/2692> [Accessed 2008].
- MATTSON, M. P. 2008. Glutamate and Neurotrophic Factors in Neuronal Plasticity and Disease. *Annals of the New York Academy of Sciences*, 1144, 97-112.
- MAYLE, K. M., LE, A. M. & KAMEI, D. T. 2012. The Intracellular Trafficking Pathway of Transferrin. *Biochimica et Biophysica Acta*, 1820, 264-281.
- MCGEER, E. G. & MCGEER, P. L. 2003. Inflammatory processes in Alzheimer's disease. *Prog Neuropsychopharmacol Biol Psychiatry*, 27, 741-9.
- MCGEER, P. L., KAWAMATA, T., WALKER, D. G., AKIYAMA, H., TOOYAMA, I. & MCGEER, E. G. 1993. Microglia in degenerative neurological disease. *Glia*, 7, 84-92.
- MCQUILLAN, K., LYNCH, M. A. & MILLS, K. H. 2010. Activation of mixed glia by A β -specific Th1 and Th17 cells and its regulation by Th2 cells. *Brain, behavior, and immunity*, 24, 598-607.
- MEDEIROS, R. & LAFERLA, F. M. 2013. Astrocytes: conductors of the Alzheimer disease neuroinflammatory symphony. *Exp Neurol*, 239, 133-8.
- MENON, D., COLL, R., O'NEILL, L. A. & BOARD, P. G. 2015. GSTO1-1 modulates metabolism in macrophages activated through the LPS and TLR4 pathway. *J Cell Sci*, 128, 1982-90.
- MICHALEK, R. D., GERRIETS, V. A., JACOBS, S. R., MACINTYRE, A. N., MACIVER, N. J., MASON, E. F., SULLIVAN, S. A., NICHOLS, A. G. & RATHMELL, J. C. 2011. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4⁺ T cell subsets. *J Immunol*, 186, 3299-303.
- MIKITA, T., CAMPBELL, D., WU, P., WILLIAMSON, K. & SCHINDLER, U. 1996. Requirements for interleukin-4-induced gene expression and functional characterization of Stat6. *Molecular and Cellular Biology*, 16, 5811-5820.
- MILLS, E. L. & O'NEILL, L. A. 2016. Reprogramming mitochondrial metabolism in macrophages as an anti-inflammatory signal. *European journal of immunology*, 46, 13-21.
- MILLS, K. H. & DUNNE, A. 2009. Immune modulation: IL-1, master mediator or initiator of inflammation. *Nature medicine*, 15, 1363-1364.

- MINCHENKO, A., LESHCHINSKY, I., OPENTANOVA, I., SANG, N., SRINIVAS, V., ARMSTEAD, V. & CARO, J. 2002. Hypoxia-inducible factor-1-mediated expression of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) gene. Its possible role in the Warburg effect. *J Biol Chem*, 277, 6183-7.
- MINCHENKO, O., OPENTANOVA, I. & CARO, J. 2003. Hypoxic regulation of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene family (PFKFB-1-4) expression in vivo. *FEBS Lett*, 554, 264-70.
- MINOGUE, A. M., JONES, R. S., KELLY, R. J., MCDONALD, C. L., CONNOR, T. J. & LYNCH, M. A. 2014a. Age-associated dysregulation of microglial activation is coupled with enhanced blood-brain barrier permeability and pathology in APP/PS1 mice. *Neurobiology of Aging*, 35, 1442-1452.
- MINOGUE, A. M., JONES, R. S., KELLY, R. J., MCDONALD, C. L., CONNOR, T. J. & LYNCH, M. A. 2014b. Age-associated dysregulation of microglial activation is coupled with enhanced blood-brain barrier permeability and pathology in APP/PS1 mice. *Neurobiol Aging*, 35, 1442-52.
- MIZUNO, T., KAWANOKUCHI, J., NUMATA, K. & SUZUMURA, A. 2003. Production and neuroprotective functions of fractalkine in the central nervous system. *Brain research*, 979, 65-70.
- MOSSER, D. M. 2003. The many faces of macrophage activation. *J Leukoc Biol*, 73, 209-12.
- MOSSER, D. M. & EDWARDS, J. P. 2008. Exploring the full spectrum of macrophage activation. *Nature reviews immunology*, 8, 958-969.
- MOUNIER, R., THERET, M., ARNOLD, L., CUVELLIER, S., BULTOT, L., GORANSSON, O., SANZ, N., FERRY, A., SAKAMOTO, K., FORETZ, M., VIOLLET, B. & CHAZAUD, B. 2013. AMPK α 1 regulates macrophage skewing at the time of resolution of inflammation during skeletal muscle regeneration. *Cell Metab*, 18, 251-64.
- MURPHY, S. 2000. Production of nitric oxide by glial cells: regulation and potential roles in the CNS. *Glia*, 29, 1-13.
- MURRAY, C. A. & LYNCH, M. A. 1998. Evidence that increased hippocampal expression of the cytokine interleukin-1 β is a common trigger for age- and stress-induced impairments in long-term potentiation. *The Journal of neuroscience*, 18, 2974-2981.
- MURRAY, P. J., ALLEN, J. E., BISWAS, S. K., FISHER, E. A., GILROY, D. W., GOERDT, S., GORDON, S., HAMILTON, J. A., IVASHKIV, L. B., LAWRENCE, T., LOCATI, M., MANTOVANI, A., MARTINEZ, F. O., MEGE, J. L., MOSSER, D. M., NATOLI, G., SAEIJ, J. P., SCHULTZE, J. L., SHIREY, K. A., SICA, A., SUTTLES, J., UDALOVA, I., VAN GINDERACHTER, J. A., VOGEL, S. N. & WYNN, T. A. 2014. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity*, 41, 14-20.
- NAG, S., SARKAR, B., BANDYOPADHYAY, A., SAHOO, B., SREENIVASAN, V. K., KOMBRABAIL, M., MURALIDHARAN, C. & MAITI, S. 2011. Nature of the amyloid- β monomer and the monomer-oligomer equilibrium. *Journal of Biological Chemistry*, 286, 13827-13833.

