Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from their thesis.

To request permissions please use the Feedback form on our webpage.
http://researchspace.auckland.ac.nz/feedback

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the Library Thesis Consent Form and Deposit Licence.
Adult Human Microglia

Phenotypic Diversity and Functional Complexity

Amy Megan Smith

Abstract

The immune system continuously monitors and protects the central nervous system (CNS) throughout health and disease. Microglia are the resident immune cells of the CNS. Present throughout the brain parenchyma and in constant communication with the peripheral immune system, microglia are essential regulators of brain physiology. The adult human brain is prone to immune dysregulation in neurodegeneration and normal ageing. Understanding the roles and mechanisms of microglia in the adult human brain will aid the understanding of normal and degenerative brain processes.

Despite increasing evidence of species differences, the majority of microglial studies are performed using rodent cells and few studies have addressed the questions of microglial function and regulation in the context of the adult human brain. This thesis explores the phenotypic diversity of microglia from adult human brain tissue – the primary microglia which are themselves part of neurological diseases. This study makes use of the unique resources of the Neurological Foundation of New Zealand Human Brain Bank and the Centre for Brain Research Biobank. Microglia were isolated from biopsy and post-mortem human adult brain tissue and grown in mixed glial cultures with astrocytes and brain derived fibroblast-like cells.

The transcription factor PU.1 is important for myeloid cell development and function, but has not previously been investigated in adult human microglia. In this study PU.1 has been identified for the first time in adult human brain tissue and microglia, and demonstrated to be critical for microglial survival and phagocytosis. Short interfering RNA (siRNA) proved to be a highly effective and useful tool for investigating primary human microglial function. Microglial phenotype is highly dynamic and changes according to their external environment. Macrophage Colony-Stimulating Factor (M-CSF) promoted a distinct microglial phenotype with increased PU.1 expression, and induced microglia to proliferate and undergo phagocytosis. Conversely, interferon-γ (IFNy) induced an activated microglial phenotype with increased Human Leukocyte Antigen (HLA) expression and secretion of pro-inflammatory cytokines and chemokines. Despite Transforming Growth Factor-β1 being reported to inhibit IFNy-induced HLA expression in rodent microglia, this response was completely absent in human microglia. Instead this mechanism was active in brain derived fibroblast-like cells. As regulators of the brain microenvironment, microglia affect many physiological processes including proliferation and fate determination of neural progenitor cells (NPCs). Microglia in the constitutively neurogenic regions of the adult human brain were found to have greater proliferative potential than microglia from cortical non-neurogenic regions. Interestingly, neurogenic region microglia showed
enhanced responses to M-CSF. NPCs were also isolated from the neurogenic regions of adult human brain tissue and the immune cytokine IFNγ was found to direct differentiation of NPCs towards an astrocytic fate.

Immune cells and molecules are essential for CNS function and the study of adult human microglia provides a unique insight into the workings of these vital cells. Targeting microglia to limit detrimental neuroinflammation, for example using siRNA directed against PU.1 or by using M-CSF molecule mimics, is a potential therapy for future treatment of neurological diseases.
Acknowledgements

Firstly I would like to thank my supervisor Professor Mike Dragunow for his enthusiastic support and approachability during my PhD studies. Thank you for letting me find my own way while giving me valuable advice and fostering my interest in science.

To my co-supervisor Dr Hannah Gibbons: thank you for instructing me so thoroughly in the lab and being such a good mentor whose high research standards were such a fine example. I am very grateful for your continued support and involvement in my PhD.

Thank you to Dr Thomas Park for passing on your neurogenesis knowledge, for scientific discussions and ‘friendly competition’.

To my co-supervisor, the wonderful director of the Centre for Brain Research, Professor Richard Faull: thank you for all your encouragement.

I am grateful too for the fabulous members of the Dragunow lab who have made my PhD such an enjoyable journey: Hannah, Tom, Pritika, Kerhan, Claire, Miranda, Hector, Natacha, Deidre, Sheryl, Inna, Casey, Imogene and Justin. Many thanks for your willing support and advice, for sharing your expertise, for analysing and proof-reading my work and, most importantly, for providing the perfect mix of squash, coffee, laughter and Archie’s lunches.

I have really appreciated the companionship of my fellow CBR PhD students Janitha, Jerusha, Amelia, Chantelle and Toby.

I extend my sincere gratitude to the donors, and their families, for the brain tissue provided for this research, as well as to the clinicians who helped co-ordinate this process. I would also like to acknowledge the University of Auckland, the Tertiary Education Commission and the National Research Centre for Growth and Development for financial support.

Thank you Mom and Dad for supporting me every step of the way, and for valiantly proof-reading my work from phagocytes to cytokines. Thanks to my uncle, Jonathan, for his support and humour-filled encouragement. Thank you Byron for not hassling your older sister too much for still being a student.

Finally, David, thank you for always being there for me.
# Table of Contents

Abstract ................................................................................................................................................... i
Table of Contents .................................................................................................................................. vi
List of Figures ...................................................................................................................................... xiv
List of Tables ...................................................................................................................................... xviii

Chapter 1 .............................................................................................................................................. 1

General Introduction .......................................................................................................................... 1

1.1. Microglia ...................................................................................................................................... 1

1.2. The CNS and the immune system ............................................................................................... 2

1.3. Functions of microglia ................................................................................................................ 3
  1.3.1. Phagocytosis ......................................................................................................................... 3
  1.3.2. Cytokine secretion ................................................................................................................ 4
  1.3.3. Migration .............................................................................................................................. 5
  1.3.4. Antigen presentation ............................................................................................................ 6
  1.3.5. Morphology .......................................................................................................................... 6

1.4. Microglia in health ...................................................................................................................... 7
  1.4.1. Renewal of the microglial population ................................................................................... 7

1.5. Microglia in disease .................................................................................................................... 8
  1.5.1. Anti-inflammatory neuroprotective mechanisms of microglia .......................................... 10
  1.5.2. The immune system as a therapeutic target ...................................................................... 11

1.6. External factors affecting microglial phenotype ....................................................................... 12
  1.6.1. Soluble molecules ............................................................................................................... 12
  1.6.2. Regulation by cell surface proteins ..................................................................................... 12
  1.6.3. Age ...................................................................................................................................... 13
  1.6.4. Brain region ........................................................................................................................ 14
  1.6.5. Species ................................................................................................................................ 14

1.7. Intrinsic microglial control mechanisms .................................................................................... 15
  1.7.1. Transcription factors ........................................................................................................... 15
  1.7.2. Epigenetics .......................................................................................................................... 16

1.8. *In vitro* models of microglia .................................................................................................... 17

1.9. Astrocytes .................................................................................................................................. 17
1.10. Brain derived fibroblast-like cells ................................................................. 18
1.11. The cells that got away .................................................................................. 20
1.12. Neurogenesis ................................................................................................. 21
   1.12.1. In vitro culture of NPCs ........................................................................... 22
   1.12.2. Microglia influence neurogenesis ............................................................. 22
1.13. Thesis outline ................................................................................................. 24
Chapter 2 .................................................................................................................. 26
General Methods ...................................................................................................... 26
   2.1. Adult human brain tissue .............................................................................. 26
   2.2. Cell culture .................................................................................................... 28
      2.2.1. Mixed glial cell cultures ........................................................................... 28
      2.2.2. Fibroblast-like cell only cultures ............................................................. 28
      2.2.3. Isolation of neural progenitor cells from adult human brain tissue ......... 29
      2.2.4. Leptomeningeal explant cultures ............................................................ 30
      2.2.5. Differentiation of NT2 precursor cells into NT2Ns .................................. 30
   2.3. Transfection .................................................................................................. 31
      2.3.1. siRNA transfection ................................................................................ 31
      2.3.2. DNA oligonucleotide transfection .......................................................... 31
   2.4. AlamarBlue cell viability assay ...................................................................... 31
   2.5. xCELLigence cell viability assay ................................................................... 32
   2.6. BrdU proliferation assay ............................................................................... 32
   2.7. Phagocytosis assay ....................................................................................... 32
   2.8. Scratch injury assay for assessing migration and scar formation ............... 33
   2.9. Cytokine and growth factor treatment ......................................................... 33
   2.10. Immunochemistry ....................................................................................... 33
      2.10.1. Immunocytochemistry .......................................................................... 33
      2.10.2. Live cell antibody labelling ................................................................... 34
      2.10.3. Immunohistochemistry ....................................................................... 34
      2.10.4. Antigen retrieval .................................................................................. 35
      2.10.5. Hoechst ............................................................................................... 35
      2.10.6. Imaging ............................................................................................... 36
   2.11. Western blot ............................................................................................... 36
      2.11.1. Cell protein lysates .............................................................................. 36
      2.11.2. Tissue protein lysates .......................................................................... 36
The Transcription Factor PU.1 is Critical for Viability and Function of Human Brain Microglia

3.1. Abstract

3.2. Introduction

3.3. Methods

3.3.1. Tissue

3.3.2. Human glial cell isolation and culture

3.3.3. siRNA transfection

3.3.4. DNA oligonucleotide transfection

3.3.5. Immunochemistry

3.3.6. Western blot

3.3.7. Reverse transcription and quantitative real time polymerase chain reaction

3.3.8. AlamarBlue cell viability assay

3.3.9. xCELLigence System cell viability measurement

3.3.10. Phagocytosis assay

3.3.11. Quantitative image analysis of cell number and phagocytosis

3.3.12. Cytokine measurement

3.3.13. Statistical analysis

3.4. Results

3.4.1. PU.1 is a marker of cultured adult human primary microglia

3.4.2. PU.1 is a marker of microglia in the adult human brain

3.4.3. PU.1 mRNA is expressed in the adult human brain

3.4.4. Reduction of in vitro PU.1 protein expression by siRNA

3.4.5. Not all siRNAs are created equal

3.4.6. PU.1 siRNA reduces microglial viability

3.4.7. PU.1 siRNA reduces microglial phagocytosis of Aβ peptide

3.4.8. PU.1 siRNA increases microglial release of IL-6

3.4.9. siRNA as a general method to investigate protein function in human adult microglia
3.5. Discussion ................................................................................................................................. 68
  3.5.1. PU.1 in the adult human brain ............................................................................................ 68
  3.5.2. Reduction of in vitro PU.1 protein expression by siRNA ................................................... 69
  3.5.3. PU.1 siRNA reduces microglial viability ............................................................................. 70
  3.5.4. PU.1 siRNA reduces phagocytosis of Aβ42 peptide .......................................................... 71
  3.5.5. PU.1 siRNA increases microglial release of IL-6 ............................................................. 72

Chapter 4 ........................................................................................................................................... 75
M-CSF is a Major Determinant of Adult Human Microglial Phenotype and Function ............ 75
  4.1. Abstract ................................................................................................................................... 75
  4.2. Introduction ............................................................................................................................ 76
  4.3. Methods ................................................................................................................................. 78
    4.3.1. Adult human brain tissue and glial cell isolation ............................................................ 78
    4.3.2. M-CSF treatment ............................................................................................................. 78
    4.3.3. BrdU proliferation assay ................................................................................................... 78
    4.3.4. Immunocytochemistry ..................................................................................................... 78
    4.3.5. Immunohistochemistry ................................................................................................... 78
    4.3.6. Western blot .................................................................................................................... 80
    4.3.7. Migration assay ............................................................................................................... 80
    4.3.8. Phagocytosis assay ......................................................................................................... 80
    4.3.9. Quantitative image analysis of cell number, microglial morphology and phagocytosis ... 80
    4.3.10. Statistical analysis ......................................................................................................... 80
  4.4. Results .................................................................................................................................... 81
    4.4.1. Adult human microglia express the M-CSF receptor in vitro .......................................... 81
    4.4.2. M-CSF increases expression of microglial transcription factor PU.1 ............................... 82
    4.4.3. M-CSF increases proliferation of adult human microglia ............................................... 83
    4.4.4. M-CSF increases adult microglial phagocytosis ............................................................. 84
    4.4.5. M-CSF induces a change in microglial morphology ....................................................... 85
    4.4.6. M-CSF decreases HLA expression ................................................................................. 86
    4.4.7. M-CSF increases microglial expression of C/EBPβ transcription factor and DAP12 adaptor protein ................................................................................................................. 87
    4.4.8. M-CSF increases microglial expression of M-CSF and IGF-1 receptors .......................... 89
    4.4.9. M-CSF increases microglial migration in a scratch wound assay .................................... 91
    4.4.10. The role of PU.1 in M-CSF-mediated microglia actions ................................................ 92
    4.4.11. The influence of other molecules on M-CSF microglial effects ..................................... 92
4.5. Discussion ......................................................................................................................... 93
4.5.1. M-CSF increases proliferation of adult human microglia ................................................... 93
4.5.2. M-CSF increases expression of PU.1 in adult human microglia ........................................... 93
4.5.3. M-CSF increases adult human microglial phagocytosis ...................................................... 94
4.5.4. M-CSF induces a change in microglial morphology ............................................................ 94
4.5.5. M-CSF decreases HLA expression ....................................................................................... 95
4.5.6. Increased expression of C/EBPβ and DAP12 in M-CSF-treated adult human microglia .... 96
4.5.7. CSF-1R and IGF1-R expression are increased in M-CSF-treated adult human microglia .... 97
4.5.8. M-CSF increases microglial migration in a scratch wound assay ........................................ 98
4.5.9. The role of PU.1 in M-CSF-mediated microglia actions ...................................................... 99
4.5.10. The influence of other small molecules on M-CSF microglial effects ............................... 99

Chapter 5 ................................................................................................................................. 102

Differential Regulation of HLA and IP-10 in Adult Human Glia ................................................. 102

5.1. Abstract ............................................................................................................................ 102
5.2. Introduction ....................................................................................................................... 103
5.3. Methods ............................................................................................................................. 107
5.3.1. Human glial cell isolation and culture ............................................................................... 107
5.3.2. Leptomeningeal explant cultures ..................................................................................... 107
5.3.3. Cytokine treatment ........................................................................................................... 107
5.3.4. Immunocytochemistry ...................................................................................................... 107
5.3.5. Quantitative cytokine and chemokine measurement ...................................................... 108
5.3.6. Western blotting of IP-10 in conditioned media .............................................................. 109
5.3.7. Scratch injury assay to assess scar formation ................................................................... 109
5.3.8. Quantitative image analysis of cell number, protein expression and microglial morphology ........................................................................................................................................... 109
5.3.9. Statistical analysis ............................................................................................................. 109

5.4. Results ................................................................................................................................ 110
5.4.1. Microglial expression of HLA-DP, DQ, DR is increased by IFNγ and reduced by M-CSF but not by TGFβ1 ........................................................................................................................ 110
5.4.2. Microglial cell number is increased by IFNγ and M-CSF, but reduced by TGFβ1 .......... 111
5.4.3. IFNγ treatment results in microglia with a more rounded morphology .......................... 113
5.4.4. Astroglial expression of HLA-DP, DQ, DR is increased by IFNγ but not affected by TGFβ1 or M-CSF ........................................................................................................................................ 114
5.4.5. IFNγ induces HLA-DP, DQ, DR expression in brain-derived fibroblast-like cells ....... 116
5.4.6. IFNy-induced fibroblast-like cell HLA-DP, DQ, DR expression is inhibited by TGFβ1 but not by M-CSF .................................................................117
5.4.7. IFNy also induces leptomeningeal fibroblast-like cells to express HLA-DP, DQ, DR.......119
5.4.8. IFNy treatment of primary adult human mixed glia results in increased pro-inflammatory cytokine and chemokine release.........................................................121
5.4.9. TGFβ1 reduces closure of a gap wound area by human adult glia ..............................125

5.5. Discussion ......................................................................................................................127
5.5.1. Regulation of microglial expression of HLA-DP, DQ, DR ...........................................127
5.5.2. Microglial cell number is increased by IFNy and M-CSF, but reduced by TGFβ1 ..........128
5.5.3. IFNy treatment results in microglia with a more rounded morphology .................128
5.5.4. Regulation of astrocytic HLA-DP, DQ, DR expression ................................................129
5.5.5. Regulation of fibroblast-like cell expression of HLA-DP, DQ, DR .............................130
5.5.6. IFNy treatment of primary adult human mixed glia results in increased pro-inflammatory cytokine and chemokine release.................................131
5.5.7. TGFβ1 reduces closure of a gap wound area by human adult mixed glia ..................134

Chapter 6 ................................................................................................................................136
6.1. Abstract .........................................................................................................................136
6.2. Introduction ....................................................................................................................137
6.3. Methods .........................................................................................................................140
6.3.1. Tissue ..........................................................................................................................140
6.3.2. Human glial cell isolation and culture .......................................................................140
6.3.3. Cytokine treatment ....................................................................................................140
6.3.4. BrdU proliferation assay ............................................................................................140
6.3.5. Immunocytochemistry ..............................................................................................140
6.3.6. Quantitative image analysis of cell number, protein expression and microglial morphology ..................................................................................141
6.3.7. Statistical analysis .....................................................................................................141
6.4. Results ..........................................................................................................................142
6.4.1. Differential proliferation of microglia from ventricular/Hp and cortical regions .........142
6.4.2. Ventricular/Hp microglia have a greater proliferation response to M-CSF than cortical microglia ..............................................................144
6.4.3. Similar expression of PU.1 transcription factor and CD45 cell surface receptor in ventricular/Hp and cortical microglia .........................................................146
6.4.4. Ventricular/Hp microglia express more CSF-1R than cortical microglia .................147
6.4.5. DAP12 adaptor protein, C/EBPβ transcription factor, and IGF-1 receptor are more highly expressed by microglia from ventricular/Hp regions ................................................................. 148
6.4.6. Ventricular/Hp microglia express higher levels of HLA-DP, DQ, DR protein ............... 150
6.4.7. Ventricular/Hp microglia have a more rounded ‘amoeboid’ morphology than cortical microglia ..................................................................................................................................... 152

6.5. Discussion ................................................................................................................................ 153
6.5.1. Microglia from neurogenic regions have greater basal proliferation than cortical microglia .................................................................................................................................................... 153
6.5.2. Microglia from neurogenic regions have heightened responses to M-CSF ...................... 153
6.5.3. Microglia from neurogenic regions express higher levels of HLA and have rounder morphology ................................................................................................................................. 155
6.5.4. Possible functional significance of increased ventricular/Hp microglial proliferation .... 156

Chapter 7 .......................................................................................................................................... 158
The Influence of Microglia and Immune Molecules on Adult Human Neurogenesis ...... 158
7.1. Abstract ................................................................................................................................... 158
7.2. Introduction ............................................................................................................................. 159
7.3. Methods .................................................................................................................................. 162
7.3.1. Differentiation of NT2 precursor cells into NT2Ns ........................................................... 162
7.3.2. Isolation of NPCs from adult human brain tissue ............................................................. 162
7.3.3. Culture and proliferation of ahNPCs ................................................................................. 162
7.3.4. Differentiation of ahNPCs into neurons and astrocytes ................................................... 163
7.3.5. Immunohistochemistry ........................................................................................................ 164
7.3.6. Quantitative image analysis .............................................................................................. 165
7.3.7. Statistical analysis ............................................................................................................. 165
7.4. Results ..................................................................................................................................... 166
7.4.1. The NT2 cell model of neurogenesis ................................................................................. 166
7.4.2. Characterisation of adult human neural progenitor cells .............................................. 171
7.4.3. Differentiation of ahNPCs into neurons and astrocytes ................................................... 172
7.4.4. Differentiation of ahNPCs in mixed glial cell conditioned media ..................................... 173
7.4.5. Effects of microglia-specific conditioned media on ahNPC differentiation .................... 176
7.4.6. IFNγ increases astrocytic differentiation of ahNPCs ......................................................... 178
7.5. Discussion ................................................................................................................................ 180
7.5.1. The NT2 cell model of neurogenesis ................................................................................. 180
7.5.2. Adult human neural progenitor cells as a model of neurogenesis ................................. 181
7.5.3. No effect of glial conditioned media was observed on ahNPC differentiation ........... 181
7.5.4. IFNγ increases astrocytic differentiation of ahNPCs .......................................................... 182

Chapter 8 ......................................................................................................................................... 185

General Discussion .......................................................................................................................... 185

8.1. Summary of major findings ....................................................................................................... 186
8.2. Microglia: species differences matter ...................................................................................... 188
8.3. Limitations of primary adult human mixed glial cultures ....................................................... 189
8.4. The potential of fibroblast-like cells ......................................................................................... 192
8.5. Future directions ....................................................................................................................... 192
8.6. Conclusions .............................................................................................................................. 193

References ....................................................................................................................................... 196
# List of Figures

Figure 2.1: Regions of the adult human brain from which glia and neural progenitor cells were cultured................................................................. 27

Figure 3.1: Primary human microglia express PU.1 in vitro. ................................................................. 50
Figure 3.2: All CD68, DAP12, CX3CR1 and Iba-1 immunoreactive adult human microglia co-express PU.1 in vitro. ...................................................................................................................... 51
Figure 3.3: GFAP-positive astrocytes in human adult mixed glial cultures do not express PU.1. .......... 52
Figure 3.4: PU.1 protein is present in biopsy adult human brain tissue and is co-expressed with other microglial markers. ................................................................. 53
Figure 3.5: Immunohistochemical staining of PU.1 in biopsy and autopsy human brain tissue is not affected by acidic or basic pre-treatment. ................................................................. 54
Figure 3.6: PU.1 mRNA is present in adult human brain tissue................................................................. 55
Figure 3.7: Adult human microglia can be transfected with PU.1 siRNA to reduce PU.1 protein expression......................................................................................... 56
Figure 3.8: Adult human microglia in mixed glial cultures are transfectable with siRNA. ...................... 57
Figure 3.9: PU.1 siRNA transfection of adult human microglia specifically reduces PU.1 protein expression but does not affect CREB or ATF2 transcription factor expression................................................................. 57
Figure 3.10: PU.1 siRNA purchased from Santa Cruz biotechnology company reduced viability of mixed glial cultures................................................................. 58
Figure 3.11: PU.1 siRNA purchased from Santa Cruz, but not from Sigma, biotechnology company non-specifically reduced cell number compared to control siRNA................................................................. 59
Figure 3.12: Reduced PU.1 protein expression decreases adult human microglial viability............... 60
Figure 3.13: Astrocytes and fibroblast-like cells in adult human mixed glial cultures are not affected by PU.1 siRNA. ................................................................................................. 61
Figure 3.14: Microglial DAP12 and CX3CR1 expression are reduced by PU.1 siRNA......................... 62
Figure 3.15: Transfection of adult human mixed glial cultures with PU.1 antisense DNA oligonucleotides results in reduced PU.1 and CD45 immunoreactive microglia. ......................... 63
Figure 3.16: Transfection of adult human mixed glial cultures with PU.1 siRNA produces a concentration response in total cell number and in PU.1 expression............................................. 64
Figure 3.17: PU.1 siRNA reduces phagocytosis of amyloid-β_{1-42} (Aβ_{42}) by human microglia. .......... 65
Figure 3.18: PU.1 siRNA increased microglial secretion of IL-6 but not IP-10........................................ 66
Figure 3.19: CREB siRNA reduces CREB expression in mixed glial cultures........................................ 67
Figure 4.1: The M-CSF receptor (CSF-1R) is expressed by adult human microglia in vitro. .................. 81
Figure 4.2: M-CSF treatment increases the number of microglia and expression of PU.1 in primary adult human mixed glial cultures. ........................................................................................................... 82
Figure 4.3: M-CSF increases the division of adult human microglia. .................................................. 83
Figure 4.4: M-CSF increases microglial phagocytosis of amyloid-β$_{1-42}$. ................................... 84
Figure 4.5: M-CSF-treated microglia assume a rod-like shape. ....................................................... 85
Figure 4.6: Microglial expression of HLA-DR, DQ, DR is reduced by M-CSF. ............................... 86
Figure 4.7: M-CSF increases adult human microglial expression of C/EBPβ. ................................. 87
Figure 4.8: M-CSF increases adult human microglial expression of DAP12. ..................................... 88
Figure 4.9: DAP12 and CSF-1R proteins are expressed by microglia in adult human brain tissue. .... 89
Figure 4.10: M-CSF increases adult human microglial expression of CSF-1R and IGF-1R. ............... 90
Figure 4.11: M-CSF increases migration of microglia into a scratch wound ‘gap’. ......................... 91

Figure 5.1: Microglial expression of HLA-DR, DQ, DR is increased by IFNγ, not changed by TGFβ$_1$, and reduced by M-CSF. .......................................................................................................................... 112
Figure 5.2: IFNγ produces a change in microglia morphology to a more rounded, less elongated form. .................................................................................................................................................. 113
Figure 5.3: Astrocytic expression of HLA-DR, DQ, DR is increased by IFNγ, and not changed by TGFβ$_1$ or M-CSF. ................................................................................................................. 115
Figure 5.4: Brain-derived fibroblast-like cells do not express HLA-DR, DQ, DR protein under basal conditions but it is induced by IFNγ in a concentration-dependent manner. ............................. 116
Figure 5.5: αSMA-positive fibroblast-like cells do not express high levels of HLA-DR, DQ, DR. ......... 117
Figure 5.6: IFNγ-induced expression of HLA-DR, DQ, DR in brain-derived fibroblast-like cells is inhibited by TGFβ$_1$ but not by M-CSF. ........................................................................................................ 118
Figure 5.7: TGFβ$_1$ reduces proliferation of fibroblast-like cells. .................................................... 119
Figure 5.8: IFNγ-induced expression of HLA-DR, DQ, DR in leptomeningeal fibroblast-like cells is completely blocked by TGFβ$_1$. .......................................................................................... 120
Figure 5.9: IFNγ increases pro-inflammatory cytokine and chemokine release from adult human mixed glial cultures and brain-derived fibroblast-like cells. ...................................................... 120
Figure 5.10: IFNγ induces IP-10 release from adult human mixed glial cultures and brain-derived fibroblast-like cells. .................................................................................................................... 122
Figure 5.11: IFNγ induces IP-10 expression in microglia, astrocytes and fibroblast-like cells in primary adult human mixed glial cultures. ...................................................................................... 123
Figure 5.12: IFNγ induces IP-10 expression in fibroblast-like cells and is not affected by TGFβ1 or M-CSF..............................................................124
Figure 5.13: IP-10 receptor CXCR3 is expressed by all cells in adult human mixed glial cultures......124
Figure 5.14: Closure of a scratch wound injury by microglia and fibroblast-like cells is inhibited by TGFβ1..................................................................................................................126
Figure 5.15: TGFβ1 alters fibronectin expression in fibroblast-like cells. .........................................126
Figure 5.16: Differential regulation of HLA and IP-10 in adult human microglia, astrocytes and fibroblast-like cells by IFNγ, TGFβ1 and M-CSF........................................................................133

Figure 6.1: Microglia from neurogenic ventricular/Hp tissue proliferate more than microglia from non-neurogenic cortical tissue. .................................................................143
Figure 6.2: M-CSF has a greater effect on ventricular/Hp than cortical microglial proliferation......145
Figure 6.3: M-CSF treatment increases the number of microglia and expression of PU.1 in primary adult human mixed glial cultures from cortical and ventricular/Hp regions......................146
Figure 6.4: CSF-1R is expressed at higher levels by neurogenic region microglia compared to cortical microglia. ........................................................................................................147
Figure 6.5: DAP12 is more highly expressed in ventricular/Hp than cortical microglia. ...................148
Figure 6.6: M-CSF increases adult human microglial expression of C/EBPβ. .................................149
Figure 6.7: IGF-1R is expressed more in ventricular/Hp microglia and is increased by M-CSF treatment......................................................................................................................150
Figure 6.8: Microglia from the ventricular/Hp region express greater levels of HLA-DP, DQ, DR than cortical microglia.................................................................151
Figure 6.9: Microglia from the cortex are more elongated and less rounded than ventricular/Hp microglia. ...............................................................................................................152

Figure 7.1: The NT2 human teratocarcinoma cell line has stem cell-like characteristics..............166
Figure 7.2: Neuronal differentiation of NT2 cells is increased with time in 96-well plates following 2 weeks RA treatment. .................................................................167
Figure 7.3: NT2N expression of neuronal markers βIII-tubulin, MAP2 and GAP-43 is increased by longer RA treatment times .................................................................168
Figure 7.4: NT2Ns derived from a shortened differentiation protocol express neuronal proteins PSA-NCAM and synaptophysin.................................................................169
Figure 7.5: NT2Ns derived from a shortened differentiation protocol are electrophysiologically active.................................................................170
Figure 7.6: ahNPCs form neurospheres and monolayers in proliferative culture media. ...............171
Figure 7.7: ahNPCs express progenitor cell markers. .................................................................172
Figure 7.8: ahNPCs differentiate into neurons and astrocytes......................................................173
Figure 7.9: Microglia are in close proximity to NPCs in the adult human SVZ.............................173
Figure 7.10: Adult human glial conditioned media does not influence ahNPC differentiation.......175
Figure 7.11: Factors produced by microglia were not identified as having an effect on ahNPC differentiation............................................................................................................177
Figure 7.12: Astrocytic differentiation of ahNPCs is increased by IFNy.......................................178
Figure 7.13: IFNy induces HLA expression in ahNPC differentiation cultures and slightly reduces their viability. ..................................................................................................................179
List of Tables

Table 3.1: siRNAs used for knock-down of protein expression. ........................................................... 45
Table 3.2: Antibodies used for immunochemistry. .............................................................................. 46
Table 4.1: Antibodies used for immunochemistry. .............................................................................. 79
Table 5.1: Antibodies used for immunocytochemistry................................................................. 108
Table 5.2: Levels of HLA protein expression differ between biopsy cases................................. 110
Table 6.1: Antibodies used for immunocytochemistry................................................................. 141
Table 7.1: Antibodies used for immunochemistry. ............................................................................ 164
Chapter 1.

General Introduction

1.1. Microglia

It was previously thought that the brain had no immune activity and that the immune system had no role to play in the brain. Now it is appreciated that the immune system plays a significant role in the central nervous system (CNS) and the study of how the immune system affects brain processes is one of the largest growing areas of neuroscience.

Microglia are the brain-resident counterpart of peripheral macrophages. They survey the brain microenvironment and act as stabilisers of the CNS (Hanisch and Kettenmann 2007). Microglia are myeloid cells which are derived from the mesenchyme, infiltrate the brain during development (Cuadros and Navascues 1998), and are present in the brain throughout life. Microglia cells produce an immune network all through the brain. Each microglia surveys its surroundings and monitors its microenvironment with extended processes (Hanisch and Kettenmann 2007). Microglia respond to disturbances in homeostasis, such as changes in ion levels and accumulation of proteins, and they act to restore balance by clearing cell debris and protein accumulations (Hanisch and Kettenmann 2007).

Microglia show astonishing phenotypic diversity (Olah et al. 2011; Saijo and Glass 2011). The functional phenotype of microglia depends on their surrounding microenvironment and microglia are said to have an acquired phenotype, reflecting their response to a collection of external signals (Butovsky et al. 2005; Schwartz et al. 2006). One of their core functions is to integrate many simultaneous messages and respond appropriately to a combination of signals. Microglia respond gradually to changes in their environment, but in diseased brains and models we often see the total end result of microglial activation without understanding the processes that have occurred to get to this point. As microglia play so many roles in both healthy and diseased brains, it is vital to unravel the mechanisms behind their various actions. One of the focuses of this thesis is to investigate these mechanisms. Using primary human brain microglia, this thesis investigates their phenotypic profile, their functions, their interactions with other primary human cells, their responses to factors in their environment, and the mechanisms controlling their actions.

Thus this thesis sets out to determine: What is the true nature of adult human microglia?
1.2. The CNS and the immune system

The CNS and the peripheral immune system are in constant communication (Kerschensteiner et al. 2009; Ransohoff and Brown 2012). Systemic immune molecules signal to the brain via both neural and humoral routes in the blood (Perry 2010). Microglia residing in the brain parenchyma are responsive to immune signals and are thus a cellular link between the peripheral immune system and the CNS (Perry 2010). For example, phenotypic changes of microglia isolated from the adult human brain have been associated with peripheral inflammation (Melief et al. 2012), and numerous studies have found microglial phenotypic changes following peripheral immune stimuli (Perry 2010). In addition, immune molecules can have brain-specific roles, such as regulation of synaptic plasticity and development (Graeber 2010).

Although the presence of a specialised blood-brain barrier and lack of some immune responses has led to the idea of the brain as an immune privileged organ, the brain is not excluded from immune surveillance. Immune privilege is not absolute and is not applicable to all contexts (Galea et al. 2007). This partial immune privilege concerns the parenchyma, but not the ventricles or the meninges. It is variable, depending on brain region and also on age (Galea et al. 2007). Immune privilege of the adaptive immune system predominantly limits the afferent arm of the immune response (antigen presentation to naive T cells) but not the efferent arm (trafficking of innate and adaptive immune cells into the brain) (Galea et al. 2007).

The CNS is indeed affected by the peripheral immune system as demonstrated by the classic example of ‘sickness behaviour’ which is triggered by interleukin (IL)-1β activity in the brain. The hippocampus has a high density of IL-1β binding sites and IL-1β inhibits hippocampal memory consolidation (Pugh et al. 2001). IL-1β also decreases hippocampal subgranular zone cell proliferation, while blocking IL-1β reverses the inhibition of neurogenesis caused by stress (Koo and Duman 2008). Immune activity in the meninges surrounding the brain parenchyma has been shown to affect learning and memory (Derecki et al. 2010). Metabolic stress is also known to be associated with immune activation and inflammatory processes and it has been shown that obese rodent mothers produce offspring with increased microglial activation (Bilbo and Tsang 2010). Chemicals normally associated with the brain can also affect cells of the peripheral immune system. For example, the neurotransmitter dopamine can reduce the suppressive activity of regulatory T cells (Kipnis et al. 2004b).

Innate immune processes are well-known to occur in the CNS, performed by microglia and other cells with immune function such as astrocytes and perivascular macrophages. Although the adaptive
immune system has been more elusive, it is becoming apparent that surveillance of the CNS by cells of the adaptive immune system is necessary for normal physiological processes as well as in response to injury (Schwartz and Ziv 2008). Immune cells, including innate monocytes and adaptive T cells, can enter the CNS from the periphery and affect neurological processes (Schwartz et al. 2009). Under normal conditions CD4+ T cells are present in cerebrospinal fluid in the brain ventricles, and in inflammatory conditions the expression of adhesion molecules and chemokines on brain endothelium and choroid plexus provides trafficking signals for circulating leukocytes to enter the CNS (Engelhardt and Ransohoff 2005). There is evidence to suggest that T cells are required to coordinate proper repair of CNS damage and the theory of ‘protective autoimmunity’ postulates that surveillance of the CNS by T cells is necessary for optimal recovery after injury (Kipnis and Schwartz 2005; Schwartz and Ziv 2008). For example, it has been demonstrated that a T cell-based vaccination can ameliorate pharmacologically-induced cognitive impairment and behavioural abnormalities (Schwartz and Kipnis 2005). Thus T cells play an active role in brain function and insufficient immunity can result in loss of CNS homeostasis (Schwartz et al. 2009). Nevertheless, T cells are tightly regulated by innate immune cells, including microglia in the brain, and many microglial functions involve signaling and cell-cell contact with adaptive immune cells (Kipnis et al. 2004a).

1.3. Functions of microglia

1.3.1. Phagocytosis

One of the principal actions of microglia is phagocytosis - the actin-dependent internalization of foreign particles and debris (Aderem and Underhill 1999). The clearance of apoptotic cells, protein accumulations and exogenous pathogens by microglia is important for CNS homeostasis (Chan et al. 2001). The clearance of cell debris following cell death is necessary to limit bystander effects on healthy cells. Debris is also inhibitory to growth and repair, thus phagocytosis is especially important both during development and for regeneration following injury.

An example of phagocytic action in the ageing human brain is the uptake of amyloid-beta (Aβ) protein by microglia. Once activated and present at the site of Aβ deposition, there is evidence for microglial phagocytosis of Aβ. Abnormally deposited, highly insoluble Aβ aggregates have properties
that stimulate phagocytosis (Streit 2004). Phagocytosis of A\(\beta\) in rodents has been demonstrated in vivo and in vitro (Simard et al. 2006) and ultrastructural studies report that microglia in the Alzheimer’s disease cortex contain intracytoplasmic A\(\beta\) fibrils (Rogers et al. 2002). Furthermore, adult human microglia phagocytose A\(\beta\) in vitro (Gibbons et al. 2011). However, activated microglia can secrete neurotoxic factors and if microglial phagocytosis is uncontrolled, their neuroprotective effect can switch towards neuroinflammation (Aderem and Underhill 1999; Bisht et al. 2009; Neumann et al. 2009).

Phagocytosis is highly controlled by many factors including cell surface receptors and extracellular cytokines/growth factors (Koenigsknecht-Talboo and Landreth 2005). For example, phagocytosis of A\(\beta\) has been shown to involve a complex of cell surface receptors including scavenger receptors, integrins, and integrin-associated proteins (Bamberger et al. 2003; Koenigsknecht and Landreth 2004). Phagocytosis of apoptotic cells involves phosphatidylserine receptors, and phagocytosis of pathogens involves Toll-like receptors (Ravichandran 2003), whilst the Fc receptor family is important for the phagocytosis of antibody-opsonised particles (Aderem and Underhill 1999). The specific receptors involved in phagocytosis have been found to determine whether a simultaneous pro-inflammatory response is stimulated. For example, Toll-like receptors, which recognise pathogens, stimulate release of pro-inflammatory cytokines, whereas receptors recognising apoptotic cells do not (Ravichandran 2003). In particular, “Triggering Receptor Expressed on Myeloid cells-2” (TREM2)-mediated signalling in microglia has been shown to facilitate debris clearance without invoking inflammation (Takahashi et al. 2005).

1.3.2. Cytokine secretion

Another significant function of microglia is to secrete soluble immune signalling molecules for communication between cells of the immune system and other cell types including neurons and astrocytes. Cytokines are a major class of small molecule immune signalling proteins. Microglia, astrocytes and neurons all express cytokines and cytokine receptors (de Haas et al. 2007). The vast array of known cytokines is ever-increasing and they display complex regulation in response to environmental factors. They not only provide a means of communication between cells within the brain, but also between the CNS and the periphery (Perry 2010).

Cytokines have many necessary functions in the brain such as directing growth and promoting survival. However, a physiological balance of multiple pro- and anti-inflammatory cytokines is important for a healthy brain. For example, IL-6 has numerous roles in the brain, both as a
neurotrophic and neurotoxic factor (Spooren et al. 2011; Vezzani et al. 2011). Elevated pro-inflammatory cytokines have been found in a number of neurological diseases such as epilepsy for which a recent meta-analysis of cytokine levels found that epileptic patients’ IL-6 plasma concentrations were significantly elevated compared to controls (Yu et al. 2012). Often the presence of one cytokine can induce production of many others, for example IFNy treatment of primary human glia can induce production of pro-inflammatory cytokines IL-6, monocyte chemotactic protein 1 (MCP-1) and IP-10 (Chapter 5).

Chemotactic cytokines (chemokines) are guidance molecules which control the migration of microglia (and other immune and neural cells) towards sites of injury or damage. For example, microglia migrate towards Aβ plaques and areas of ischemia in response to MCP-1 (Mildner et al. 2007). Other cells can also migrate in response to microglial-produced cytokines, such as T cells and monocytes infiltrating the parenchyma from the periphery. Newly born neuronal cells are also guided by chemokines to their destination during adult neurogenesis in response to injury (Gordon et al. 2009).

1.3.3. Migration

Microglia have the ability to move to sites of injury or disturbance where they can then clear up debris and assist with repair. Microglia respond to chemokine gradients via receptors on their cell surface (Dijkstra et al. 2004; Wang et al. 2008). Migration is a function of chemokine gradients, expression of chemokine receptors, and a cell’s position in time (Flynn et al. 2003). Where over-activation of microglia is evident in disease processes, there are reports of upregulated chemokine and chemokine receptor expression, for example in multiple sclerosis (Simpson et al. 2000; Tanuma et al. 2006). Reports have also documented chemotaxis of microglia in the Alzheimer’s disease brain towards Aβ plaques (Xia et al. 2000). Chemoattraction not only of brain-resident microglia but also of blood-borne monocytes is evident in Alzheimer’s disease. Rodent bone marrow cells are attracted to Aβ and have been shown to cross the blood-brain barrier and migrate to plaques (Simard et al. 2006).
1.3.4. Antigen presentation

The expression of Human Leukocyte Antigen (HLA) cell surface antigen presentation proteins by microglia in the brain suggests a role for microglia in communication with other immune cells and monitoring the extracellular space. However, the details of this process in the brain are not well-understood. Immunohistochemical studies have not found evidence for the presence of cells with the conventional immunophenotype of dendritic antigen-presenting cells in the normal brain, although they are present in the meninges and choroid plexus (Galea et al. 2007). Unlike peripheral tissues, the afferent arm of the adaptive immune response in the brain lacks a cellular pathway for antigen transport, and seems to rely on soluble antigen drainage to local lymph nodes instead (Galea et al. 2007). Nevertheless, increased levels of HLA expression have been observed on human microglia in neurologically diseased brain tissue (McGeer et al. 1988; Sapp et al. 2001). HLA expression appears to be involved in the process of microglial activation as it is often associated with increased cytokine production and phagocytic activity (Huizinga et al. 2012; van Horssen et al. 2012).

Recently the leptomeninges has been found to be a location of T cell contact with phagocytic antigen-presenting cells (Bartholomaus et al. 2009; Kivisäkk et al. 2009). Live cell two-photon imaging of rats revealed T cells moving out of leptomeningeal blood vessels and into the subarachnoid space where they interacted with antigen-presenting cells and subsequently invaded the CNS parenchyma (Bartholomaus et al. 2009; Kivisäkk et al. 2009). This complex neuro-immune process involves the actions of phagocytosis, migration involving chemokines, and antigen presentation.

1.3.5. Morphology

Different morphologies of microglia have been shown to correlate with different phenotypes and functions (Graeber 2010). Microglia are found throughout normal brain tissue generally with non-overlapping ramified processes allowing sensing of their environment (Hanisch and Kettenmann 2007). In general a ‘ramified’ morphology with extended processes is thought to represent a ‘resting’ phenotype, whereas an ‘amoeboid’ rounded morphology more frequently relates to an ‘activated’ or phagocytic phenotype (Graeber 2010). Microglia change their morphology in response to a number of environmental cues including cytokines which influence their function or induce them to migrate, and microglia are able to shift from one morphology state to another when environmental
cues change (Kettenmann et al. 2011). Thus quantification of microglial morphology is a valuable tool to assess changes in microglial activation state.

1.4. Microglia in health

There is increasing awareness of the functions that microglia perform in the healthy, normal brain under non-pathological conditions. Microglia play crucial regulatory roles in the brain right from development. Microglia and immune proteins play a role in synaptogenesis (Bessis et al. 2007; Boulanger 2009) and continue to perform many homeostatic roles in the CNS throughout life (Saijo and Glass 2011). They perform constant immune surveillance and communication with other brain cells. The finding that lack of microglial activity itself causes disease provides a strong argument for normal microglia being necessary for a healthy brain (Graeber 2010).

Exciting advances were recently made in the area of ‘microglia in health’ using in vivo two-photon microscopy to image microglia in the intact, live brain. Observing the fluorescent microglia of transgenic mice revealed that microglia in the ‘resting’ state (in the absence of injury) are not inactive (Nimmerjahn et al. 2005). On the contrary, microglial processes were found to continuously scan the parenchyma by extension and withdrawal and subsequent de novo formation (Nimmerjahn et al. 2005). Microglia processes directly contact other brain cells and blood vessels (Nimmerjahn et al. 2005). Subsequent experiments have discovered that microglia directly contact neuronal synapses, and even more astonishingly, that these contacts are activity-dependent, with fewer contacts made when neuronal activity is reduced (Wake et al. 2009). Furthermore, microglia appear to be involved in determining loss of synapses following ischemia (Wake et al. 2009). These results demonstrate that microglial cells are highly dynamic during the resting state and that they respond to injury and actively modulate synaptic connections. This discovery leads the way for exploring the exciting possibilities of microglial influence on neuronal activity and behavior.

1.4.1. Renewal of the microglial population

There is evidence to support both the local proliferation and renewal of the microglial population within the brain itself (Ajami et al. 2007; Ginhoux et al. 2010), and the replacement of microglia by bone-marrow derived circulating monocytic cells (Chen et al. 2010; Xu et al. 2007). It is possible that
subpopulations of microglia may have different origins and perform different roles. Furthermore, ‘subpopulations’ of microglia could in fact represent different stages of development of microglia. It seems likely that under normal conditions, where the blood-brain barrier is intact, there is little infiltration of bone-marrow precursors into the CNS. However, with disruption of the blood-brain barrier (as occurs during head trauma, degenerative disease and irradiation experiments) there is replacement of microglia with peripheral bone marrow-derived cells. Under pathological conditions the contribution of microglia and monocytes to neuroinflammation is likely to differ temporally and spatially (Ajami et al. 2011; Schilling et al. 2003).

1.5. Microglia in disease

An immune response, and a role for microglia as the predominant immune effector cells of the CNS, is a well-established factor in acute and chronic neurological disease (Saijo and Glass 2011). When microglia are activated over a long period of time, their over-activation seems to tip the balance from a neuroprotective to a neurotoxic state. Neuroinflammation has been a growing area of research over the past several decades (McGeer et al. 1988). Microglia are involved, for good or bad, in both progressive degenerative diseases such as Huntington’s disease (Sapp et al. 2001) and those with discrete, acute episodes such as seizures and traumatic injury (Vezzani et al. 2011; Yang et al. 2010). There is evidence for inflammation and microglial activation in early as well as progressive stages of disease (Sapp et al. 2001; Tai et al. 2007; Yang et al. 2010). Monitoring microglial activity by imaging patients throughout disease progression is useful to help us understand the role that they play in disease and may also be used as a biomarker of disease progression (Politis et al. 2012).

Some microglial activities appear to exacerbate disease processes. As mentioned previously, phagocytic removal of debris can be associated with production of potentially toxic factors (Rogers et al. 2002). In multiple sclerosis this has been associated with neuronal damage (Huijinga et al. 2012). Aggressive phagocytosis of viable neurons has been reported and phagocytic cells could also play a role in augmenting autoimmunity to neuronal antigens (Neher et al. 2011).

As previously alluded to, microglia are important players in all brain disease processes and will have distinct roles depending on the disease process, causative agent and cells involved. Brain tumours represent a specialised disease process which involves a major immune component. A variety of immune factors are produced by tumours, such as chemokines, anti-inflammatory cytokines and other immunosuppressant agents (Charles et al. 2011; Li and Graeber 2012). Expression of antigen
presentation proteins by microglia suggests the ability to present tumour antigens and recruit peripheral immune cells. Tumour microglia/macrophages probably play a dual role in tumour immune evasion and progression as it is thought that they initially fight against tumour cells but later are used by the same tumour cells to promote their own survival (Ghosh and Chaudhuri 2010).

HIV/AIDS is also interesting to study as a model of perturbed immune homeostasis. HIV-associated dementia (HAD) is a condition that develops in a significant proportion of infected individuals. There is documented cell loss in the hippocampus of HIV-positive patients, which may result from a number of immune factors, including cytokine release (Castelo et al. 2006). There may be similar neurochemical mechanisms underlying age- and HIV-related hippocampal impairment. The principal pathway for HIV entry into the CNS is through infected macrophages and microglia. MCP-1, a chemoattractant for monocyctic cells, is elevated in the cerebrospinal fluid of patients with HAD and may correlate with cerebrospinal fluid viral load (Kaul et al. 2001).

An interesting and continually developing discovery is that our immune systems greatly influence our behaviour. Disturbances in peripheral and central immune cells and immune molecules have been reported for a range of psychological diseases including Down’s syndrome, autism, schizophrenia and depression (Ashwood et al. 2011; Beumer et al. 2012; Garey 2010). In a seminal study by Chen et al. (2010) it was found that a compulsive behavioural disorder in rodents is associated with mutant microglia, providing a direct link between behavioural deficit and dysfunctional microglia (Chen et al. 2010). Immune theories of depression are supported by several lines of evidence including increased HLA-DR antigen presentation protein found on microglia of the hippocampus and prefrontal cortex of depressed patients (Bayer et al. 1999); increased inflammatory gene expression in monocytes of depressed patients (Beumer et al. 2012); and anti-inflammatory effects of commonly used anti-depressants such as fluoxetine (Hashioka et al. 2007; Lee et al. 2012; Lim et al. 2009).

It is unclear in many disease contexts whether microglial activity is predominantly good or bad, and it may be a dynamic process which is initially beneficial and later becomes detrimental. Therefore, the brain must have a balance between maintaining adequate protective immunity and avoiding detrimental inflammation.
1.5.1. Anti-inflammatory neuroprotective mechanisms of microglia

Although microglia (and the immune system in general) can clearly contribute to neurodegeneration through inflammatory mechanisms, there is also evidence that they can be neuro-protective through anti-inflammatory mechanisms. Microglia can provide neuroprotection through expression of anti-inflammatory cytokines such as IL-10 and growth factors (Xin et al. 2011). For example, a neuroprotective role of microglia as a source of insulin-like growth factor-1 has been demonstrated after ischemic injury in mice (Lalancette-Hebert et al. 2007). In the absence of proliferating microglia there was found to be an increase in pro-inflammatory cytokine expression, an increase in lesion size and more apoptotic neurons in the area of ischemia (Lalancette-Hebert et al. 2007). Microglia have also been shown to reduce glutamate excitotoxicity through functional uptake of glutamate via glutamate transporter-1 (Persson et al. 2009).

During disease processes microglia also co-ordinate with other immune cells, both within the brain parenchyma such as astrocytes, and with peripheral monocytes and T cells. For example, in neurological disease microglia up-regulate cell surface molecules, such as HLA-DR, which interact with T cells (McGeer et al. 1988). A number of studies have demonstrated that T cell deficiency is detrimental to brain function and recovery from injury, and there is evidence to suggest that enhancing adaptive immunity may assist repair processes (Schwartz et al. 2009).

The microglial dysfunction hypothesis has been put forward to describe microglia in the aged human brain as being senescent cells with impaired ability to phagocytose and clear debris (Streit 2006). Microglial dysfunction may also be accompanied by impaired protective capacity, further leading to neurodegeneration (Hanisch and Kettenmann 2007). This hypothesis suggests that improving beneficial microglial functions will be of therapeutic advantage in neurodegenerative disorders (Streit 2006).

Pathological processes of the CNS can occur in microglia themselves and glial cells may also degenerate in ‘neuro’-degenerative diseases. Genetic mutations which result in neurological disease (such as the CAG repeat in Huntington’s disease, superoxide dismutase-1 mutation in Amyotrophic Lateral Sclerosis (ALS), and presenilin-1 mutation in Alzheimer’s disease) are also present in microglial cells. Even if microglia are not the predominant cells which are lost in the disease process, the genetic defect may play an important role in these cells as well. This theory has been tested in transgenic animal models of disease. Beers et al. (2006) found that a mouse model of familial ALS which lacked macrophages and microglia (PU.1 knockout mouse) had improved outcome with
transplantation of wild-type microglia without the ALS-causing mutation (Beers et al. 2006). A genetic mutation in the presenilin-1 gene is responsible for a subset of familial Alzheimer’s disease cases. Presenilin-1 mutant microglia were found to impair proliferation of neural precursor cells in vitro compared to wild-type microglia (Choi et al. 2008a).

It is likely that glial and immune activation are not always the initial causative factors of disease, but that without inflammatory processes the underlying disease pathology would not manifest in neurological symptoms, i.e. immune mechanisms may exacerbate disease processes. There is a great need to understand the immune processes involved leading up to clinical neurological symptoms. The pre-symptomatic stage harbours a wealth of possibilities for intervention and changing disease course. It is recognised that many immune processes are occurring at this stage and we need to identify which of these actions ward off disease and which progress it.

1.5.2. The immune system as a therapeutic target

The inflammatory component to neurological diseases provides an opportunity to manipulate this process pharmacologically. Cells of the immune system predominantly function in defence and repair of the body’s tissues. Therefore, targeting microglia is a logical strategy for maximizing neuroprotection in neurological disorders (Popovich and Longbrake 2008) and new methods of selectively targeting microglia are continuously being developed (Minami et al. 2012).

Movement of pharmacological agents across the blood-brain barrier is a limitation for many therapeutics for the brain. However, targeting the immune response has the advantage of not necessarily requiring direct contact with the brain parenchyma as immune cells can enter the brain from the periphery. Disruption of the blood-brain barrier is a major aspect of many brain disorders and can itself be a therapeutic target (Shlosberg et al. 2010). Immune cells residing in the meninges can also be targeted for treatment of brain disorders rather than targeting cells in the brain parenchyma per se. Furthermore, macrophages and microglia can fuse with other cell types offering another avenue for delivery of therapeutic agents to other brain cells such as neurons (Ackman et al. 2006).

However, we need to gain more knowledge about immune responses in the brain, to more effectively treat brain disorders. Different immune mechanisms occur in different neurological diseases, and one type of immune activity can be beneficial in one disease context whilst being detrimental in another (Vaknin et al. 2011). Thus, targeted, as opposed to global, immune
manipulation is likely to be of most benefit for neurological disorders. One of the aims of this thesis is to better understand the processes involved in turning a supportive microglial cell into a harmful one, with the potential of targeting this process for therapeutic effect.

1.6. External factors affecting microglial phenotype

1.6.1. Soluble molecules

Microglia clearly perform different actions in different contexts. The microenvironment of a microglial cell shapes its phenotype and they are highly responsive to soluble factors, as well as to other cells, in their surroundings (Kettenmann et al. 2011; Olah et al. 2011). Microglia sense their environment through expression of a whole host of cell surface receptors which initiate intracellular signaling pathways when their ligands are present. Microglia respond to numerous growth factors such as the Macrophage Colony-Stimulating Factor (M-CSF). M-CSF stimulates signalling pathways in microglia which influence transcription factor expression, cell surface receptor expression, and many downstream cellular functions including proliferation and phagocytosis (Chapter 4).

In conjunction with the ability to produce soluble cytokine molecules (discussed above), microglia also respond to many cytokines and their behaviour is largely controlled by the balance of pro- and anti-inflammatory cytokines with which they are in contact. Response to cytokines can in turn determine further cytokine production. For example, Interferon-y (IFNy) can influence microglial cytokine/chemokine production as well as other cellular processes such as cell surface receptor expression (Klegeris et al. 2005). Microglia also respond to many other soluble molecules such as ATP (Lambert et al. 2010). In the brain, a microglia cell will have to integrate signals from a multitude of soluble molecules simultaneously and the combined effect of all the soluble molecules in its surrounding will determine the ultimate phenotype of the microglia.

1.6.2. Regulation by cell surface proteins

The presence of other cells also affects microglial phenotype, not only because of the soluble signalling molecules they produce, but also due to direct cell-cell contact and signalling (Neumann
Microglia are regulated by other cells in their environment through cell surface receptors. Ligands on other cells which bind to receptors on microglia provide a focused, local mechanism for microglia regulation, without the more general effects obtained with soluble factors.

CD200 is a membrane protein expressed by neurons and endothelial cells. The receptor for CD200 is only found on monocyctic cells including microglia and it is probable that CD200 provides a steady-state control mechanism for microglia in the brain as it has been shown to downregulate microglial activation (Barclay et al. 2002; Hoek et al. 2000). CD47 is a more widely distributed membrane protein which interacts with its receptor CD172a on microglia and reduces microglial activation (Barclay et al. 2002). Neurodegeneration (i.e. loss of neurons) could lead to a more ‘activated’ microglial phenotype, as regulatory neuronal factors are lost. This could lead to a vicious cycle where inflammation further exacerbates neuronal loss. A decrease in both CD200 and CD47 has been reported in chronic active and inactive multiple sclerosis lesions compared to control white matter, with relatively little change in their expression of receptors (Koning et al. 2007). CD200 and CD200R have also been reported to be decreased in regions of the brain affected by Alzheimer’s disease (Walker et al. 2009).

1.6.3. Age

The peripheral immune systems of elderly people are not as robust as those of young people. As in the periphery, ageing can lead to unpredictable responsiveness and/or senescence of the brain’s immune cells (Streit 2006). Markers of inflammation and glial activation are increased in the aged brain even when there are no symptoms of neurological disease (Lynch et al. 2010). It remains unclear to what extent phenotypic changes occur within microglia populations during ageing, and it is possible that age-related immune changes are indicative of a shift in immune cell populations to a more perivascular/peripheral cell origin.

Concurrent with increased inflammation, signs of microglial dystrophy have been noted in the ageing human brain (Lopes et al. 2008; Streit 2006). It is possible that dystrophic microglia are unable to carry out normal house-keeping functions such as accumulation and storage/breakdown of extracellular ions (e.g. Fe) and proteins (e.g. Aβ) (Avagyan et al. 2009). Thus microglial dysfunction is also likely to be a factor in neurodegenerative processes and neurological decline as the microglial functions of phagocytosis, migration and cell-cell communication become less efficient. Whereas human microglia show signs of ageing-related structural deterioration, rodent microglia appear to remain functionally active throughout their relatively short lives. This calls into question whether
rodent microglia can adequately model microglia of the ageing human brain. Another external factor which affects microglial phenotype is brain region, and microglial activation has been shown to be differentially affected by age in different brain regions (Hart et al. 2012).

### 1.6.4. Brain region

The phenotypes and functions of microglia vary across brain regions in response to differing composition of cell types, proximity to blood vessels and access to cerebrospinal fluid. A defining regional difference has been reported for microglia in grey vs white matter with a greater number of microglia present in myelinated white matter (Mittelbronn et al. 2001; Olah et al. 2011). Microglia isolated from adult human brain tissue were also reported to have greater expression of cell surface protein CD45 if they are from white matter rather than grey matter (Melief et al. 2012). The presence of more oligodendrocytes or more neurons in white and grey matter respectively is likely to affect the phenotype of microglia in those areas. Region-specific expression of other microglial immune proteins has been documented for the healthy adult mouse CNS (de Haas et al. 2008). As cells of immune origin, microglia are very responsive to plasma proteins in the blood and cerebrospinal fluid. Microglia residing in regions with a less defined blood-brain barrier, and hence greater contact with plasma proteins, appear to be basally more activated and less ramified than microglia in other regions (Cuadros and Navascues 1998; Olah et al. 2011).

### 1.6.5. Species

Rodents are the predominant experimental model in neuroscience research despite numerous species differences to humans. It is important to validate *in vivo* and *in vitro* rodent findings in human models and our inability to do this effectively is reflected in the failure of translating neurological treatments to the clinic (Dragunow 2008a). Immunology is another area of biomedical research where species differences appear to carry a lot of weight (Davis 2012). Likely reasons for differences between results in rodent and human studies include inbreeding/lack of heterozygosity in rodent models; humans’ exposure to disease in contrast with the sterile environment of laboratory animals; and greater evolutionary investment in the human immune system (Davis 2012).

As an example, a commonly assessed neuroinflammatory end-point is production of nitric oxide (NO). Although rodent microglia readily produce NO upon stimulation, adult human microglia do
not. Conversely, human astrocytes appear to express NO instead, and human macrophages and microglia do not share the same NO regulation and production processes as rodent macrophages and microglia (Denis 1994; Liu et al. 1996). Recent investigation in our laboratory has led to the finding that rodent and human microglia also do not have the same responses to the neuroactive drug valproic acid (VPA). VPA is used clinically for the treatment of bipolar and epilepsy disorders. Whilst rodent microglia exhibit increased phagocytosis and undergo apoptosis in response to VPA treatment \textit{in vitro} (Chen et al. 2007; Smith et al. 2010), adult human microglia were found to have the opposite response of decreased phagocytosis and no evidence of apoptosis (Gibbons et al. 2011). This information is critical for our understanding of VPA’s mechanisms of action in the brain, and for the future design of more targeted and effective treatments.

1.7. Intrinsic microglial control mechanisms

1.7.1. Transcription factors

Transcription factor control of gene expression is a major point of regulation of microglial phenotype. This thesis investigates the role of the transcription factor PU.1 in adult human microglia (Chapter 3). PU.1 is dynamically and heterogeneously expressed within hematopoietic lineages where high PU.1 expression activates genes of the myeloid lineage and is expressed primarily in monocytic cells (Back et al. 2005). In rodents, high levels of PU.1 protein are expressed in resting microglia as well as in activated microglia following injury (Walton et al. 2000). PU.1 controls a vast network of myeloid genes which are relevant to neuroimmunology and brain inflammation. PU.1 is essential for myeloid cell differentiation (Feng et al. 2008; Forsberg et al. 2010) and regulates genes involved in a range of monocytic functions such as phagocytosis and chemotaxis (Lloberas et al. 1999), for example cytokines and cell surface receptors. Importantly, cells expressing PU.1 are responsive to the monocytic growth factor M-CSF (Henkel et al. 2002) due to PU.1 regulation of the M-CSF receptor gene \textit{c-fms} (Zhang et al. 1994).

PU.1 has been shown to interact with members of the CCAAT enhancer-binding protein (C/EBP) family of transcription factors (Jin et al. 2011). C/EBP transcription factors also play important roles within immune cells including proliferation, differentiation and inflammation (Ramji and Foka 2002; Walton et al. 2000). From the C/EBP transcription factor family this thesis investigates C/EBPβ
expression within human adult microglia (Chapter 4). C/EBPβ protein expression has been demonstrated in microglia in human spinal cord tissue from ALS patients, but was rarely found in control spinal cord tissue (Valente et al. 2011). C/EBPβ forms heterodimers with members of its own family and interacts with several other transcription factors (Ramji and Foka 2002) including PU.1 (Tissieres et al. 2009; Yang et al. 2000). C/EBPβ plays numerous roles in activation and differentiation of macrophages (Ramji and Foka 2002) and interestingly, combined expression of PU.1 and C/EBPβ in fibroblasts can induce a macrophage phenotype (Feng et al. 2008).

1.7.2. Epigenetics

Another level of transcriptome regulation comes from epigenetic modification of the genetic code. Epigenetic modifications include acetylation of histone proteins which organise DNA structure, and methylation of DNA bases (Coppieters and Dragunow 2011; Narayan and Dragunow 2010). A large area of research focuses on epigenetic modifications in neurological disease. There are reports specifically investigating perturbed microglial epigenetic states, and epigenetic pharmacology of microglia is a promising research area (Faraco et al. 2009; Zhang et al. 2007). Findings of altered epigenetic states in neurological disorders such as Alzheimer’s and Huntington’s disease have provided a rationale for treatment of these disorders with epigenetic modifying drugs (Best and Carey 2010; Narayan and Dragunow 2010). However the efficacy of these treatments in human patients remains to be shown (Narayan and Dragunow 2010).

As mentioned above, the drug VPA has numerous effects on microglia as well as other brain cells. A major mechanism of action of VPA is as a histone deacetylase inhibitor, i.e. it produces a global increase in histone acetylation (Phiel et al. 2001). With VPA treatment, histone acetylation is observed along with reduced phagocytosis in human adult microglia (Gibbons et al. 2011). Further investigation into the precise downstream effects of histone deacetylase inhibition in human microglia is warranted, especially in the advent of epigenetic drugs used to treat neurological disorders (Best and Carey 2010).
1.8. *In vitro* models of microglia

To study the extrinsic and intrinsic control mechanisms of microglia functions, several *in vitro* models of microglia are available. However, due to the species differences previously mentioned, rodent microglia do not represent an ideal platform for microglial studies. Additionally, no adult human microglia cell lines are currently available. Recently a method for differentiating ‘human monocyte-derived microglia’ has been published (Etemad et al. 2012). However, this protocol involves treatment of cells with a variety of cytokines which will prime the cells towards a particular phenotype and must be taken into account for subsequent experiments. Some studies have used a human fetal microglial cell line: CHME-5. However, the developing human brain and the adult/ageing human brain undergo markedly different processes *in vivo* and the microglia in each of these states will have very different roles and are not likely to be completely modelled by one another. Due to inherent differences between cell lines and primary cells, such as senescence and clonal origin, they may respond differently to a number of experimental paradigms.

The use of primary adult human microglia for research is a significant advancement on other models of microglia. Primary human cells provide the advantages of being derived from the brain (as opposed to the periphery), being without transformation or chemical differentiation, and being from humans not animal models (Gibbons and Dragunow 2010). To investigate the actions of adult human microglia, this thesis has used primary cultured cells and immunohistochemical staining of human brain tissue. Dissociated adult human brain tissue cell cultures comprise the glial cells microglia and astrocytes, and fibroblast-like cells (discussed below). Microglia are identified and monitored within the mixed glial cultures using microglial-specific markers. One disadvantage of using primary human microglia is that they are not highly proliferative in culture and will predominantly only divide in response to growth factors ((Gibbons et al. 2007); Chapter 4). Thus, unlike a cell line, these cells are not replenishing and a limited use is obtained from each culture. Nevertheless, the information gained from their use is invaluable to the field of neuroimmunology.

1.9. Astrocytes

Along with microglia, astrocytes are another crucial glial cell type. Astrocytes are highly abundant brain glial cells of neuroectodermal origin (Matyash and Kettenmann 2010). These large cells, which can be up to several hundred microns in length, have a star-like morphology with multiple processes,
and are present throughout the grey and white matter (Matyash and Kettenmann 2010). Astrocytes perform many homeostatic functions in the brain and are also an important element of neurological synapses. Astrocytes are a major component of the neurovascular unit – the cells comprising the blood vessel-brain parenchyma interface (Abbott et al. 2006). The blood-brain barrier is integral to CNS homeostasis, and proper astrocyte functioning is important for regulation of immune cell trafficking into the brain and microglial activation (Abbott et al. 2006). Another role that they play in homeostatic regulation is uptake of the excitatory neurotransmitter glutamate which is necessary to prevent excitotoxicity. Astrocytes themselves have many immune functions (Farina et al. 2007; Ransohoff and Brown 2012). They respond to a variety of immune signalling molecules (e.g. IFN\(\gamma\)) and produce them too (e.g. M-CSF, TGF\(\beta\) and IP-10; Chapter 5). Astrocyte immune activity has been shown to play a specific role in several diseases including Alzheimer’s disease (Li et al. 2011) and epilepsy (Vezzani et al. 2011), partially through upregulated expression of pro-inflammatory cytokines.

The quintessential characteristic of astrocytes is expression of glial fibrillary acidic protein (GFAP). However, this distinction is not as simple as it first appears as there are multiple subtypes of GFAP and it has been shown that although GFAP is expressed in the cytoplasm it may not label the entire cell (Sofroniew and Vinters 2010). Furthermore, it is possible that not all cells with astrocytic function express GFAP. In our primary adult human mixed glial cultures, astrocytes account for a small proportion (~1%) of cells. They do not divide and the number obtained is variable between cultures. While cultures from biopsy tissue usually contain some astrocytes, post-mortem cultures generally do not (Gibbons et al. 2007). Nevertheless, they represent an important brain cell type and add complexity and \textit{in vivo} characteristics to primary mixed glial cultures.

\section*{1.10. Brain derived fibroblast-like cells}

The third cell population in our mixed glial cultures is a proliferating cell type of indefinite character. These cells do not express microglial or blood cell markers (such as CD45, PU.1 or Iba-1) nor do they express GFAP, neuronal or oligodendrocytic markers. They have a distinct morphology from microglia and astrocytes with little extension of processes and flat, spread-out cell bodies (Gibbons et al. 2007). This cell type has been previously identified as having fibroblast characteristics by expression of the collagen-synthesising enzyme prolyl-4-hydroxylase (Gibbons et al. 2007). There are ongoing studies in our research group to elucidate the phenotype of these cells but they are referred
to here as ‘fibroblast-like cells’ for simplicity. Due to the confusion over the phenotype of these cells, other research groups have labelled similar cell types as ‘astrocytes’ or ‘neural progenitor cells’ (described below). Previous publications from our research group have aimed to clarify the nature of these cells and identify them as a separate cell population (Gibbons et al. 2007; Park et al. 2012). These cells have largely been ignored in the literature, however many interesting questions surround their origin and purpose as a proliferative cell type in the adult human brain.

The presence of fibroblast-like cells in isolations from adult human brain tissue is not region specific. Although the origin of fibroblast-like cells is uncertain, they express markers previously ascribed to pericytes and mesenchymal stem cells (Karow et al. 2012; Park et al. 2012; Paul et al. 2012). They are likely of meningeal or blood vessel origin and are thus an intriguing cell type to investigate as they may be situated throughout the brain parenchyma (Kang et al. 2010).

The fibroblast-like cells appear to be a rather ‘plastic’ cell type with the potential for a wide range of functions. This thesis explores their immune capabilities and finds that they can respond to cytokines and be induced to express antigen presentation molecules and secrete their own cytokines, suggesting that they can communicate and interact with microglia and other brain cells (Chapter 5). Stand-in phagocytic fibroblasts have also been reported in mice lacking monocytic cells (Martin et al. 2003). This cell type is also likely to play a role in scar formation as it has the properties of proliferation and migration. The fibrotic scar has been examined in detail in other organs of the body and is likely to be a part of brain injury too (Kawano et al. 2012). Meningeal fibroblasts may migrate from the brain surface into a wound and form fibrotic tissue (Kawano et al. 2012). Importantly, pericytes have been found to play a role in spinal cord scar formation (Goritz et al. 2011). They were found to proliferate in response to injury and to form the inner layer of scar tissue, surrounded by astrocytes. Thus the fibroblast-like cell type could interact with astrocytes during disease processes such as scar formation and also during normal homeostasis such as in maintenance of the blood-brain barrier. The plasticity of fibroblast-like cells also makes them amenable to reprogramming and differentiation into other cell types including neurons (Karow et al. 2012). However they are a cell population distinct from neural precursor cells (described below).
1.11. The cells that got away

In the mixed glial cell cultures prepared from human brain tissue (white and grey matter) two major brain cell types are not found – oligodendrocytes and neurons (Gibbons et al. 2007). Oligodendrocytes are the third major glial cell population (along with astrocytes and microglia) and are derived from the ectoderm like astrocytes and neurons (Emery 2010). Oligodendrocytes generate myelin which insulates axons and promotes transmission of action potentials (Emery 2010). They are therefore found in the white matter surrounding axon tracts. Like microglia and astrocytes, oligodendrocytes are intimately connected to the actions of neurons and other glia in their environment. During development, myelination of axons is activity-dependent and action potentials control the initial events of myelination (Emery 2010; Wake et al. 2011). Oligodendrocytes are not observed in primary adult human mixed glial cultures by immunostaining for myelin basic protein or the oligodendroglial lineage marker Olig2 (De Groot et al. 2000; Yokoo et al. 2004). The isolation procedure may damage the oligodendrocytes beyond repair, or the protocol used in our laboratory may not select for this cell type.

In these cultures mature neurons are not present either. This could be due to the isolation process being harsh on the cells, or because neurons are particularly sensitive to glucose and oxygen deprivation and thus do not survive in the tissue following death or removal from the body. A limited amount of βIII-tubulin (an immature neuronal marker) protein expression is found in mixed glial cultures (Park et al. 2012). However this βIII-tubulin is most often expressed with GFAP and it is not clear whether these cells represent an immature neuronal phenotype, or are the recently classified hybrid ‘asterons’ (Laywell et al. 2005). Although neurons are the most studied brain cell type and considered to be the predominant functional cell of the brain, there are at least equal numbers of glial cells in the human brain as neurons (Azevedo et al. 2009). As demonstrated above, glia are functionally integrated with neurons and their study provides increased depth of understanding of neuron physiology. Microglia, astrocytes and fibroblast-like cells provide plentiful and unique human brain cell material with which to discover the workings of the brain.
1.12. Neurogenesis

There is one more specialised brain cell type that this thesis explores: the neural progenitor cell (NPC) of the adult human brain. An intriguing aspect of the adult human brain is the recent discovery of its continual production of new neuronal cells, even in the aged brain (Eriksson et al. 1998). This phenomenon of adult neurogenesis involves many stages - proliferation, differentiation, migration and functional integration. These processes of neurogenesis are by no means exempt from immune influence.

Although neocortical neurogenesis in humans is restricted to development (Bhardwaj et al. 2006), there are two known constitutively neurogenic regions in the adult brain: the subventricular zone (SVZ) lining the lateral ventricles, and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus (Alvarez-Buylla et al. 2002; Curtis et al. 2007; Eriksson et al. 1998). Proliferative NPCs have been extensively characterised in the ‘neurogenic niches’ of rodent and human adult brains. Other cells which are in contact with NPCs, including microglia which are also present in rodent and human neurogenic niches, play significant roles in the process of neurogenesis.

The SGZ of the hippocampus is a brain region known to be involved in the dynamic processes of learning and memory. Whilst the functional significance of newly-born adult neurons remains unclear, they may play a role in these processes. In several rodent studies a positive link between the amount of SGZ adult neurogenesis and learning ability has been shown (Leuner et al. 2006). Neurogenesis in the hippocampus may contribute to encoding of temporal information in the hippocampus, such as formation of trace memories in which one stimulus is associated with a second at a later time (Aimone et al. 2006; Shors et al. 2001). Nevertheless, the question of whether an aged brain is able to integrate new neuronal resources successfully has no definitive answer yet, and adult neurogenesis may serve different roles to neurogenesis in a young brain, such as through immunomodulation of their environment (Couillard-Despres et al. 2011; Pluchino et al. 2005).

Disturbances in adult neurogenesis have been found for human neurological disorders including epilepsy (Liu et al. 2008), stroke (Jin et al. 2006; Martí-Fàbregas et al. 2010), Alzheimer’s disease (Marlatt and Lucassen 2010) and Huntington’s disease (Curtis et al. 2005a); reviewed by (Thompson et al. 2008). Furthermore, adult neurogenesis can be influenced by early life events including prenatal inflammation and early life infection (Bilbo and Tsang 2010; Graciarena et al. 2010). Decreased neurogenesis also naturally occurs with ageing (Villeda and Wyss-Coray 2012). It may be that the same capacity for neural progenitor cell proliferation is still present in older adults but is repressed by factors such as corticosteroids (Cameron and McKay 1999). Loss of supportive growth
factors from the local microenvironment may also occur in normal ageing, and in disease processes. In fact transplant experiments have shown that the microenvironment of the neurogenic niche is important for neurogenic potential as it contains important cells, extracellular matrix molecules, and signalling molecules which direct neurogenesis (Alvarez-Buylla and Lim 2004; Robel et al. 2011).