- NAGELE, R. G., WEGIEL, J., VENKATARAMAN, V., IMAKI, H., WANG, K.-C. & WEGIEL, J. 2004. Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease. *Neurobiology of aging*, 25, 663-674.
- NAIRZ, M., FRITSCH, G., BRUNNER, P., TALASZ, H., HANTKE, K. & WEISS, G. 2008. Interferon-gamma limits the availability of iron for intramacrophage *Salmonella typhimurium*. *Eur J Immunol*, 38, 1923-36.
- NAMGALADZE, D. & BRUNE, B. 2014. Fatty acid oxidation is dispensable for human macrophage IL-4-induced polarization. *Biochim Biophys Acta*, 1841, 1329-35.
- NATH, N., KHAN, M., RATTAN, R., MANGALAM, A., MAKAR, R. S., DE MEESTER, C., BERTRAND, L., SINGH, I., CHEN, Y., VIOLLET, B. & GIRI, S. 2009. Loss of AMPK exacerbates experimental autoimmune encephalomyelitis disease severity. *Biochem Biophys Res Commun*, 386, 16-20.
- NATHAN, C. F. & HIBBS, J. B., JR. 1991. Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr Opin Immunol*, 3, 65-70.
- NATHAN, C. F., MURRAY, H. W., WIEBE, M. E. & RUBIN, B. Y. 1983. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J Exp Med*, 158, 670-89.
- NEMETH, B., DOCZI, J., CSETE, D., KACSO, G., RAVASZ, D., ADAMS, D., KISS, G., NAGY, A. M., HORVATH, G., TRETTNER, L., MOCSAI, A., CSEPANYI-KOMI, R., IORDANOV, I., ADAM-VIZI, V. & CHINOPOULOS, C. 2016. Abolition of mitochondrial substrate-level phosphorylation by itaconic acid produced by LPS-induced Irg1 expression in cells of murine macrophage lineage. *FASEB J*, 30, 286-300.
- NEMETH, E., RIVERA, S., GABAYAN, V., KELLER, C., TAUDORF, S., PEDERSEN, B. K. & GANZ, T. 2004. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest*, 113, 1271-6.
- NEUMANN, H., KOTTER, M. R. & FRANKLIN, R. J. 2009. Debris clearance by microglia: an essential link between degeneration and regeneration. *Brain*, 132, 288-95.
- NEWMAN, E. A. 2003. New roles for astrocytes: regulation of synaptic transmission. *Trends in neurosciences*, 26, 536-542.
- NEWMAN, M., MUSGRAVE, F. & LARDELLI, M. 2007. Alzheimer disease: amyloidogenesis, the presenilins and animal models. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1772, 285-297.
- NIKODEMOVA, M. & WATTERS, J. J. 2011. Outbred ICR/CD1 mice display more severe neuroinflammation mediated by microglial TLR4/CD14 activation than inbred C57Bl/6 mice. *Neuroscience*, 190, 67-74.
- NJIE, E. G., BOELEN, E., STASSEN, F. R., STEINBUSCH, H. W., BORCHELT, D. R. & STREIT, W. J. 2012a. Ex vivo cultures of microglia from young and aged rodent brain reveal age-related changes in microglial function. *Neurobiol Aging*, 33, 195 e1-12.

- NJIE, E. G., BOELEN, E., STASSEN, F. R., STEINBUSCH, H. W. M., BORCHELT, D. R. & STREIT, W. J. 2012b. Ex vivo cultures of microglia from young and aged rodent brain reveal age-related changes in microglial function. *Neurobiology of Aging*, 33, 195.e1-195.e12.
- NOMURA, M., LIU, J., ROVIRA, I. I., GONZALEZ-HURTADO, E., LEE, J., WOLFGANG, M. J. & FINKEL, T. 2016. Fatty acid oxidation in macrophage polarization. *Nat Immunol*, 17, 216-217.
- NORDEN, D. M. & GODBOUT, J. P. 2013. Microglia of the Aged Brain: Primed to be Activated and Resistant to Regulation. *Neuropathology and applied neurobiology*, 39, 19-34.
- O'NEILL, L. A. 2008. The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. *Immunological reviews*, 226, 10-18.
- O'NEILL, L. A. 2011. A critical role for citrate metabolism in LPS signalling. *Biochem J*, 438, e5-6.
- O'NEILL, L. A. & HARDIE, D. G. 2013. Metabolism of inflammation limited by AMPK and pseudo-starvation. *Nature*, 493, 346-55.
- OBACH, M., NAVARRO-SABATE, A., CARO, J., KONG, X., DURAN, J., GOMEZ, M., PERALES, J. C., VENTURA, F., ROSA, J. L. & BARTRONS, R. 2004. 6-Phosphofructo-2-kinase (pfkfb3) gene promoter contains hypoxia-inducible factor-1 binding sites necessary for transactivation in response to hypoxia. *J Biol Chem*, 279, 53562-70.
- ODEGAARD, J. I. & CHAWLA, A. 2011. Alternative macrophage activation and metabolism. *Annu Rev Pathol*, 6, 275-97.
- OH, Y. T., LEE, J. Y., YOON, H., LEE, E. H., BAIK, H. H., KIM, S. S., HA, J., YOON, K. S., CHOE, W. & KANG, I. 2008. Lipopolysaccharide induces hypoxia-inducible factor-1 alpha mRNA expression and activation via NADPH oxidase and Sp1-dependent pathway in BV2 murine microglial cells. *Neurosci Lett*, 431, 155-60.
- OLABARRIA, M., NORISTANI, H. N., VERKHRATSKY, A. & RODRIGUEZ, J. J. 2010. Concomitant astroglial atrophy and astrogliosis in a triple transgenic animal model of Alzheimer's disease. *Glia*, 58, 831-8.
- OLEFSKY, J. M. & GLASS, C. K. 2010a. Macrophages, inflammation, and insulin resistance. *Annual review of physiology*, 72, 219-246.
- OLEFSKY, J. M. & GLASS, C. K. 2010b. Macrophages, inflammation, and insulin resistance. *Annu Rev Physiol*, 72, 219-46.
- ORIHUELA, R., MCPHERSON, C. A. & HARRY, G. J. 2016. Microglial M1/M2 polarization and metabolic states. *Br J Pharmacol*, 173, 649-65.
- OSHIRO, S., MORIOKA, M. S. & KIKUCHI, M. 2011. Dysregulation of Iron Metabolism in Alzheimer's Disease, Parkinson's Disease, and Amyotrophic Lateral Sclerosis. *Advances in Pharmacological Sciences*, 2011, 8.
- OSTHUS, R. C., SHIM, H., KIM, S., LI, Q., REDDY, R., MUKHERJEE, M., XU, Y., WONSEY, D., LEE, L. A. & DANG, C. V. 2000. Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. *J Biol Chem*, 275, 21797-800.