1.12.1. *In vitro* culture of NPCs

Isolation and culture of adult NPCs can be routinely performed from rodent brain tissue and, with greater technical difficulty, from human brain tissue (Rietze et al. 2006). A handful of research groups, including our own, have demonstrated the *in vitro* culture and proliferation of human NPCs, and their subsequent differentiation into neurons and astrocytes (Coras et al. 2010; Leonard et al. 2009; Park et al. 2012). The main technical challenges involve isolating a pure NPC population, free from other proliferating cells such as fibroblast-like cells (Park et al. 2012; Paul et al. 2012), and assessing the functional maturation of differentiated neuronal cells. By studying endogenous human NPCs not only can we gain increased understanding of adult neurogenesis, but differentiation of these precursors into functional neurons will provide an invaluable tool for neuroscience research as large numbers of adult human neurons cannot at present be isolated and cultured from biopsy or post-mortem tissue. Thus, despite the lack of mature neurons in cell cultures, adult human neuronal cells can still be derived from primary human brain tissue. It is also hoped that with better understanding of endogenous NPC production of new neurons, we may be able to enhance this process for therapeutic benefit.

1.12.2. Microglia influence neurogenesis

The above literature review illustrates how the immune system, and particularly microglia as predominant immune effector cells of the CNS, influence many neurological processes. The study of the impact of the immune system on adult neurogenesis is an exciting field as both neuroimmunology and adult mammalian neurogenesis are relatively new concepts which have transformed our understanding of the CNS. Microglia and astrocytes are found in the vicinity of NPCs in the adult human brain (Curtis et al. 2005b; Quiñones-Hinojosa et al. 2006). Moreover, immune cells and blood vessels are linked to NPCs via a connective tissue basal lamina containing macrophages and fibroblasts (Kerever et al. 2007; Mercier et al. 2002).
A link between immune processes and neurogenesis has been demonstrated in many inflammatory and disease settings. For example, following an inflammatory stimulus and subsequent microglial activation, minocycline (an inhibitor of microglial activation) ameliorated neurogenesis (Ekdahl et al. 2003). In a disease model of status epilepticus, activation of microglia also correlated with decreased neurogenesis after an epileptic insult (Ekdahl et al. 2003). In addition, mutation-carrying microglia have been shown to have negative effects on neurogenesis, such as microglia with an AD-associated mutation in the presenilin-1 gene which are inhibitory to neurogenesis (Baron et al. 2008; Choi et al. 2008a). Immune molecules and cells may also be important mediators of environmental effects on neurogenesis such as exercise which has been shown to enhance neurogenesis and which also has multiple effects on the immune system (van Praag et al. 2005; Wu et al. 2007).

The effects of immune cells on neurogenesis have been found to be partly through secreted soluble molecules, the same cytokines and growth factors which affect glial and immune cell function. Monje et al. (2003) reported that peripheral injection of the inflammatory stimulus lipopolysaccharide (LPS) to rats resulted in central pro-inflammatory cytokine production which increased the number of activated microglia and decreased hippocampal neurogenesis (Monje et al. 2003). This result was replicated in vitro by Butovsky et al. (2006) who found that LPS-activated microglia reduced neurogenesis, in part via the cytokines IL-6 and TNFα (Butovsky et al. 2006). The anti-inflammatory drug indomethacin restored neurogenesis in rats injected with LPS, indicating that neuroinflammation inhibits neurogenesis (Monje et al. 2003). Battista et al. (2006) showed for the first time that the anti-inflammatory cytokine Transforming Growth Factor (TGF)-β can promote neurogenesis. It was found in vivo and in vitro that the number of activated microglia, and amount of TGFβ, correlated with the number of newly born neurons (Battista et al. 2006). This finding highlighted a functional role for immune activity in the neurogenic niche and, as for all other immune processes, demonstrates a need for balance between immune suppression and immune control. Thus understanding more about immune regulation of neurogenesis holds the possibility of modulating adult neurogenesis therapeutically.
1.13. Thesis outline

Microglia play major roles in health and disease of the CNS. They regulate crucial homeostatic functions but they can also greatly exacerbate disease processes, leading to neurodegeneration and cognitive decline. Further study of this important brain immune cell is needed to further define its diverse actions and activation states. Primary adult human microglia are a unique, but rarely used, tool for neuroimmunological research. Their use enables exclusive insight into the functioning of the adult human brain.

This thesis aims to expand our understanding of primary adult human microglia and to extend their use in biomedical research. Chapter 3 begins by investigating one of the key regulators of monocytic immune cells – the transcription factor PU.1. Key functions of microglia, including phagocytosis and cytokine production, are examined. Chapters 4 and 5 then explore the effects of immune growth factors and cytokines M-CSF, IFNy and TGFβ1 on microglia protein expression and function whilst aiming to understand the role that PU.1 plays in the responses of microglia to various external factors. Chapter 6 explores the diversity of the microglial phenotype by comparing microglia from two distinct regions of the adult human brain. Lastly, brain immune cell functions are investigated in the context of other brain cells in the process of neurogenesis (Chapter 7).

The findings of this thesis contribute to our understanding of the role that microglia play in the adult human brain and pave the way forward for targeting of microglia in the treatment of neurological disorders.
Chapter 2.
General Methods

2.1. Adult human brain tissue

To investigate the actions of adult human microglia, cells have been cultured and immunohistochemical staining has been performed using human brain tissue acquired by the Centre for Brain Research at the University of Auckland. Human brain tissue was collected from two generous sources:

1) Autopsy adult human brain tissue from a range of neurologically diseased (Alzheimer’s, Huntington’s and Parkinson’s disease) and normal individuals was obtained from the Neurological Foundation of New Zealand Human Brain Bank. This Brain Bank is run under the guidelines of the University of Auckland Human Participants Ethics Committee.

2) Biopsy adult human brain tissue was obtained from patients of Auckland City Hospital undergoing surgery for intractable temporal lobe epilepsy. This research was approved by the Northern Regional Ethics Committee and informed consent was obtained from all tissue donors. All biopsy specimens were from temporal lobe epilepsy cases with mesial temporal sclerosis. Temporal lobe epilepsy is a complex disorder involving genetic determinants and environmental factors (Salzmann and Malafosse 2012). It is characterised by recurrent epileptic seizures arising from the temporal lobe and involves a substantial immune and inflammatory component (Vezzani et al. 2011). Surgical resection of the epileptic focal point is a common treatment for drug-resistant epilepsy patients (Al-Otaibi et al. 2012). Tissue was received from patients undergoing standard anterior temporal lobectomy which involves removal of the middle temporal gyrus (allowing access to the hippocampus) and the hippocampus on one side of the brain. Hippocampal sclerosis was pathologically confirmed following resection and most specimens were graded 3-4 by neuropathological examination (where 4 is the highest grade). Patients had taken a range of medications alone or in combination, including phenytoin, tegretol, topiramate, lamotrigine, and sodium valproate. There were no obvious associations between the results of any of the reported experiments and drug use or degree of sclerosis.
Cells were cultured from 2 regions of autopsy and biopsy adult human brain tissue: 1) the middle temporal gyrus; 2) the hippocampus and overlying wall of the lateral ventricle (Figure 2.1).

Figure 2.1: Regions of the adult human brain from which glia and neural progenitor cells were cultured.

A coronal section of one side of the adult human brain at the level of the hippocampus. The purple box outlines the temporal lobe, the region of the brain from which tissue was obtained. Mixed glial cultures containing microglia, astrocytes and fibroblast-like cells (for use in Chapters 3-6) were isolated from middle temporal gyrus grey and white matter (outlined in green). Microglia (Chapter 6) and adult human neural progenitor cells (Chapter 7) were isolated from the hippocampus including the dentate gyrus (outlined in red), and the overlying wall of the lateral ventricle (the temporal horn of the lateral ventricle is outlined in blue).
2.2. Cell culture

2.2.1. Mixed glial cell cultures

This protocol was used for isolating cells for experimentation in Chapters 3-5. Microglia were isolated and cultured in ‘mixed glial cultures’ which also contained astrocytes and fibroblast-like cells.

Gliial cells were isolated from adult human brain tissue using methods previously described by Gibbons et al. (Gibbons and Dragunow 2010; Gibbons et al. 2011) with minor modifications. Approximately 2 g of white and grey matter from the middle temporal gyrus (Figure 2.1) was washed twice in Hanks balanced salt solution (HBSS; Ca2+ and Mg2+ free, Gibco). The meninges and visible blood vessels were removed, then the tissue was diced into small (approx. 1 mm³) cubes and placed in 10 ml/g tissue warm enzyme dissociation mix containing 2.5 U/ml papain (Worthington) and 10 U/ml DNase (Invitrogen) in HBSS and incubated for 30 min at 37 °C with agitation. An equal volume of DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin-Glutamine (Gibco) (final concentrations: penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (0.29 mg/ml)) was added and the tissue gently triturated. The cell suspension was passed through a 100 μm nylon cell strainer (Becton Dickinson), centrifuged for 10 min at 160 g, the pellet resuspended in 20 ml medium and plated into 75 cm² tissue culture flask (Nunc) which was incubated at 37 °C in 95% air/5% CO₂. After 24 h, the debris and unattached cells were removed, centrifuged for 10 min at 160 g and replated onto the adhered cells for a further 24 h. Finally, the debris was removed and the cells carefully washed with medium. Cells were cultured for 1-2 weeks, detached by trypsinization with 0.25% Trypsin/1 mM EDTA, and plated at 50,000 cells/ml on 96-well plates for experiments.

2.2.2. Fibroblast-like cell only cultures

To assess the effect of treatments on the fibroblast-like cell population alone, or to provide a control for microglia and astrocyte effects, cultures of fibroblast-like cells only were generated.

The initial passage of cells from mixed glial cell cultures consisted of microglia, astrocytes and fibroblast-like cells. To obtain cultures of fibroblast-like cells only, 3 or 4 subsequent passages were made (roughly 1 week apart, when cells had reached ~90% confluence) at which point the negligibly dividing microglia and astrocytes were no longer present. Fibroblast-like cell only cultures were
maintained as for mixed glial cultures in DMEM/F12 supplemented with 10% FBS and 1% Penicillin-Streptomycin-Glutamine.

2.2.3. Isolation of neural progenitor cells from adult human brain tissue

This protocol was used for isolating microglia and neural progenitor cells (NPCs) for experimentation in Chapters 6 and 7. The initial isolation procedure for NPCs is similar to that for the mixed glial cultures with a few exceptions, the major difference being the growth media used. The method for adult human NPC (ahNPC) isolation and culture is similar to that detailed by Park et al. (2012).

~1 g of tissue from the hippocampus and overlying wall of the lateral ventricle (Figure 2.1) was washed twice in Hanks balanced salt solution (HBSS; Ca\(^{2+}\) and Mg\(^{2+}\) free, Gibco). The meninges and visible blood vessels were removed. The tissue was then diced into small (approx. 1 mm\(^3\)) cubes and placed in 10 ml/g tissue warm enzyme dissociation mix containing 2.5 U/ml papain (Worthington) and 100 U/ml DNase (Invitrogen) in HBSS and incubated for 30 min at 37 °C with agitation. Enzymatic digestion was halted by addition of neural progenitor cell proliferation media; DMEM:F12 containing B27, Penicillin/Streptomycin, GlutaMAX (all from Gibco), 40 ng/ml Fibroblast Growth Factor-2 (FGF-2; Peprotech), 40 ng/ml Epidermal Growth Factor (EGF; Peprotech) and 2 mg/ml Heparin (Sigma). The tissue was then gently triturated and passed through a 70 μm nylon cell strainer (Becton Dickinson). Cells were collected by centrifugation at 160 g for 10 min, resuspended in 6 ml neural progenitor cell proliferation media and plated into uncoated 25 cm\(^2\) tissue culture flasks (Nunc) which were incubated at 37 °C in 95% air/5% CO\(_2\). The following day, culture flasks were gently agitated to detach any loosely adhered cells (including neural progenitor cells) and all the media was collected and replated onto a new T25 culture flask (Chapter 7).

The adherent cells (including microglia) were carefully washed with glial maintenance medium (DMEM/F12 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Gibco)) and maintained for microglial studies (Chapter 6).

Further culture and proliferation of ahNPCs is detailed in Chapter 7. Differentiation of ahNPCs into neurons and astrocytes was achieved by removal of proliferative growth factors (EGF and FGF-2) and addition of serum and the neuronal growth factors Nerve Growth Factor (NGF) and Brain-Derived Growth Factor (BDNF) (i.e. DMEM:F12 containing 1% FBS, 40 ng/ml NGF and 40 ng/ml BDNF (Peprotech)). Specific differentiation conditions are also detailed in Chapter 7.
2.2.4. Leptomeningeal explant cultures

The leptomeninges is usually removed from cortical middle temporal gyrus tissue for mixed glial cultures to prevent contamination of the sample with non-parenchymal cells. This same leptomeninges tissue can be cultured as explants and the leptomeningeal cells growing out of the explants can be examined. These cultures were performed by Sheryl Feng, research technician in the Dragunow laboratory.

Leptomeninges covering the middle temporal gyrus was carefully removed from underlying tissue using forceps. Small pieces of leptomeningeal tissue, ~2 x 3 mm, were placed into wells of a 6-well plate with ~850 μl (not so much that they were floating, but enough to surround them with nutrients) DMEM/F12 media supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin-Glutamine (Gibco) (final concentrations: penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (0.29 mg/ml)). Half the volume of media was changed twice in the first week, and then a full media change was done every 3-4 days. Cells started to grow out of the tissue after ~1 week. Leptomeningeal explants were passaged by moving to a new plate with forceps. For cytokine treatment, the explants were moved to a 24-well plate for 2 weeks to generate cells. The explants were then passaged into a new plate and the cells in the 24-well plate were left for 2 days before beginning cytokine treatment.

2.2.5. Differentiation of NT2 precursor cells into NT2Ns

NT2 precursor cells (Stratagene) were grown in DMEM/F-12 (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.29 mg/ml L-glutamine (complete DMEM/F-12; all from Gibco). Neuronal differentiation was induced using a method modified from Hill et al. (2008) and optimized as described in Chapter 7.
2.3. Transfection

2.3.1. siRNA transfection

2-3 days after plating, primary human mixed glial cultures were transfected with 25-100 nM siRNA delivered by Lipofectamine RNAiMax (Invitrogen) (5 μl per 1 μM siRNA) in DMEM/F12 supplemented with FBS and L-glutamine. The transfection mix was left at room temperature (RT) for 15 min prior to addition to cells. Antibiotics were not included in the transfection media and were only re-introduced 2 days after transfection. Cells were cultured for up to 7 days post-transfection. Protein knock-down was assessed by immunocytochemistry and western blot. Table 3.1 in Chapter 3 provides details of the siRNA sequences used.

2.3.2. DNA oligonucleotide transfection

Transfection of mixed glial cultures with sense and anti-sense PU.1 DNA oligonucleotides followed a protocol similar to siRNA transfection and is detailed in Chapter 3.

2.4. AlamarBlue cell viability assay

AlamarBlue® (AbD Serotec) is a cell viability indicator which takes advantage of the ability of metabolically active cells to convert the reagent into a fluorescent and colorimetric indicator (Hamid et al. 2004). The active ingredient of AlamarBlue (resazurin) is a nontoxic, cell permeable compound that is blue in colour and nonfluorescent. Upon entering cells, resazurin is reduced to resorufin by the reduced environment of metabolically active cells. Resorufin produces red fluorescence and is pink in colour. Viable cells continuously convert resazurin to resorufin, thereby generating a quantitative measure of viability.

At the conclusion of an experiment, 10 μl of AlamarBlue solution was added to each well and cells were incubated at 37 °C for 1 h. Fluorescence was measured on a FLUOStar Optima plate reader (BMG Labtech) with an excitation of 544 nm and an emission of 590 nm.
2.5. xCELLigence cell viability assay

The xCELLigence System (Roche Applied Science) was used to gain a real time measure of cell viability (Ke et al. 2011). Cells were cultured as usual but plated into specialized ‘E-plates’ fitted with microelectrodes on the bottom of the wells. Electrical impedance (resistance to flow of current through a circuit) was measured from the electrodes using the xCELLigence System. Impedance was measured throughout normal culture conditions at 37 °C. As cells settle onto the bottom of the tissue culture plate on top of the electrodes, they increase electrode impedance. Impedance is therefore a measure of cell number and cell viability (adherence to the tissue culture plate) (Ke et al. 2011). See Chapter 3 for details of xCELLigence cell viability assay during siRNA transfection experiments.

2.6. BrdU proliferation assay

At the conclusion of an experiment, 10 μM 5-bromo-2'-deoxyuridine (BrdU) was added to the cells for 24 h. Cells were washed twice with PBS to remove excess BrdU and fixed with 4% paraformaldehyde (PFA) for 15 min at RT. For immunocytochemistry, cells were first incubated with 2 M HCl at 37 °C for 30 min. Cells were then washed twice in 0.1 M borate buffer (pH 8.5) and three times in PBS before applying anti-BrdU antibody and performing immunocytochemistry.

2.7. Phagocytosis assay

To evaluate the effect of a treatment on primary adult human microglial phagocytosis, mixed glial cultures were incubated with amyloid-β1–42 amino acid peptide (Aβ42; Bachem) and phagocytosis assays were performed as previously described (Gibbons et al. 2011; Smith et al. 2010). At the conclusion of an experiment, 5 μM Aβ42 (dissolved in H2O and diluted 1:1 in PBS) was added to the cells for 24 h. Cells were washed twice with PBS to remove excess Aβ42 and fixed with 4% PFA for 15 min at RT. Thioflavin S stain was used to visualise phagocytosed Aβ42 (Smith et al. 2010). Cells were incubated with a 0.01% solution of Thioflavin S in 50% ethanol for 10 min at RT in the dark. Cells were then washed in 50% ethanol, followed by distilled H2O, for 10 min each. Thioflavin S-labelled
Aβ$_{42}$ was visualised by fluorescence microscopy using a fluorescein isothiocyanate filter. Cells were visualized with Hoechst nuclear stain or by immunocytochemistry for microglial proteins.

2.8. **Scratch injury assay for assessing migration and scar formation**

The *in vitro* scratch assay is a simple and well-characterised method to measure both cell migration (Liang et al. 2007) and scar formation (Lim et al. 2007; Moon et al. 2012). These two responses of the cells to scratch injury are comparable to events that occur *in vivo* following injury.

In Chapter 4 this assay is used to measure migration, and in Chapter 5 it is used to measure scar formation. The scratch injury assay was performed by scratching a monolayer of mixed primary human glia with a 10 μl pipette tip down the centre of a well of a 96-well plate. Cells were treated before and after scratch injury as detailed in Chapters 4 and 5. Cells were cultured and allowed to recover for 3 - 7 days before fixation.

2.9. **Cytokine and growth factor treatment**

Primary human brain cell cultures were treated in 96-well plates 1 or 2 days after plating. 1 μl cytokine was added to 100 μl media. Specific cytokine/growth factor diluents and treatment times are specified in each chapter.

2.10. **Immunochemistry**

2.10.1. **Immunocytochemistry**

To label cells cultured *in vitro*, cells were fixed in 4% PFA for 15 min at RT then washed for 10 min with phosphate-buffered saline containing 0.2% Triton X-100 (PBS-T). Antibodies were diluted in immuno-buffer (PBS-T containing 1% goat serum and 0.04% merthiolate). Cells were incubated with primary antibody (see tables in each results chapter for antibody details) overnight at 4 °C with gentle rocking. Alexa Fluor-conjugated and biotinylated secondary antibodies (see tables in each results
chapter for antibody details) were applied for 3 h at RT with gentle rocking. For colourimetric protein detection, ExtrAvidin-HRP was applied for 2 h at RT followed by 3,3’-diaminobenzidine tetrahydrochloride (DAB) reaction. PBS-T was used for all washes. For fluorescent double-labelling of cells using two primary antibodies (of different species), cells were incubated with both primary antibodies simultaneously, followed by both secondary antibodies (of different fluorophores) simultaneously.

2.10.2. Live cell antibody labelling

Some extracellular antigens (microglial cell surface receptors) were found to be better detected using a live cell antibody labelling technique. This technique allows antibody binding to extracellular epitopes prior to fixation (which may alter epitope structure). At the end of the experiment, primary antibody was diluted in cell culture media. Experimental media was carefully removed and replaced with antibody-containing media (50 μl per well) and incubated for 40 min at 37 °C. Antibody was then removed and cells were washed briefly 2x with fresh media to remove unbound antibody. Cells were fixed as usual and immunocytochemistry was continued with secondary antibody as above.

2.10.3. Immunohistochemistry

Adult human brain tissue donated to the Neurological Foundation of New Zealand Human Brain Bank was processed as described by Waldvogel et al. (Waldvogel et al. 2008). Fixed-frozen adult human brain autopsy and biopsy tissue was cut on a freezing microtome (Microm HM450) into 50 μm sections and processed for light and confocal microscopy according to procedures outlined by Waldvogel et al. (Waldvogel et al. 2007). Free-floating sections were incubated for 20 min in 50% methanol with 1% H₂O₂ in order to block endogenous peroxidase activity. Sections were then washed with PBS-T, and incubated in primary antibody for 3 days at 4 °C with gentle rocking. Following the primary antibody incubation, sections were washed and incubated overnight at RT in biotinylated secondary antibody before washing and incubating for 4 h at RT in ExtrAvidin-HRP. Sections were then washed and exposed to DAB with 0.01% H₂O₂ for 15–20 min to produce a brown reaction product. 0.4% nickel ammonium sulfate was added to the DAB to produce a darker reaction product when needed. After washing, the sections were mounted onto glass slides using a gelatin and chromic potassium sulfate solution and left to dry overnight. The slides were dehydrated through a graded alcohol series to xylene and coverslipped with DPX mounting medium (Merck).
Fluorescent labelling of tissue sections was carried out as above except that sections were mounted using PBS and coverslipped directly with Prolong Gold (Invitrogen). A Leica SP2 confocal laser-scanning microscope was used to obtain confocal images.

For double-labelling with two primary antibodies (from different species), sections were incubated with both primary antibodies simultaneously. For fluorescent detection, both secondary antibodies (of different fluorophores) were also applied simultaneously. For colourimetric detection, secondary and tertiary antibodies for each antibody species were applied sequentially; the first primary antibody was detected from secondary antibody through to DAB, then the second primary antibody was detected from secondary antibody through to DAB-Nickel.

2.10.4. Antigen retrieval

Antigen retrieval is the restoration of an antigen’s optimal immune reactivity (required for antibody binding) after formalin fixation (Dapson 2007). The process of tissue fixation with formalin modifies biological molecules by reacting with end groups to produce addition products. These addition products are reactive and can cross-link with other end groups within reach (Leong and Leong 2007). Antigen retrieval aims to break the cross-links formed by fixation which mask epitopes of interest. Thus antigen retrieval methods can be used for epitopes which fail to be detected by an antibody.

Specific procedures used for optimization of PU.1 protein detection in adult human brain tissue are described in Chapter 3.

2.10.5. Hoechst

To label nuclei, cells and tissue were stained with Hoechst 33258 (Sigma-Aldrich) for 30 min at RT protected from light. Cells were washed in TNE buffer (pH 7.4) containing 10 mM Tris, 200 mM NaCl and 1 mM EDTA prior to, and following, incubation in 20 μM Hoechst 33258 diluted in TNE buffer. Tissue sections were incubated in 1 μM Hoechst 33258 diluted in PBS-T buffer and PBS-T was used for washes.
2.10.6. Imaging

Microscopy of cells cultured and immunolabelled in 96-well plates was predominantly performed using an automated Discovery-1 fluorescence and brightfield microscope (Molecular Devices). See below for details on automated image acquisition and analysis.

Manual imaging of cells was occasionally used for cells that grew in clumps and for which automatic focusing was not successful (such as NT2N cells, Chapter 7). A Leica DMIRB inverted microscope with Leica DC100 camera was used for this purpose.

Immunohistochemistry slides were viewed and imaged on a Leica DMRB microscope with Nikon Digital Sight DS-U1 camera attached.

2.11. Western blot

2.11.1. Cell protein lysates

Cells intended for Western Blot analysis were cultured in 6-well plates. Wells were washed with PBS and 120 μl lysis buffer (2% sodium dodecyl sulphate, 4% glycerol and 62.5 mM Tris, pH 6.8; supplemented with protease inhibitor (Roche Diagnostics)) was added per well and cells were collected using a cell scraper (Nunc). Samples were heated to 95 °C for 10 min, cooled on ice and stored at -20 °C. Protein concentrations were determined using the Biorad DC assay.

2.11.2. Tissue protein lysates

Protein lysates were extracted from brain tissue using the Bullet Blender (Next Advance) homogenisation protocol. ~100 μg fresh-frozen brain tissue was put into a microcentrifuge tube and kept on ice. An equal volume (~100 μl) of glass beads (Next Advance) was added to the tube. 2x the mass of tissue (~200 μl) lysis buffer was added to the tube (Lysis buffer: 150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl pH 8, 5 mM EDTA; supplemented with protease inhibitor (Roche Diagnostics)). The tube was closed and placed in the Bullet Blender and the sample was blended at Speed 8 for 3 min. The tube was then visually inspected for complete homogenisation. If required, the tube was blended for another 2 min at Speed 9. After homogenisation, the sample was kept for 1 h on ice with interval vortexing every 10-15 min. The sample was then centrifuged for 10 min at 14000 rpm at 4 °C
and then the supernatant was collected and stored at -20 °C. Protein concentrations were determined using the Biorad DC assay.

### 2.11.3. Gel electrophoresis

All gel electrophoresis was performed using Invitrogen systems and buffers according to the manufacturer’s instructions. Samples with equal amounts of protein were loaded into pre-cast Bis–Tris gels and resolved by gel electrophoresis using the Xcell SureLock Mini Cell. Proteins were then electrotransferred onto polyvinylidene difluoride membrane (PVDF, Hybond-P; Amersham) using the Xcell Blot Module. After protein transfer, PVDF membranes were blocked for 1 h with 0.1% Tween-20 (Sigma) Tris Buffered Saline solution (TBST) supplemented with 5% skim milk on a rocker, and incubated with primary antibody (see tables in results chapters) diluted in 5% bovine serum albumin (Gibco)-TBST overnight at 4 °C with gentle shaking. PVDF membranes were subsequently washed 3 × 10 min with TBST and probed with species-specific secondary antibody (see tables in results chapters) linked to horseradish peroxidase diluted in 5% skim milk TBST for 2 h at RT with gentle shaking. Membranes were washed again and proteins visualised using the Amersham ECL Plus kit (GE Healthcare) and LAS-3000 imager (Fujifilm).

### 2.12. Reverse transcription and quantitative real time polymerase chain reaction

Quantitative real time reverse transcription-polymerase chain reaction (qRT-PCR) was carried out as described by Monzo et al. (Monzo et al. 2012). Total RNA was extracted from cells and tissue using Trizol® (Invitrogen) and RNeasy Mini Kit (Qiagen). ~100 mg fresh-frozen tissue was homogenised with 1 ml Trizol. Cells intended for qRT-PCR analysis were cultured in 6-well plates. Cells were washed with PBS and lysed directly in the culture dish by adding 1 ml of Trizol per well. The cell/tissue lysate was passed several times through a pipette and transferred to a microcentrifuge tube. After 5 min at RT, 0.2 ml chloroform per 1 ml Trizol was added to the tubes and they were shaken by hand for 3 min. Samples were then centrifuged at 12,000 g for 15 min at 4 °C. The upper aqueous phase was then transferred to a fresh tube with the addition of an equal volume of 70%
ethanol. From this point the RNA extraction procedure followed the Qiagen RNeasy Mini Kit instructions. RNA was stored at \(-80\,\text{°C}\) until use.

RNA samples were treated with DNase (Promega) and cDNA was formed using random hexamers and SuperScript III First-Strand Synthesis System (Invitrogen). RNA was quantified using the ‘Platinum SYBR Green qPCR SuperMix-UDG with ROX reference dye’ kit (Invitrogen) and Applied Biosystems 7900HT Fast Real-Time PCR System. Negative reverse transcriptase and non-template controls were run alongside test samples. Primers for PU.1 and \(\beta\)-actin are specified in Chapter 3.

### 2.13. Quantitative cytokine and chemokine measurement

The B.D. Cytometric Bead Array is a multiplexed, bead-based immunoassay that allows analysis of multiple analytes from a small sample. It is a flow cytometry method which can quantify multiple proteins simultaneously with great sensitivity (Morgan et al. 2004). It uses antibody-coated beads, each with a unique fluorescence intensity, to capture and quantify molecules of interest (in this case secreted cytokines and chemokines).

Conditioned media from experiments was collected after 4 days of treatment. The media was filtered using a 0.2 \(\mu\)m filter (Pall Life Sciences) and stored at \(-80\,\text{°C}\) until use. A Cytometric Bead Array (BD Biosciences) was performed according to the manufacturer’s instructions using a FACSAria II flow cytometer (BD Biosciences) (Burkert et al. 2012). A custom array of 16 cytokines/chemokines was measured from these samples.

### 2.14. Automated image acquisition and quantitative image analysis

Images of cells immunolabelled in 96-well plates were taken automatically and at high throughput (4 or 9 sites per well) by a Discovery-1 automated fluorescence microscope (Molecular Devices) using either brightfield or fluorescence microscopy. Images were analysed within experiments using the image analysis software Metamorph (6.2.6 software, Molecular Devices). Immunocytochemical, phagocytic and morphological observations have been quantified in this way. This system of high-throughput quantification has been previously described by our laboratory (Dragunow 2008b; Smith et al. 2010).
Immunocytochemical detection of protein expression was quantified using the *Count Nuclei* journal in MetaMorph (for round, nuclear staining patterns such as for the transcription factor PU.1) or the custom made journal *Standard Area Count* (for irregularly-shaped staining patterns such as the cell surface protein HLA). Phagocytosis was quantified using the *Count Nuclei* journal by adjusting the intensity threshold to segment the Thioflavin S-positive cells from background and adjusting the cell size thresholds to segment individual whole cells and exclude extracellular Thioflavin S-stained Aβ42 blobs. For quantification of microglial morphology, the Journal “Microglial Shape” was written in Metamorph. The journal automatically thresholded each image to isolate CD45-positive microglia, then applied the *Integrated Morphometry Analysis* tools *Elliptical Form Factor* (length/breadth) and *Shape Factor* \( \frac{4\pi A}{P^2} \), \( P = \) cell perimeter, \( A = \) cell area) to determine cell shape. The *Neurite Outgrowth* journal in MetaMorph was used to quantify number of cell processes. Results were logged automatically to Microsoft Excel spreadsheets. Where it was not possible to analyse images automatically, such as where cells were in clumps instead of monolayers, or had particularly complex morphology, cell number was counted by eye.

### 2.15. Statistical analysis

Data from representative experiments are displayed as mean ± standard error of the mean (SEM). SEM was used to indicate variability as it takes into account sample size as well as standard deviation. Each experiment contained a number of replicates (e.g. individual wells of a 96-well plate), usually 12-20 per condition. Experiments were also repeated separately with cells from different individuals. The number of cases used for a particular experiment varies throughout the thesis, due to inconsistent supply of primary human tissue, and is indicated throughout the results chapters. Statistical analysis was carried out using paired or unpaired t-tests where appropriate, or One-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. The F-test (for t-tests) and Bartlett’s test (for one-way ANOVA) were used to check for equal variances. The equivalent non-parametric test was used in cases of unequal variance (Mann Whitney test or Kruskal-Wallis test). The Kolmogorov-Smirnov test was used to check for normality. P values of <0.05 were considered statistically significant differences. Unless otherwise stated, data was collected and analysed for each case separately, rather than aggregated and analysed across the population.
Chapter 3.

The Transcription Factor PU.1 is Critical for Viability and Function of Human Brain Microglia

3.1. Abstract

Microglia are the predominant resident immune cells of the brain and can assume a range of phenotypes. They are critical for normal brain development and function but can also contribute to many disease processes. Although they are widely-studied, the transcriptional control of microglial phenotype and activation requires further research. PU.1 is a key myeloid transcription factor expressed by peripheral macrophages and rodent microglia. This chapter reports the presence of PU.1 specifically in microglia of the adult human brain and examines its functional role in primary human microglia. Using siRNA, substantial PU.1 protein knock-down was achieved in vitro. By assessing a range of characteristic microglial proteins, decreased viability of adult human microglia was found with reduced PU.1 protein expression. This observation was confirmed with PU.1 antisense DNA oligonucleotides. An important function of microglia is to clear debris by phagocytosis. The impact of loss of PU.1 on microglial phagocytosis was assessed and it was found that PU.1 siRNA reduces the ability of adult human microglia to phagocytose amyloid-beta_{1-42} peptide. Furthermore, from a screen of 16 cytokines, it was found that interleukin-6 concentration alone was increased in media from PU.1-depleted microglia compared to control microglia. These results show that PU.1 controls human microglial viability and function and suggest PU.1 as a molecular target for manipulation of human microglial phenotype.
3.2. Introduction

As the main resident immune cells of the brain, microglia are involved in most, if not all, brain diseases. Microglia are surveyors of the brain micro-environment and their motile processes constantly search for areas of local injury and disturbances in homeostasis where they respond to ameliorate the damage (Hanisch and Kettenmann 2007). Microglia are of myeloid origin (Ginhoux et al. 2010) and perform a variety of functions in the brain: cell-cell communication via secreted molecules; monitoring extracellular ion and neurotransmitter levels; surveying synapses; and clearing extracellular debris by phagocytosis (Graeber 2010; Hanisch and Kettenmann 2007). In the healthy brain, microglial protrusions have been shown to directly contact astrocytes, neuronal cell bodies, and blood vessels (Nimmerjahn et al. 2005). It is well documented that microglia have a spectrum of activation states under different conditions with different stimuli (Graeber 2010). However, we have a limited understanding of how they come to assume these different phenotypes and functions in the brain. Thus, the basic molecular mechanisms of microglial activation and their role in neurological disease require further research, as microglia are a potential target for treatments for many neurodegenerative diseases including Epilepsy, Alzheimer’s disease, Motor Neuron disease and Parkinson’s disease, as well as for acute insults such as Stroke and Traumatic Brain Injury (Klegeris et al. 2007; Saijo and Glass 2011; Tai et al. 2007; Yang et al. 2010).

Transcription factors are regulators of gene expression and therefore of cell phenotype. To address the question of control of microglial phenotype, the microglial transcription factor PU.1 was investigated. PU.1 is dynamically and heterogeneously expressed within hematopoietic lineages where high PU.1 expression activates genes of the myeloid lineage and PU.1 is expressed primarily in monocytic cells (Back et al. 2005). It has previously been shown in rodents that high levels of PU.1 protein are expressed in resting microglia as well as in activated microglia following injury (Walton et al. 2000).

Transcription of the PU.1 gene is regulated by other hematopoietic transcription factors, predominantly C/EBPα, as well as by PU.1 itself (Cai et al. 2007; Kummalue and Friedman 2003; Wang et al. 1999). In myeloid cells, C/EBPα facilitates PU.1 binding to its enhancer (Leddin et al. 2011; Yeamans et al. 2007). PU.1 can activate its own promoter elements in an autoregulatory loop (Chen et al. 1995) and PU.1 sense mRNA is further regulated by its own non-coding antisense transcript that controls translation of PU.1 sense mRNA (Ebralidze et al. 2008).
PU.1 transcription factor binds to a purine-rich sequence known as the PU-box found near the promoters of target genes, and regulates their expression in coordination with other transcription factors and cofactors. PU.1 controls a vast network of myeloid genes which are relevant to neuroimmunology and brain inflammation. PU.1 activates Erg2 transcription which in turn leads to up-regulation of several macrophage genes (Weigelt et al. 2009). PU.1 is essential for myeloid cell differentiation (Feng et al. 2008; Forsberg et al. 2010) and has been found to regulate genes involved in a range of monocytic functions such as phagocytosis and chemotaxis (Lloberas et al. 1999). For example, PU.1 regulates expression of secreted molecules tumour necrosis factor (TNF)-α (Fukai et al. 2009), interleukin-18 (Koyama et al. 2004) and CXCL9 (Ellis et al. 2010); cell surface receptors CXCR1 (Wilkinson and Navarro 1999) and CD11b (Pahl et al. 1993); and intracellular protein DAP12 (Henkel et al. 2002). Importantly, cells expressing PU.1 are responsive to Macrophage Colony-Stimulating Factor (M-CSF) (Henkel et al. 2002) due to PU.1 regulation of the M-CSF receptor gene c-fms (Zhang et al. 1994). Ghani et al. (2011) have provided evidence that PU.1 directly controls expression of at least four microRNAs (miR-146a, 342, 338, and 155), implicating PU.1 as a master coordinator of myeloid microRNA expression (Ghani et al. 2011).

Although most of the research on PU.1 and microglia has been carried out using rodent cells and models, it is becoming increasingly clear that there are important differences between rodent microglia and their human counterpart (Davis 2012; Dragunow 2008a). Age has also been shown to have an impact on immune cell responses and activation (Lynch et al. 2010; Streit 2006). Furthermore, the micro-environment of the brain may affect immune responses in ways that are different to those of the periphery. While the majority of research on PU.1 has been carried out on peripheral immune cells, PU.1 function has not been investigated in human microglia. In this study PU.1 expression is demonstrated in the adult human brain and the role of this transcription factor is investigated in adult human microglia. Using small interfering RNA (siRNA), it is shown that PU.1 is involved in adult human microglial viability, phagocytosis, and cytokine production.
3.3. Methods

3.3.1. Tissue

As outlined in Chapter 2 (General Methods), autopsy adult human brain tissue was obtained from the Neurological Foundation of New Zealand Human Brain Bank under the University of Auckland Human Subjects Ethics Committee. Biopsy adult human brain tissue was obtained from patients undergoing surgery for intractable temporal lobe epilepsy. This research was approved by the Northern Regional Ethics Committee and informed consent was obtained from all tissue donors.