- PALOP, J. J. & MUCKE, L. 2010. Amyloid- β Induced Neuronal Dysfunction in Alzheimer's Disease: From Synapses toward Neural Networks. *Nature neuroscience*, 13, 812-818.
- PALSSON-MCDERMOTT, E. M., CURTIS, A. M., GOEL, G., LAUTERBACH, M. A., SHEEDY, F. J., GLEESON, L. E., VAN DEN BOSCH, M. W., QUINN, S. R., DOMINGO-FERNANDEZ, R., JOHNSTON, D. G., JIANG, J. K., ISRAELSEN, W. J., KEANE, J., THOMAS, C., CLISH, C., VANDERHEIDEN, M., XAVIER, R. J. & O'NEILL, L. A. 2015. Pyruvate kinase M2 regulates Hif-1 α activity and IL-1 β induction and is a critical determinant of the warburg effect in LPS-activated macrophages. *Cell Metab*, 21, 65-80.
- PAPANDREOU, I., CAIRNS, R. A., FONTANA, L., LIM, A. L. & DENKO, N. C. 2006. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell Metab*, 3, 187-97.
- PARADKAR, P. N., DE DOMENICO, I., DURCHFORT, N., ZOHAN, I., KAPLAN, J. & WARD, D. M. 2008. Iron depletion limits intracellular bacterial growth in macrophages. *Blood*, 112, 866-74.
- PARKHURST, C. N., YANG, G., NINAN, I., SAVAS, J. N., YATES, J. R., 3RD, LAFAILLE, J. J., HEMPSTEAD, B. L., LITTMAN, D. R. & GAN, W. B. 2013. Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor. *Cell*, 155, 1596-609.
- PEARCE, E. J. & EVERTS, B. 2015. Dendritic cell metabolism. *Nat Rev Immunol*, 15, 18-29.
- PEARCE, E. L. & PEARCE, E. J. 2013. Metabolic pathways in immune cell activation and quiescence. *Immunity*, 38, 633-43.
- PEARSE, B. M. & ROBINSON, M. S. 1990. Clathrin, adaptors, and sorting. *Annu Rev Cell Biol*, 6, 151-71.
- PERRY, R. T., COLLINS, J. S., WIENER, H., ACTON, R. & GO, R. C. 2001. The role of TNF and its receptors in Alzheimer's disease. *Neurobiology of aging*, 22, 873-883.
- PERRY, V. H. & TEELING, J. 2013. Microglia and macrophages of the central nervous system: the contribution of microglia priming and systemic inflammation to chronic neurodegeneration. *Semin Immunopathol*, 35, 601-12.
- PESTKA, S., KRAUSE, C. D. & WALTER, M. R. 2004. Interferons, interferon-like cytokines, and their receptors. *Immunol Rev*, 202, 8-32.
- PEYSSONNAUX, C., DATTA, V., CRAMER, T., DOEDENS, A., THEODORAKIS, E. A., GALLO, R. L., HURTADO-ZIOLA, N., NIZET, V. & JOHNSON, R. S. 2005. HIF-1 α expression regulates the bactericidal capacity of phagocytes. *J Clin Invest*, 115, 1806-15.
- PFEIFFER, T., SCHUSTER, S. & BONHOEFFER, S. 2001. Cooperation and competition in the evolution of ATP-producing pathways. *Science*, 292, 504-507.
- PILKIS, S. J., EL-MAGHRABI, M. R., MCGRANE, M. M., PILKIS, J. & CLAUS, T. H. 1981. The role of fructose 2,6-bisphosphate in regulation of fructose-1,6-bisphosphatase. *J Biol Chem*, 256, 11489-95.

- POLTORAK, A., HE, X., SMIRNOVA, I., LIU, M. Y., VAN HUFFEL, C., DU, X., BIRDWELL, D., ALEJOS, E., SILVA, M., GALANOS, C., FREUDENBERG, M., RICCIARDI-CASTAGNOLI, P., LAYTON, B. & BEUTLER, B. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*, 282, 2085-8.
- PONOMAREV, E. D., MARESZ, K., TAN, Y. & DITTEL, B. N. 2007. CNS-derived interleukin-4 is essential for the regulation of autoimmune inflammation and induces a state of alternative activation in microglial cells. *J Neurosci*, 27, 10714-21.
- PRATT, B. M. & MCPHERSON, J. M. 1997. TGF- β in the central nervous system: potential roles in ischemic injury and neurodegenerative diseases. *Cytokine & growth factor reviews*, 8, 267-292.
- PRILLER, C., BAUER, T., MITTEREGGER, G., KREBS, B., KRETZSCHMAR, H. A. & HERMS, J. 2006. Synapse formation and function is modulated by the amyloid precursor protein. *J Neurosci*, 26, 7212-21.
- PROLLA, T. A. 2002a. DNA microarray analysis of the aging brain. *Chem Senses*, 27, 299-306.
- PROLLA, T. A. 2002b. DNA microarray analysis of the aging brain. *Chemical senses*, 27, 299-306.
- QIU, C., KIVIPELTO, M. & VON STRAUSS, E. 2009. Epidemiology of Alzheimer's disease: occurrence, determinants, and strategies toward intervention. *Dialogues in Clinical Neuroscience*, 11, 111-128.
- QUERFURTH, H. W. & LAFERLA, F. M. 2010. Alzheimer's disease. *N Engl J Med*, 362, 329-44.
- RAHA, A. A., VAISHNAV, R. A., FRIEDLAND, R. P., BOMFORD, A. & RAHA-CHOWDHURY, R. 2013. The systemic iron-regulatory proteins hepcidin and ferroportin are reduced in the brain in Alzheimer's disease. *Acta Neuropathol Commun*, 1, 55.
- RANSOHOFF, R. M. & BROWN, M. A. 2012. Innate immunity in the central nervous system. *J Clin Invest*, 122, 1164-71.
- RECALCATI, S., LOCATI, M., MARINI, A., SANTAMBROGIO, P., ZANINOTTO, F., DE PIZZOL, M., ZAMMATARO, L., GIRELLI, D. & CAIRO, G. 2010. Differential regulation of iron homeostasis during human macrophage polarized activation. *Eur J Immunol*, 40, 824-35.
- REED-GEAGHAN, E. G., SAVAGE, J. C., HISE, A. G. & LANDRETH, G. E. 2009. CD14 and Toll-like receptors 2 and 4 are required for fibrillar A β -stimulated microglial activation. *The Journal of neuroscience: the official journal of the Society for Neuroscience*, 29, 11982-11992.
- REZAIE, P. & MALE, D. 2002. Differentiation, ramification and distribution of microglia within the central nervous system examined. *Neuroembryology and Aging*, 1, 29-43.
- RICCIOTTI, E. & FITZGERALD, G. A. 2011. Prostaglandins and Inflammation. *Arteriosclerosis, thrombosis, and vascular biology*, 31, 986-1000.