3.3.2. Human glial cell isolation and culture

Glial cells were isolated from adult human brain tissue using methods previously described by Gibbons et al. (Gibbons and Dragunow 2010; Gibbons et al. 2011) and detailed in Chapter 2. Cells were cultured for 1-2 weeks, detached by trypsinization with 0.25% Trypsin/1 mM EDTA, and plated at 50,000 cells/ml on 96-well plates for experiments.

The yield of cells per tissue sample varied between cases, with an average of 2.2 +/- 0.4 x 10^6 cells per gram tissue (mean +/- SEM, n = 5 cases). The proportion of different cell types is also variable between cultures, with an average of 13.2 +/- 1.3% PU.1-positive microglia, and 1.1 +/- 0.02% GFAP-positive astrocytes (mean +/- SEM, n = 3 cases). Routine validation of cell phenotypes was conducted as per Gibbons et al. (Gibbons et al. 2007).

3.3.3. siRNA transfection

siRNAs used in this study are listed in Table 3.1 below. PU.1 and control siRNAs from Sigma-Aldrich were used for viability and functional studies. 2-3 days after plating, primary human mixed glial cultures were transfected with siRNA delivered by Lipofectamine RNAiMax (Invitrogen) (5 μl per 1 μM siRNA) in DMEM/F12 supplemented with FBS and L-glutamine. The transfection mix was left at room temperature (RT) for 15 min prior to adding to cells. No antibiotics were used in the transfection media and were only re-introduced 2 days after transfection. Cells were cultured for up to 7 days post-transfection.
siRNA | Company | Catalogue # |
--- | --- | --- |
PU.1 | Sigma-Aldrich | SASI_Hs02_00315880 |
Universal Negative Control #1 | Sigma-Aldrich | SiC001 |
PU.1 | Santa Cruz | SC-36330 |
Scrambled Negative Control | Santa Cruz | SC-37007 |
ATF2 | Santa Cruz | SC-29205 |
CREB | Santa Cruz | SC-29281 |

**Table 3.1: siRNAs used for knock-down of protein expression.**

### 3.3.4. DNA oligonucleotide transfection

PU.1 antisense (5’aacccctttcatcttgA), and control sense (5’tgcaaatggaaggtT), DNA oligonucleotides were purchased from Sigma-Aldrich. 2-3 days after plating, primary human mixed glial cultures were transfected with 150 nM oligonucleotides delivered by Fugene HD (Roche) (2.5 µl per 1 µM oligonucleotide) as for siRNA above.

### 3.3.5. Immunochemistry

To label cells cultured in vitro, immunocytochemistry and Hoechst staining of nuclei were performed as per Chapter 2. Table 3.2 below lists the antibodies used.

Tissue from 2 biopsy cases and 6 autopsy cases was used for immunohistochemistry. DAB and fluorescent labeling of tissue sections was carried out as in Chapter 2.

For acid and base antigen retrieval, sections from 3 autopsy cases were washed in H₂O and then incubated in either 2 or 4 M hydrochloric acid (HCl) or 1 mM ethylenediaminetetraacetic acid (EDTA) for 2 h at RT. Sections were then washed and processed as normal. No immunohistochemical labeling was seen when primary antibody was omitted.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Catalogue #</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-PU.1</td>
<td>Cell Signaling</td>
<td>2258</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-CD45</td>
<td>Abcam</td>
<td>ab8216</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-HLA-DP, DQ, DR</td>
<td>Dako</td>
<td>M0775</td>
<td>1:500 (cells); 1:1000 (tissue)</td>
</tr>
<tr>
<td>Mouse anti-GFAP</td>
<td>Sigma-Aldrich</td>
<td>G3893</td>
<td>1:5000</td>
</tr>
<tr>
<td>Rabbit anti-GFAP</td>
<td>Dako</td>
<td>Z0334</td>
<td>1:5000</td>
</tr>
<tr>
<td>Mouse anti-Prolyl 4-hydroxylase</td>
<td>Dako</td>
<td>M0877</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit anti-DAP12</td>
<td>Santa Cruz</td>
<td>Sc-20783</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-CX3CR1</td>
<td>Abcam</td>
<td>Ab8021</td>
<td>1:100</td>
</tr>
<tr>
<td>Goat anti-Iba-1</td>
<td>Abcam</td>
<td>Ab5076</td>
<td>1:1000</td>
</tr>
<tr>
<td>Mouse anti-CD68</td>
<td>Abcam</td>
<td>Ab955</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-CREB</td>
<td>Cell Signaling</td>
<td>9197</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-ATF2</td>
<td>Santa Cruz</td>
<td>Sc-187</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-NeuN</td>
<td>Chemicon</td>
<td>MAB377</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-MAP2</td>
<td>Sigma-Aldrich</td>
<td>M4403</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG Alexa Fluor® 594</td>
<td>Invitrogen</td>
<td>A11012</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-mouse IgG Alexa Fluor® 488</td>
<td>Invitrogen</td>
<td>A11001</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-mouse IgG Alexa Fluor® 594</td>
<td>Invitrogen</td>
<td>A11005</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG Alexa Fluor® 488</td>
<td>Invitrogen</td>
<td>A11008</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit biotinylated</td>
<td>Sigma-Aldrich</td>
<td>B7389</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-mouse biotinylated</td>
<td>Sigma-Aldrich</td>
<td>B7264</td>
<td>1:500</td>
</tr>
<tr>
<td>ExtrAvidin-HRP</td>
<td>Sigma-Aldrich</td>
<td>E2886</td>
<td>1:500 (cells); 1:1000 (tissue)</td>
</tr>
<tr>
<td>Mouse anti-β-actin</td>
<td>Abcam</td>
<td>A86276</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-rabbit horseradish peroxidase</td>
<td>GE Healthcare</td>
<td>NA934V</td>
<td>1:2000</td>
</tr>
<tr>
<td>Anti-mouse horseradish peroxidase</td>
<td>GE Healthcare</td>
<td>NA934V</td>
<td>1:2000</td>
</tr>
</tbody>
</table>

Table 3.2: Antibodies used for immunochemistry.
3.3.6. Western blot

Cells intended for Western blot analysis were cultured in 6-well plates and protein lysates were prepared as in Chapter 2. Protein concentrations were determined using the Biorad DC assay. All gel electrophoresis was performed using Invitrogen systems and buffers according to the manufacturer’s instructions (see Chapter 2). Proteins were visualized using the Amersham ECL Plus kit (GE Healthcare) and LAS-3000 imager (Fujifilm).

3.3.7. Reverse transcription and quantitative real time polymerase chain reaction

Quantitative real time reverse transcription-polymerase chain reaction (qRT-PCR) was carried out as described by Monzo et al. (Monzo et al. 2012) and detailed in Chapter 2. Briefly, total RNA was extracted from cells and tissue using Trizol (Invitrogen) and cDNA was formed using SuperScript III First-Strand Synthesis System (Invitrogen). PU.1 RNA was quantified using ‘Platinum SYBR Green qPCR SuperMix-UDG with ROX reference dye’ kit (Invitrogen) and Applied Biosystems 7900HT Fast Real-Time PCR System. Primers for PU.1 were obtained from Invitrogen (Forward: 5’ CGACCATTACTGGGACTTCCA; Reverse: 5’ GGAGCTCCGTGAAGTTGTTCTC). PU.1 RNA amount was made relative to β-ACTIN internal control.

3.3.8. AlamarBlue cell viability assay

Following treatment of cells with PU.1 siRNA, 10 μl of AlamarBlue solution (AbD Serotec) was added to each well and cells were incubated at 37 °C for 1 h. Fluorescence was measured on a FLUOStar Optima plate reader (BMG Labtech) with an excitation of 544 nm and an emission of 590 nm.

3.3.9. xCELLigence System cell viability measurement

Cells were plated as normal into ‘E-plates’ fitted with microelectrodes. They were immediately transfected with siRNA (PU.1, ATF2 or control) purchased from Santa Cruz, and impedance (as a measure of cell viability) was recorded using the xCELLigence System from day 0 to 2.5 weeks after transfection.
3.3.10. Phagocytosis assay
To evaluate the effect of PU.1 siRNA on primary adult human microglial phagocytosis of amyloid-β_{42} amino acid peptide (Aβ_{42}; Bachem), phagocytosis assays were performed as previously described (Gibbons et al. 2011; Smith et al. 2010). 4 days after siRNA transfection, 5 μM Aβ_{42} was added to the cells for 24 h. Cells were washed twice with PBS to remove excess Aβ_{42} before fixing with 4% PFA for 15 min at RT. Thioflavin S was used to visualize phagocytosed Aβ_{42} (Smith et al. 2010).

3.3.11. Quantitative image analysis of cell number and phagocytosis
Quantification of PU.1-immunopositive, CD45-immunopositive and total Hoechst-positive cell number, and phagocytosis of Aβ_{42} was achieved using a Discovery-1 automated fluorescence microscope (Molecular Devices) and Metamorph (6.2.6 software, Molecular Devices) image analysis system as previously described (Smith et al. 2010) (see Chapter 2 for details).

3.3.12. Cytokine measurement
Conditioned media from PU.1 siRNA experiments was collected 4 days after transfection. The media was filtered using a 0.2 µm filter (Pall Life Sciences) and stored at -80 °C until use. A Cytometric Bead Array (B.D Biosciences) was performed according to the manufacturer’s instructions using a FACSAria II flow cytometer (B.D Biosciences).

3.3.13. Statistical analysis
Representative data are displayed as mean ± standard error of the mean (SEM). Experiments were performed in at least triplicate. Statistical analysis was carried out using unpaired t-tests and One-way ANOVA followed by Tukey's multiple comparison test. P values of <0.05 were considered statistically significant differences.
3.4. Results

3.4.1. PU.1 is a marker of cultured adult human primary microglia

Isolation of cells from human autopsy and biopsy cortical brain tissue gives rise to a mixed population of cells including microglia, astrocytes and fibroblast-like cells (Gibbons and Dragunow 2010; Gibbons et al. 2011). With specific cell markers, microglia-specific effects can be identified and quantified within this mixed glia population (Gibbons et al. 2011). Primary microglia isolated from both biopsy and post-mortem adult human brain tissue, selectively express the transcription factor PU.1 and it was found that microglia always co-express PU.1 and leukocyte common antigen (the protein tyrosine phosphatase CD45), a pan microglia marker (Figure 3.1A-C). Levels of PU.1 expression in microglia are highly consistent between different cultures (representative images are shown in Figure 3.1). HLA-DP,-DQ,-DR is a highly inducible marker of adult human microglia and was expressed by some, but not all, PU.1-immunopositive microglia, suggesting that it may label a subset of ‘activated’ microglia in vitro (Figure 3.1D). However, all microglia expressing HLA-DP,-DQ,-DR also expressed PU.1 (Figure 3.1D) and no significant difference was found in PU.1 staining intensity between HLA-DP,-DQ,-DR positive or negative microglia. A range of other commonly used microglial markers was used to confirm PU.1 as a microglial-specific protein and found PU.1 to be co-expressed with all CD68 (a cytoplasmic glycoprotein), DAP12 (an adaptor protein expressed by cells of the monocytic lineage), CX3CR1 (fractalkine receptor) and Iba-1 (allograft inflammatory factor 1) immunoreactive microglia (Figure 3.2). PU.1 is specific for microglia in mixed glial cell populations isolated from human brain tissue as it was not found in glial fibrillary acidic protein (GFAP)-positive astrocytes (Figure 3.1E; Figure 3.3) or in fibroblast-like cells that express the collagen-synthesising enzyme prolyl 4-hydroxylase, a known marker of fibroblasts (Figure 3.1F).
Figure 3.1: Primary human microglia express PU.1 in vitro.

A) PU.1 (red) expression in microglial nuclei in mixed glial cell cultures isolated from adult human brain tissue. B) All microglia in mixed glial cell cultures co-express PU.1 (red) in their nuclei and CD45 (green) on their cell surface. C) Figure 3.1B overlaid with Hoechst to label all nuclei (blue). PU.1 is specifically expressed by CD45-positive microglia in mixed glial cultures. D) HLA (green) labels a subset of PU.1-positive microglia. E) GFAP-positive astrocytes (green) do not express PU.1 (pink). F) Prolyl 4-hydroxylase-positive fibroblast-like cells (green) also do not express PU.1 (pink). Scale bar = 50 µm.
Figure 3.2: All CD68, DAP12, CX3CR1 and Iba-1 immunoreactive adult human microglia co-express PU.1 in vitro.

A) CD68-positive microglia (green) express PU.1 (pink). B) Microglia expressing DAP12 (red) also express PU.1 (turquoise). C) CX3CR1-positive microglia (brown) are PU.1-positive (turquoise). D) Microglia co-express Iba-1 (brown) and PU.1 (turquoise). For C) and D) PU.1 primary antibody was first detected with goat anti-rabbit IgG Alexa Fluor® 488. CX3CR1 and Iba-1 primary antibodies were then applied and detected with goat anti-rabbit biotinylated secondary antibody, ExtrAvidin-HRP and DAB. All nuclei in the mixed glial cultures are labelled with Hoechst (blue). Scale bar = 50 µm.
Figure 3.3: GFAP-positive astrocytes in human adult mixed glial cultures do not express PU.1.

A) GFAP-positive astrocytes (green) identified with Sigma mouse anti-GFAP antibody. B) GFAP-positive astrocytes in same field of view (red) identified using Dako rabbit anti-GFAP antibody. C) Overlay of A and B with Hoechst shows that Sigma and Dako anti-GFAP antibodies label identical cells. D) Astrocytes (red) labelled with Dako anti-GFAP antibody are not PU.1-positive (turquoise). Scale bar = 50 µm.
3.4.2. PU.1 is a marker of microglia in the adult human brain

To confirm that PU.1 is expressed by microglia in the adult human brain, as well as in primary human microglia in vitro, immunohistochemistry for PU.1 was performed on fixed-frozen sections of human brain tissue. PU.1 was found to be ubiquitously expressed in biopsy human brain tissue in both the grey and white matter (Figure 3.4A). Double-labeling immunofluorescence studies were carried out with a range of glial and neuronal markers to confirm PU.1 as a microglia-specific marker. In adult human brain tissue, PU.1 was co-expressed by cells labeled with the other widely-used microglia markers HLA-DP,-DQ,-DR and CD45 (Figure 3.4B and C). However, as in vitro, HLA-DP,-DQ,-DR was only co-expressed by a subset of PU.1-immunopositive microglia. Co-labeling of PU.1 was not found with astrocytic (GFAP) or neuronal (Neuronal Nuclei (NeuN) and Microtubule-Associated Protein 2 (MAP2)) markers.

Figure 3.4: PU.1 protein is present in biopsy adult human brain tissue and is co-expressed with other microglial markers.

A) PU.1 (DAB, brown) is abundantly expressed in both the grey and white matter of biopsy adult human brain tissue. B) PU.1 (darker DAB-Ni nucleus indicated by arrow) and HLA-DP,-DQ,-DR (lighter DAB processes) label the same microglial cell. C) Confocal image of PU.1 (red) and CD45 (green) immunoreactive microglia. Hoechst (blue) stains all nuclei. D) Post-mortem adult human brain tissue does not show any PU.1 immunochemical staining. Scale bar = 20 μm.
In contrast to the strong PU.1 protein expression in microglia from adult human biopsy brain tissue, the same immunochemical method could not detect PU.1 protein in post-mortem brain tissue from a number of neurologically normal and diseased patients (Figure 3.4D). To determine whether this might be due to fixation-related effects, acidic (2 M and 4 M HCl) and basic (1 mM EDTA, pH 8) antigen retrieval methods were tested but did not result in specific PU.1 labeling (Figure 3.5).

**Figure 3.5: Immunohistochemical staining of PU.1 in biopsy and autopsy human brain tissue is not affected by acidic or basic pre-treatment.**

A) PU.1 is present in the grey (GM) and white (WM) matter of adult human biopsy brain tissue. B) PU.1 cannot be detected by immunohistochemistry in autopsy human brain tissue. Pre-treatment of tissue sections with 1 mM EDTA basic solution does not change the immunochemical detection of PU.1 in biopsy (C) or autopsy (D) tissue. Pre-treatment of tissue sections with 2 M or 4 M HCl acidic solutions also does not change the immunochemical detection of PU.1 in biopsy (E and G respectively) or autopsy (F and H respectively) tissue. Omission of the primary antibody does not result in any PU.1 staining in adult human biopsy (I) or autopsy (J) tissue. Scale bar = 50 µm.
3.4.3. PU.1 mRNA is expressed in the adult human brain

qRT-PCR was performed to determine whether, despite PU.1 protein being elusive in autopsy human brain tissue, PU.1 mRNA is present in the adult human brain. Levels of PU.1 mRNA expression were compared to those in a PU.1-positive human monocytic cell line (THP-1 cells), and to PU.1-negative fibroblast-like human brain cells (Figure 3.1F). PU.1 mRNA is indeed found at a considerable level in autopsy adult human brain tissue (Figure 3.6). Thus it is likely that PU.1 protein is degraded relatively quickly, prior to fixation of autopsy human brain tissue, compared to biopsy tissue.

![Figure 3.6: PU.1 mRNA is present in adult human brain tissue.](image)

qRT-PCR analysis reveals that PU.1 mRNA is expressed in autopsy adult human brain tissue. The THP-1 human monocytic cell line was used as a positive control for PU.1 mRNA expression. As a negative control, pure cultures of human brain fibroblast-like cells were obtained after 3-4 passages of mixed cell cultures and did not contain microglia or astrocytes.

3.4.4. Reduction of *in vitro* PU.1 protein expression by siRNA

In order to assess the function of PU.1 protein in adult human microglia, siRNA was used to reduce PU.1 protein expression. High transfection efficiencies were achieved in these primary adult cells and it was found that PU.1 siRNA produced 70.6 +/- 2.4% (mean +/- SEM; n=10) PU.1 protein knock-down 4-7 days post-transfection (Figure 3.7A, B and C). Quantification of PU.1 expression shows a significant decrease in the number of PU.1-positive cells (Figure 3.7D). This method of PU.1 protein reduction has produced consistent results in microglia from more than 10 biopsy and post-mortem tissue samples, demonstrating the efficacy of this method on microglia from a range of human cases, both diseased (Epilepsy, Huntington’s and Parkinson’s disease) and normal. Transfection of adult human microglia with siRNA was confirmed using a fluorescent-tagged siRNA to verify the uptake of siRNA by these cells (Figure 3.8).
Figure 3.7: Adult human microglia can be transfected with PU.1 siRNA to reduce PU.1 protein expression.

A) Microglia isolated from adult human brain tissue express high constitutive levels of PU.1 in their nuclei. B) Microglial PU.1 expression is greatly reduced at 7 days following transfection with PU.1 siRNA. C) Western blot showing reduced PU.1 expression after specific siRNA-targeted knock-down. β-actin was used as a loading control. D) Quantification of immunocytochemical PU.1 expression shows that PU.1 siRNA significantly decreases the number of PU.1-expressing cells after transfection. Scale bar = 50 μm.

Specificity of the loss of PU.1 protein expression was tested immunocytochemically using markers of other transcription factors expressed by adult human microglial cells: cAMP Response Element-binding protein (CREB) and Activating Transcription Factor 2 (ATF2). The levels of CREB and ATF2 transcription factor expression were not affected by PU.1 siRNA (Figure 3.9).
Figure 3.8: Adult human microglia in mixed glial cultures are transfectable with siRNA.

Fluorescent-labelled siRNA (green) is co-localised with adult human microglia immunolabelled for CD45 (red). Arrows indicate examples of transfected microglia. Other cells in the mixed glial culture are also transfected with siRNA (arrowheads). Scale bar = 50 μm.

Figure 3.9: PU.1 siRNA transfection of adult human microglia specifically reduces PU.1 protein expression but does not affect CREB or ATF2 transcription factor expression.

7 days following transfection, PU.1 protein expression (red bars) is decreased by PU.1 siRNA compared to control siRNA. Neither CREB (blue bars) nor ATF2 (green bars) expression is significantly changed by PU.1 siRNA.
3.4.5. Not all siRNAs are created equal

Whilst optimising the method for siRNA knock-down of PU.1 protein, it was found that a PU.1 siRNA sequence from the company Santa Cruz reduced cell viability markedly compared to the corresponding control siRNA sequence. The reduction in cell viability was verified by AlamarBlue assay, total cell number counts and xCELLigence cell viability measurements (Figure 3.10).

![Graph](image.png)

**Figure 3.10:** PU.1 siRNA purchased from Santa Cruz biotechnology company reduced viability of mixed glial cultures.

Mixed glial cultures containing microglia were transfected with control siRNA, PU.1 siRNA or siRNA targeting ATF2, a transcription factor expressed by adult human mixed glial cells. A) Cell viability measured by AlamarBlue assay is reduced by PU.1 siRNA, but not by ATF2 siRNA, compared to control siRNA. B) This effect is mirrored by reduced total cell number with PU.1 siRNA compared to control and ATF2 siRNA. C) An xCELLigence System was used to investigate this effect in more detail. The trace of electrical impedance (a reflection of cell viability) shows that while control siRNA (green line) and ATF2 siRNA (pink line) transfected cells have increased viability over time, PU.1 siRNA transfected cells (blue line) have reduced viability. Arrow indicates time at which cells were assessed for AlamarBlue viability, cell number, and immunochemistry.

To investigate whether this was a specific effect resulting from reduced PU.1 expression, fibroblast-like cell only cultures (which do not express PU.1; Figure 3.6) were transfected with PU.1 siRNA. Even without microglia, and therefore without PU.1 expression, reduced cell viability was observed with PU.1 siRNA compared to control siRNA. This signalled the possibility that the viability effect was a non-specific response of the cells to either the PU.1 siRNA or the negative control siRNA. A PU.1 siRNA sequence from a different company (Sigma) was then trialled and significant differences in cell
viability between the PU.1 siRNA and corresponding control siRNA were no longer observed (Figure 3.11). As the Sigma siRNA produced an impressive knock-down of PU.1 protein (Figure 3.7) without a non-specific reduction in cell viability, all further functional studies were performed with PU.1 and control siRNA from Sigma.

Isolations of mixed glial cells from adult human brain tissue were cultured until passage 3 or more when they consisted only of fibroblast-like cells and did not contain PU.1-positive microglia. Fibroblast-like cells were transfected for 5 days with Santa Cruz or Sigma control and PU.1 siRNA. Cell viability (A) and total cell number (B) were reduced by Santa Cruz siRNA but not by Sigma siRNA.

3.4.6. PU.1 siRNA reduces microglial viability

To determine the functional effects of loss of PU.1 expression with PU.1 siRNA, the long-term viability of mixed glial cultures was measured 7 days following transfection. At this time point PU.1 expression was greatly reduced (Figure 3.12A and B) while total glial cell number was not notably altered (Figure 3.12C and D). Using AlamarBlue solution to assess functional mitochondria in viable cells, it was found that PU.1 siRNA does not significantly affect overall primary glial cell viability (Figure 3.12G). Total cell counts of all Hoechst-labeled nuclei for control and PU.1 siRNA-treated cultures were also not significantly different (Figure 3.12H). Astrocytes and fibroblast-like cells are not affected by PU.1 siRNA (Figure 3.13).

Despite no significant loss in total cell viability, there was still a reduced number of CD45-immunoreactive microglia amongst cells with PU.1 knock-down (Figure 3.12E and F). The number of CD45-positive microglia was significantly decreased by PU.1 siRNA (Figure 3.12I), as was total CD45 protein expression measured by Western blot (Figure 3.12J).
**Figure 3.12: Reduced PU.1 protein expression decreases adult human microglial viability.**

A and B) PU.1 siRNA reduces PU.1 protein expression (green) 7 days following transfection. C and D) Total glial cell number (all nuclei labeled with Hoechst) is not affected by PU.1 siRNA. E and F) CD45 microglial cell surface protein expression (red) is reduced 7 days following transfection with PU.1 siRNA. Arrows and inset indicate disintegrating microglia. G) Total glial cell viability as measured by AlamarBlue assay was not changed by PU.1 siRNA. H) Quantification of total Hoechst nuclei does not show a significant difference between control and PU.1 siRNA. I) Quantification of CD45-positive microglial cell number shows a significant reduction in microglia number after PU.1, but not control, siRNA transfection. J) Western blot showing reduced CD45 expression with siRNA-induced PU.1 protein knock-down. β-actin was used as a loading control. Scale bar = 50 μm.
Figure 3.13: Astrocytes and fibroblast-like cells in adult human mixed glial cultures are not affected by PU.1 siRNA.

A) Astrocyte cell number, as measured by GFAP-labelled cells, is not changed by PU.1-specific siRNA. B) Viability of fibroblast-like cell cultures is comparable following control and PU.1-specific siRNA transfection.

Immunocytochemistry for other microglial proteins showed the same result as for CD45. It was found that the number of DAP12 and CX3CR1 immunoreactive microglia was decreased with PU.1 siRNA compared to control siRNA (Figure 3.14). There did not appear to be a specific decrease in staining intensity for DAP12 or CX3CR1 (Figure 3.14A and B; C and D respectively), only a decreased number of microglia expressing these proteins 7 days following transfection (Figure 3.14E and F respectively). In general, the PU.1-depleted microglia were less spread-out (insets in Figure 3.14C and D) and some showed signs of disintegration (Figure 3.12F, arrows and inset). These observations suggest that even though the microglia are still present with PU.1 siRNA, reduced levels of PU.1 expression are affecting their viability and phenotype.
Figure 3.14: Microglial DAP12 and CX3CR1 expression are reduced by PU.1 siRNA.

Adult human microglia in a mixed glial culture express DAP12 adaptor protein (A) and CX3CR1 fractalkine receptor (C). 7 days following transfection with PU.1 siRNA there is reduced expression of DAP12 (B) and CX3CR1 (D). Insets in C and D show representative morphology of cells. Quantification of immunocytochemical images shows a reduction in DAP12-immunoreactive (E) and CX3CR1-immunoreactive (F) microglia following PU.1 siRNA transfection compared to control siRNA transfection. Scale bar = 50 μm.
The finding that PU.1 siRNA reduces microglial viability was validated using DNA oligonucleotides against PU.1 as an alternative method of reducing PU.1 protein expression. As with siRNA, the antisense DNA oligonucleotides targeting PU.1 (150 nM) successfully reduced PU.1 protein expression compared to control sense oligonucleotides (Figure 3.15A and B). This was again followed by a reduction in the number of CD45-immunoreactive microglia (Figure 3.15B). Whilst the number of PU.1-expressing microglia was significantly reduced from 3 days post-transfection, the reduction in CD45-positive microglia was only evident 6 days post-transfection (Figure 3.15). The number of microglia is decreased at 6 days compared to 3 days with both sense and antisense oligonucleotides, but the specific reductions in PU.1 (at 3 and 6 days) and CD45 (at 6 days) expression are significantly greater for antisense oligonucleotides.

![Figure 3.15: Transfection of adult human mixed glial cultures with PU.1 antisense DNA oligonucleotides results in reduced PU.1 and CD45 immunoreactive microglia.](image)

A) 3 days after transfection, PU.1 expression in adult human mixed glial cultures is significantly reduced by PU.1 antisense DNA oligonucleotides, compared to sense oligonucleotides. However, CD45 expression is unaffected. B) At 6 days after transfection, both PU.1 and CD45 expression is significantly reduced by PU.1 antisense DNA oligonucleotides.

To determine the optimal siRNA concentration for subsequent functional studies and to investigate the effects of high siRNA concentration on overall cell viability, concentration-response experiments were carried out using 25-100 nM siRNA. Concentrations of PU.1 siRNA above 50 nM caused a general decrease in cell number, probably through non-specific actions. For this reason 50 nM siRNA was chosen for later experiments as it provided more than 50% PU.1 protein reduction (Figure 3.16A) without a significant decrease in total cell number (Figure 3.16B).
Figure 3.16: Transfection of adult human mixed glial cultures with PU.1 siRNA produces a concentration response in total cell number and in PU.1 expression.

A) PU.1 expression in mixed glial cultures is reduced concentration-dependently by PU.1 siRNA. A significant reduction in PU.1 expression, compared to control siRNA, is produced from 50 nM or more PU.1 siRNA. B) Hoechst-labelled nuclei provide a measure of total cell number. For 50 nM of siRNA, there is not a significant reduction in total glial cell number for control versus PU.1 siRNA.
3.4.7. PU.1 siRNA reduces microglial phagocytosis of Aβ42 peptide

Phagocytosis assays were performed with adult human microglia to investigate the role of PU.1 in this process. Cells were incubated with Aβ42 peptide following PU.1 siRNA transfection. With control siRNA transfection, phagocytosed Aβ42 was seen surrounding the microglial nuclei and filled up almost the entire cytoplasm (Figure 3.17A and C). With PU.1 siRNA treatment, there was a dramatic reduction in the amount of Aβ42 that had been phagocytosed by the microglia, concomitant with a reduction in PU.1 expression (Figure 3.17B and D). PU.1 knock-down significantly reduced the percentage of microglia that undertook phagocytosis of Aβ42 (Figure 3.17E).

Figure 3.17: PU.1 siRNA reduces phagocytosis of amyloid-β1-42 (Aβ42) by human microglia.

A) Microglial nuclei labelled with PU.1 (red) are surrounded by fluorescently labelled Aβ42 peptide (green) which has been phagocytosed by the microglia. B) PU.1 siRNA reduces PU.1 expression and dramatically reduces the amount of Aβ42 that has been phagocytosed by the microglia. C) and D) Microglial cells are labelled with cell surface marker CD45 (red). Hoechst (blue) labels all nuclei in the mixed glial culture. Aβ42 (green) can be seen inside control microglial cells (arrows, C) but not in those treated with PU.1 siRNA (D). E) PU.1 siRNA significantly reduces the percentage of microglia that undergo phagocytosis. Scale bar = 50 μm.
3.4.8. PU.1 siRNA increases microglial release of IL-6

Another important function of microglia is production and secretion of cytokines. To assess the role of PU.1 in the production of these immune signalling molecules, an array of cytokines in the conditioned media of control and PU.1 siRNA-treated microglia was measured using a Cytometric Bead Array (B.D Biosciences). A total of 16 cytokines were assessed, 10 of which (GM-CSF, IFNγ, TNF, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-7, IL-12p70 and IL-13) were not detected in conditioned media from siRNA transfected, nor untreated, adult human mixed glia cultures. IL-10 and MIP-1α were detected at very low levels (< 5pg/ml) in both transfected and non-transfected cells’ conditioned media. IL-6, IL-8, IP-10 and MCP-1 were expressed at moderate levels in non-transfected control cells’ conditioned media (0.5-10 ng/ml). Expression of these cytokines was reduced, but still present at moderate levels, in control and PU.1 siRNA treated cells’ conditioned media. The only significant difference in cytokine concentration was a notable increase in the amount of IL-6 in the conditioned media of PU.1 siRNA-treated cells compared to control siRNA-treated cells (Figure 3.18). Control siRNA-treated cultures produced 72.6 +/- 9.5 pg/ml (mean +/- SD; n=3) IL-6 and this increased to 225.8 +/- 54.8 pg/ml (mean +/- SD; n=3) when PU.1 expression was reduced by PU.1 siRNA.

![Figure 3.18: PU.1 siRNA increased microglial secretion of IL-6 but not IP-10.](image)

A) A B.D. Cytometric Bead Array was used to measure the concentration of IL-6 in conditioned media from primary adult human mixed glial cells 4 days after siRNA transfection. Control siRNA transfected glia produce basal levels of 72.6 +/- 9.5 pg/ml (mean +/- SD; n=3) IL-6, while PU.1 siRNA transfection increased the amount of IL-6 to 225.8 +/- 54.8 pg/ml (mean +/- SD; n=3). B) Analysis of the concentration of IP-10 in the same conditioned media did not show a difference between control and PU.1 siRNA-treated cells.
3.4.9. siRNA as a general method to investigate protein function in human adult microglia

CREB is another transcription factor expressed by human adult microglia (Figure 3.19A). CREB is also expressed by fibroblast-like cells in mixed glial cultures. CREB siRNA produces a substantial reduction in CREB protein expression, in microglia as well as in other cells in a mixed glial culture (Figure 3.19).

Figure 3.19: CREB siRNA reduces CREB expression in mixed glial cultures.

A) CREB transcription factor is highly expressed in mixed glial cultures. B-D) Transfection of mixed glial cells with siRNA targeted to CREB reduces CREB protein expression in a concentration-dependent manner. Scale bar = 50 μm.
3.5. Discussion

This study establishes the presence of PU.1 and investigates its function in microglia in the adult human brain. Microglia are known to play a significant role in central nervous system homeostasis and disease. Thus, learning more about the factors, such as PU.1, that control the flexibility of microglial phenotype could provide strategies for manipulating microglia actions in the brain.

Most research on PU.1 in microglia has been conducted on rodent models despite increasing evidence of important distinctions between rodent microglia and their human counterparts (Davis 2012; Dragunow 2008a; Gibbons et al. 2011; Smith et al. 2010). It may not always be appropriate to directly translate findings across different species or age groups. Thus, this chapter attempts to extend the current knowledge of PU.1 into the human context by investigating the role of PU.1 in microglia from the adult human brain. In doing so, these results may shed more light onto the role of PU.1 and microglia in the context of human neurodegenerative disease.

The importance of studying PU.1 in the context of the brain is highlighted in a paper by Ponomarev et al. (2010) which identifies a brain-specific microglia/macrophage deactivating C/EBPα-PU.1 pathway (Ponomarev et al. 2010). Furthermore, Durafourt et al. (2012) have found several differences between human adult microglia and blood-derived macrophages regarding cell surface protein expression, cytokine production and phagocytosis (Durafourt et al. 2012). Nevertheless, an increasing number of studies are investigating connections between the central nervous system and the peripheral immune system (Frankenberg et al. 2008; Perry 2010) and PU.1 may also influence the brain through its expression in peripheral immune cells (Weigelt et al. 2011).

### 3.5.1. PU.1 in the adult human brain

It has previously been shown that PU.1 is constantly expressed in rodent microglia (Walton et al. 2000). Here the results further demonstrate 1) PU.1 protein expression in primary adult human microglia cultured in vitro from biopsy and post-mortem brain tissue; 2) in situ expression of PU.1 protein in adult human biopsy brain tissue; 3) PU.1 mRNA expression in post-mortem adult human brain tissue (Figures 3.1-3.6).

The possibility that PU.1 expression in biopsy tissue is due to the underlying epileptic condition can likely be ruled out from the in vitro cell culture studies, in which PU.1 expression is seen in microglia from all cases regardless of disease state. A difference in levels of PU.1 in microglia from...
neurologically normal and neurologically diseased individuals was not observed, however, it would be interesting to investigate whether alterations in PU.1 expression are seen in affected areas of the brain for particular diseases. The lack of PU.1 immunoreactivity in autopsy human brain tissue (Figure 3.4D), despite PU.1 mRNA expression (Figure 3.6), is possibly due to fast degradation of the protein following death. A previous report of immunohistochemical staining of human post-mortem brain tissue did not find nuclear localization of several transcription factors despite nuclear staining in biopsy tissue (MacGibbon et al. 1997). As expected if the PU.1 protein degrades during post-mortem delay, antigen retrieval methods for immunohistochemistry in autopsy adult human brain tissue did not result in positive PU.1 labelling (Figure 3.5). The average post-mortem delay of tissue in the Neurological Foundation of New Zealand Human Brain Bank is greater than 10 h, but with shorter post-mortem delays, it may be possible to achieve PU.1 labelling in autopsy human brain tissue. Of course, it is also possible that the non-epileptic brain does not express PU.1 protein and that the observed expression of PU.1 protein in a range of non-epileptic and epileptic cell culture cases observed in this study is due to infiltration of peripheral monocytes that are known to express this protein. Although this is a possibility, it is unlikely as monocyte infiltration (and hence PU.1 protein) would also be expected in post-mortem tissue but no PU.1 protein was observed in this tissue. Furthermore, the pattern of expression of PU.1 in the epileptic brain is very widespread in regions that are unlikely to be part of the epileptic focus. While more inducible markers such as HLA-DP,-DQ,-DR may provide a measure of particular subsets of microglia, PU.1 identifies a larger microglial population and PU.1 can thus serve as a marker for microglia cultured from the adult human brain.

3.5.2. Reduction of in vitro PU.1 protein expression by siRNA

The tool used to investigate PU.1 function in adult human microglia was siRNA, which specifically targets and degrades PU.1 mRNA, reducing PU.1 protein levels. High transfection efficiencies were achieved in these primary adult cells with high levels of PU.1 protein knock-down 4-7 days post-transfection (Figure 3.7). Direct targeting of PU.1 protein found only in microglia brain cells allows analysis of microglia-specific functions within a mixed primary glial cell culture. While a pure microglial culture would remove the possibility of stand-by effects from other cells, a mixed glial culture better models the mix of cells present in vivo (Gibbons and Dragunow 2010). The outstanding efficiency with which these primary adult human cells are transfected demonstrates their usefulness as a model system for investigating basic cell biology as well as for drug discovery (Dragunow 2008a). If the ability of siRNA to knockdown PU.1 expression in human microglia in vitro
translates to human microglia in vivo, then this approach may also serve as a possible therapeutic strategy to modulate neuroinflammation in neurodegenerative disorders.

Nevertheless it is vital to rule out non-specific effects on cell viability which may occur as a result of extracellular or intracellular actions. As demonstrated in Figures 3.10 and 3.11, not all siRNA sequences are created equal and it is likely that the control or PU.1 siRNA sequence from Santa Cruz matched to an additional, undesired RNA target to produce non-specific effects on cell viability. By assessing expression of other transcription factors (Figure 3.9), and measuring astrocyte and fibroblast-like cell number (Figure 3.13), the specificity of Sigma control siRNA and PU.1 siRNA sequences used in this study has been verified.

It was also established that antisense DNA oligonucleotides give the same result of reduced PU.1 protein expression as siRNA (Figure 3.15). Both siRNA and antisense DNA oligonucleotides specific for PU.1 result in a reduced number of CD45-immunoreactive microglia (Figure 3.12 and 3.15 respectively). However, siRNA was more effective at reducing PU.1 protein expression at a lower concentration (50 nM) compared to DNA oligonucleotides (150 nM).

### 3.5.3. PU.1 siRNA reduces microglial viability

PU.1 has been shown to be an important factor in rodent myeloid cell differentiation, proliferation and survival (Anderson et al. 1998; Lloberas et al. 1999). Here it is shown that reduced PU.1 expression leads to reduced adult human microglial viability. In this chapter, the PU.1-depleted microglia were characterised with antibodies to CD45, DAP12 and CX3CR1, and a reduced number of microglia expressing these proteins was found (Figure 3.12 and 3.14). Anderson et al. (2001) have shown that CD45 is not expressed by PU.1-deficient myeloid cells and that PU.1 regulates murine CD45 gene expression (Anderson et al. 2001). The result of reduced DAP12 expression with reduced levels of PU.1 was similarly reported by Henkel et al. (2002) using mouse monocytic precursor cells and by Weigelt et al. (2007) using murine macrophage RAW264.7 cells (Henkel et al. 2002; Weigelt et al. 2007). While a reduction in the number of CD45 and DAP12 immunopositive microglia was observed with PU.1 siRNA, reduced staining intensity of CD45 or DAP12 was not observed in individual microglia treated with PU.1 siRNA. Either this experimental paradigm does not allow us time to see specific reductions in CD45 and DAP12 expression, or compensatory mechanisms/low turn-over rates result in stable CD45 and DAP12 protein expression in these adult human microglia.

Specific regulation of CX3CR1 gene transcription by PU.1 has not been reported, although several other transcription factor binding sites have been found for the human CX3CR1 gene promoter.
regions (Barlic et al. 2004; Garin et al. 2002). Nevertheless a reduction in the number of CX3CR1-expressing microglia was found when PU.1 expression was reduced by siRNA, suggesting that a general decrease in microglial cell number and reduced microglial viability result from loss of PU.1 expression. Furthermore, disintegration and rounding of some microglia after PU.1 siRNA transfection suggest a loss in membrane integrity (insets in Figure 3.12F, 3.14C and 3.14D).

3.5.4. PU.1 siRNA reduces phagocytosis of Aβ42 peptide

To assess the long-term effect of reduced PU.1 on human microglia function, their phagocytosis of Aβ42 was assessed. Phagocytosis is an important innate function of microglia during development and throughout adulthood. In Alzheimer’s disease microglia may be helpful in clearing the brain of extracellular Aβ42 deposits (Bard et al. 2000; Boche et al. 2010). However, microglial phagocytosis can also be detrimental in the case of ‘primary phagocytosis’ which actively induces cell death (Neher et al. 2011).

It was found that PU.1 siRNA consistently decreased microglial phagocytosis of Aβ42 (Figure 3.17). The PU.1-regulated genes which are involved in this process are yet to be confirmed but some potential candidates are cytoskeletal genes (Berclaz et al. 2002; Weigelt et al. 2009) and DAP12 adapter protein (Henkel et al. 2002). Berclaz et al. (2002) have previously shown that PU.1 rescues phagocytosis in mouse macrophages in a process involving cytoskeletal changes. Additionally, down-regulation of the M-CSF receptor in vivo could reduce M-CSF-stimulated phagocytosis (Zhang et al. 1994). There are also likely to be previously unidentified PU.1-regulated proteins or microRNAs which can impact the phagocytic process.

A phagocytosis assay was used which has previously been validated by confocal microscopy and cytochalasin D inhibition of actin polymerisation (Smith et al. 2010). However it is possible that some of the Aβ42 associated with microglia is adhered to the cell surface as opposed to phagocytosed.

It is possible that decreased phagocytic ability of microglia is a consequence of overall decreased microglial viability. However, previous reports demonstrate that decreased viability is not always associated with decreased phagocytosis. We have reported that whilst Valproic acid (VPA) treatment of BV2 microglia reduces their viability, it also increases their phagocytic capacity (Smith et al. 2010). Conversely, 1 mM VPA treatment of primary adult human microglia does not reduce their viability, but does reduce their phagocytic capacity alongside a decrease in PU.1 expression (Gibbons et al. 2011). Altogether this data suggests that there is a more specific mechanism(s) at play whereby reduced PU.1 leads to decreased phagocytosis in primary human microglia.
3.5.5. PU.1 siRNA increases microglial release of IL-6

Another significant function of microglia is to secrete soluble immune signalling molecules for communication between cells of the immune system and other cell types including neurons and astrocytes. An array of 16 cytokines/chemokines from the conditioned media of control and PU.1 siRNA-treated microglia was assessed. Of those cytokines and chemokines that were expressed at a consistently detectable level (IL-6, IL-8, IP-10 and MCP-1), there was a significant increase in the amount of IL-6 secreted into the conditioned media of PU.1 siRNA-treated cells compared to control siRNA-treated cells (Figure 3.18).

That cells with lower PU.1 expression produced greater amounts of IL-6, suggests that PU.1 normally functions to suppress IL-6 expression. It cannot be ascertained from the current results whether PU.1 is having a direct effect on IL-6 gene expression, or whether some downstream effect of reduced PU.1 is affecting IL-6 production, stability or release. As PU.1 is known to interact with many other transcription factors, it is possible that PU.1 usually binds to proteins which regulate IL-6 expression (Dendorfer et al. 1994), and that without PU.1, these proteins are free to activate IL-6 transcription.

Karlstetter et al. (2010) found a similar increase in IL-6 mRNA transcript levels in BV2 microglia transfected with siRNA to the PU.1-regulated protein Activated Microglia/Macrophage WAP Domain Protein (AMWAP) (Karlstetter et al. 2010). It is possible that the increase in IL-6 released from PU.1 siRNA treated cells in the current study involves reduced AMWAP activity. However, in BV2 cells Karlstetter et al. (2010) also found increased MCP-1 and IL-1β mRNA with AMWAP siRNA, whereas here no change was found in MCP-1 concentration, and no detectable IL-1β, in media from primary adult human microglia with PU.1 siRNA.

On the contrary, Fukai et al. (2009) noted a lack of effect on IL-6 with PU.1 siRNA (Fukai et al. 2009). Our differing results could be due to differences in species, tissue origin, age, and experimental time points. Nishiyama et al. (2004) found that bone marrow-derived cells require PU.1 in order to produce IL-6 in response to LPS (Nishiyama et al. 2004). In this context PU.1 may act by establishing a monocytic phenotype, rather than by a direct effect of PU.1 on IL-6 gene expression. The highly specific change in IL-6 expression presented in the current chapter is important for our understanding of this essential and multifunctional brain cytokine. IL-6 has numerous roles in the brain, both as a neurotrophic and neurotoxic factor (Spooren et al. 2011; Vezzani et al. 2011) and is of particular interest to epilepsy research in light of a recent meta-analysis of cytokine levels in epileptic patients which found that their IL-6 plasma concentrations were significantly elevated compared to controls (Yu et al. 2012).
siRNA is a technique that can theoretically be used to knock-down any protein expressed by human adult microglia to investigate its function in these cells. However, optimisation of each siRNA is necessary and the efficiency of protein knock-down is dependent on the particular RNA sequence targeted, the corresponding sequence of siRNA, and the dynamics of protein expression such as stability and turn-over rate.

Using siRNA, this chapter establishes the importance of the transcription factor PU.1 to microglia in adult human brains. It is shown here that PU.1 has many important roles in the function of human microglia. Ultimately we may be able to harness PU.1, or downstream targets of PU.1, to modulate microglial activity for treatment of brain disorders. PU.1 expression may be modulated by many external factors, and one such growth factor, Macrophage Colony-Stimulating Factor, forms the focus of the next chapter.
Chapter 4.

M-CSF is a Major Determinant of Adult Human Microglial Phenotype and Function

4.1. Abstract

Microglia are the primary immune cells of the brain whose phenotype largely depends on their surrounding micro-environment. Microglia respond to a multitude of soluble molecules produced by a variety of brain cells. Macrophage Colony-Stimulating Factor (M-CSF) is a cytokine found in the brain whose receptor is expressed by microglia. Previous studies suggest a critical role for M-CSF in brain development and normal functioning as well as in several disease processes.

Using biopsy tissue from patients with intractable temporal epilepsy and autopsy tissue, primary adult human microglia were cultured to investigate their response to M-CSF. Microglial phenotype was markedly changed following 96 hours exposure to 25 ng/ml M-CSF. A greater number of microglia were present in the M-CSF treated cultures as the percentage of proliferating (BrdU and Ki67-positive) microglia was greatly increased. A number of changes in protein expression occurred following M-CSF treatment including increased transcription factors PU.1 and C/EBPβ, increased DAP12 adaptor protein, increased M-CSF receptor (CSF-1R) and IGF-1 receptor, and reduced HLA-DP, DQ, DR antigen presentation protein. Furthermore, a distinct morphological change was observed with elongation of microglial processes. These changes in phenotype were accompanied by a functional increase in phagocytosis of Aβ1-42 peptide and increased migration in a scratch wound assay.

This chapter demonstrates that the cytokine M-CSF dramatically influences the phenotype of adult human microglia. Targeting the M-CSF receptor, for example with small molecule mimics, may provide a mechanism by which to modulate microglial functions for therapeutic benefit.
4.2. Introduction

Microglial cells are brain-resident immune cells that have many protective roles but can also contribute to neurological disease processes. The functional phenotype of microglia depends on the cell types and specific activating factors in their surroundings, and for this reason microglia are said to have an adaptive or acquired phenotype, reflecting their response to a collection of external signals (Butovsky et al. 2005). Secreted small molecules, such as cytokines and growth factors, allow microglia to communicate with each other, and with other immune and brain cells which express these receptors (Boulanger 2009). In this way microglia can be recruited to sites of injury, where they can respond by ameliorating the damage (Dijkstra et al. 2004; Lambert et al. 2010). However, there have also been numerous reports of detrimental microglial activity in response to injury, and neuroinflammation is thought to play a major role in pathogenesis of many neurological disorders, including epilepsy and Alzheimer’s disease (Akiyama et al. 2000; Saijo and Glass 2011; Sapp et al. 2001; Tai et al. 2007; Yang et al. 2010). The activation of microglia to a beneficial or detrimental phenotype can be described in terms of inducible protein expression, morphology, and functional outcomes such as cytokine production and phagocytosis (Graeber 2010; Saijo and Glass 2011). Modulating microglial phenotype towards a protective role is a potential strategy for the treatment of many brain disorders and thus the factors influencing microglial activation require further research.

One molecule that can influence microglial phenotype is the cytokine Macrophage Colony-Stimulating Factor (M-CSF). M-CSF is found in the brain and its receptor is expressed by microglia (Akiyama et al. 1994; Du Yan et al. 1997). M-CSF is produced by a range of cells in the developing and adult brain (Du Yan et al. 1997; Thery et al. 1990). M-CSF mRNA and protein have been found to be constitutively expressed by human fetal astrocytes and low levels of M-CSF mRNA and protein were detected in unstimulated microglia cultures (Lee et al. 1993). Du Yan et al. (1997) have also demonstrated M-CSF expression in neurons of the adult human brain.

Signaling through the M-CSF receptor (CSF-1R) is required for the development and differentiation of microglia (Ginhoux et al. 2010) and M-CSF has been shown to increase division of rodent and human fetal microglia (Lee et al. 1993; Vidyadaran et al. 2009; Yamamoto et al. 2010). M-CSF also has the ability to change microglial morphology (Brummer and Stevens 1994; Liu et al. 1994) and influence microglial activation (Imai and Kohsaka 2002; Liu et al. 2011). M-CSF signalling has been shown to be dependent on the transcription factor PU.1 (Henkel et al. 2002), a vital myeloid transcription factor expressed by microglia in the adult human brain (Chapter 3). Celada et al. (1996)
found that sense PU.1 expression constructs increased M-CSF-dependent proliferation in mouse bone-marrow macrophages, and antisense PU.1 constructs reduced proliferation in response to M-CSF (Celada et al. 1996). They also found that sense PU.1 constructs gave rise to increased M-CSF receptor expression, and it has previously been demonstrated that PU.1 binds to the c-fms promoter (Zhang et al. 1994).

While M-CSF is important for normal brain development and function (Michaelson et al. 1996), several studies have found abnormal levels of M-CSF associated with neurological diseases. M-CSF was demonstrated to be upregulated in brain tumors (Alterman and Stanley 1994; Papavasiliou et al. 1997) and a correlation was found between levels of M-CSF and HIV-associated cognitive impairment (Lentz et al. 2010). Furthermore, within 3 months of HIV therapy, levels of both M-CSF and viral RNA in the CSF were reduced (Lentz et al. 2010). Despite Boissonneault et al. (2009) reporting beneficial effects of M-CSF on cognitive impairment and Aβ1-42 deposition in a mouse model of Alzheimer’s disease, it has been suggested that increased M-CSF expression could contribute to Alzheimer’s disease pathogenesis (Boissonneault et al. 2009; Du Yan et al. 1997). Lue et al. (2001) cultured glia from adult human brains and found that M-CSF was elevated in Alzheimer’s disease compared to non-demented microglia (Lue et al. 2001). M-CSF receptor expression in microglia in human brain tissue was upregulated in lesions of Alzheimer’s disease and Amyotrophic Lateral Sclerosis (Akiyama et al. 1994). On the contrary, while microglia are found associated with multiple sclerosis lesions, the relative number of microglia expressing M-CSF and its receptor have been found to decrease (Werner et al. 2002). M-CSF is therefore considered a key factor in regulating microglial inflammatory responses in the damaged brain.

Despite this comprehensive body of literature, there are several caveats which may prohibit the linking of functional studies to observations in human brain tissue. Although most of the research on M-CSF and microglia has been carried out using rodent cells and models, it is becoming increasingly clear that there are important differences between rodent microglia and their human counterpart (Davis 2012; Dragunow 2008a). Age has also been shown to have an impact on immune cell responses and activation (Hart et al. 2012; Lynch et al. 2010; Streit 2006). Furthermore, the microenvironment of the brain may affect immune responses in ways that are different to those of the periphery (Lambert et al. 2010) and while the majority of research on M-CSF has been carried out on peripheral immune cells, the effects of M-CSF on adult human microglia have not been fully investigated. Thus the phenotypic profile of adult human microglia after exposure to M-CSF has been assessed and it was found that this cytokine dramatically influences their phenotype and activation state.
4.3. Methods

4.3.1. Adult human brain tissue and glial cell isolation

The primary adult human cells used for these experiments were obtained as described in Chapter 2 (General Methods).

4.3.2. M-CSF treatment

Mixed primary human glial cell cultures were given 2 concentrations of 25 ng/ml M-CSF at 0 and 48 h. Total time of M-CSF treatment was 96 h.

4.3.3. BrdU proliferation assay

Following 72 h exposure to 25 ng/ml M-CSF, 10 μM BrdU was added to the cells for 24 h. Cells were washed twice with PBS to remove excess BrdU and fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature (RT). For immunocytochemistry, cells were first incubated with 2 M HCl at 37 °C for 30 min. Cells were then washed twice in 0.1 M borate buffer (pH 8.5) and three times in phosphate-buffered saline (PBS) before applying anti-BrdU antibody.

4.3.4. Immunocytochemistry

Detection of proteins by immunocytochemistry was performed as in Chapter 2. The extracellular antigens CSF-1R and IGF-1R were found to be better detected using the live cell antibody labelling technique. Antibody details are listed in Table 4.1.

4.3.5. Immunohistochemistry

Fixed-frozen adult human brain tissue was immunolabelled with antibodies to DAP12 and CSF-1R (see Table 4.1) as outlined in Chapter 2. Proteins were visualised by HRP and DAB colourimetric detection.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Catalogue #</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-PU.1</td>
<td>Cell Signaling</td>
<td>2258</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-CD45</td>
<td>Abcam</td>
<td>ab8216</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-CSF-1R</td>
<td>Santa Cruz</td>
<td>Sc-692</td>
<td>1:50 (cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:500 (tissue)</td>
</tr>
<tr>
<td>Mouse anti-HLA-DP, DQ, DR</td>
<td>Dako</td>
<td>M0775</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-GFAP</td>
<td>Sigma-Aldrich</td>
<td>G3893</td>
<td>1:5000</td>
</tr>
<tr>
<td>Mouse anti-Prolyl 4-hydroxylase</td>
<td>Dako</td>
<td>M0877</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit anti-DAP12</td>
<td>Santa Cruz</td>
<td>Sc-20783</td>
<td>1:500 (cells)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:1000 (tissue)</td>
</tr>
<tr>
<td>Mouse anti-C/EBPβ</td>
<td>Santa Cruz</td>
<td>Sc-7962</td>
<td>1:250</td>
</tr>
<tr>
<td>Mouse anti-IGF-1R</td>
<td>Millipore</td>
<td>MAB1120</td>
<td>1:50</td>
</tr>
<tr>
<td>Mouse anti-BrdU</td>
<td>Roche</td>
<td>11170376001</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-Ki67</td>
<td>Dako</td>
<td>A0047</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG Alexa Fluor® 594</td>
<td>Invitrogen</td>
<td>A11012</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-mouse IgG Alexa Fluor® 488</td>
<td>Invitrogen</td>
<td>A11001</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-mouse IgG Alexa Fluor® 594</td>
<td>Invitrogen</td>
<td>A11005</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG Alexa Fluor® 488</td>
<td>Invitrogen</td>
<td>A11008</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit biotinylated</td>
<td>Sigma-Aldrich</td>
<td>B7389</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-mouse biotinylated</td>
<td>Sigma-Aldrich</td>
<td>B7264</td>
<td>1:500</td>
</tr>
<tr>
<td>ExtrAvidin-HRP</td>
<td>Sigma-Aldrich</td>
<td>E2886</td>
<td>1:500</td>
</tr>
</tbody>
</table>

Table 4.1: Antibodies used for immunochemistry.
4.3.6. Western blot

Protein was extracted from adult human brain biopsy tissue for Western blot analysis as detailed in Chapter 2. Gel electrophoresis and C/EBPβ protein detection were carried out using Invitrogen systems and buffers according to the manufacturer’s instructions.

4.3.7. Migration assay

The scratch injury migration assay involved scratching a monolayer of mixed primary human glia with a 10 μl pipette tip down the centre of a well of a 96-well plate. Cells were treated with either vehicle or M-CSF for 48 h prior to, and 48 h following, scratch injury. Cells were cultured and allowed to migrate for 3 - 5 days before fixation.

4.3.8. Phagocytosis assay

To evaluate the effect of M-CSF on primary adult human microglial phagocytosis of amyloid-β_{1–42} amino acid peptide (Aβ_{1-42}; Bachem), phagocytosis assays were performed as per Chapter 2 following 72 h exposure to 25 ng/ml M-CSF.

4.3.9. Quantitative image analysis of cell number, microglial morphology and phagocytosis

Immunocytochemical, phagocytic and morphological observations have been quantified using a Discovery-1 automated fluorescence microscope (Molecular Devices) and Metamorph (6.2.6 software, Molecular Devices) image analysis system as previously described (Dragunow 2008b; Smith et al. 2010) and detailed in Chapter 2.

4.3.10. Statistical analysis

Representative data are displayed as mean ± standard error of the mean (SEM). Experiments were replicated with cells from at least 6 different individuals. Statistical analysis was carried out using t-tests and P values of <0.05 were considered statistically significant differences.
4.4. Results

4.4.1. Adult human microglia express the M-CSF receptor in vitro

Primary mixed glial cultures containing microglia were prepared from biopsy and autopsy adult human brain tissue as previously described (Gibbons et al. 2007; Gibbons et al. 2011).

In mixed cultures of adult human glial cells, the M-CSF receptor (CSF-1R) was expressed by microglia. Protein expression of CSF-1R coincided with microglial cell surface protein CD45 (leukocyte common antigen) and microglial transcription factor PU.1 (Figures 4.1A-D). No CSF-1R expression was found on glial fibrillary acidic protein (GFAP)-positive astrocytes or collagen synthesising enzyme prolyl-4-hydroxylase-positive fibroblast-like cells in the mixed glial cultures (Figures 4.1E and F respectively).

Figure 4.1: The M-CSF receptor (CSF-1R) is expressed by adult human microglia in vitro.

A) Adult human microglia in culture labelled for CD45 cell surface protein (green). B) Same field of view showing microglial expression of PU.1 transcription factor (red). C) Brightfield image showing DAB-labelling of CSF-1R in the same field of view. D) Overlay of images A, B and C demonstrating co-localisation of CSF-1R with microglial markers CD45 and PU.1. E) CSF-1R (brown) is not co-expressed by GFAP-positive astrocytes (green) in mixed glial cultures (all nuclei labelled with Hoechst, blue). F) CSF-1R (brown) is not expressed by prolyl-4-hydroxylase-positive fibroblast-like cells (green) in mixed glial cultures (all nuclei labelled with Hoechst, blue). Scale bar = 50 µm.
4.4.2. M-CSF increases expression of microglial transcription factor PU.1

To assess the response of adult human microglia to M-CSF, the mixed glial cultures were treated with 25 ng/ml M-CSF for 96 h. The microglial transcription factor PU.1 and the pan microglial cell surface protein CD45 were used to identify and count the number of microglia (Figures 4.2A, B and 4.2C, D respectively). Significantly more microglia were observed and quantified in the cultures treated with M-CSF than vehicle-treated cultures (Figure 4.2E).

The number of PU.1-positive and CD45-positive cells was increased by M-CSF, reflecting the increase in microglial number. However, there was also a significant increase in PU.1 protein expression in microglia treated with M-CSF (Figure 4.2F). Thus, M-CSF increases the amount of PU.1 within microglia as well as PU.1-positive microglial number.

![Figure 4.2: M-CSF treatment increases the number of microglia and expression of PU.1 in primary adult human mixed glial cultures.](image)

A) PU.1-positive microglia (red nuclei) are present in glial cultures from human brain tissue. B) There are more PU.1-positive cells with M-CSF treatment compared to vehicle treatment. The increase in microglia number is also seen by immunostaining for microglial surface receptor CD45 (C and D). E) Treatment of human glial cultures with 25 ng/ml M-CSF for 72 h significantly increases the number of microglia present. F) M-CSF significantly increases the intensity of PU.1 expression (amount of PU.1 protein) in adult human microglia. Scale bar = 100 µm.
4.4.3. M-CSF increases proliferation of adult human microglia

To further investigate the finding that more PU.1-positive cells are present in cultures treated with M-CSF, microglial proliferation was assessed. Adult human microglia basally proliferate at a very low rate (% dividing microglia as measured by BrdU incorporation = 4.2 +/- 1.2% [mean +/- SEM; n = 6 cases]). However, treatment with M-CSF markedly increased microglial division (% dividing microglia after M-CSF treatment = 12.6 +/- 2.0% [mean +/- SEM; n = 6 cases]). M-CSF treatment resulted in a greater number of microglia expressing the cell division protein Ki67 (Figures 4.3A and B) and significantly increased BrdU incorporation by microglia (Figure 4.3C).

![Image](image.png)

**Figure 4.3: M-CSF increases the division of adult human microglia.**

A and B) Immunocytochemical images of CD45 microglial cell surface marker (red) and Ki67 cell division marker (green). Adult human microglia undergo limited proliferation in culture, as demonstrated by a lack of co-expression of Ki67 by CD45-immunoreactive microglia in (A). However, M-CSF treatment increases the number of dividing microglia, as shown by microglial nuclear expression of Ki67 (B). Arrows indicate examples of Ki67-immunopositive microglia. C) Quantification of the percentage of microglia that incorporate BrdU under control conditions and with M-CSF treatment showing a significant increase in microglial division with M-CSF. Scale bar = 50 μm.
4.4.4. M-CSF increases adult human microglial phagocytosis

Phagocytosis is a key innate function of microglia. Their ability to perform efficient phagocytosis is important for the healthy, as well as diseased, brain. In an assay of microglial phagocytosis of Aβ<sub>1-42</sub> peptide, M-CSF-treated microglia were more phagocytic than vehicle-treated microglia (Figures 4.4A and C, compared to 4.4B and D). A greater proportion of highly phagocytic microglia were found amongst M-CSF-treated cells compared to vehicle-treated cells and the percentage of phagocytic microglia from the total PU.1-positive microglia population is significantly increased with M-CSF (Figure 4.4C).

Figure 4.4: M-CSF increases microglial phagocytosis of amyloid-β<sub>1-42</sub>.

A) Microglial nuclei labelled with PU.1 (red) are surrounded by fluorescently labelled amyloid-β<sub>1-42</sub> peptide (green) that has been phagocytosed by the microglia. B) M-CSF treatment for 72 h increases the amount of amyloid-β<sub>1-42</sub> that has been phagocytosed by microglia. Another demonstration of this is shown in C) and D) where all nuclei are labelled with Hoechst and microglia are labelled with cell surface marker CD45 (red). Arrows indicate highly phagocytic microglia. E) M-CSF significantly increases the percentage of microglia that undergo phagocytosis. Scale bar = 50 µm.
4.4.5. M-CSF induces a change in microglial morphology

The morphology of untreated adult human microglia *in vitro* is heterogeneous, with cells having variable protrusions and extensions (Figure 4.5A). A striking effect of M-CSF on adult human microglia was a change in their morphology to elongated, slender, bipolar cells (Figure 4.5B). A shift in microglial shape to a ‘rod-like’ morphology is evident under a light microscope after 48 h of M-CSF treatment and is more pronounced at 96 h. This effect can be quantified using the Metamorph image analysis tools *Elliptical Form Factor* and *Shape Factor*. These measures of microglial shape show a significant morphological difference with M-CSF treatment (Figures 4.5C and D).

![Figure 4.5: M-CSF-treated microglia assume a rod-like shape.](image)

A and B) Immunocytochemical labelling of CD45 microglial cell surface protein shows the morphology of adult human microglia *in vitro*. A) Basal adult human microglial morphology is heterogeneous – some microglia are more rounded and others are more ramified. B) A shift in microglial shape to a rod-like morphology is evident after 48 h M-CSF treatment. This significant elongation of microglia with M-CSF can be quantified using Metamorph Elliptical Form Factor (elongation) (C) and Shape Factor (roundness) (D) image analysis tools. Scale bar = 100 µm.
4.4.6. M-CSF decreases HLA expression

To further characterise the phenotype of M-CSF-treated microglia and elucidate the functional significance of the M-CSF-induced change in morphology, the levels of HLA-DP, DQ, DR expressed by microglia were investigated. Human microglial expression of HLA-DP, DQ, DR is inducible and is highly variable between cases (from a sample of 10 cases; 4 cases had high basal microglial expression of HLA-DP, DQ, DR, 3 cases had moderate expression and 3 cases had no expression). In the majority (6 out of 7) of cases which had basal microglial HLA-DP, DQ, DR expression, M-CSF treatment was found to decrease the level of expression (Figures 4.6A and B). Quantification of microglial HLA-DP, DQ, DR expression clearly shows a significant reduction with M-CSF (Figure 4.6C).

When the morphological analysis quantification tools were applied to CD45-immunopositive and HLA-DP, DQ, DR-immunopositive microglia under basal conditions, there was no difference in Elliptical Form Factor or Shape Factor morphology parameters (data not shown).

A) Representative image of basal HLA expression (green) in cultured adult human microglia. HLA expression is highly variable between cases and is expressed by some, but not necessarily all, microglia (PU.1-positive nuclei, red). B) Following 96 h exposure to M-CSF, the level of microglial HLA expression was found to be decreased compared to basal levels. C) Quantification of the effect of M-CSF on HLA expression shows a significant reduction in the number of microglia expressing HLA.

Scale bar = 100 μm.

Figure 4.6: Microglial expression of HLA-DP, DQ, DR is reduced by M-CSF.

A) Representative image of basal HLA expression (green) in cultured adult human microglia. HLA expression is highly variable between cases and is expressed by some, but not necessarily all, microglia (PU.1-positive nuclei, red). B) Following 96 h exposure to M-CSF, the level of microglial HLA expression was found to be decreased compared to basal levels. C) Quantification of the effect of M-CSF on HLA expression shows a significant reduction in the number of microglia expressing HLA. Scale bar = 100 μm.
4.4.7. M-CSF increases microglial expression of C/EBPβ transcription factor and DAP12 adaptor protein

To investigate the molecular events precipitated by M-CSF treatment, changes in factors known to be associated with PU.1 were assessed. From the CCAAT enhancer-binding protein (C/EBP) transcription factor family, an increase in C/EBPβ expression within microglia in mixed glial cultures was detected with M-CSF treatment (Figure 4.7). A quantifiable increase in the percentage of PU.1-immunoreactive microglia expressing C/EBPβ was found following M-CSF treatment (Figure 4.7G). While the percentage of CD45-positive microglia expressing PU.1 (100%) did not change with M-CSF treatment, the percentage of CD45-positive microglia expressing C/EBPβ was increased with M-CSF treatment. C/EBPβ is known to interact with PU.1 and may be associated with the increase in PU.1 expression levels (Figure 4.2F). The presence of C/EBPβ protein in adult human brain tissue was confirmed by western blot. A clear band for C/EBPβ protein ~40 kDa was visible in blots with biopsy adult human brain tissue (Figure 4.7H).

![Images of immunostaining and western blot](image-url)

**Figure 4.7: M-CSF increases adult human microglial expression of C/EBPβ.**

A-C) C/EBPβ is expressed by PU.1-immunopositive adult human microglia in mixed glial cultures. D-F) M-CSF increases the intensity of C/EBPβ labelling in adult human microglia and the number of microglia which express C/EBPβ. G) Quantification of microglial C/EBPβ expression showing that M-CSF increases the percentage of microglia which express C/EBPβ. H) Western blot showing detection of C/EBPβ in adult human biopsy brain tissue from two different donors. Scale bar = 100 µm.
DAP12 is a myeloid adapter protein found in microglia in the human brain (Satoh et al. 2011). DAP12 protein expression was clearly detected in microglia in sections of adult human brain tissue (Figure 4.9A). Signalling through DAP12 has been associated with the PU.1 transcription factor (Henkel et al. 2002). It was found that M-CSF increased expression of DAP12 in adult human microglia such that, concurrent with the increase in PU.1 and C/EBPβ, there is a significant, quantifiable increase in intensity of DAP12 staining (Figure 4.8).

Figure 4.8: M-CSF increases adult human microglial expression of DAP12.

A) DAP12 monocyte/microglial adaptor protein is expressed by human microglia isolated from biopsy tissue. B) Treatment of microglia with M-CSF increases their expression of DAP12 as seen by increased intensity of immunochemical staining. C) Quantification of DAP12 staining intensity shows a significant increase in DAP12 expression with M-CSF treatment. Scale bar = 100 µm.
4.4.8. M-CSF increases microglial expression of M-CSF and IGF-1 receptors

As shown in Figure 4.1D, adult human microglia express the M-CSF receptor, CSF-1R, *in vitro*. Furthermore, on exposure to M-CSF (25 ng/ml) for 96 h microglial expression of CSF-1R was increased (Figure 4.10C and G). Again an increase in microglial number was observed after exposure to M-CSF but an increase in intensity of CSF-1R staining was also seen (Figure 4.10I).

Levels of another mitogenic growth factor receptor, Insulin-like Growth Factor 1 receptor (IGF-1R), were also assessed. IGF-1R has previously been reported to be linked to M-CSF signalling (Gow et al. 2010). M-CSF treatment co-incidentally increased microglial expression of both IGF-1 (Figure 4.10D, H and J) and M-CSF receptors (Figure 4.10C, G and I).

CSF-1R was detected on cells with microglial morphology in adult human brain tissue by immunohistochemistry (Figure 4.9B). However, no IGF-1R protein was detected in fixed adult human brain tissue with the antibody from Millipore used for immunocytochemistry.
Figure 4.10: M-CSF increases adult human microglial expression of CSF-1R and IGF-1R.

M-CSF treatment does not significantly alter the total number of Hoechst-labelled nuclei in human adult mixed glial cultures (A and E), but it does increase the number of microglia (B and F), labelled for PU.1 transcription factor. Together with an increase in microglia number, M-CSF produces an increase in microglial expression of its receptor CSF-1R (C and G) and the receptor for IGF-1 (D and H). A significant increase in intensity of receptor labelling is found for both CSF-1R (I) and IGF-1R (J) following M-CSF treatment. Scale bar = 50 µm.
4.4.9. M-CSF increases microglial migration in a scratch wound assay

Microglia can be highly motile cells when recruited to sites of injury. A measure for cell migration into an injury site is the scratch wound assay (Lim et al. 2007). Microglia were treated with vehicle or 25 ng/ml M-CSF for 48 h prior to the scratch injury being made. More M-CSF-primed PU.1- and CD45-positive microglia were found in the wound ‘gap’ area after 3 days than vehicle-treated microglia (Figure 4.11). Thus, M-CSF increases the ability of adult human microglia to enter into a scratch wound area.

Figure 4.11: M-CSF increases migration of microglia into a scratch wound ‘gap’.

Migration of microglia back into a scratch wound area is accelerated by M-CSF treatment. Microglia are labelled with PU.1 (A and B) and CD45 (C and D). More microglia are present in the scratch wound area (i.e. between the dotted lines) with M-CSF treatment (B and D) compared to vehicle (A and C). Scale bar = 250 µm.
4.4.10. The role of PU.1 in M-CSF-mediated microglia actions

Given the increase in amount of PU.1 protein expression in microglia with M-CSF (Figure 4.2), it was investigated whether PU.1 protein was necessary for the other effects of M-CSF on adult human microglia. siRNA was used to target PU.1 and knock-down PU.1 protein expression (as in Chapter 3). Following PU.1 protein knock-down (4 days after transfection) microglia were assessed for a range of responses to M-CSF.

Despite reduced PU.1 protein expression due to PU.1 siRNA, M-CSF was still able to have a multitude of effects on adult human microglia. PU.1 expression was still increased with M-CSF and microglial proliferation still increased (data not shown). In some experiments there was a slight difference in response to M-CSF between PU.1 siRNA and control siRNA-treated microglia. However, these differences were not consistent when repeated on microglia from multiple cases. For example, the effect of M-CSF on microglial morphology (Figure 4.5) was reduced in PU.1 siRNA-treated microglia in only half of the cases studied (data not shown).

4.4.11. The influence of other molecules on M-CSF microglial effects

As microglia in the brain have to respond to many signals simultaneously, not to just one growth factor or cytokine at a time, it was investigated whether the effects of M-CSF on microglia were altered after microglia had prior exposure to other compounds. Microglia were pre-treated with either Transforming Growth Factor (TGF)-β1 (10 ng/ml) or IGF-1 (10 ng/ml) growth factors, or the drug Valproic Acid (VPA; 1 mM) for 48 h (2 concentrations, at 0 and 24 h) and then treated with M-CSF in the standard way for 96 h. The M-CSF effects of increased PU.1 expression, increased proliferation, and morphology change were assessed. These effects of M-CSF on microglia were still apparent after treatment with TGFβ1, IGF-1 or VPA (data not shown).
4.5. Discussion

M-CSF has numerous interesting effects on adult human microglia, many of which may have significance for a range of neurological diseases.

4.5.1. M-CSF increases proliferation of adult human microglia

The numbers of PU.1 and CD45 immunopositive cells were increased by M-CSF treatment and it was shown that this is at least partially through an increase in microglial division. This division effect was immediately apparent as adult human microglia do not frequently divide when cultured in basic medium (DMEM/F12 + FBS + PSG). This observation is in line with previous studies noting a proliferation effect with M-CSF for human fetal microglia (Lee et al. 1993), and up-regulation of M-CSF following axotomy of the rat facial nucleus which triggered microglia to proliferate (Yamamoto et al. 2010). Many myeloid cells have been shown to have M-CSF growth dependence (Feng et al. 2008). It may be that adult human microglia require M-CSF for division, but not for survival, in vitro. The proportion of microglia that undergo division is specific to each individual patient. However, the result of increased microglial division with M-CSF treatment is consistent between cases.

4.5.2. M-CSF increases expression of PU.1 in adult human microglia

It has previously been shown that microglia in the rat brain, as well as the BV2 rodent microglial cell line, constitutively express a high level of PU.1 in both the ‘normal’ and post-injury state (Walton et al. 2000). It was shown in Chapter 3 that microglia in the adult human brain also express the transcription factor PU.1. Previous studies have demonstrated that PU.1 regulates the M-CSF receptor (Zhang et al. 1994), upstream of M-CSF signalling, and it was found here that PU.1 also acts down-stream of M-CSF signalling. PU.1 has been shown to be involved in M-CSF-dependent proliferation of mouse bone-marrow macrophages (Celada et al. 1996) and thus it is likely that PU.1 is involved in the M-CSF-induced proliferation of adult human microglia, as well as many of the other processes discussed below.
4.5.3. M-CSF increases adult human microglial phagocytosis

Phagocytosis is an important innate function of microglia, as part of their role to respond to cell injury and regulate the extracellular environment. It was found that M-CSF consistently increased adult human microglial phagocytosis of Aβ_{1-42} peptide by microglia from different cases, regardless of the starting level of phagocytosis. In the context of Alzheimer’s disease it is thought that microglia may be helpful in clearing the brain of extracellular deposits of Aβ_{1-42} protein (Bard et al. 2000; Rogers and Lue 2001). M-CSF has been shown to be beneficial in Alzheimer’s disease mouse models and one mechanism that may be responsible for this effect is increased phagocytosis of Aβ_{1-42} (Boissonneault et al. 2009). In another recent study using human microglia, M-CSF treatment (in combination with IL-4 and IL-13) increased microglial phagocytic ability for myelin debris compared to microglia stimulated with a combination of GM-CSF, IFNγ and LPS (Durafourt et al. 2012).