- RIDER, MARK H., BERTRAND, L., VERTOMMEN, D., MICHELS, PAUL A., ROUSSEAU, GUY G. & HUE, L. 2004. 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase: head-to-head with a bifunctional enzyme that controls glycolysis. *Biochemical Journal*, 381, 561-579.
- RODRIGUEZ-PRADOS, J. C., TRAVES, P. G., CUENCA, J., RICO, D., ARAGONES, J., MARTIN-SANZ, P., CASCANTE, M. & BOSCA, L. 2010. Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation. *J Immunol*, 185, 605-14.
- ROUAULT, T. A. 2013. Iron metabolism in the CNS: implications for neurodegenerative diseases. *Nat Rev Neurosci*, 14, 551-564.
- RUIZ-GARCIA, A., MONSALVE, E., NOVELLASDEMUNT, L., NAVARRO-SABATE, A., MANZANO, A., RIVERO, S., CASTRILLO, A., CASADO, M., LABORDA, J., BARTRONS, R. & DIAZ-GUERRA, M. J. 2011. Cooperation of adenosine with macrophage Toll-4 receptor agonists leads to increased glycolytic flux through the enhanced expression of PFKFB3 gene. *J Biol Chem*, 286, 19247-58.
- SAG, D., CARLING, D., STOUT, R. D. & SUTTLES, J. 2008. Adenosine 5'-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. *J Immunol*, 181, 8633-41.
- SAIJO, K. & GLASS, C. K. 2011a. Microglial cell origin and phenotypes in health and disease. *Nature Reviews Immunology*, 11, 775-787.
- SAIJO, K. & GLASS, C. K. 2011b. Microglial cell origin and phenotypes in health and disease. *Nat Rev Immunol*, 11, 775-787.
- SAKAKIBARA, R., KATO, M., OKAMURA, N., NAKAGAWA, T., KOMADA, Y., TOMINAGA, N., SHIMOJO, M. & FUKASAWA, M. 1997. Characterization of a human placental fructose-6-phosphate, 2-kinase/fructose-2,6-bisphosphatase. *J Biochem*, 122, 122-8.
- SAWCER, S., HELLENTHAL, G., PIRINEN, M., SPENCER, C. C., PATSOPOULOS, N. A., MOUTSIANAS, L., DILTHEY, A., SU, Z., FREEMAN, C., HUNT, S. E., EDKINS, S., GRAY, E., BOOTH, D. R., POTTER, S. C., GORIS, A., BAND, G., OTURAI, A. B., STRANGE, A., SAARELA, J., BELLENGUEZ, C., FONTAINE, B., GILLMAN, M., HEMMER, B., Gwilliam, R., ZIPP, F., JAYAKUMAR, A., MARTIN, R., LESLIE, S., HAWKINS, S., GIANNOULATOU, E., D'ALFONSO, S., BLACKBURN, H., MARTINELLI BONESCHI, F., LIDDLE, J., HARBO, H. F., PEREZ, M. L., SPURKLAND, A., WALLER, M. J., MYCKO, M. P., RICKETTS, M., COMABELLA, M., HAMMOND, N., KOCKUM, I., MCCANN, O. T., BAN, M., WHITTAKER, P., KEMPPINEN, A., WESTON, P., HAWKINS, C., WIDAA, S., ZAJICEK, J., DRONOV, S., ROBERTSON, N., BUMPSTEAD, S. J., BARCELLOS, L. F., RAVINDRARAJAH, R., ABRAHAM, R., ALFREDSSON, L., ARDLIE, K., AUBIN, C., BAKER, A., BAKER, K., BARANZINI, S. E., BERGAMASCHI, L., BERGAMASCHI, R., BERNSTEIN, A., BERTHELE, A., BOGGILD, M., BRADFELD, J. P., BRASSAT, D., BROADLEY, S. A., BUCK, D., BUTZKUEVEN, H., CAPRA, R., CARROLL, W. M., CAVALLA, P., CELIUS, E. G., CEPOK, S., CHIAVACCI, R., CLERGET-DARPOUX, F., CLYSTERS, K., COMI, G., COSSBURN, M., COURNU-REBEIX, I., COX, M. B., COZEN, W., CREE, B. A., CROSS, A. H., CUSI, D., DALY, M. J., DAVIS, E., DE BAKKER, P. I., DEBOUVERIE, M., D'HOOGE M. B., DIXON, K., DOBOSI, R., DUBOIS, B., ELLINGHAUS, D., ELOVAARA, I., ESPOSITO, F., et al. 2011. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature*, 476, 214-9.

- SAWYER, R., STRAUSBAUCH, P. & VOLKMAN, A. 1982. Resident macrophage proliferation in mice depleted of blood monocytes by strontium-89. *Laboratory investigation; a journal of technical methods and pathology*, 46, 165-170.
- SCHAER, C. A., VALLELIAN, F., IMHOF, A., SCHOEDON, G. & SCHAER, D. J. 2008. Heme carrier protein (HCP-1) spatially interacts with the CD163 hemoglobin uptake pathway and is a target of inflammatory macrophage activation. *J Leukoc Biol*, 83, 325-33.
- SCHRODER, K., HERTZOG, P. J., RAVASI, T. & HUME, D. A. 2004. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol*, 75, 163-89.
- SCHWANDNER, R., DZIARSKI, R., WESCHE, H., ROTHE, M. & KIRSCHNING, C. J. 1999. Peptidoglycan-and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *Journal of Biological Chemistry*, 274, 17406-17409.
- SCHWARTZ, R. H. 2003. T cell anergy*. *Annual review of immunology*, 21, 305-334.
- SCOTTON, C. J., MARTINEZ, F. O., SMELT, M. J., SIRONI, M., LOCATI, M., MANTOVANI, A. & SOZZANI, S. 2005. Transcriptional profiling reveals complex regulation of the monocyte IL-1 beta system by IL-13. *J Immunol*, 174, 834-45.
- SEMBA, H., TAKEDA, N., ISAGAWA, T., SUGIURA, Y., HONDA, K., WAKE, M., MIYAZAWA, H., YAMAGUCHI, Y., MIURA, M., JENKINS, D. M., CHOI, H., KIM, J. W., ASAGIRI, M., COWBURN, A. S., ABE, H., SOMA, K., KOYAMA, K., KATOH, M., SAYAMA, K., GODA, N., JOHNSON, R. S., MANABE, I., NAGAI, R. & KOMURO, I. 2016. HIF-1alpha-PDK1 axis-induced active glycolysis plays an essential role in macrophage migratory capacity. *Nat Commun*, 7, 11635.
- SEMENZA, G. L. 2007. Hypoxia-inducible factor 1 (HIF-1) pathway. *Sci. Stke*, 2007, cm8-cm8.
- SHAO, D., LIU, Y., LIU, X., ZHU, L., CUI, Y., CUI, A., QIAO, A., KONG, X., LIU, Y., CHEN, Q., GUPTA, N., FANG, F. & CHANG, Y. 2010. PGC-1 beta-regulated mitochondrial biogenesis and function in myotubes is mediated by NRF-1 and ERR alpha. *Mitochondrion*, 10, 516-27.
- SHI, L. Z., WANG, R., HUANG, G., VOGEL, P., NEALE, G., GREEN, D. R. & CHI, H. 2011. HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *J Exp Med*, 208, 1367-76.
- SIEGERT, I., SCHÖDEL, J., NAIRZ, M., SCHATZ, V., DETTMER, K., DICK, C., KALUCKA, J., FRANKE, K., EHRENSCHWENDER, M., SCHLEY, G., BENEKE, A., SUTTER, J., MOLL, M., HELLERBRAND, C., WIELOCKX, B., KATSCHINSKI, DÖRTHE M., LANG, R., GALY, B., HENTZE, MATTHIAS W., KOIVUNEN, P., OEFNER, PETER J., BOGDAN, C., WEISS, G., WILLAM, C. & JANTSCH, J. 2015. Ferritin-Mediated Iron Sequestration Stabilizes Hypoxia-Inducible Factor-1α upon LPS Activation in the Presence of Ample Oxygen. *Cell Reports*, 13, 2048-2055.
- SIERRA, A., GOTTFRIED-BLACKMORE, A. C., MCEWEN, B. S. & BULLOCH, K. 2007. Microglia derived from aging mice exhibit an altered inflammatory profile. *Glia*, 55, 412-24.
- SMITH-GARVIN, J. E., KORETZKY, G. A. & JORDAN, M. S. 2009. T Cell Activation. *Annual review of immunology*, 27, 591-619.

- SMITS, H. A., RIJSMUS, A., VAN LOON, J. H., WAT, J. W., VERHOEF, J., BOVEN, L. A. & NOTTET, H. S. 2002. Amyloid-beta-induced chemokine production in primary human macrophages and astrocytes. *J Neuroimmunol*, 127, 160-8.
- ST-PIERRE, J., LIN, J., KRAUSS, S., TARR, P. T., YANG, R., NEWGARD, C. B. & SPIEGELMAN, B. M. 2003. Bioenergetic analysis of peroxisome proliferator-activated receptor gamma coactivators 1alpha and 1beta (PGC-1alpha and PGC-1beta) in muscle cells. *J Biol Chem*, 278, 26597-603.
- STAHL, P., SCHLESINGER, P. H., SIGARDSON, E., RODMAN, J. S. & LEE, Y. 1980. Receptor-mediated pinocytosis of mannose glycoconjugates by macrophages: characterization and evidence for receptor recycling. *Cell*, 19, 207-215.
- STANSLEY, B., POST, J. & HENSLEY, K. 2012. A comparative review of cell culture systems for the study of microglial biology in Alzheimer's disease. *Journal of Neuroinflammation*, 9, 115-115.
- STEEN, E., TERRY, B. M., RIVERA, E. J., CANNON, J. L., NEELY, T. R., TAVARES, R., XU, X. J., WANDS, J. R. & DE LA MONTE, S. M. 2005. Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes? *J Alzheimers Dis*, 7, 63-80.
- STEIN, M., KESHAV, S., HARRIS, N. & GORDON, S. 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med*, 176, 287-92.
- STREIT, W. J. 2006. Microglial senescence: does the brain's immune system have an expiration date? *Trends in Neurosciences*, 29, 506-510.
- STREIT, W. J., BRAAK, H., XUE, Q. S. & BECHMANN, I. 2009. Dystrophic (senescent) rather than activated microglial cells are associated with tau pathology and likely precede neurodegeneration in Alzheimer's disease. *Acta Neuropathol*, 118, 475-85.
- STREIT, W. J. & XUE, Q. S. 2009. Life and death of microglia. *J Neuroimmune Pharmacol*, 4, 371-9.
- STUMPO, D. J., LAI, W. S. & BLACKSHEAR, P. J. 2010. Inflammation: Cytokines and RNA-based Regulation. *Wiley interdisciplinary reviews. RNA*, 1, 60-80.
- TAKAHASHI, K., ROCHFORD, C. D. & NEUMANN, H. 2005. Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. *J Exp Med*, 201, 647-57.
- TAKEDA, N., O'DEA, E. L., DOEDENS, A., KIM, J. W., WEIDEMANN, A., STOCKMANN, C., ASAGIRI, M., SIMON, M. C., HOFFMANN, A. & JOHNSON, R. S. 2010. Differential activation and antagonistic function of HIF-1alpha isoforms in macrophages are essential for NO homeostasis. *Genes Dev*, 24, 491-501.
- TANG, Y. & LE, W. 2016. Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases. *Mol Neurobiol*, 53, 1181-94.