In Chapter 3 it was found that PU.1 is necessary for basal phagocytosis in human adult microglia. DAP12 also appears to be involved in the phagocytic process (Weigelt et al. 2007) and associates with proteins which have been found to play a role in phagocytosis, for example CD68, TREM2 and SIRPB1 (Satoh et al. 2011; Takahashi et al. 2005). Given the M-CSF-induced increases in PU.1 and DAP12 expression, it is likely that these proteins are involved in M-CSF-dependent phagocytosis in adult human microglia.

4.5.4. M-CSF induces a change in microglial morphology

One of the most prominent effects of M-CSF on adult human microglia is their change in morphology to bipolar, elongated (‘ramified’) cells. Microglial morphology is presumed to relate to their function, although exactly how is currently unclear. Round ‘amoeboid’ microglia are traditionally viewed as activated, inflammatory microglia. The M-CSF-induced morphology change could be a sign of microglia being ‘primed’ towards a particular activation state. However, microglial phenotype is multifaceted and M-CSF-induced elongation doesn’t prohibit microglia from changing morphology when exposed to other molecules, for example Aβ_{1-42} which induces changes to their cytoskeletons necessary for phagocytosis. Even though the M-CSF-treated microglia are more rod-shaped and ‘ramified’ than vehicle-treated microglia, they have an increased propensity to phagocytose compared to control microglia of heterogeneous morphology.

Durafourt et al. (2012) used the same concentration of M-CSF as part of an ‘alternative’ macrophage/microglia activation polarising protocol. Whereas macrophages under these conditions had more extended processes, no change was observed for microglia. It may be that the CD45
marker of microglia used here is better for observing morphological differences, or that their addition of IL-4 and IL-13 reduced the morphology effect seen in microglia (Durafourt et al. 2012). The M-CSF-induced ‘rod’-like effect has also been noted for human fetal microglia (Lee et al. 1993).

Elongated rod-shaped microglia have also been reported in situ in rodent models of neurological injury including ischemia (Lambertsen et al. 2011; Zhan et al. 2008). Graeber (2010) has reported on microglial rod cells observed in human brain tissue associated with cognitive symptoms and psychopathologies (Graeber 2010). Rod-shaped, elongated microglia have been reported in the Huntington’s disease cortex (Sapp et al. 2001) and in Subacute Sclerosing Panencephalitis, Alzheimer’s disease and Wilson’s disease brains (Wierzba-Bobrowicz et al. 2002). In concordance with the present findings, Wierzba-Bobrowicz et al. (2002) also noticed Proliferating Cell Nuclear Antigen co-labelling with rod microglia and reduced expression of HLA compared to more rounded microglia.

Rod-shaped microglia are relatively under investigated (Graeber 2010) and this study presents some of the first functional results relating to the findings of rod-microglia in human brain tissue. If M-CSF-treated adult human microglia are indeed in vitro correlates of the ‘rod’ microglia reported in diseased adult human brain tissue, this will provide an invaluable tool for investigation into this particular microglial phenotype. Furthermore, the method used here for quantifying microglial morphology is a quick, high throughput and unbiased way of assessing these changes, compared to laborious and subjective quantification by eye.

4.5.5. M-CSF decreases HLA expression

To determine the functional relevance of the morphology change and to look further at the activation state of these M-CSF-exposed microglia, it was investigated whether their expression of HLA was affected. HLA-DP, DQ, DR is an inducible protein and its expression by microglia varies widely between cases. It was asked whether this inducible expression of HLA-DP, DQ, DR was modulated by M-CSF and it was found that, despite an increased number of microglia, fewer microglia express high levels of HLA-DP, DQ, DR with M-CSF. HLA-DP, DQ, DR expression by microglia is often taken to represent an ‘activated’ or inflammatory microglial phenotype. These results suggest that M-CSF-treated microglia may be alternatively activated and have reduced antigen presentation capacity.
This M-CSF effect of reduced HLA-DR has also been noted for human fetal microglia (Lee et al. 1993). Furthermore, expression of HLA class II molecules was noted to be less intensive on the surface of microglial rod cells compared to neighbouring ramified microglia in neurologically diseased human brain tissue (Wierzba-Bobrowicz et al. 2002). In the different context of mouse monocytic precursor cells, Henkel et al., (2002) found that M-CSF-induced maturation increased MHC class II expression with IFNγ (Henkel et al. 2002). This finding indicates that the effect of M-CSF on HLA may be dependent on cell differentiation stage and/or species.

An HLA-DQ-derived peptide has been found to have anti-proliferative effects (Ling et al. 2000), suggesting that the decrease in HLA-DP, DQ, DR seen in the present study may be mechanistically related to the increase in microglial proliferation also seen with M-CSF. Thus decreased HLA may not only have implications for antigen presentation, but perhaps for other cellular functions like proliferation.

4.5.6. Increased expression of C/EBPβ and DAP12 in M-CSF-treated adult human microglia

To decipher the mechanisms by which these phenotypic changes occur in microglia following exposure to M-CSF, changes in factors known to be associated with the transcription factor PU.1 were explored.

CCAAT enhancer-binding protein (C/EBP) transcription factors are expressed throughout the body including the brain (Ramji and Foka 2002; Walton et al. 2000). The C/EBP family has already been shown to have a number of species-specific regulation processes and expression patterns (Ramji and Foka 2002). From the C/EBP transcription factor family an increase in C/EBPβ expression was detected within microglia in human mixed glial cultures.

C/EBPβ plays numerous roles in activation and differentiation of macrophages (Ramji and Foka 2002). It has been reported to have a role in inflammatory processes in rodents (Cortes-Canteli et al. 2008; Straccia et al. 2011; Valente et al. 2011) and may play a role in differentiated macrophage morphology (Gutsch et al. 2011). Although the results presented here find increased C/EBPβ along with increased microglial proliferation, C/EBPβ has been reported to inhibit proliferation of human THP-1 monocytic cells and murine macrophage-like cells (Gutsch et al. 2011). However, other reports have shown that C/EBPβ can promote proliferation (Ramji and Foka 2002) and furthermore, be involved in M-CSF-directed mechanisms in tumours (Marigo et al. 2010) and HIV infection
(Komuro et al. 2003). C/EBP transcription factors including C/EBPβ can transactivate the CSF-1R promoter in mammalian cell line COS-7 cells (Zhang et al. 1996), and could also be involved in the increase in CSF-1R expression observed here. C/EBPβ has recently been demonstrated in human spinal cord tissue from Amyotrophic Lateral Sclerosis patients, but rarely in controls, colocalised with the microglial marker CR3 (Valente et al. 2011). In this study C/EBPβ was also detected in adult human brain biopsy tissue from epilepsy patients (Figure 4.7H).

C/EBPβ forms heterodimers with members of its own family and interacts with several other transcription factors (Ramji and Foka 2002) including PU.1 (Tissieres et al. 2009; Yang et al. 2000). M-CSF-mediated enhanced PU.1 and C/EBPβ transcription factor protein expression have also been reported for a murine myeloblastic cell line (Carney et al. 2009). Furthermore, co-expression of PU.1 and C/EBPβ in fibroblasts can induce a macrophage phenotype (Feng et al. 2008). PU.1 and C/EBPβ transcription factors together may be responsible for many of the M-CSF induced effects found in adult human microglia.

DAP12 is a myeloid adapter protein found in microglia in the brain (Figure 4.9A). M-CSF treatment of adult human microglia increased their DAP12 expression. It has been shown to be involved in M-CSF-induced proliferation and survival of mouse bone marrow-derived macrophages (Otero et al. 2009). The concurrent increase in DAP12 protein expression with increased adult human microglia number and proliferation suggest a role for DAP12 in this M-CSF-induced mechanism. DAP12 could also be involved in the process of phagocytosis as primary mouse microglia transduced with mutant DAP12 have reduced phagocytic ability (Takahashi et al. 2005).

Henkel et al., (2002) demonstrated an upregulation of DAP12 in PU.1-rescued monocytic precursor cells and Weigelt et al., (2007) have shown that DAP12 expression is dependent on PU.1 via a binding site in the DAP12 proximal promoter (Henkel et al. 2002; Weigelt et al. 2007). Thus the M-CSF-induced increase in DAP12 expression may be directly mediated by the increase in PU.1. Furthermore, the role of M-CSF in determining PU.1 and DAP12 expression in microglia may have implications for many neurological diseases (Weigelt et al. 2011).

4.5.7. CSF-1R and IGF1-R expression are increased in M-CSF-treated adult human microglia

Immunocytochemical results show here that CSF-1R expression was restricted to microglia, and not detected on other cell types in these cultures, both basally and with M-CSF treatment (Figures 4.1 and 4.10). CSF-1R is also expressed by adult human microglia in situ (Figure 4.9B). In addition, it was
found that M-CSF increased the expression of its receptor on microglia. The increase in PU.1 found with M-CSF treatment is likely to directly increase CSF-1R levels as it has been reported to regulate c-fms transcript expression (Zhang et al. 1994). Yamamoto et al. (2010) have also found that M-CSF increases microglial CSF-1R expression in the context of the rat axotomized facial nucleus (Yamamoto et al. 2010).

Co-incidently M-CSF treatment also increased microglial expression of the IGF-1 receptor. There have been a number of associations previously reported between the growth factors M-CSF and IGF-1 (Gow et al. 2010). Both factors are mitogenic, play critical roles in development and have similar regulation mechanisms. In a study of mouse macrophage tumor cells, Wessells et al. (2004) found C/EBPβ to have a critical role in cell survival, in part by regulating expression of IGF-I. Furthermore, M-CSF was found to compensate for IGF-1 and could rescue IGF-1-deficient cells (Wessells et al. 2004). The overlapping functions of these ligands may explain the simultaneous increase in both the CSF-1 and IGF-1 receptors in response to M-CSF.

Another CSF-1R ligand, IL-34, has recently been discovered and is expressed throughout the body, including the brain (Lin et al. 2008). Like M-CSF, IL-34 is involved in human monocytic proliferation and viability (Chihara et al. 2010; Lin et al. 2008) but its biological activity and signal activation are not identical to that of M-CSF (Chihara et al. 2010). Furthermore, it has been suggested that the in vivo role of IL-34 may differ between rodents and humans (Chihara et al. 2010), and research into the effects of this cytokine on microglia in the human brain is warranted.

4.5.8. M-CSF increases microglial migration in a scratch wound assay

Microglia can be highly motile cells when recruited to sites of injury and their migration requires changes in morphology and extension of membrane protrusions (Kettenmann et al. 2011). Using a scratch wound assay, it was found that M-CSF promoted microglial migration into a wound site (Figure 4.11). As M-CSF also increases proliferation of microglia and thus microglia number, it is not clear from this assay whether M-CSF has a direct effect on microglial migration ability, or whether increased numbers of microglia are found in the gap area due to an overall increase in their number. The increase in microglial filling of the gap wound area with M-CSF was seen for the majority of cases on which this experiment was performed (3 out of 4 cases). However, limitations of this assay include variability in scratch area, and combined with variability inherent in adult human primary cultures, this means that the effect of M-CSF treatment is not seen every time.
**4.5.9. The role of PU.1 in M-CSF-mediated microglia actions**

To assess the role of the transcription factor PU.1 in the mechanism of M-CSF action on adult human microglia, PU.1 protein expression was reduced using siRNA (as in Chapter 3). Reduced PU.1 protein expression did not prevent M-CSF-induced microglial proliferation, morphology change, or an increase in PU.1 expression itself. Thus it seems that M-CSF can over-ride the lack of PU.1 produced by PU.1 siRNA. PU.1 does not appear to be crucial for the effects of M-CSF on microglia but it does seem to be involved due to its increase with M-CSF treatment. Perhaps the outcome of whether or not the M-CSF effect is inhibited by lack of PU.1 depends on the kinetics of de novo PU.1 production. Reduced PU.1 may be compensated for by other molecules which allow the M-CSF effect to still occur. Further experiments to delineate this issue could include a time course of M-CSF action and time course of PU.1 replacement. In light of the experiments with PU.1 siRNA alone, where knock-down of PU.1 protein expression results in reduced phagocytosis (Chapter 3), it is inviting to speculate that M-CSF increases microglial phagocytosis by a process involving the simultaneous increase in PU.1 expression.

**4.5.10. The influence of other small molecules on M-CSF microglial effects**

Although a single cytokine or growth factor can have an effect on microglial phenotype, at any one time microglia in vivo are exposed to a plethora of soluble molecules and other environmental cues. In an attempt to investigate combined effects of soluble molecules on microglia, microglia were pre-treated with TGFβ, IGF-1 or VPA and assessed for their subsequent response to M-CSF.

We have previously shown that VPA has several opposite effects to M-CSF on adult human microglia. VPA reduces microglial expression of PU.1 and reduces microglial phagocytosis (Gibbons et al. 2011). Despite these opposing actions, M-CSF still produced increased microglial PU.1 expression and increased microglial number following VPA treatment. The growth factor IGF-1 also did not alter the effects of M-CSF on microglia. This may be due to the overlapping, complementary functions of M-CSF and IGF-1. TGFβ similarly did not change the response of microglia to M-CSF. Although in the next chapter it is revealed that TGFβ reduces microglial number, subsequent M-CSF treatment still increased microglial number and proliferation. These findings demonstrate the flexibility of the microglial phenotype in response to opposing signals and show that M-CSF is a powerful modulator of microglial phenotype. Microglia are able to integrate many different environmental cues and respond dynamically to multiple factors in their environment.
M-CSF dramatically influences the phenotype of adult human microglia. These studies have found that M-CSF has many fascinating effects on these human brain cells and has an important role in determining microglial phenotype and function in the context of the adult human brain. Therapeutically, it would be desirable to have the ability to modulate microglial phenotype towards a protective role, and to this end, M-CSF-induced microglia are worthy of further investigation. Microglial phenotype can be differentially affected by many other soluble molecules. In the following chapter it is found that the cytokine interferon-γ produces a distinct microglial phenotype.
Chapter 5.

Differential Regulation of HLA and IP-10 in Adult Human Glia

5.1. Abstract

Human leukocyte antigen (HLA) is widely used as an indicator of glial activation. HLA expression is up-regulated in many neurological disease states, but the functional outcomes of this are not clear. Although most research on neuroinflammatory disorders uses rodent models, there are important differences between rodent glia and their adult human counterparts. This study investigates the effects of cytokines IFNγ, TGFβ1 and M-CSF on adult human glial inflammatory mechanisms, namely the inducible expression of HLA and production of cytokines and chemokines by microglia, astrocytes and brain-derived fibroblast-like cells.

Human microglial expression of HLA is inducible and highly variable between cases. Expression of HLA on human microglia was increased by exposure to IFNγ (1 ng/ml for 96 h), regardless of the level of basal HLA protein expression. Contrary to data in rodents, the anti-inflammatory cytokine TGFβ1 (10 ng/ml) did not affect this increase in HLA, nor did TGFβ1 affect basal microglial HLA expression. However, M-CSF (25 ng/ml) decreased both IFNγ-induced and basal microglial HLA expression. Brain-derived fibroblast-like cells do not basally express HLA but have a marked induction on exposure to IFNγ. Despite TGFβ1 having no effect on microglial HLA expression, TGFβ1 blocked the IFNγ-induced expression of HLA by brain-derived fibroblast-like cells. Conversely, M-CSF had no effect. Astrocytic expression of HLA was also increased by IFNγ, but was not modulated by TGFβ1 or M-CSF. IFNγ also increased adult human glial expression of pro-inflammatory cytokines and chemokines, particularly IP-10. TGFβ1 did not block the IFNγ-induced increase in IP-10 as it did for HLA induction in fibroblast-like cells. In addition, the role of TGFβ1 in scar formation was examined and found to inhibit microglia and fibroblast-like cell presence in a scratch wound area.

This study highlights species differences, cell type specificity and differential regulation in response to pro- and anti-inflammatory cytokines with major impact on their role in neuroinflammation in the adult human brain.
5.2. Introduction

Although the brain was long thought to have limited immunological activity, it is now appreciated that substantial immune activity occurs in the brain at a homeostatic level as well as during disease (Ransohoff and Brown 2012). Markers of immune activation are ubiquitously used to track disease progress, correlate with symptomology, and have become a major target for disease therapies (Politis et al. 2012). Brain-resident microglia are immune cells of myeloid origin. Microglia are the predominant antigen-presenting cell types of the brain and they perform a variety of functions including phagocytosis of debris, production of signalling molecules and monitoring extracellular ion levels (Hanisch and Kettenmann 2007). Immune surveillance of the CNS is important for many homeostatic processes. However, neuroinflammation is thought to contribute to the pathogenesis of many neurological disorders (Khandelwal et al. 2011; Klegeris et al. 2007; Walker and Sills 2012). A complete understanding of the phenotype of microglia in the adult human brain is still lacking as there is evidence that human adult microglia are different to fetal microglia and blood monocytes (Abutbul et al. 2012; Lambert et al. 2008). Dystrophic microglia have been identified in the aged human brain and ‘microglial senescence’ is a possible contributor to neurological decline (Lopes et al. 2008; Streit 2006). Furthermore, immune responsiveness changes with age and over time microglia may become increasingly activated (Perry 2010). The “activated” microglial phenotype can be assessed in multiple ways, including expression of proteins involved in functional activities such as antigen presentation, morphological changes, and functional activation such as production of cytokines and chemokines.

Other cells apart from microglia have immune roles in the brain. Astrocytes perform many homeostatic functions which impact on immune activity in the CNS, for example maintaining blood-brain barrier (BBB) integrity, glutamate recycling, and potassium buffering (Ransohoff and Brown 2012). Astrocytes also have many direct roles in the innate immunity of the CNS. They express innate immune receptors (e.g. TLR3 and CXCR3) and secrete soluble mediators which affect immune responses (e.g. TGFβ1, IL-6, and IL-10) (Farina et al. 2007; Goldberg et al. 2001). Astrocyte immune activity has been shown to play a specific role in several diseases including Alzheimer’s disease (AD) (Li et al. 2011) and epilepsy (Vezzani et al. 2011), partially through upregulated expression of pro-inflammatory cytokines.

Many other cells contribute to immune responses in the CNS, including cells at the blood-brain barrier such as pericytes (Dore-Duffy 2008; Pardridge et al. 1989), perivascular macrophages, perivascular mesenchymal stem cells (Paul et al. 2012) and other cells adjacent to the CNS.
parenchyma such as cells of the meninges (Ransohoff and Brown 2012). We have previously identified and characterized a population of fibroblast-like cells in cultures of adult human brain tissue that express the fibroblast markers prolyl-4-hydroxylase and fibronectin (Gibbons et al. 2007; Park et al. 2012). Although the exact origin of these proliferative and phenotypically diverse cells is unknown, they are likely to be of neurovascular and/or leptomeningeal origin as they express markers of pericytes and fibroblast-like cells (Park et al. 2012; Paul et al. 2012). Indeed whether these are one cell type with varied phenotype, or a mixture of distinct cells is presently unclear. These cells are referred to collectively as “fibroblast-like cells” for simplicity. This cell population exhibits distinct immune characteristics and is likely distributed throughout the CNS in ideal locations for immune interaction, both with the periphery and CNS (Paul et al. 2012).

An essential aspect of neuroinflammation is cross-talk between different cells of the immune and central nervous systems via cell surface proteins and secreted molecules. Human leukocyte antigen (HLA) is a cell surface antigen presentation protein. HLA-DP, DQ and DR classes present extracellular antigens to T cells and are the human-specific versions of the class II Major Histocompatibility (MHC) complex in vertebrates. There are numerous reports of increased HLA and MHC class II expression with brain injury and disease processes in both rodent models and human post-mortem brain tissue. Expression of HLA-DR has been reported in neurologically diseased human brain tissue for more than two decades (McGeer et al. 1988). For example, an increased number of HLA-DR positive microglia have been found in epileptic hippocampus compared to control human brain (Beach et al. 1995) and progressive accumulation and correlation to disease has been found for HLA-positive microglia in Huntington’s disease brain tissue (Sapp et al. 2001). MHC class II expression is increased in response to neuronal injury (Neumann et al. 1996) and dense focal clusters of HLA-DR immunoreactivity are visible at senile plaques in AD gray matter (Styren et al. 1990). While microglia are the predominant resident cell type in the brain to express HLA both in vitro and in situ, Styren et al. (1990) have shown that astrocytes in control and AD brains can also express HLA-DR (Styren et al. 1990). Given its upregulation in so many diseases, the regulation of HLA in the adult human brain is of great interest. Substantial research has been conducted on the regulation of HLA expression (O’Keefe et al. 2002), and it has become apparent that there can be species and cell type specific differences in its regulation.

A major cytokine known to influence HLA expression is the T-cell cytokine Interferon-γ (IFNy). IFNy acts through the MHC Class II Transactivator (CIITA), the master regulator of MHC II gene expression. It has been demonstrated in primary mouse microglia that IFNy induces CIITA mRNA expression and
subsequent expression of class II MHC (O'Keefe et al. 1999). Two other molecules which can affect HLA expression are Transforming Growth Factor β1 (TGFβ1) and Macrophage Colony-Stimulating Factor (M-CSF). The predominantly anti-inflammatory cytokine TGFβ1 has been shown to counteract the upregulation of HLA by IFNγ via inhibition of the expression of IFNγ-induced CIITA mRNA (O'Keefe et al. 1999; Piskurich et al. 1999). Mouse microglial cell lines have been shown to have differential activation of the CIITA gene by IFNγ and TGFβ1 (Pazmany and Tomasi 2006), and TGFβ1 blocks IFN-γ-induced CIITA mRNA accumulation in human cell lines (Lee et al. 1997). The effect of M-CSF on basal and IFNγ-induced HLA-DR has previously been investigated in human fetal astrocytes and microglia (Lee et al. 1993), where it was found that M-CSF reduced HLA-DR in microglia but not astrocytes (Lee et al. 1993). However, the relevance of these findings to the adult human brain is still to be determined.

The cytokine IFNγ not only affects immune responses by inducing expression of cell surface proteins but also produces changes in glial cytokine and chemokine production. The Interferon gamma-induced protein 10 (IP-10; CXCL10) is produced by a variety of cells in the brain in response to IFNγ. IP-10 belongs to a family of IFNγ-induced proteins including two other chemokines MIG (monokine induced by interferon gamma; CXCL9) and I-TAC (interferon-inducible T-cell chemoattractant; CXCL11). These chemokines function in selective trafficking of leukocytes, migration of glia and proliferation of various cell types (de Haas et al. 2007). IP-10 binds the G protein–coupled receptor CXCR3 which also has affinity for MIG and I-TAC (Weng et al. 1998). CXCR3 expression has been reported in the developing human brain (Van Der Meer et al. 2001) and in human and rodent cultured microglia and astrocytes (Biber et al. 2002; Flynn et al. 2003).

IP-10 and CXCR3 have been shown to be increased in several neurological disease states (Goldberg et al. 2001). IP-10 plays a particular role in viral infection as it is induced by the T-cell anti-viral cytokine IFNγ. As such, IP-10 was found to be elevated in the cerebrospinal fluid (CSF) from patients with viral meningitis (Sorensen 2004). In a study of human brain tissue, IP-10 immunoreactivity was not detected in HIV-negative brains, but was present in HIV-positive brains and further found to be induced in human neurons by HIV infection in vitro (Maingat et al. 2010). Many chemokines, including IP-10, play a role in tumours. CXCR3 expression, IP-10 release, and proliferation in response to IP-10 were all found to be higher in transformed grade III and IV glioma cells than in primary human astrocytes (Maru et al. 2008). Multiple sclerosis (MS) is an autoimmune demyelinating disease which involves a large recruitment of lymphocytes into the brain parenchyma. CXCR3-positive T cells are increased in blood (Balashov et al. 1999) and brain tissue (Simpson et al.
2000) of MS patients compared with healthy controls. Blocking IP-10 in the experimental autoimmune encephalomyelitis (EAE) mouse model of MS reduces the severity of the disease and the number of pathogenic T-cells in the inflamed CNS (Sorensen 2004). IP-10 is a common feature of other neurodegenerative conditions including AD (Xia et al. 2000). It is clear that IP-10 plays a profound role in neurological disease and the extracellular factors regulating IP-10 expression in the adult human brain require further investigation.

This study investigates the effects of cytokines IFNγ, TGFβ1 and M-CSF on adult human glial inflammatory mechanisms, namely the inducible expression of HLA-DP, DQ, DR and production of cytokines and chemokines by microglia, astrocytes and brain-derived fibroblast-like cells.
5.3. Methods

5.3.1. Human glial cell isolation and culture

Autopsy and biopsy adult human brain tissue was obtained as described in Chapter 2 (General Methods). Glial cells were isolated from adult human brain tissue according to the standard protocol (Chapter 2). Cells were cultured for ~1 week prior to plating for experiments at 50,000 cells/ml in 96-well plates. This initial passaging of cells consisted of a mixed glial culture containing microglia, astrocytes and fibroblast-like cells. To obtain cultures of fibroblast-like cells only, 3 or 4 subsequent passages were made (roughly 1 week apart, when cells had reached ~90% confluence) and the negligibly dividing microglia and astrocytes were no longer present.

5.3.2. Leptomeningeal explant cultures

Leptomeninges (from the same tissue as above) covering the middle temporal gyrus was cultured to obtain leptomeningeal fibroblast-like cells as described in Chapter 2. These cultures and experiments (Figure 5.8) were performed by Sheryl Feng, research technician in the Dragunow laboratory.

5.3.3. Cytokine treatment

Mixed primary human glial cell cultures were treated in 96-well plates. 1 µl cytokine was added to 100 µl media. Cells were treated with 1 ng/ml IFNγ (in PBS with 0.1% BSA) at 0 and 48 h. Total time of IFNγ treatment was 96 h. Cells were pre-treated with 10 ng/ml TGFβ1 (in 1 mM citric acid pH 3 with 0.1% BSA) or 25 ng/ml M-CSF (in H2O) at 0, 24 and 48 h. The last pre-treatment (at 48 h) was given at the same time as the first IFNγ treatment.

For experiments assessing the effects of TGFβ1 alone, cells were treated with 10 ng/ml TGFβ1 for a total of 96 h, applied every 24 h.

5.3.4. Immunocytochemistry

Chapter 2 details the protocol used for immunocytochemistry. In addition, cells were stained with Hoechst 33258 (Sigma-Aldrich) to label all nuclei.
### Table 5.1: Antibodies used for immunocytochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Catalogue #</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-HLA-DP, DQ, DR</td>
<td>Dako</td>
<td>M0775</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-PU.1</td>
<td>Cell Signaling</td>
<td>2258</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-CD45</td>
<td>Abcam</td>
<td>ab8216</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-GFAP</td>
<td>Dako</td>
<td>Z0334</td>
<td>1:5000</td>
</tr>
<tr>
<td>Mouse anti-Prolyl 4-hydroxylase</td>
<td>Dako</td>
<td>M0877</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit anti-αSMA</td>
<td>Abcam</td>
<td>ab5694</td>
<td>1:100</td>
</tr>
<tr>
<td>Rabbit anti-iP-10</td>
<td>Abcam</td>
<td>ab9807</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-CXCR3</td>
<td>Abcam</td>
<td>ab64714</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG Alexa Fluor® 594</td>
<td>Invitrogen</td>
<td>A11012</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-mouse IgG Alexa Fluor® 488</td>
<td>Invitrogen</td>
<td>A11001</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-mouse IgG Alexa Fluor® 594</td>
<td>Invitrogen</td>
<td>A11005</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG Alexa Fluor® 488</td>
<td>Invitrogen</td>
<td>A11008</td>
<td>1:500</td>
</tr>
</tbody>
</table>

#### 5.3.5. Quantitative cytokine and chemokine measurement

Conditioned media from experiments was collected after 96 h IFNγ treatment. The media was filtered using a 0.2 µm filter (Pall Life Sciences) and stored at -80 °C until use. A Cytometric Bead Array (B.D Biosciences) was performed according to the manufacturer’s instructions using a FACSArray II flow cytometer (B.D Biosciences) (Burkert et al. 2012).
5.3.6. Western blotting of IP-10 in conditioned media

Conditioned media was collected from cells as above and run directly through gel electrophoresis. 20 µl media was run per sample and Western blot was performed as for protein lysate samples described in Chapter 2.

5.3.7. Scratch injury assay to assess scar formation

The scratch injury assay was performed to assess scar formation. Monolayers of mixed primary human glia were scratched with a 10 µl pipette tip down the centre of wells of a 96-well plate. Cells were treated with either vehicle or TGFβ1 (10 ng/ml) for 48 h prior to, and 48 h following, scratch injury. Cells were allowed to recover for 4 - 7 days before fixation.

5.3.8. Quantitative image analysis of cell number, protein expression and microglial morphology

Immunocytochemical and morphological observations have been quantified as per Chapter 2 using a Discovery-1 automated fluorescence microscope (Molecular Devices) and Metamorph (6.2.6 software, Molecular Devices) image analysis system as previously described (Dragunow 2008b; Smith et al. 2010).

5.3.9. Statistical analysis

Representative data are displayed as mean ± standard error of the mean (SEM). Experiments were replicated with cells from at least 3 different individuals. Statistical analysis was carried out using t-tests and ANOVA. P values of <0.05 were considered statistically significant differences. Significant differences from vehicle (no cytokine treatment) are indicated.
5.4. Results

Primary mixed glial cultures containing microglia, astrocytes and fibroblast-like cells were prepared from biopsy and autopsy adult human brain tissue as previously described (Gibbons et al. 2007; Gibbons et al. 2011).

5.4.1. Microglial expression of HLA-DP, DQ, DR is increased by IFNγ and reduced by M-CSF but not by TGFβ1

Microglia are the predominant HLA-DP, DQ, DR-expressing cell type in human adult mixed glial cultures. Microglia from different cases express differing basal amounts of HLA-DP, DQ, DR. From 10 biopsy cases, 5 had high basal microglial HLA expression, 4 had moderate expression and 1 had low HLA expression (Table 5.2).

<table>
<thead>
<tr>
<th>Case number</th>
<th>Microglia</th>
<th>Astrocytes</th>
<th>Fibroblast-like cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>High</td>
<td>High</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>High</td>
<td>High</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>High</td>
<td>Moderate</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>High</td>
<td>Moderate</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>Moderate</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>Low</td>
<td>Low</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>Moderate</td>
<td>Low</td>
<td>None</td>
</tr>
<tr>
<td>8</td>
<td>Moderate</td>
<td>Low</td>
<td>None</td>
</tr>
<tr>
<td>9</td>
<td>Moderate</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>10</td>
<td>High</td>
<td>Moderate</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 5.2: Levels of HLA protein expression differ between biopsy cases.

In adult human mixed glial cultures, microglia and astrocytes express variable basal levels of HLA. However, HLA expression is not observed on untreated brain-derived fibroblast-like cells in culture.
HLA expression in adult human microglia was increased by exposure to IFNy (1 ng/ml), regardless of basal levels of expression (Figure 5.1A and B). Adult human glial cultures were immunostained for the microglial transcription factor PU.1 and the percentage of HLA-immunopositive microglia was found to significantly increase with IFNy (Figure 5.1G). The number of HLA-immunopositive microglia and the intensity of HLA expression were both increased by IFNy.

Contrary to the literature on rodent studies, TGFβ₁ (10 ng/ml) treatment of human adult microglia did not reduce (or enhance) IFNy-induced HLA expression. Furthermore, no effect of TGFβ₁ was observed for basal (vehicle-treated) microglial HLA expression (Figure 5.1C, D and G). The previous chapter demonstrates that M-CSF-treated adult human microglia have reduced expression of HLA compared to vehicle-treated microglia. This chapter further shows that M-CSF (25 ng/ml) in combination with IFNy significantly reduced the IFNy-mediated increase in microglial HLA expression (Figure 5.1E, F and G).

Furthermore, the role of PU.1 transcription factor in IFNy-induced microglial HLA expression was assessed using siRNA. As detailed in Chapter 3, PU.1 siRNA effectively reduced PU.1 protein expression. However, there was no evidence for the induction of HLA by IFNy being reduced in the absence of PU.1 (data not shown).

5.4.2. Microglial cell number is increased by IFNy and M-CSF, but reduced by TGFβ₁

The previous chapter reports an increase in microglia number following M-CSF treatment. Despite increased numbers of microglia, HLA expression was simultaneously reduced (Figure 5.1G and H). IFNy was also found to slightly increase microglia number compared to vehicle. However, the increase in microglial cell number produced by IFNy was not as great as for M-CSF (Figure 5.1H).

Although TGFβ₁ did not influence microglial expression of HLA, it did slightly reduce microglial cell number (Figure 5.1H). As TGFβ₁ reduced microglial cell number (as observed by both PU.1 and CD45 immuno-labelling), it was investigated whether TGFβ₁ had an effect on microglial expression of the critical transcription factor PU.1. Measures of PU.1 staining intensity showed no significant differences between vehicle and TGFβ₁ treatment (data not shown). Thus it does not appear that TGFβ₁ affects levels of PU.1 within individual microglia, only the number of PU.1-expressing cells.
Figure 5.1: Microglial expression of HLA-DP, DQ, DR is increased by IFNγ, not changed by TGFβ_1, and reduced by M-CSF.

A) Adult human PU.1+ve microglia (pink) express variable levels of HLA-DP, DQ, DR (green) in basal conditions without any treatment. B) IFNγ (1 ng/ml, 96 h) increased microglial expression of HLA-DP, DQ, DR, as well as HLA-DP, DQ, DR expression by astrocytes and fibroblast-like cells in the mixed glial culture. C) TGFβ_1 (10 ng/ml) did not affect microglial HLA-DP, DQ, DR expression alone, or when enhanced by IFNγ treatment (D). E) M-CSF (25 ng/ml) reduced basal HLA-DP, DQ, DR expression in microglia and also decreased IFNγ-enhanced HLA-DP, DQ, DR expression in microglia (F). Scale bar = 100 μm. G) A significant increase in percentage of HLA-positive microglia is found with IFNγ treatment. No change in microglial HLA-DP, DQ, DR expression is seen for TGFβ_1 treatment, but M-CSF significantly reduces microglial HLA-DP, DQ, DR protein expression. H) The number of microglia in culture, as measured by PU.1-immunopositive cells, is significantly increased by IFNγ and M-CSF. However, TGFβ_1 significantly reduces microglial cell number.
5.4.3. IFNy treatment results in microglia with a more rounded morphology

The morphology of untreated adult human microglia in vitro is heterogeneous, with cells having variable protrusions and extensions. Microglial morphology is presumed to relate to their function, although exactly how is currently unclear. Round ‘amoeboid’ microglia are traditionally viewed as activated, inflammatory microglia (Graeber 2010). A quantifiable change in microglial morphology was observed following 96 h IFNy treatment toward a rounded, less ramified shape (Figure 5.2A and B). The ‘elongation’ of microglia was significantly reduced by IFNy as shown using the Elliptical Form Factor image analysis tool in MetaMorph software (Figure 5.2C).

Figure 5.2: IFNy produces a change in microglia morphology to a more rounded, less elongated form.

A) Adult human microglia immunolabelled with the cell surface marker CD45 have a heterogeneous morphology in basal conditions without any treatment. B) IFNy (1 ng/ml, 96 h) resulted in microglia with a rounder morphology (arrows). Insets in A) and B) show representative morphology of cells. Scale bar = 100 μm. C) Quantification of the ‘rounding’ effect using Metamorph Elliptical Form Factor (a measure of elongation) image analysis demonstrates a significant shift in microglia shape following IFNy treatment to a more rounded and less elongated form.
5.4.4. Astroglial expression of HLA-DP, DQ, DR is increased by IFN\(\gamma\) but not affected by TGF\(\beta_1\) or M-CSF

Astrocytes from different cases express differing basal amounts of HLA-DP, DQ, DR. From 10 biopsy cases, 2 had high basal astrocytic HLA expression, 3 had moderate expression and 5 had low or no HLA expression (Table 5.2). Basal astrocytic expression of HLA was generally higher when microglial HLA expression was high (Table 5.2), but the percentage of astrocytes expressing HLA (<10%) was lower than for microglia (40%, Figure 5.1G and 5.3G).

Astrocytes were identified in human adult mixed glial cultures by expression of glial fibrillary acidic protein (GFAP). The number of astrocytes expressing HLA, and the amount of HLA expressed, was greatly increased in all cases by exposure to IFN\(\gamma\) (Figure 5.3A, B and G). TGF\(\beta_1\) and M-CSF had no effect on IFN\(\gamma\)-induced astrocytic HLA expression (Figure 5.3D, F and G). TGF\(\beta_1\) and M-CSF also did not influence basal HLA expression by astrocytes (Figure 5.3C, E and G).