- TANNAHILL, G. M., CURTIS, A. M., ADAMIK, J., PALSSON-MCDERMOTT, E. M., MCGETTRICK, A. F., GOEL, G., FREZZA, C., BERNARD, N. J., KELLY, B., FOLEY, N. H., ZHENG, L., GARDET, A., TONG, Z., JANY, S. S., CORR, S. C., HANEKLAUS, M., CAFFREY, B. E., PIERCE, K., WALMSLEY, S., BEASLEY, F. C., CUMMINS, E., NIZET, V., WHYTE, M., TAYLOR, C. T., LIN, H., MASTERS, S. L., GOTTLIEB, E., KELLY, V. P., CLISH, C., AURON, P. E., XAVIER, R. J. & O'NEILL, L. A. 2013. Succinate is an inflammatory signal that induces IL-1 β through HIF-1 α . *Nature*, 496, 238-42.
- TAWAKOL, A., SINGH, P., MOJENA, M., PIMENTEL-SANTILLANA, M., EMAMI, H., MACNABB, M., RUDD, J. H. F., NARULA, J., ENRIQUEZ, J. A., TRAVÉS, P. G., FERNÁNDEZ-VELASCO, M., BARTRONS, R., MARTÍN-SANZ, P., FAYAD, Z. A., TEJEDOR, A. & BOSCA, L. 2015. HIF-1 α and PFKFB3 mediate a tight relationship between pro-inflammatory activation and anaerobic metabolism in atherosclerotic macrophages. *Arteriosclerosis, thrombosis, and vascular biology*, 35, 1463-1471.
- TAYLOR, P. R., MARTINEZ-POMARES, L., STACEY, M., LIN, H. H., BROWN, G. D. & GORDON, S. 2005. Macrophage receptors and immune recognition. *Annu Rev Immunol*, 23, 901-44.
- THEURL, I., THEURL, M., SEIFERT, M., MAIR, S., NAIRZ, M., RUMPOLD, H., ZOLLER, H., BELLMANN-WEILER, R., NIEDEREGGER, H., TALASZ, H. & WEISS, G. 2008. Autocrine formation of hepcidin induces iron retention in human monocytes. *Blood*, 111, 2392-9.
- THOMSEN, M. S., ANDERSEN, M. V., CHRISTOFFERSEN, P. R., JENSEN, M. D., LICHOTA, J. & MOOS, T. 2015. Neurodegeneration with inflammation is accompanied by accumulation of iron and ferritin in microglia and neurons. *Neurobiol Dis*, 81, 108-18.
- TOWN, T., NIKOLIC, V. & TAN, J. 2005. The microglial "activation" continuum: from innate to adaptive responses. *Journal of Neuroinflammation*, 2, 1-10.
- TREMBLAY, M.-È., STEVENS, B., SIERRA, A., WAKE, H., BESSIS, A. & NIMMERJAHN, A. 2011. The role of microglia in the healthy brain. *The Journal of neuroscience*, 31, 16064-16069.
- TURVEY, S. E. & BROIDE, D. H. 2010. Innate immunity. *Journal of Allergy and Clinical Immunology*, 125, S24-S32.
- URRUTIA, P., AGUIRRE, P., ESPARZA, A., TAPIA, V., MENA, N. P., ARREDONDO, M., GONZALEZ-BILLAULT, C. & NUNEZ, M. T. 2013. Inflammation alters the expression of DMT1, FPN1 and hepcidin, and it causes iron accumulation in central nervous system cells. *J Neurochem*, 126, 541-9.
- VALLELIAN, F., SCHAER, C. A., KAEMPFER, T., GEHRIG, P., DUERST, E., SCHOEDON, G. & SCHAER, D. J. 2010. Glucocorticoid treatment skews human monocyte differentiation into a hemoglobin-clearance phenotype with enhanced heme-iron recycling and antioxidant capacity. *Blood*, 116, 5347-56.
- VAN FURTH, R. & COHN, Z. A. 1968. The origin and kinetics of mononuclear phagocytes. *The Journal of experimental medicine*, 128, 415-435.
- VARIN, A., MUKHOPADHYAY, S., HERBEIN, G. & GORDON, S. 2010. Alternative activation of macrophages by IL-4 impairs phagocytosis of pathogens but potentiates microbial-induced signalling and cytokine secretion. *Blood*, 115, 353-62.

- VATS, D., MUKUNDAN, L., ODEGAARD, J. I., ZHANG, L., SMITH, K. L., MOREL, C. R., WAGNER, R. A., GREAVES, D. R., MURRAY, P. J. & CHAWLA, A. 2006. Oxidative metabolism and PGC-1 β attenuate macrophage-mediated inflammation. *Cell Metab*, 4, 13-24.
- VIANA DA SILVA, S., HABERL, M. G., ZHANG, P., BETHGE, P., LEMOS, C., GONÇALVES, N., GORLEWICZ, A., MALEZIEUX, M., GONÇALVES, F. Q., GROSJEAN, N., BLANCHET, C., FRICK, A., NÄGERL, U. V., CUNHA, R. A. & MULLE, C. 2016. Early synaptic deficits in the APP/PS1 mouse model of Alzheimer's disease involve neuronal adenosine A(2A) receptors. *Nature Communications*, 7, 11915.
- VILHARDT, F. 2005. Microglia: phagocyte and glia cell. *The international journal of biochemistry & cell biology*, 37, 17-21.
- VODOVOTZ, Y., LUCIA, M. S., FLANDERS, K. C., CHESLER, L., XIE, Q. W., SMITH, T. W., WEIDNER, J., MUMFORD, R., WEBBER, R., NATHAN, C., ROBERTS, A. B., LIPPA, C. F. & SPORN, M. B. 1996. Inducible nitric oxide synthase in tangle-bearing neurons of patients with Alzheimer's disease. *J Exp Med*, 184, 1425-33.
- VOLOBOUEVA, L. A., EMERY, J. F., SUN, X. & GIFFARD, R. G. 2013. Inflammatory response of microglial BV-2 cells includes a glycolytic shift and is modulated by mitochondrial glucose-regulated protein 75/mortalin. *FEBS letters*, 587, 756-762.
- VON BERNHARDI, R., TICHAUER, J. E. & EUGENIN, J. 2010. Aging-dependent changes of microglial cells and their relevance for neurodegenerative disorders. *J Neurochem*, 112, 1099-114.
- WAICKMAN, A. T. & POWELL, J. D. 2012. mTOR, metabolism, and the regulation of T-cell differentiation and function. *Immunol Rev*, 249, 43-58.
- WANG, L., HARRINGTON, L., TREBICKA, E., SHI, H. N., KAGAN, J. C., HONG, C. C., LIN, H. Y., BABITT, J. L. & CHERAYIL, B. J. 2009. Selective modulation of TLR4-activated inflammatory responses by altered iron homeostasis in mice. *J Clin Invest*, 119, 3322-8.
- WANG, L., JOHNSON, E. E., SHI, H. N., WALKER, W. A., WESSLING-RESNICK, M. & CHERAYIL, B. J. 2008. Attenuated inflammatory responses in hemochromatosis reveal a role for iron in the regulation of macrophage cytokine translation. *J Immunol*, 181, 2723-31.
- WANG, Q., LIU, Y. & ZHOU, J. 2015. Neuroinflammation in Parkinson's disease and its potential as therapeutic target. *Translational Neurodegeneration*, 4, 19.
- WANG, T., MARQUARDT, C. & FOKER, J. 1976. Aerobic glycolysis during lymphocyte proliferation.
- WARBURG, O. 1924. Über den stoffwechsel der carcinomzelle. *Naturwissenschaften*, 12, 1131-1137.
- WARBURG, O. 1956. On the origin of cancer cells. *Science*, 123, 309-14.
- WEISS, G., WACHTER, H. & FUCHS, D. 1995. Linkage of cell-mediated immunity to iron metabolism. *Immunol Today*, 16, 495-500.

- WEISS, G., WERNER-FELMAYER, G., WERNER, E. R., GRUNEWALD, K., WACHTER, H. & HENTZE, M. W. 1994. Iron regulates nitric oxide synthase activity by controlling nuclear transcription. *J Exp Med*, 180, 969-76.
- WESTWOOD, A. J., BEISER, A., DECARLI, C., HARRIS, T. B., CHEN, T. C., HE, X. M., ROUBENOFF, R., PIKULA, A., AU, R., BRAVERMAN, L. E., WOLF, P. A., VASAN, R. S. & SESHADRI, S. 2014. Insulin-like growth factor-1 and risk of Alzheimer dementia and brain atrophy. *Neurology*, 82, 1613-9.
- WILCOCK, D. M., LEWIS, M. R., VAN NOSTRAND, W. E., DAVIS, J., PREVITI, M. L., GHARKHOLONAREHE, N., VITEK, M. P. & COLTON, C. A. 2008. Progression of amyloid pathology to Alzheimer's disease pathology in an amyloid precursor protein transgenic mouse model by removal of nitric oxide synthase 2. *J Neurosci*, 28, 1537-45.
- WILCOCK, D. M., ROJANI, A., ROSENTHAL, A., LEVKOWITZ, G., SUBBARAO, S., ALAMED, J., WILSON, D., WILSON, N., FREEMAN, M. J., GORDON, M. N. & MORGAN, D. 2004. Passive amyloid immunotherapy clears amyloid and transiently activates microglia in a transgenic mouse model of amyloid deposition. *J Neurosci*, 24, 6144-51.
- WINDER, W. W., HOLMES, B. F., RUBINK, D. S., JENSEN, E. B., CHEN, M. & HOLLOSZY, J. O. 2000. Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle. *J Appl Physiol* (1985), 88, 2219-26.
- WU, C. Y., KAUR, C., SIVAKUMAR, V., LU, J. & LING, E. A. 2009. Kv1.1 expression in microglia regulates production and release of proinflammatory cytokines, endothelins and nitric oxide. *Neuroscience*, 158, 1500-8.
- WU, N., ZHENG, B., SHAYWITZ, A., DAGON, Y., TOWER, C., BELLINGER, G., SHEN, C. H., WEN, J., ASARA, J., MCGRAW, T. E., KAHN, B. B. & CANTLEY, L. C. 2013. AMPK-dependent degradation of TXNIP upon energy stress leads to enhanced glucose uptake via GLUT1. *Mol Cell*, 49, 1167-75.
- WU, Z., PUIGSERVER, P., ANDERSSON, U., ZHANG, C., ADELMANT, G., MOOTHA, V., TROY, A., CINTI, S., LOWELL, B., SCARPULLA, R. C. & SPIEGELMAN, B. M. 1999. Mechanisms Controlling Mitochondrial Biogenesis and Respiration through the Thermogenic Coactivator PGC-1. *Cell*, 98, 115-124.
- WYNN, T. A. & BARRON, L. Macrophages: master regulators of inflammation and fibrosis. *Seminars in liver disease*, 2010. NIH Public Access, 245.
- WYNNE, A. M., HENRY, C. J., HUANG, Y., CLELAND, A. & GODBOUT, J. P. 2010. Protracted downregulation of CX3CR1 on microglia of aged mice after lipopolysaccharide challenge. *Brain Behav Immun*, 24, 1190-201.
- YALCIN, A., CLEM, B., IMBERT-FERNANDEZ, Y., OZCAN, S., PEKER, S., O'NEAL, J., KLARER, A., CLEM, A., TELANG, S. & CHESNEY, J. 2014. 6-Phosphofructo-2-kinase (PFKFB3) promotes cell cycle progression and suppresses apoptosis via Cdk1-mediated phosphorylation of p27. *Cell death & disease*, 5, e1337.
- ZHANG, B., GAITERI, C., BODEA, L. G., WANG, Z., MCELWEE, J., PODTELEZHNIKOV, A. A., ZHANG, C., XIE, T., TRAN, L., DOBRIN, R., FLUDER, E., CLURMAN, B., MELQUIST, S., NARAYANAN,

- M., SUVER, C., SHAH, H., MAHAJAN, M., GILLIS, T., MYSORE, J., MACDONALD, M. E., LAMB, J. R., BENNETT, D. A., MOLONY, C., STONE, D. J., GUDNASON, V., MYERS, A. J., SCHADT, E. E., NEUMANN, H., ZHU, J. & EMILSSON, V. 2013. Integrated systems approach identifies genetic nodes and networks in late-onset Alzheimer's disease. *Cell*, 153, 707-20.
- ZHANG, G. X., LI, J., VENTURA, E. & ROSTAMI, A. 2002. Parenchymal microglia of naïve adult C57BL/6J mice express high levels of B7.1, B7.2, and MHC class II. *Experimental and Molecular Pathology*, 73, 35-45.
- ZHANG, X. & MOSSER, D. 2008. Macrophage activation by endogenous danger signals. *The Journal of pathology*, 214, 161-178.
- ZHENG, Y., DELGOFFE, G. M., MEYER, C. F., CHAN, W. & POWELL, J. D. 2009. Anergic T cells are metabolically anergic. *J Immunol*, 183, 6095-101.
- ZOTOVA, E., BHARAMBE, V., CHEAVEAU, M., MORGAN, W., HOLMES, C., HARRIS, S., NEAL, J. W., LOVE, S., NICOLL, J. A. R. & BOCHE, D. 2013. Inflammatory components in human Alzheimer's disease and after active amyloid- β 42 immunization. *Brain*, 136, 2677-2696.

Appendix I

Poly-D-lysine coated coverslips: Poly-D-lysine (10mg; Sigma Aldrich, UK) was reconstituted to a stock solution (1mg/mL) in sterile H₂O and sterilised using a syringe filter (0.2µm). This stock solution was aliquoted and stored at -20°C. Poly-D-lysine was diluted to a working concentration (50ng/mL) in dH₂O. Glass coverslips (10mm diameter; VWR, UK) were sterilised by autoclaving and exposure to UV light. The following day, the coverslips were placed in a petri-dish containing poly-D-lysine (50ng/mL) and placed in an incubator for 1h at 37°C. Coverslips were carefully removed using a sterile forceps, rinsed with sterile dH₂O and air dried in a sterile fume hood. They were then placed on tissue paper in the flow hood to dry and coated coverslips were stored at 4°C for up to 2 weeks.

Macrophage colony-stimulating factor (M-CSF): Recombinant mouse M-CSF (50µg; *E. coli*-derived; R&D Systems, UK) was reconstituted to a stock solution (100µg/mL) in bovine serum albumin (BSA; 0.1%) in sterile PBS and stored at -20°C. Stock solution was diluted to a working concentration (100ng/mL) in pre-warmed cDMEM prior to use.