While IFN\(\gamma\) increased HLA expression in astrocytes, it did not influence the number of GFAP-immunopositive astrocytes. TGF\(\beta_1\) and M-CSF did not affect GFAP-immunopositive astrocyte cell number either (Figure 5.3H).
Figure 5.3: Astrocytic expression of HLA-DP, DQ, DR is increased by IFNy, and not changed by TGFβ1 or M-CSF.

A) Adult human GFAP +ve astrocytes (red) express variable levels of HLA-DP, DQ, DR (green) in basal conditions without any treatment. B) IFNy (1 ng/ml, 96 h) increased astroglial expression of HLA-DP, DQ, DR. C) TGFβ1 (10 ng/ml) did not affect astrocyte HLA-DP, DQ, DR expression alone, or when enhanced by IFNy treatment (D). E) M-CSF (25 ng/ml) also did not affect basal HLA-DP, DQ, DR expression in astrocytes or IFNy-enhanced HLA-DP, DQ, DR expression in astrocytes (F). Insets show close-up examples of astrocytes indicated by arrows. Scale bar = 100 µm. G) A significant increase in percentage of HLA-DP, DQ, DR-immunopositive astrocytes is found with IFNy treatment. Neither TGFβ1 nor M-CSF significantly affect astrocyte HLA-DP, DQ, DR protein expression. H) Quantification of GFAP-immunopositive astrocyte cell number following treatment with IFNy, TGFβ1 or M-CSF does not result in any significant differences compared to vehicle-treated cells.
5.4.5. IFNγ induces HLA-DP, DQ, DR expression in brain-derived fibroblast-like cells

HLA induction was next investigated in the third population of cells in mixed human glial cultures: fibroblast-like cells (Gibbons et al. 2007). Pure cultures of brain-derived fibroblast-like cells were obtained after 3-4 passages of mixed glial cultures as they are the predominant cell type to divide basally in culture (Gibbons et al. 2007). These prolyl-4-hydroxylase (P4H)-immunopositive fibroblast-like cells do not express HLA basally without stimulation (Table 5.2 and Figure 5.4A). However, upon exposure to IFNγ they elicit a robust response by increasing HLA expression in a concentration-dependent fashion (Figure 5.4). This response was seen for cultures of fibroblast-like cells from both biopsy and post-mortem tissue from a range of neurologically diseased (Epilepsy, Alzheimer’s, Huntington’s and Parkinson’s disease) and normal individuals. Fibroblast-like cells had the same response whether in mixed cultures with microglia and astrocytes, or in cultures of fibroblast-like cells alone.

Figure 5.4: Brain-derived fibroblast-like cells do not express HLA-DP, DQ, DR protein under basal conditions but it is induced by IFNγ in a concentration-dependent manner.

A) In normal culture conditions of DMEM/F12 + 10% FBS + 1% PSG fibroblast-like cells do not express HLA-DP, DQ, DR protein. However, IFNγ (0.1-10 ng/ml, 96 h) induced a concentration-dependent increase in HLA-DP, DQ, DR expression (B-D). Scale bar = 50 µm.
To further characterise this population of ‘fibroblast-like cells’ which express HLA in response to IFNγ, the cells were double-labelled with antibodies to α-smooth muscle actin (α-SMA) and HLA-DP, DQ, DR. α-SMA is used as a marker of pericytes - pluripotent cells found adjacent to the vasculature (Dore-Duffy 2008). As seen in Figure 5.5, fibroblast-like cells expressing α-SMA did not necessarily express high levels of HLA-DP, DQ, DR following IFNγ stimulation.

Figure 5.5: αSMA-positive fibroblast-like cells do not express high levels of HLA-DP, DQ, DR.

Cultures of fibroblast-like cells contain αSMA-expressing cells (red) but this subset of cells is not induced to express particularly high levels of HLA-DP, DQ, DR (green) with IFNγ. Cells with high αSMA expression are indicated by arrows and cells with high HLA expression are indicated by arrowheads. Scale bar = 100 µm.

5.4.6. IFNγ-induced fibroblast-like cell HLA-DP, DQ, DR expression is inhibited by TGFβ1 but not by M-CSF

Whereas no effect of TGFβ1 on HLA induction was seen for adult human microglia, there was a major inhibition effect of TGFβ1 on fibroblast-like cells (Figure 5.6D and G). This response was seen for fibroblast-like cells alone and within mixed glial cultures with microglia and astrocytes present. Conversely, whereas microglial HLA induction was reduced by M-CSF, fibroblast-like cells were unaffected (Figure 5.6F and G). This is expected from previous findings of the M-CSF receptor (CSF-1R) being expressed only on microglia in primary human mixed glial cultures (Chapter 4).
Figure 5.6: IFNγ-induced expression of HLA-DP, DQ, DR in brain-derived fibroblast-like cells is inhibited by TGFβ1 but not by M-CSF.

A) Vehicle-treated fibroblast-like cells (Hoechst-labelled nuclei) do not express HLA-DP, DQ, DR protein. B) IFNγ induces a major up-regulation of HLA-DP, DQ, DR protein (green) in fibroblast-like cells. C) TGFβ1 treatment alone does not induce expression of HLA-DP, DQ, DR in these cells. However, TGFβ1 completely inhibits the IFNγ-stimulated increase in HLA-DP, DQ, DR (D). M-CSF affects neither basal (E) nor IFNγ-induced (F) HLA-DP, DQ, DR expression in fibroblast-like cells. Scale bar = 100 µm. G) HLA-DP, DQ, DR is induced by treatment with IFNγ, and inhibited by simultaneous exposure to TGFβ1 but not M-CSF. H) Fibroblast-like cell number is not influenced by IFNγ or M-CSF but is significantly decreased by TGFβ1.

IFNγ or M-CSF treatment had no effect on total number of fibroblasts as measured by Hoechst staining of nuclei in fibroblast only cultures (Figure 5.6H). However, TGFβ1 was found to reduce fibroblast cell number (Figure 5.6H). AlamarBlue cell viability assays showed a significant reduction in viability of fibroblasts following TGFβ1 treatment (Figure 5.7A). Furthermore, in BrdU cell proliferation assays a smaller number of dividing cells was observed with TGFβ1 compared to vehicle (Figure 5.7C). Thus TGFβ1 reduces fibroblast cell number by reducing fibroblast proliferation. An increase in apoptotic-looking, blebbing nuclei was not observed.
Cultures of fibroblast-like cells were treated with TGFβ1 for 96 h. A) TGFβ1 reduces viability of fibroblast-like cells as measured by AlamarBlue viability assay. B) Total cell number, measured by Hoechst-labelled nuclei, is also reduced by TGFβ1. C) TGFβ1 decreases the number of cells that incorporate BrdU in a cell proliferation assay.

5.4.7. IFNy also induces leptomeningeal fibroblast-like cells to express HLA-DP, DQ, DR

To further study the induction of HLA-DP, DQ, DR in fibroblast-like cells, explant culture studies were performed. Explant cultures were generated from leptomeninges overlying the middle temporal gyrus from both biopsy epilepsy specimens and autopsy specimens. The explant cultures generated cells over 1-2 weeks in 24-well plates. Once confluent, the explants were removed (and placed in a new 24-well plate) and the remaining adherent cells were characterised using antibodies to prolyl-4-hydroxylase and fibronectin for fibroblast-like cells, and CD45 and PU.1 for leptomeningeal/perivascular macrophages. The majority of cells (> 95%) were prolyl-4-hydroxylase and fibronectin-immunopositive meningeal fibroblast-like cells, with scattered CD45 and PU.1-immunopositive leptomeningeal/perivascular macrophages. HLA-DP, DQ, DR was absent in untreated leptomeningeal-explant fibroblast-like cells, but present in leptomeningeal/perivascular macrophages (Figure 5.8A). This pattern of staining matches closely that found in the fibroblast-like cells and microglia derived from dissociated mixed glial cultures used in this study. IFNy induced strong expression of HLA in leptomeningeal-explant derived fibroblast-like cells (Figure 5.8B and E). This expression was again completely blocked in fibroblast-like cells by TGFβ1 (Figure 5.8D and E).
Figure 5.8: IFNy-induced expression of HLA-DP, DQ, DR in leptomeningeal fibroblast-like cells is completely blocked by TGFβ₁.

A) In vehicle-treated leptomeningeal explant cultures only leptomeningeal/perivascular macrophages express HLA-DP, DQ, DR (indicated by arrows). B) IFNy increases intensity of HLA expression on macrophage cells and greatly induces HLA expression in leptomeningeal fibroblast-like cells. C) TGFβ₁ has no effect on basal leptomeningeal/perivascular macrophage or fibroblast-like cell HLA expression. D) However, TGFβ₁ completely inhibits IFNy-induced fibroblast-like cell HLA expression, without affecting leptomeningeal/perivascular macrophage HLA expression. DAB brightfield images have been inverted for image analysis. Scale bar = 100 µm. E) Quantification of HLA expression shows a massive increase in HLA expression in leptomeningeal explant cultures with IFNy but not with TGFβ₁ + IFNy.
5.4.8. IFNγ treatment of primary adult human mixed glia results in increased pro-inflammatory cytokine and chemokine release

Another important function of microglia is production and secretion of cytokines. To assess the effect of IFNγ on the production of these immune signalling molecules, an array of cytokines and chemokines in the conditioned media of vehicle control and IFNγ-treated mixed glial cultures (containing microglia, astrocytes and fibroblast-like cells) was measured using a Cytometric Bead Array (B.D Biosciences). A total of 16 cytokines was assessed, of which 10 (GM-CSF, IFNγ, TNF, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-7, IL-12p70 and IL-13) were not detected in conditioned media from IFNγ-treated, nor vehicle-treated, adult human mixed glia cultures. IL-10 and MIP-1α were detected at very low levels (< 5 pg/ml) in both IFNγ-treated and non-treated cells’ conditioned media. IL-6, IL-8, IP-10 and MCP-1 were expressed at moderate levels in vehicle-treated cells’ conditioned media (0.5-10 ng/ml). With IFNγ treatment, there was no change in IL-8 concentration. IL-6 concentration was increased with IFNγ (418 ± 42 [mean ± SEM] pg/ml for vehicle treatment vs 576 ± 39 pg/ml for IFNγ treatment, n=3; P=0.0519), though not to statistical significance (Figure 5.9A). MCP-1 concentration was significantly increased with IFNγ (8104 ± 608 pg/ml for vehicle treatment vs 10190 ± 437 pg/ml for IFNγ treatment, n=3; P=0.0493) (Figure 5.9B). However, the biggest IFNγ-induced change was an increase in IP-10 concentration from 2021 ± 782 pg/ml for vehicle treatment to 36860 ± 10140 pg/ml for IFNγ treatment (n=3; P=0.0267) (Figure 5.9C).

Cultures of fibroblast-like cells alone did not secrete IP-10 under vehicle conditions but did with IFNγ (13003 ± 5798 pg/ml, n=3), albeit to a lesser extent than the mixed glial cultures (Figure 5.9F). Fibroblast-only cultures also had lower basal secretion of IL-6 and MCP-1 but whereas IL-6 concentration was not changed by IFNγ treatment (Figure 5.9D), MCP-1 concentration was increased as for mixed glial cultures (4293 ± 735 pg/ml for vehicle treatment vs 10190 ± 387 pg/ml for IFNγ treatment, n=3; P=0.0021) (Figure 5.9E).
Figure 5.9: IFNγ increases pro-inflammatory cytokine and chemokine release from adult human mixed glial cultures and brain-derived fibroblast-like cells.

Here each data point indicates an individual case (n=3). Control and IFNγ-treated samples from the same case are indicated by connecting lines. A) IL-6 secretion is slightly, but not significantly, increased by IFNγ treatment of mixed glia (microglia, astrocytes and brain-derived fibroblast-like cells). B) MCP-1 production is significantly increased by IFNγ in mixed glial cultures. C) Adult human mixed glia produce a low basal level of IP-10 which is markedly increased by IFNγ. D) Relatively low concentrations of IL-6 production by fibroblast-like cells are not changed by IFNγ. E) Fibroblast-like cell cultures produce comparable levels of MCP-1 to mixed glial cultures when stimulated with IFNγ. F) Fibroblast-like cells release IP-10 upon IFNγ stimulation only.

As a means to further verify the secretion of IP-10 into the extracellular environment, media from cells treated with IFNγ was run on a gel and subjected to immunoblotting. Clear bands were evident at the molecular weight for IP-10 (10 kDa), confirming that IP-10 is indeed secreted from mixed glia cultures as well as fibroblast-only cultures (Figure 5.10). The increase in IP-10 production with IFNγ treatment can also be visualised by immunocytochemistry. This revealed that IP-10 is produced by all cell types in the mixed glial cultures (microglia, astrocytes and fibroblast-like cells) and thus is likely to be secreted into the extracellular environment by all of these cell types (Figure 5.11). In fibroblast-only cultures, IP-10 staining is increased by IFNγ. Unlike the inhibition of HLA expression by TGFβ1, the IFNγ-induction of IP-10 expression was not blocked by TGFβ1 (Figure 5.12). An effect of M-CSF on IP-10 levels in fibroblast-like cells was not observed either (Figure 5.12).
Figure 5.10: IFNγ induces IP-10 release from adult human mixed glial cultures and brain-derived fibroblast-like cells.

Mixed glial cells (A) and fibroblast-like cells (B) were treated with IFNγ for 96 h before collecting conditioned media to analyse by Western blot. Anti-IP-10 antibody revealed that IP-10 is greatly induced by IFNγ in both mixed glia (A) and fibroblast-like cells (B).

Figure 5.11: IFNγ induces IP-10 expression in microglia, astrocytes and fibroblast-like cells in primary adult human mixed glial cultures.

A) Following IFNγ treatment (1 ng/ml, 96 h) IP-10 expression (green) is co-localised with CD45-immunopositive microglia (red). All nuclei are labelled with Hoechst (blue). B) GFAP-immunopositive astrocytes (red) express IP-10 following IFNγ treatment. C) Prolyl-4-hydroxylase (red) marker of fibroblast-like cells co-localises with IP-10. Scale bar = 100 µm. Arrows indicate high levels of IP-10 expression and insets show close-up examples of cells expressing IP-10.
Figure 5.12: IFNγ induces IP-10 expression in fibroblast-like cells and is not affected by TGFβ1 or M-CSF.

Following IFNγ treatment, IP-10 expression is significantly increased in fibroblast-like cells from basal levels. Simultaneous treatment with either TGFβ1 or M-CSF does not affect the levels of IP-10 expression induced by IFNγ.

To validate the relevance of IP-10 secretion into the extracellular milieu of these adult human brain cells, IP-10 receptor CXCR3 protein expression was assessed by immunocytochemistry. CXCR3 protein was detected in all cell types in the mixed glial cultures (Figure 5.13A). CXCR3 expression was not noticeably changed by exposure to IFNγ (Figure 5.13B) or TGFβ1 (data not shown).

Figure 5.13: IP-10 receptor CXCR3 is expressed by all cells in adult human mixed glial cultures.

A) CXCR3 protein expression (green) is found on all cell types in mixed glial cultures. Microglia, identified by PU.1-labelled nuclei (pink), express CXCR3 on their cell surface. CXCR3 is also expressed by flat, round fibroblast-like cells (asterisk) and astrocytes with long, fine processes (arrow). B) Exposure to IFNγ for 96 h does not alter CXCR3 expression by microglia, astrocytes or fibroblast-like cells. Scale bar = 100 µm.
5.4.9. TGFβ₁ reduces closure of a gap wound area by human adult glia

As the previous results have shown intriguing actions (and lack thereof) of TGFβ₁ on adult human brain cells, another previously characterised effect of TGFβ₁ was evaluated - this time in the process of scar formation. A role for TGFβ₁ in scar formation and wound repair has previously been postulated in rodent cells and models (Kimura-Kuroda et al. 2010). Here it was assessed whether the same was true for primary adult human brain cells.

A scratch wound injury assay was used to assess the ability of human brain cells to migrate/proliferate in response to injury and form a ‘scar’ in the wound area. Cells were treated with TGFβ₁ (10 ng/ml) or vehicle 2 days before and 2 days after the injury. TGFβ₁ clearly reduced the presence of cells in the gap area 4-7 days following injury (Figure 5.14). The number of cells in the gap area was assessed by Hoechst staining of total nuclei and also with immunostaining for markers of different cell types. A decrease in microglia and fibroblast-like cells in the gap wound area was seen with TGFβ₁ treatment compared to vehicle (Figure 5.14A and B; C and D respectively). This robust effect was present even when cells were also treated with M-CSF, which contrastingly increases microglial migration compared to vehicle (Chapter 4; data not shown). However IFNγ did not affect closure of the gap wound area (data not shown). Astrocytes were not seen to occupy the gap area in the time course evaluated.

Another aspect of scar formation is deposition of extracellular matrix molecules such as collagen and fibronectin (Kawano et al. 2012). As TGFβ₁ has been shown to increase fibronectin expression in rodent studies of scar formation (Kimura-Kuroda et al. 2010), fibronectin expression by fibroblast-like cells was assessed. As shown in Figure 5.15, the distribution of fibronectin expression within fibroblast-like cells was altered by TGFβ₁. However, the intensity/amount of fibronectin did not appear to change.
Figure 5.14: Closure of a scratch wound injury by microglia and fibroblast-like cells is inhibited by TGFβ1.

A) Some CD45-positive microglia (green; arrowheads) migrate into the gap created by a scratch wound injury after 4-7 days. B) Very few microglia are found in the scratch wound area with TGFβ1 treatment. C) Fibroblast-like cells expressing prolyl-4-hydroxylase (green) fill a scratch wound injury in 4-7 days. However their ability to do so is greatly decreased by TGFβ1 (D). Hoechst (blue) labels all nuclei. Scale bar = 250 µm.

Figure 5.15: TGFβ1 alters fibronectin expression in fibroblast-like cells.

A) A high level of fibronectin expression is found in fibroblast-like cells, mostly around the edges of the cells. B) After 96 h TGFβ1 treatment, fibroblast expression of fibronectin protein has a different pattern of organisation and appears more uni-directional. Scale bar = 100 µm.
5.5. Discussion

5.5.1. Regulation of microglial expression of HLA-DP, DQ, DR

These findings demonstrate that HLA-DP, DQ, DR is an inducible protein which is not expressed constitutively by all microglia, and that levels of HLA expression vary between individuals (Table 5.2). These results also reiterate the findings of others that HLA can be expressed by other brain cell types apart from microglia. Despite variable basal HLA expression, microglia from all cases consistently showed increased HLA with IFNγ treatment (Figure 5.1). In these studies an antibody which targets HLA classes DP, DQ and DR has been used. It will be interesting to see if the changes in expression observed are due to one or more particular classes. B7 costimulatory molecules have also been shown to be regulated by IFNγ but human and murine regulation differ; IFNγ induces the expression of B7-1 in human, but not murine, microglia (De Simone et al. 1995; Iglesias et al. 1997).

The results of the present study, together with previous work, suggest that the effects of TGFβ1 on IFNγ-induced HLA expression are species specific as well as cell type specific. TGFβ1 did not affect HLA expression in adult human microglia, either at basal levels or with IFNγ-induction (Figure 5.1). Conversely, TGFβ1 blocked IFN-γ-induced enhancement of CIITA in murine macrophages and microglia (Abutbul et al. 2012; Delvig et al. 2002; O’Keefe et al. 1999), and human macrophage U937 cells (Nandan and Reiner 1997). These results highlight the importance of cell type specificity of HLA regulation.

Although TGFβ1 did not affect microglial HLA, M-CSF significantly reduced HLA expression by microglia (Figure 5.1 and 5.16). The effect of M-CSF on basal and IFNγ-induced HLA-DR has previously been investigated in human fetal astrocytes and microglia (Lee et al. 1993). Similar to the present results, they found reduced HLA-DR with M-CSF for microglia but not astrocytes (Lee et al. 1993). It has been found that IFNγ-mediated MHC-II induction in rodents was significantly muted in tumor microglia/macrophages compared with normal brain (Schartner et al. 2005). As M-CSF has been demonstrated to be upregulated in brain tumors (Alterman and Stanley 1994; Papavasiliou et al. 1997), it could be a possible mediator of decreased HLA expression within tumors.
5.5.2. Microglial cell number is increased by IFNy and M-CSF, but reduced by TGFβ1

Both M-CSF and IFNy increased the number of microglia in culture, although M-CSF had a greater effect (Figure 5.1H). An increase in microglial cell number with M-CSF treatment has previously been shown (Lee et al. 1993) (Chapter 4). Given that M-CSF reduced microglial HLA expression whereas IFNy increased HLA, it was surprising to find a similar effect of M-CSF and IFNy on microglial cell number. As M-CSF has been shown to increase microglial number by increasing their proliferation (Chapter 4), the effect of IFNy on microglial cell number was further investigated by BrdU proliferation assays (data not shown). However, no change in the percentage of dividing microglia was found with IFNy treatment suggesting that IFNy may have a survival, rather than a proliferative, effect on adult human microglia.

On the other hand it was found that TGFβ1 reduced microglia cell number. This could be a mechanism by which TGFβ1 exerts anti-inflammatory effects. Previous reports in rodents have shown that TGFβ1 inhibits microglial proliferation (Jones et al. 1998; Suzumura et al. 1993). The current results show a similar effect of TGFβ1 on human microglial cell number to that previously reported for rodent microglial cell number, but a differential effect of TGFβ1 on microglial HLA expression.

5.5.3. IFNy treatment results in microglia with a more rounded morphology

Immunocytochemistry and morphological analysis show increased rounding of IFNy-treated adult human microglia, with increased HLA-DP, DQ, DR staining (Figure 5.2). Immunohistochemistry of brains of adult humans with MS has shown HLA-DR+ve cells with oval morphology within MS lesions, whereas cells just outside the lesion and in the normal appearing parenchyma had a more ramified morphology (Ulvestad et al. 1994). Furthermore, expression of HLA class II molecules was noted to be less intensive on rod-shaped microglia compared to neighbouring ramified microglia in neurologically diseased human brain tissue (Wierzba-Bobrowicz et al. 2002). To complement this finding it is reported here that IFNy treatment produces the opposite effect of rounded microglial morphology with increased HLA expression. ‘Activated microglia’ cannot be solely defined by morphology or expression of a single cell surface marker (Perry 2010). However, together with increased HLA-DP, DQ, DR and IP-10 expression, this change in morphology is suggestive of a pro-inflammatory microglial phenotype.
5.5.4. Regulation of astrocytic HLA-DP, DQ, DR expression

The results demonstrate that HLA is expressed by a small percentage of astrocytes under basal culture conditions and that they readily increase HLA expression upon IFNγ stimulation (Figure 5.3). Early studies of HLA-DR expression on cultured human adult astrocytes similarly found that a small proportion expressed HLA-DR and that there was a concentration-dependent increase in HLA-positive astrocytes with IFNγ stimulation (Ulvestad et al. 1994; Yong et al. 1991). While microglia are the predominant cell type to express HLA both in vitro and in situ, Styren et al. (1990) have shown that astrocytes in control and AD brains can express HLA-DR, although they are reported to be rare compared to HLA-DR-positive microglia (Styren et al. 1990).

Astrocytes were not responsive to either TGFβ1 or M-CSF when analysed for HLA expression (Figure 5.3). It was shown in Chapter 4 that GFAP-positive astrocytes in mixed human adult glial cultures are negative for M-CSF receptor protein. Astrocytes have however been shown to produce TGFβ1 and M-CSF which then act on other brain cells (Lee et al. 1993; Weiss et al. 2011). These differential cell type responses to TGFβ1 and M-CSF show that astrocytes have a distinct immune phenotype and have an important role in brain immune responses.

A study investigating the expression of the IFNγ receptor on human cells and tissue found astrocytes to be the predominant cell type with IFNγ receptor expression (Hashioka et al. 2010). Astrocytes, but not microglia or oligodendrocytes, expressed IFNγ receptor in diseased and normal human brain tissue (Hashioka et al. 2010). On the other hand, cultured human microglia, astrocytes and oligodendrocytes showed constitutive expression of IFNγ receptor protein. While confirming IFNγ receptor expression on microglia in vitro, this finding calls into question the physiological in vivo relevance of the effect of IFNγ on microglia. However it will be important to confirm these immunohistochemical double-label results with in situ hybridization and a range of antisera to the IFNγ receptor. If this result is validated by other studies it suggests that astrocytes are the main cells contributing to IFNγ-mediated neuroinflammation in the brain. The presence of IFNγ receptors on microglia in vitro but not in vivo might occur because factors in the brain microenvironment may decrease IFNγ receptor expression on microglia as a safe-guard against neuroinflammation. Future studies are required to resolve these important issues.

We have previously distinguished between GFAP-positive astrocytes and P4H-positive fibroblast-like cells in mixed glial cultures (Gibbons et al. 2007; Park et al. 2012). Here the results demonstrate further differences between these two cell populations; a proportion of astrocytes express basal HLA whereas fibroblast-like cells do not, and TGFβ1 affects HLA expression of fibroblast-like cells but not astrocytes.
5.5.5. Regulation of fibroblast-like cell expression of HLA-DP, DQ, DR

The fibroblast-like cell population did not express HLA in basal culture conditions, either in mixed glial cultures or in later passage (passage 3-5) fibroblast-like cell only cultures (Table 5.2). Exposure to IFNγ resulted in a concentration-dependent increase in HLA expression by these fibroblast-like cells (Figure 5.4), showing that these cells have the capacity to be directed towards an immune role.

Despite TGFβ1 not affecting microglial HLA response, TGFβ1 had a dramatic effect on HLA induction in fibroblast-like cells (Figure 5.16). TGFβ1 completely blocked IFNγ-induced HLA expression in these cells (Figure 5.6). Similar reports of TGFβ1 modulation of HLA expression have been made for human cells with fibroblast characteristics from other regions of the body (Armendariz-Borunda et al. 1996; Navarrete Santos et al. 1998). The finding that M-CSF does not influence fibroblast-like cell expression of HLA is consistent with the previous observation that these cells don’t express the receptor for M-CSF (Chapter 4).

To further study the cellular basis of the induction of HLA in fibroblast-like cells, explant culture studies of leptomeninges were undertaken. Leptomeningeal-explant derived cells appeared very similar to dissociated fibroblast-like cells (with both expressing the fibroblast markers prolyl-4-hydroxylase and fibronectin) and responded to IFNγ by expressing HLA in a similar fashion to the dissociated cells (Figure 5.8B). Furthermore, TGFβ1 abolished this induction (Figure 5.8D and E). These results suggest that the fibroblast-like cells are likely derived from leptomeninges but whether they are classical leptomeningeal fibroblasts, pericytes or a mixture of cells is still not clear. Further investigation of cell markers beyond α-SMA (Figure 5.5) is currently underway.

The upregulation of HLA in individuals with neurological disease identifies HLA as an important molecule in the adult human brain. Increased intercellular adhesion molecule-1 (ICAM-1) expression has been found in epileptic and AD brains, and increased infiltration of CD8- and CD4- positive T lymphocytes was found in the hippocampus of patients with hippocampal sclerosis (Akiyama et al. 1993; Nakahara et al. 2010). ICAM-1 may aid T cell infiltration into the brain parenchyma where they could interact with antigen-presenting cells. However it is still unknown to what extent T cell activation occurs in the brain, and what factors govern this immune activation. The leptomeninges has been demonstrated to be a location of T cell contact with phagocytic antigen-presenting cells and a point of entry of encephalogenic T cells into the CNS (Bartholomaus et al. 2009; Kivisäkk et al. 2009). Live cell two-photon imaging of rats has revealed T cells moving out of leptomeningeal blood vessels and into the subarachnoid space where they interact with antigen-presenting cells and
subsequently invade the CNS parenchyma (Bartholomaus et al. 2009). In addition, the T cells became reactivated and upregulated pro-inflammatory cytokines and receptors including IFNγ and CXCR3.

5.5.6. IFNγ treatment of primary adult human mixed glia results in increased pro-inflammatory cytokine and chemokine release

Cytokines and chemokines are a major system of brain communication as there is mounting evidence that endogenous cytokines and chemokines in the brain act together with neurotransmitter and neuropeptide systems to control brain function (Adler et al. 2005). This chapter reports extensive release of pro-inflammatory chemokines IP-10 and MCP-1 following IFNγ treatment of adult human mixed glial cultures (Figure 5.9B and C). IL-6 was present at lower levels under control conditions and not significantly increased by IFNγ (Figure 5.9A). Pure cultures of fibroblast-like cells had lower basal cytokine/chemokine expression but also demonstrated a massive increase in MCP-1 and IP-10 release with IFNγ treatment (Figure 5.9E and F). Within mixed glial cultures the increase in MCP-1 appears to be largely from the fibroblast-like cells as the relative increase in MCP-1 is much greater for fibroblast-like cell only cultures than for mixed glial cultures. IL-6 levels were higher in the mixed glial cultures than in the pure fibroblast-like cell cultures, suggesting that astrocytes or microglia are the main source of this cytokine. The increase in IP-10 release from mixed glial cultures is likely produced by all cell types present as immunocytochemical labelling of IP-10 production is demonstrated in microglia, astrocytes and fibroblast-like cells after IFNγ treatment (Figure 5.11). Leptomeningeal fibroblast-like cells grown as explant cultures also expressed IP-10 in response to IFNγ (data not shown), suggesting that leptomeningeal fibroblast-like cells are a potential source of this chemokine in the inflamed brain.

Whereas TGFβ1 completely blocked IFNγ-induced HLA expression in brain-derived fibroblast-like cells (Figure 5.6), TGFβ1 did not affect IFNγ-induced IP-10 expression (Figure 5.12). Thus TGFβ1 has specific effects on different inflammatory pathways. A study using human gingival fibroblasts found that IFNγ (1 ng/ml) also stimulated IP-10 release, but that TGFβ1 treatment (10 ng/ml) further enhanced IP-10 secretion (Hosokawa et al. 2009). This illustrates the complexity of context and cell-type specificity in response to cytokine stimulation.

IP-10 and MCP-1 can also be released by human fetal and simian adult astrocytes in response to IFNγ (Croitoru-Lamoury et al. 2003). Astrocytes and microglia have increased expression of IP-10 in several infectious and neurotoxic contexts including AD, ischemia and LPS-challenge (Kremlev et al.
2004; Uddin et al. 2005; Wang et al. 1998; Xia et al. 2000). There is also evidence to suggest that not only glial cells but neuronal cells too can release chemokines to attract T cells into the brain parenchyma (Klein et al. 2005). Adult human brain microvascular endothelial cells have been shown to upregulate IP-10 in response to IFNγ (Salmaggi et al. 2002). The brain-derived fibroblast-like cells are also likely to be in ideal locations (e.g. blood vessels, meninges) to convey systemic inflammatory signals to brain glia and neurons, acting as a gate-way between peripheral physiology and the CNS (Wu et al. 2005). In cases of viral infection, Dionne et al. (2011) have demonstrated, using a brain slice culture model, that at least some of the IP-10 production and functional effects induced by viral infection are brain specific (Dionne et al. 2011). Interestingly, Durafourt et al. (2012) found IP-10 to be upregulated following activation in human microglia, but not in human macrophages, suggesting that IP-10 may be expressed by brain microglia more than peripheral macrophages in adult humans (Durafourt et al. 2012).

IP-10 expression is generally associated with loss of neuronal viability, however a direct mechanism has not always been established (Klein et al. 2005; Nelson and Gruol 2004; Sui et al. 2004). As the IP-10 receptor CXCR3 is expressed by numerous cell types including adult human microglia, astrocytes and fibroblast-like cells (Figure 5.13), IP-10 could act on a variety of cell types to eventuate in neuronal cell death. However, astrocytes and microglia have been found to respond differently to IP-10, and cellular background has been shown to determine CXCR3 signaling, highlighting cell type specificity in response to chemokines (Dijkstra et al. 2004; Flynn et al. 2003).

IP-10 protein is expressed by macrophages in MS lesions and IP-10 and MCP-1 are expressed by astrocytes at the rim of MS lesions, while both microglia and astrocytes express the IP-10 and MCP-1 receptors CXCR3 and CCR2 respectively (Simpson et al. 2000; Tanuma et al. 2006). CXCR3-positive astrocytes were also found to be increased in the CNS of HIV-positive patients, in ischaemic infarcts and in astrocytic neoplasms (Goldberg et al. 2001). Furthermore, IP-10-positive cell clusters have been observed in the cortex of rodent models of traumatic brain injury and neurodegeneration (Israelsson et al. 2010). It has been suggested that these cells may represent a novel population of cells to target pharmacologically in a broad range of neurodegenerative conditions (Israelsson et al. 2010). Increased IP-10 mRNA was inhibited by noradrenaline reuptake inhibitors in the neonatal rat brain following a systemic inflammatory challenge (O'Sullivan et al. 2010). The effect of IP-10 on neuronal viability in the adult human brain remains unknown and pharmacologic reduction of IP-10 expression should be further explored in the context of the adult human brain.
Figure 5.16: Differential regulation of HLA and IP-10 in adult human microglia, astrocytes and fibroblast-like cells by IFNy, TGFβ1 and M-CSF.

The T cell pro-inflammatory cytokine IFNy upregulates HLA and IP-10 protein expression in adult human brain glial cells. Microglial HLA was increased by IFNy (1 ng/ml for 96 h). M-CSF (25 ng/ml), but not TGFβ1 (10 ng/ml), was found to decrease microglial HLA expression. Astrocytic expression of HLA was also increased by IFNy, and not modulated by TGFβ1 or M-CSF. Fibroblast-like cells do not basally express HLA but have a marked induction on exposure to IFNy, which was blocked by TGFβ1. IFNy increased adult human glial expression and release of pro-inflammatory cytokines and chemokines, particularly IP-10. IP-10 may be involved in leukocyte trafficking into the CNS.
5.5.7. TGFβ1 reduces closure of a gap wound area by human adult mixed glia

TGFβ1 was also found to profoundly affect the process of scar formation by primary human mixed glia. TGFβ1 clearly reduced the presence of cells within the wound area in this in vitro scar assay (Figure 5.14). As TGFβ1 also reduces microglia and fibroblast cell number (Figures 5.1; 5.6 and 5.7 respectively), it is not clear whether reduced proliferation/survival or reduced migration of these cells is more responsible for inhibition of gap filling. Nevertheless, a clear decrease in the number of microglia and fibroblast-like cells in the wound area was seen with TGFβ1.

In contrast, in an in vitro rat model of CNS scar formation TGFβ1 increased meningeal fibroblast proliferation and promoted scar formation (Kimura-Kuroda et al. 2010). An in vitro model of human corneal fibrosis also found that at lower concentrations of 0.25 ng/ml TGFβ1, thickness of the 3D model constructs was increased as was the number of Ki67-positive cells (Karamichos et al. 2010). However, they noted that at concentrations greater than 1 ng/ml (up to the concentrations used in the present experiments) TGFβ1 caused contraction of the constructs. An increase in fibronectin protein was observed with TGFβ1 in both the human corneal fibrosis model and rat model of CNS scar formation. Although an absolute increase in fibronectin expression was not clear in the TGFβ1–treated brain-derived fibroblast-like cells, the change in fibronectin expression pattern depicted in Figure 5.15 is reminiscent of the striking directional arrangement of fibronectin in the human corneal fibrosis model (Karamichos et al. 2010). It is not clear what this change in expression pattern means in terms of fibronectin function, but it is likely to influence scar formation and tissue remodelling.

In summary, this study used primary human adult glia to demonstrate species and cell type specificity in response to IFNγ, TGFβ1 and M-CSF. While IFNγ induced inflammatory responses in all human brain cell types studied, TGFβ1 and M-CSF have anti-inflammatory effects on specific cell populations (Figure 5.16). This data is likely to have relevance for neuroinflammation in the adult human brain and more studies are warranted to determine the regulators of this neuroinflammation. The opposing results presented here from rodent models of brain injury and inflammation indicate the need for constant validation of neurological findings in human contexts.

Given the distinct phenotypes of microglia in response to different activating agents, the next chapter investigates whether microglia from different brain regions have comparable phenotypes and similar responses to these soluble molecules.
Chapter 6.

Microglia From Neurogenic Regions of the Adult Human Brain are More Proliferative Than Their Cortical Counterparts

6.1. Abstract

The adult human brain has two consistently neurogenic regions – the subventricular zone of the lateral ventricles and the dentate gyrus of the hippocampus (Hp). These special areas (the neurogenic niche) contain extracellular and cellular components which are instructive for proliferation of neural progenitor cells and differentiation into neurons. Environmental cues present in different areas of the brain also regulate microglial activity. This chapter investigates whether environmental cues of adult neurogenic regions influence microglia phenotype.

Biopsy tissue was obtained from epileptic patients undergoing surgery and consisted of both non-neurogenic cortical areas and neurogenic ventricular/Hp areas. Microglia were separately isolated from both regions and compared. A greater number of microglia resulted from isolation and culture of ventricular/Hp tissue than cortical tissue. This was found to be due to a greater proliferative capacity of microglia from neurogenic regions compared to the cortex. Additionally, ventricular/Hp microglia had a greater proliferative response to the microglial mitogen Macrophage Colony-Stimulating Factor (M-CSF). This enhanced response was found to be associated with higher M-CSF receptor expression and higher intracellular, putative M-CSF signalling, proteins DAP12 and C/EBPβ. Microglia from the ventricular/Hp region also displayed higher expression of the receptor for Insulin-like Growth Factor-1, a molecule with some functional similarity to M-CSF. Compared to microglia isolated from the cortex, ventricular/Hp microglia had a more ‘activated’ phenotype of increased HLA-DP, DQ, DR antigen presentation protein expression, and rounded morphology.