Granulocyte macrophage colony-stimulating factor (M-CSF): Recombinant mouse GM-CSF (50µg; *E. coli*-derived; R&D Systems, UK) was reconstituted to a stock solution (100µg/mL) in bovine serum albumin (BSA; 0.1%) in sterile PBS and stored at -20°C. Stock solution was diluted to a working concentration (100ng/mL) in pre-warmed cDMEM prior to use.

Interferon-γ (IFN-γ): Recombinant mouse IFN-γ (100µg; *E. coli*-derived; R&D Systems, UK) was reconstituted to a stock solution (100µg/mL) in bovine serum albumin (BSA; 0.1%) in sterile PBS and stored at -20°C. Stock solution was diluted to a working concentration (50ng/mL) in pre-warmed cDMEM prior to use.

Interleukin-4 (IL-4): Recombinant mouse IL-4 (100µg; *E. coli*-derived; R&D Systems, UK) was reconstituted to a stock solution (100µg/mL) in BSA (0.1%) in sterile PBS and stored at -20°C. Stock solution was diluted to a working concentration (20ng/mL) in pre-warmed cDMEM prior to use.

HiLyte Fluor® 488-labelled Aβ₁₋₄₂: Lyophilised peptide (0.1mg) was dissolved in ammonium hydroxide (NH₄OH; 1%) and diluted to a stock solution (0.25µg/mL) using PBS. In order to prepare more aggregated species, stock solutions were incubated for 24h at 37°C with continuous shaking (220rpm). Stock solutions were stored at -20°C and diluted to a working concentration (0.5µM) in pre-warmed cDMEM prior to use.

Solutions

Phosphate buffered saline (PBS):

PBS tablets (Sigma Aldrich, UK): 5

dH₂O: 1L

Complete Roswell park memorial institute medium (cRMPI):

RPMI: 500mL

Foetal bovine serum (FBS): 50mL

Penicillin-streptomycin: 5mL

B-mercaptoethanol: 50µM

L-glutamine 2.5mM

Complete Dulbecco's modified Eagle's medium (cDMEM):

DMEM: 500mL

FBS: 50mL

Penicillin-streptomycin: 5mL

cDMEM was filter sterilised and stored at 4°C for up to 2 weeks

Medium A:

HBSS: 500mL

45% D-(+)-glucose solution: 6.5mL

1M HEPES: 7.5mL

RBC lysis buffer:

NH₄Cl: 8.3g

KHCO₃: 1g

EDTA: 0.037g

dH₂O: 1L

Filter sterilised

Freezing buffer:

FBS: 45mL

Dimethyl sulfoxide: 5mL

Neuronal dissection solution:

HBSS: 9.8mL

100mM sodium pyruvate solution: 100μL

1M HEPES pH 7.4: 100μL

Neuronal plating media:

Neurobasal media: 100mL

Penicillin-streptomycin: 1mL

200mM L-glutamine solution: 250μL

B27 + antioxidants (2mL)

Neuronal replenishing media:

Neurobasal media: 100mL

200mM L-glutamine solution:: 250µL

B27 without antioxidants: 2mL

Papain inhibiting solution:

Plating media: 20mL

DNase: 400µL

Papain solution:

Neurobasal media: 8.5mL

Papain (approx. 15U/mL): 1.5mL

MACS buffer:

Sterile PBS: 200mL

BSA: 1g

EDTA: 2mM

Filter sterilised

FACS buffer:

PBS: 500mL

FBS: 10mL (2%)

NaN₃: 0.5g (0.1%)

4% paraformaldehyde (PFA):

PFA: 20g

dH₂O: 250mL

Dissolved for 1h at @60°C

NaOH: 1 drop

2X PBS: 250mL

The solution was brought to a pH of 7.4 and filter sterilised

PBT:

PBS: 500mL

Triton-X: 500µL

Assay media for glycolytic flux test:

XF base medium: 50mL

200mM L-glutamine solution: 500µL (2mM)

pH to 7.35 and filter sterilise

Assay media for mitochondrial stress test:

XF assay medium: 50mL

200mM L-glutamine solution: 500µL (2mM)

100mM sodium pyruvate solution: 500µL

Glucose: 0.09g

pH to 7.4 and filter sterilise

ELISA wash buffer:

PBS: 1L

Tween: 500 μ L

Lysis buffer:

Tris HCl: 0.394g

NaCl: 0.438g

NP-40 (IGEPAL): 500 μ L

dH₂O: 50mL

Phosphatase inhibitor: 1% (Sigma # P5726, #P0044)

Protease inhibitor: 1% (Sigma # P8340)

4X sample buffer for SDS-PAGE:

1M Tris HCl pH6.8: 1.25mL

Glycerol: 5mL

25% SDS: 1.6mL

dH₂O: 1.15mL

β -mercaptoethanol: 1mL

Bromophenol blue: a few granules

SPA buffer for SDS-PAGE:

Tris base: 90.8g

dH₂O: 450mL

pH to 8.8

SDS: 1g

7% polyacrylamide gel for SDS-PAGE:

30% bis/acrylamide solution: 2.3mL

dH₂O: 5.1mL

SPA buffer: 2.5mL

10% APS: 100μL

TEMED: 50μL

10% polyacrylamide gel for SDS-PAGE:

30% bis/acrylamide solution: 3.3mL

dH₂O: 4.1mL

SPA buffer: 2.5mL

10% APS: 100μL

TEMED: 50μL

12% polyacrylamide gel for SDS-PAGE:

30% bis/acrylamide solution: 4mL

dH₂O: 3.4mL

SPA buffer: 2.5mL

10% APS: 100μL

TEMED: 50μL

15% polyacrylamide gel for SDS-PAGE:

30% bis/acrylamide solution: 5mL

dH₂O: 2.4mL

SPA buffer: 2.5mL

10% APS: 100μL

TEMED: 50μL

Stacking buffer for SDS-PAGE:

Tris base: 15.13g

dH₂O: 200mL

pH to 6.8

SDS: 1g

4% polyacrylamide gel for SDS-PAGE:

30% bis/acrylamide solution: 1.3mL

dH₂O: 6.1mL

Stacking buffer: 2.5mL

10% ammonium persulphate (APS): 50μL

TEMED: 10μL

10X electrode running buffer for SDS-PAGE:

Tris base: 30g

Glycine: 144g

SDS: 10g

10X western transfer buffer for SDS-PAGE:

Tris base: 30g

Glycine: 144g

dH₂O: 1L

20% methanol added when making 1X transfer buffer

10X TBS:

Tris HCl, pH 7.5: 31.52g

NaCl: 87.66g

dH₂O 1L

TBS-T:

TBS: 1L

Tween: 500μL