These findings show that microglia from adult human brain neurogenic regions are more proliferative than cortical microglia and have a unique protein expression profile. The data present a case for differential microglial phenotype and function in neurogenic vs non-neurogenic regions of the adult human brain.
6.2. Introduction

Microglia are the brain’s primary immune cells. They play important homeostatic roles and can modulate the functions of other brain cells. Microglia actively monitor neuronal synapses (Nimmerjahn et al. 2005; Wake et al. 2009), phagocytose debris (Chan et al. 2001; Simard et al. 2006), and can secrete both supportive and detrimental factors into the extracellular environment (Olah et al. 2011). Microglia communicate with other brain cells via cytokines and growth factors and are important cell types during development, in normal physiological states, and during injury/degenerative processes (de Haas et al. 2007). In these different situations, microglia can express specific cell surface receptors, have specific morphology, and produce different soluble molecules (Olah et al. 2011). Thus, microglia have marked phenotypic diversity which is influenced by their microenvironment including other cell types and soluble molecules in their surroundings. An important factor regulating microglial phenotype is the growth factor Macrophage Colony-Stimulating Factor (M-CSF). Development and differentiation of microglia is dependent on M-CSF signalling (Ginhoux et al. 2010) and M-CSF also increases microglial division (Lee et al. 1993; Vidyadaran et al. 2009; Yamamoto et al. 2010).

Normal rodent and human adult brains have regional differences in microglia density and immune protein expression (de Haas et al. 2008; Mittelbronn et al. 2001). For example, it has been demonstrated that white matter contains more microglia than grey matter (Mittelbronn et al. 2001). Furthermore, different brain regions show different microglial responses to ageing (Hart et al. 2012) and different phenotypes in vitro (Melief et al. 2012). We are starting to appreciate the diverse nature of these brain immune cells, but it is not fully known to what extent microglia vary in different brain regions and what factors/mechanisms produce these differences.

Two highly specialised areas of the adult brain are the neurogenic regions of the subventricular zone (SVZ) of the lateral ventricles and the dentate gyrus (DG) of the hippocampus. Here neural progenitor cells (NPCs) proliferate throughout life and give rise to new neurons (Curtis et al. 2007; Eriksson et al. 1998). Isolation and culture of adult NPCs can be routinely performed from rodent brain tissue and, with greater technical difficulty, from human brain tissue. A handful of research groups have demonstrated the in vitro culture and proliferation of human NPCs, and their subsequent differentiation into neurons and astrocytes (Coras et al. 2010; Leonard et al. 2009; Park et al. 2012).

Just as microglia are shaped by their environment, the microenvironment (‘neurogenic niche’) of these two regions is thought to be important for maintaining NPC proliferation and the cells of the
neurogenic niche have been found to greatly influence neurogenesis (Hellstrom et al. 2011; Morrens et al. 2012). Microglia are present in both the rodent and human SVZ and DG (Curtis et al. 2005b; Goings et al. 2006; Hellstrom et al. 2011; Morrens et al. 2012) where they perform many important roles including cytokine production and phagocytosis (Morrens et al. 2012; Sierra et al. 2010; Walton et al. 2006).

There is now a large body of evidence to support the notion that microglia regulate adult neurogenesis in pathological and non-pathological conditions (Carpentier and Palmer 2009; Martino et al. 2011; Molina-Holgado and Molina-Holgado 2010; Morrens et al. 2012; Villeda and Wyss-Coray 2008; Walton et al. 2006; Ziv and Schwartz 2008). Initial studies investigating the effects of microglia on neurogenesis revealed a negative effect for these immune cells on production of new neurons. Monje et al. reported that peripheral injection of LPS to rats resulted in central pro-inflammatory cytokine production which increased the number of activated microglia and decreased hippocampal neurogenesis (Monje et al. 2003). Battista et al. showed for the first time that the anti-inflammatory cytokine TGFβ can promote neurogenesis. It was found in vivo and in vitro that the number of activated microglia, and amount of TGFβ, correlated with the number of newly born neurons (Battista et al. 2006). This finding highlighted a physiological role for microglia in the neurogenic niche. In vitro studies have demonstrated that differentially activated microglia have different effects on neurogenesis (Butovsky et al. 2006), demonstrating that a fine balance of appropriate levels of immune activation is required for successful neurogenesis. During disease and injury processes, activated microglia can produce molecules which have been shown to aid neurogenesis and neuroblast migration (Deierborg et al. 2010; Hellstrom et al. 2011; Starossom et al. 2011; Young et al. 2011). Neurogenesis is also modulated by a number of environmental factors, such as exercise and stress, which can affect immune functions including cytokine production. For example, stress causes increased levels of IL-1β which decreases hippocampal cell proliferation (Koo and Duman 2008). It has been shown that young and aged mice have differential immune regulation of NPC proliferation in response to injury, and that a young systemic environment enhances neurogenesis in an aged brain (McPherson et al. 2011; Villeda and Wyss-Coray 2012). The influence of immune factors on neurogenesis is thus context dependent and reflects the combined effect of all factors present in a specific spatial and temporal pattern.

Investigation of the role of microglia in adult neurogenesis has led to findings of specific microglial characteristics within the SVZ and DG neurogenic regions. Microglia of the SVZ were found to have a higher level of basal activation and proliferation, and greater expression of CD45 and CD11b, than non-neurogenic regions of striatum and corpus callosum (Goings et al. 2006). Following cortical
injury, SVZ microglia did not however become more activated, despite being closer to the injury than
the striatum and corpus callosum where microglia greatly increased activation compared to non-
injured brains (Goings et al. 2006). Further evidence of a special phenotype of microglia in
neurogenic regions is the unique proliferative capacity of microglia found in the SVZ of neonatal
mice whereby SVZ cultures produced 20-fold greater yields of microglia than corresponding cortical
cultures (Marshall et al. 2008). However, adult brain cultures gave 10 times fewer microglia
(Marshall et al. 2008) and it is unknown whether this unique proliferative capacity of subventricular
microglia is true for the adult human brain as several other differences exist between the culture of
rodent and human microglia. While both rodent and human microglia can be isolated by their
differential adhesion properties in vitro, rodent microglia sit on top of other cells in culture (Marshall
et al. 2008; Moussaud and Draheim 2010), whereas human microglia adhere quickly to culture
surfaces (De Groot et al. 2000; Klegeris et al. 2005). Furthermore, in the presence of other cells
including astrocytes, adult rodent microglia proliferate in culture without addition of mitogens
(Moussaud and Draheim 2010), however adult human microglia have much lower basal rates of
proliferation ((Gibbons et al. 2007); Chapter 4).

The use of primary human microglia is an invaluable tool for neuroscience research (Dragunow
2008a; Gibbons and Dragunow 2010). However, their use is limited by tissue source and also by the
number of cells obtained per isolation. It is therefore of great interest to investigate whether
microglia from neurogenic regions of the adult human brain also have increased proliferative
capacity compared to cortical brain regions. Using human biopsy tissue from temporal epilepsy
surgeries, the microglia of the hippocampus and of the temporal horn of the lateral ventricle (herein
referred to as ‘ventricular/Hp’ microglia) were compared to microglia from the cortical middle
temporal gyrus. Major distinctions were found between microglia from these two separate human
adult brain regions in terms of proliferation and immune protein expression.
6.3. Methods

6.3.1. Tissue

Biopsy adult human brain tissue used for this study was obtained from patients undergoing surgery for intractable temporal lobe epilepsy. This research was approved by the Northern Regional Ethics Committee and the University of Auckland Human Participants Ethics Committee and informed consent was obtained from all tissue donors.

6.3.2. Human glial cell isolation and culture

Microglia were isolated from neurogenic regions of adult human brain tissue using the neural progenitor cell isolation protocol from Park et al. (2012) described in Chapter 2 (General Methods). A piece of cortical middle temporal gyrus tissue of equivalent weight was processed concurrently by the same method. The isolated glial cells were maintained as described for mixed glial cultures (Gibbons et al. 2007; Gibbons et al. 2011).

6.3.3. Cytokine treatment

Mixed primary human glial cell cultures were treated in 96-well plates. 1 μl cytokine was added to 100 μl media. Cells were treated with 25 ng/ml M-CSF (in H2O) or 1 ng/ml IFNy (in PBS with 0.1 % BSA) at 0 and 48 h. Total time of cytokine treatment was 96 h.

6.3.4. BrdU proliferation assay

Following 72 h exposure to 25 ng/ml M-CSF, 10 μM BrdU was added to the cells for 24 h and BrdU proliferation assays were performed as described in Chapter 2.

6.3.5. Immunocytochemistry

Live cell antibody labelling was performed for CSF-1R and IGF-1R antigens. All other immunocytochemistry used the standard protocol (see Chapter 2 for details).
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Catalogue #</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-PU.1</td>
<td>Cell Signaling</td>
<td>2258</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-CD45</td>
<td>Abcam</td>
<td>ab8216</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-CSF-1R</td>
<td>Santa Cruz</td>
<td>Sc-692</td>
<td>1:50</td>
</tr>
<tr>
<td>Mouse anti-HLA-DP, DQ, DR</td>
<td>Dako</td>
<td>M0775</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-DAP12</td>
<td>Santa Cruz</td>
<td>Sc-20783</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-C/EBPβ</td>
<td>Santa Cruz</td>
<td>Sc-7962</td>
<td>1:250</td>
</tr>
<tr>
<td>Mouse anti-IGF-1R</td>
<td>Millipore</td>
<td>MAB1120</td>
<td>1:50</td>
</tr>
<tr>
<td>Mouse anti-BrdU</td>
<td>Roche</td>
<td>11170376001</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-Ki67</td>
<td>Dako</td>
<td>A0047</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG Alexa Fluor® 594</td>
<td>Invitrogen</td>
<td>A11012</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG Alexa Fluor® 488</td>
<td>Invitrogen</td>
<td>A11001</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-mouse IgG Alexa Fluor® 594</td>
<td>Invitrogen</td>
<td>A11005</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG Alexa Fluor® 488</td>
<td>Invitrogen</td>
<td>A11008</td>
<td>1:500</td>
</tr>
</tbody>
</table>

Table 6.1: Antibodies used for immunocytochemistry.

6.3.6. Quantitative image analysis of cell number, protein expression and microglial morphology

Immunocytochemical and morphological observations were quantified using a Discovery-1 automated fluorescence microscope (Molecular Devices) and Metamorph (6.2.6 software, Molecular Devices) image analysis system as previously described (Dragunow 2008b; Smith et al. 2010) and outlined in Chapter 2.

6.3.7. Statistical analysis

Representative data are displayed as mean ± standard error of the mean (SEM). Experiments were replicated with cells from at least 4 different individuals. Statistical analysis was carried out using t-tests and one-way ANOVA. P values of <0.05 were considered statistically significant differences.
6.4. Results

6.4.1. Differential proliferation of microglia from ventricular/Hp and cortical regions

Microglia were cultured from two anatomical regions of biopsy human brain tissue. Microglia isolated from the middle temporal gyrus (as in Chapters 3-5) were compared to microglia isolated from the neurogenic regions of the hippocampal DG and the overlying SVZ of the lateral ventricle.

It was found that the yields of microglia from the ventricular/Hp region (580,000 microglia per gram tissue, n = 3) were greater than those derived from the cortical middle temporal gyrus region (290,000 microglia per gram tissue, n = 3). Microglia were visualised using antibodies to cell surface antigen CD45 and nuclear transcription factor PU.1 (Figure 6.1A and B).

Adult human microglia cultured from middle temporal gyrus cortical regions have very low rates of proliferation in vitro (3.2 +/- 0.7%; n = 9), confirming previous results (Gibbons et al. 2007); Chapter 4). The majority (6 out of 9 cases) of glial cultures from lateral ventricle/Hp tissue had an increased proportion of dividing microglia than their corresponding middle temporal gyrus cultures from the same case. The endogenous cell division marker Ki67 and the exogenous proliferation indicator BrdU were both used to confirm differences in proliferation and gave equivalent results (Figure 6.1 C-E). The average basal percentage of dividing microglia in ventricular/Hp cultures was 9.1 +/- 2.3% (n = 9). The increase in ventricular/Hp microglial division, compared to cortical microglial division, was variable between cases, but microglia from ventricular/Hp regions were found to have a 3.5-fold (n = 9; p = 0.0424; Paired t-test) higher proliferation rate than cortical microglia (Figure 6.1E).
Figure 6.1: Microglia from neurogenic ventricular/Hp tissue proliferate more than microglia from non-neurogenic cortical tissue.

Each data point indicates an individual case (n=9). Cortex and SVZ/Hp samples from the same case are indicated by connecting lines. A) PU.1 (green nuclei) and CD45 (red) double-positive microglia are present in glial cultures from cortical human brain tissue. B) There are a greater number of microglia in ventricular/Hp cultures. C and D) Immunocytochemical images of CD45 microglial cell surface marker (green) and Ki67 cell division marker (red) showing minimal proliferation of cortical microglia (C) and greater basal proliferation of ventricular/Hp microglia (D). Arrows indicate examples of Ki67-immunopositive microglia. Scale bar = 100 μm. E) Quantification of the percentage of microglia cultured from cortical and ventricular/Hp regions that incorporate BrdU, showing a significantly greater percentage of dividing microglia in ventricular/Hp regions.
6.4.2. Ventricular/Hp microglia have a greater proliferation response to M-CSF than cortical microglia

Chapter 4 reports an increase in adult human microglial cell number with M-CSF treatment. This increase in microglia cell number was due to increased microglial proliferation. Here it was assessed whether microglia cultured from the ventricular/Hp region would also respond to M-CSF by increasing proliferation, given their higher level of basal proliferation. It was found that M-CSF treatment increased proliferation of microglia cultured from the ventricular/Hp region (7 out of 7 cases) as for microglia from the cortex. Furthermore, ventricular/Hp microglia had a larger proliferation response to M-CSF than cortical microglia (Figure 6.2). The percentage increase in proliferating microglia with M-CSF treatment was consistently greater for ventricular/Hp microglia than for cortical microglia (5 out of 7 cases) (Figure 6.2E). Although 3 out of 9 cases did not have higher basal ventricular/Hp microglial proliferation compared to cortical microglia (Figure 6.1E), these cases still had a greater proliferation response to M-CSF in ventricular/Hp microglia compared to cortical microglia (Figure 6.2E).
Figure 6.2: M-CSF has a greater effect on ventricular/Hp than cortical microglial proliferation.

Each data point indicates an individual case (n=7). A and B) Immunocytochemical images of CD45 microglial cell surface marker (green) and Ki67 cell division marker (red) showing minimal proliferation of cortical microglia (A) and greater basal proliferation of ventricular/Hp microglia (B). M-CSF treatment increases the number of dividing microglia in cortical cultures (C), but the effect of M-CSF on microglial proliferation is greater for ventricular/Hp microglia (D). Arrows indicate examples of Ki67-immunopositive microglia. Scale bar = 100 μm. E) Quantification of the percentage of microglia that incorporate BrdU under control conditions and with M-CSF treatment showing a significant increase in microglial division with M-CSF and an enhanced effect for ventricular/Hp microglia.
6.4.3. Similar expression of PU.1 transcription factor and CD45 cell surface receptor in ventricular/Hp and cortical microglia

PU.1 is a microglial transcription factor which has been shown to be involved in microglial responses to M-CSF ((Celada et al. 1996; Zhang et al. 1994); Chapter 4). No consistently significant difference was found between cortical and ventricular/Hp microglia in levels of PU.1 protein expression (Figure 6.3A, B and E). However, as previously reported for cortical microglia, M-CSF increased the amount of PU.1 protein expressed by microglia from both cortical and ventricular/Hp regions (Figure 6.3E). CD45 expression was also assessed as it has previously been shown that rodent SVZ microglia have a higher constitutive expression of CD45 than microglia in non-neurogenic regions (Goings et al. 2006). It has previously been reported that adult human microglia constitutively express high levels of CD45 in vitro (Gibbons et al. 2007) and here no obvious difference in CD45 expression was found between ventricular/Hp and cortical microglia (Figure 6.3A and B).

Figure 6.3: M-CSF treatment increases the number of microglia and expression of PU.1 in primary adult human mixed glial cultures from cortical and ventricular/Hp regions.

A) PU.1 (green nuclei) and CD45 (red) double-positive microglia are present in glial cultures from cortical human brain tissue. B) There is a greater number of microglia in ventricular/Hp cultures. M-CSF treatment increases the number of microglia in both cortical (C) and ventricular/Hp (D) glial cultures compared to vehicle treatment. Concurrently, M-CSF treatment increases the intensity of PU.1 expression in microglia from both cortical (C) and ventricular/Hp (D) cultures. Scale bar = 100 µm. E) M-CSF significantly increases the intensity of PU.1 expression (amount of PU.1 protein) in adult human microglia from neurogenic (ventricular/Hp) and non-neurogenic (cortical) regions of the adult human brain.
6.4.4. Ventricular/Hp microglia express more CSF-1R than cortical microglia

To determine whether ventricular/Hp microglia had increased endogenous ability to respond to M-CSF, M-CSF receptor (CSF-1R) expression was investigated. Chapter 4 reports the expression of CSF-1R protein on microglia in human adult mixed glial cultures. As expected given their proliferation response to M-CSF, CSF-1R was expressed by microglia cultured from ventricular/Hp as well as cortical regions (Figure 6.4A and B).

Basal levels of CSF-1R protein expression on cortical and ventricular/Hp microglia were compared and it was found that there was a higher amount of CSF-1R protein expressed by ventricular/Hp microglia than by cortical microglia (Figure 6.4A, B and E). Chapter 4 demonstrates that treatment of adult human microglia with M-CSF increases their expression of CSF-1R. Both ventricular/Hp and cortical microglia increased expression of CSF-1R following exposure to M-CSF (Figure 6.4).

Figure 6.4: CSF-1R is expressed at higher levels by neurogenic region microglia compared to cortical microglia.

A) CSF-1R is expressed by microglia in cortical mixed glial cultures. B) Levels of CSF-1R are higher in ventricular/Hp microglia. M-CSF increases both cortical (C) and ventricular/Hp (D) microglial CSF-1R expression. Scale bar = 100 µm. E) CSF-1R is more highly expressed in ventricular/Hp microglia than cortical microglia. A significant increase in intensity of receptor labelling is found for CSF-1R following M-CSF treatment.
6.4.5. DAP12 adaptor protein, C/EBPβ transcription factor, and IGF-1 receptor are more highly expressed by microglia from ventricular/Hp regions

DAP12 is an adaptor protein found in microglia in the adult human brain. DAP12 expression was assessed in microglia from ventricular/Hp regions and from the cortex. Basal DAP12 expression levels are higher in ventricular/Hp microglia than cortical microglia for most cases (6 out of 7 cases; Figure 6.5A, B and E). As previously reported in Chapter 4, M-CSF produced an increase in DAP12 expression for cortical microglia (7 out of 7 cases; Figure 6.5A and C) and this was also observed for ventricular/Hp microglia (Figure 6.5B and D). Thus ventricular/Hp microglia have higher basal levels of DAP12 expression than cortical microglia (Figure 6.5A and B), and furthermore M-CSF treatment of cortical microglia increases their levels of DAP12 expression similar to basal levels in ventricular/Hp microglia (Figure 6.5E).

![Figure 6.5](image)

**Figure 6.5: DAP12 is more highly expressed in ventricular/Hp than cortical microglia.**

A) DAP12 monocyte/microglial adaptor protein is expressed by human microglia isolated from cortical human brain tissue. B) Higher levels of DAP12 are expressed by microglia isolated from ventricular/Hp tissue. C and D) Treatment of microglia with M-CSF increases their expression of DAP12 as seen by increased intensity of immunohistochemical staining. Scale bar = 100 µm. E) Quantification of DAP12 staining intensity shows a significantly greater level of expression in ventricular/Hp microglia compared to cortical microglia, and a significant increase in DAP12 expression with M-CSF treatment.
C/EBPβ is a transcription factor expressed by microglia which has been shown to be involved in M-CSF-mediated effects (Chapter 4). Higher C/EBPβ protein expression was found in ventricular/Hp microglia than in cortical microglia (Figure 6.6). M-CSF produced an increase in C/EBPβ expression in microglia from both cortex and ventricular/Hp regions (Figure 6.6).

**Figure 6.6: M-CSF increases adult human microglial expression of C/EBPβ.**

Quantification of microglial C/EBPβ expression showing differential basal expression by cortical and ventricular/Hp microglia, and significant increases in the percentage of cortical and ventricular/Hp microglia which express C/EBPβ with M-CSF.

Chapter 4 demonstrates IGF-1R protein expression on microglia in human adult mixed glial cultures. Here it is found that basal levels of IGF-1R protein expression are higher in ventricular/Hp microglia than cortical microglia (Figure 6.7A and B). In Chapter 4 it was also observed that IGF-1R expression increased on microglia from the cortex following M-CSF treatment. This response was assessed in ventricular/Hp microglia and found that it was similar (Figure 6.7E).
Figure 6.7: IGF-1R is expressed more in ventricular/Hp microglia and is increased by M-CSF treatment.

A) Cortical adult human microglia express relatively little IGF-1R. B) In comparison, ventricular/Hp microglia express higher basal levels of IGF-1R. M-CSF increases IGF-1R expression in both cortical (C) and ventricular/Hp (D) microglia. Scale bar = 100 µm. E) Ventricular/Hp microglia express significantly more IGF-1R than cortical microglia, and a significant increase in intensity of IGF-1R is evident following M-CSF treatment.

6.4.6. Ventricular/Hp microglia express higher levels of HLA-DP, DQ, DR protein

HLA is a widely used marker of microglial ‘activation’. A subset of adult human microglia in vitro express HLA and the proportion of microglia expressing HLA basally in vitro is very variable between cases (Chapter 5). Ventricular/Hp and cortical microglia, from the same cases, were compared to see whether their basal HLA expression differed.
It was found that ventricular/Hp microglia had a greater propensity to express HLA than cortical microglia. In the majority of cases (6 out of 7), more HLA was expressed by ventricular/Hp microglia than cortical microglia (Figure 6.8A, B and E). Chapter 5 reported that IFNγ increases adult human microglial expression of HLA. This was also found to be true for the ventricular/Hp microglia populations (Figure 6.8B and D). However, due to higher basal HLA expression in ventricular/Hp microglia, this effect was not as pronounced as for cortical microglia (Figure 6.8E).

Figure 6.8: Microglia from the ventricular/Hp region express greater levels of HLA-DP, DQ, DR than cortical microglia.

A) In basal conditions without any treatment, a variable level of HLA-DP, DQ, DR is expressed by cortical adult human microglia. B) Microglia from ventricular/Hp regions have a higher basal level of HLA-DP, DQ, DR expression. C) IFNγ (1 ng/ml, 96 h) increased cortical microglial expression of HLA-DP, DQ, DR, as well as that of ventricular/Hp microglia (D). Scale bar = 100 µm. E) Quantification of HLA expression showing differential expression by cortical and ventricular/Hp microglia, and a significant increase in HLA expression with IFNγ treatment.
6.4.7. Ventricular/Hp microglia have a more rounded ‘amoeboid’ morphology than cortical microglia

Human adult microglia are not uniformly shaped in vitro. Some are rounded and others have longer processes and extensions. In most of the cases (5 out of 7 cases) it was observed that microglia cultured from the ventricular/Hp region had rounder morphology than cortical microglia (Figure 6.9A and B). This difference in cell morphology was quantified and found to be significant (Figure 6.9C).

A morphological response to M-CSF was observed for both cortical and ventricular/Hp microglia populations. M-CSF caused microglia to become more elongated, confirming previous results (Chapter 4). The response was found to be quantitatively similar for ventricular/Hp and cortical microglia (data not shown).

Figure 6.9: Microglia from the cortex are more elongated and less rounded than ventricular/Hp microglia.

A) Adult human microglia isolated from cortical tissue immunolabelled with the cell surface marker CD45 have a heterogeneous morphology with various extended processes. B) Microglia isolated from ventricular/Hp regions have a rounder morphology with fewer processes. Insets in A) and B) show representative morphology of cells. Scale bar = 100 µm. C) Quantification of microglial morphology using Metamorph Elliptical Form Factor (a measure of elongation) image analysis demonstrates a significant difference in microglia shape between cortical and adjacent neurogenic regions.
6.5. Discussion

6.5.1. Microglia from neurogenic regions have greater basal proliferation than cortical microglia

The results of this chapter report differential proliferation and protein expression of adult human microglia from two distinct brain regions – 1) the cortex and 2) the hippocampus and overlying ventricular lining.

The initial observation of spontaneously dividing ventricular/Hp microglia in normal culture conditions led to the further investigation of microglia from this region of the adult human brain in comparison to microglia from the cortical temporal lobe. Interestingly, microglia from the ventricular/Hp region were found to proliferate at a relatively high rate without growth factor stimulation, whereas cortical microglia have very low rates of basal proliferation (Figure 6.1). Using Ki67 immunocytochemistry and BrdU proliferation assays it was demonstrated that the increasing number of ventricular/Hp microglia in culture was due to cell division and not just a survival effect. This finding suggests that there are differences in intrinsic cell division mechanisms in these two microglial populations. Marshall et al. (2008) found that the huge expansive capacity of neonatal rodent SVZ microglia was diminished in the adult brain, whereas the results of the present study still found increased proliferative capacity of human adult ventricular/Hp microglia.

It was next asked whether ventricular/Hp and cortical microglia would have differential proliferation responses to the monocytic mitogen M-CSF.

6.5.2. Microglia from neurogenic regions have heightened responses to M-CSF

Even more pronounced than the differences in basal proliferation was the difference in M-CSF-stimulated proliferation of ventricular/Hp microglia compared to cortical microglia. The majority of cases exhibited a significantly larger increase in microglial proliferation with M-CSF for the ventricular/Hp region compared to the cortex (Figure 6.2).

To identify the mechanisms behind this differential proliferative response in microglia from two distinct brain regions, expression of the receptor for M-CSF (CSF-1R) was assessed. In concordance with their heightened response to M-CSF, higher expression of CSF-1R protein was found on ventricular/Hp microglia (Figure 6.4). Higher CSF-1R expression by ventricular/Hp microglia was not paralleled by relatively high PU.1 expression, even though PU.1 has been shown to regulate CSF-1R...
gene expression (Zhang et al. 1994). However, greater expression of the C/EBPβ transcription factor in ventricular/Hp microglia could be mechanistically involved in their increased response to M-CSF. C/EBPβ has been shown to regulate CSF-1R gene expression (Zhang et al. 1996) and to be involved in M-CSF actions in disease states (Komuro et al. 2003; Marigo et al. 2010). Increased DAP12 expression in ventricular/Hp microglia could also be related to their increased response to M-CSF as it is shown in Chapter 4 that M-CSF treatment increases DAP12 expression in adult human microglia, and M-CSF has been found to induce macrophage proliferation via DAP12 (Otero et al. 2009). Thus the machinery for M-CSF signalling – CSF-1R, DAP12 adaptor protein and C/EBPβ transcription factor – are more highly expressed in ventricular/Hp microglia, associated with a massive proliferative response to M-CSF.

The mitogenic growth factor IGF-1 may share some functional effects with M-CSF (Gow et al. 2010; Wessells et al. 2004) and it was found that the receptor for IGF-1 (IGF-1R) was also at higher basal levels in ventricular/Hp microglia compared to cortical microglia (Figure 6.7). The finding that protein levels of IGF-1R are increased upon M-CSF stimulation confirms the results of Chapter 4. IGF-1 has been reported numerous times within neurogenic regions (Anderson et al. 2002). Given the important role that IGF-1 is thought to play in adult neurogenesis, this intriguing finding of increased IGF-1R expression on ventricular/Hp microglia shows that IGF-1 may act through microglia, as well as other cell types including NPCs (Aberg et al. 2003), in neurogenic regions to influence neurogenesis.

Regional differences in the response of rodent microglia to cytokine receptor-stimulation have previously been reported, and together these findings raise the question of whether there are truly different ‘sub-populations’ of microglia in different brain regions or whether all microglia will respond similarly if placed in the same environment (van Weering et al. 2011). The results from this study show that microglia from different brain regions retain differential phenotype and function in vitro, but to what extent the microglial phenotype is retained or reversible in vivo is still unknown.

Another glial cell type of the SVZ, the astrocytic NPC, is also capable of proliferation unique to neurogenic regions (Ihrie and Alvarez-Buylla 2008). As mentioned in the introduction, adult neurogenesis requires immune support (Martino et al. 2011; Morrens et al. 2012) but exactly how the finding of increased proliferation of microglia in stem cell niches has an effect on the NPCs residing there is unknown. NPCs and microglia both release trophic and immunomodulatory molecules (Martino and Pluchino 2007; Pluchino et al. 2005). In fact NPCs are being discovered to have remarkable influence over immune activity and were found to have a distinct secretory protein profile (Mosher et al. 2012). This recent discovery is similar to those of other stem cell types such as
mesenchymal stem cells which have been found to regulate microglial function through release of soluble molecules such as CX3CL1 (fractalkine) (Giunti et al. 2012). Conditioned medium from primary mouse NPCs was found to induce microglial proliferation, chemotaxis and phagocytosis, while transplantation of NPCs or NPC conditioned medium significantly increased the numbers of dividing microglia in vivo (Mosher et al. 2012). Thus it seems that microglia and NPC have a two-way relationship, both contributing to maintenance of the neurogenic niche.

6.5.3. Microglia from neurogenic regions express higher levels of HLA and have rounder morphology

Ventricular/Hp microglia were found to have a rounder morphology and higher levels of HLA protein expression than their cortical counterparts (Figures 6.8 and 6.9). These characteristics are thought to be indicative of an ‘activated’ microglial phenotype (Graeber 2010). Chapter 5 reports that IFNγ treatment of adult human microglia increases HLA expression and induces morphological rounding. These two observations were concurrent with increased IL-6, MCP-1 and IP-10 cytokine and chemokine production. Conversely, adult human microglial responses to M-CSF have been shown to result in microglia with ‘resting’ characteristics. M-CSF induces a major morphological response in adult human microglia whereby they become elongated and bipolar (Chapter 4). Furthermore, M-CSF has been shown to reduce microglial HLA expression, including that of primary adult human microglia (Chapter 5). Greater CSF-1R expression, and therefore propensity to respond to M-CSF, would suggest a less activated state of ventricular/Hp microglia compared to cortical microglia. However, microglia respond to many factors in their environment simultaneously and the phenotypic plasticity of microglia is highly evident here, where ventricular/Hp microglia are shown to be basally ‘activated’ but have massive responses to M-CSF through proliferation, morphology change and multiple gene expression changes. A possible explanation for heightened ventricular/Hp microglial activation is their proximity to immune protein-containing cerebrospinal fluid in the lateral ventricles. In fact, it has been demonstrated that microglia in regions of the brain with a less defined blood-brain barrier, and thus increased exposure to plasma proteins, have a less ramified morphology than microglia from other regions (Cuadros and Navascues 1998; Galea et al. 2007).
6.5.4. Possible functional significance of increased ventricular/Hp microglial proliferation

Functional microglial diversity in specific brain regions is likely necessary to accommodate the requirements of different brain regions, for example different energy requirements and cell types. Increased microglial proliferation in neurogenic regions may reflect their role as modulators of neurogenesis. Aside from the immunomodulatory effects of microglia on NPCs, microglia of the ventricular/Hp brain region have the task of clearing away an excess of newly-formed cells that do not mature or integrate functionally (Sierra et al. 2010). Ventricular/Hp microglia appear to be equipped with a phenotype which is apt for phagocytosis of dying cells. M-CSF increases phagocytosis of adult human microglia (Chapter 4), and ventricular/Hp microglia have increased expression of CSF-1R. Moreover, these microglia have increased DAP12 expression and an amoeboid morphology which have both been associated with phagocytosis (Graeber 2010; Takahashi et al. 2005). Further studies are now needed to explore the influence of microglia on NPCs and vice versa in the context of the adult human brain in health and disease (Chapter 7).

In conclusion, this chapter reports fundamental differences in two regional populations of microglia in the human adult brain. Microglia from the hippocampus and overlying ventricular lining have greater basal and M-CSF-stimulated proliferation compared to cortical microglia from the middle temporal gyrus. Ventricular/Hp microglia express higher levels of the cell surface proteins CSF-1R, IGF-1R and HLA; the transcription factor C/EBPβ; and the adaptor molecule DAP12. These findings highlight the important role of microglia in the neurogenic niche and advance our understanding of regionally diverse microglial phenotypes. Given this differential microglial phenotype, the role of microglia in the process of neurogenesis was further studied in the next chapter.
Chapter 7.
The Influence of Microglia and Immune Molecules on Adult Human Neurogenesis

7.1. Abstract

Neurogenesis is the formation of new neurons. This process occurs predominantly during development but the adult human brain harbours two specialised areas of continued neurogenesis. In the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles, neural progenitor cells (NPCs) maintain proliferative capacity and can generate new neurons throughout adulthood. The immune system has great influence over neurogenic potential and cells of the immune system produce soluble signalling molecules which influence the fate of neurogenesis.

In vitro models of the complex neurogenesis process involve weeks of differentiation of precursor cells into cells with neuronal characteristics. This chapter describes the NT2 cell line as a model of neurogenesis and produces a shortened version of the standard protocol for neuronal differentiation. The resulting neurons are shown to express neuronal proteins βIII-tubulin, MAP2, GAP-43, PSA-NCAM and synaptophysin, as well as having electrophysiological activity.

Given the valuable resource of adult human brain tissue, neurogenesis was also modelled using primary human NPCs. These cells were isolated, characterised, and differentiated into GFAP-positive astrocytes as well as βIII-tubulin and MAP-2-positive neurons. This astrocytic and neuronal differentiation was then used as an end-point to assess the effect of immune molecules on the process of neurogenesis. Conditioned media was collected from mixed glial cells and applied to differentiating NPCs. However, no difference in astrocytic or neuronal differentiation was observed in the presence of glial conditioned media. The direct effect of immune cytokines/growth factors IFNγ, TGFβ1 and IGF-1 was then evaluated. It was found that IFNγ promoted astrocytic differentiation of adult human NPCs by increasing GFAP-positive cell number and the number of processes on GFAP-positive cells.

Thus this chapter establishes two models of adult human neurogenesis on which the impact of immune cells and molecules can be tested.
7.2. Introduction

The formation of new neurons from precursor cells is called ‘neurogenesis’. It occurs during development to produce the majority of neurons formed throughout life. However, using C\textsuperscript{14} dating techniques and BrdU incorporation into the DNA of dividing cells, neurogenesis has also been shown to continue into adulthood (Bhardwaj et al. 2006; Eriksson et al. 1998; Spalding et al. 2005). In human adult brains the birth of new neurons continues to occur in the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Curtis et al. 2007; Eriksson et al. 1998; Quiñones-Hinojosa et al. 2006). Newly born adult astrocytes are also derived from a common precursor cell and thus the process of adult astrogenesis is closely linked to neurogenesis (Alvarez-Buylla et al. 2001). Astrogenesis is thought to be involved in the important processes of regeneration and scar formation, as well as the replacement of dead cells. The process of neurogenesis requires many steps. Firstly, proliferation of neural progenitor cells (NPCs) produces new cells to turn into neurons. Next, the NPCs differentiate into specific neuronal phenotypes and migrate to their final location. And lastly, the new neurons functionally integrate into existing brain circuits. The first two stages of neurogenesis, proliferation and differentiation, are able to be adequately assessed \textit{in vitro}.

The NT2 human teratocarcinoma cell line is a commonly used \textit{in vitro} model of neurogenesis. NT2 precursor cells can be differentiated into neurons and astrocytes using retinoic acid and mitotic inhibitors in a protocol ranging from 4-8 weeks. The NT2 cell line has been well characterised in its ability to produce mature, functional neurons (Jain et al. 2007; Paquet-Durand et al. 2003). The differentiation protocol has been optimised by several groups and different approaches can be used for different applications including toxicology studies, assessing neurite outgrowth, and synapse formation (Paquet-Durand et al. 2003; Pleasure et al. 1992; Podrygajlo et al. 2010; Radio and Mundy 2008; Serra et al. 2007).

Cells with neuronal characteristics arising from NT2 cell differentiation (NT2Ns) express various neuronal proteins and differentiate into several different neurotransmitter sub-types including GABAergic, glutamatergic, cholinergic and cannabinoid (Goodfellow et al. 2011; Podrygajlo et al. 2009). Electrical activity of NT2Ns has been examined by electrophysiology (Podrygajlo et al. 2010) and they have been found to have spontaneous electrical activity as well as inhibitory post-synaptic currents and excitatory post-synaptic currents. Although previous protocols have required 7-8 weeks differentiation to obtain NT2Ns (Pleasure et al. 1992), the time for differentiation can be reduced considerably by growing cells as non-adherent spheres prior to mitotic inhibition (Paquet-Durand et al. 2003). The method of neuronal differentiation (‘neurogenesis’) used in the present study was
based on the neurosphere protocol by Hill et al. (2008). NT2 precursor cells can also be differentiated into astrocytes expressing Glial Fibrillary Acidic Protein (GFAP), Connexin 43, vimentin and glutamine synthetase (Lim et al. 2007). However NT2-derived astrocytes were not used in the current study.

Advantages of using the NT2 cell line as a model of neurogenesis include its human origin and potential for high-throughput quantification methods such as for drug screening (Hill et al. 2008). Additionally, NT2Ns are a valid model on which to test the role of immune factors in neurogenesis as they have been shown to express various cytokine receptors (Froyland et al. 2008). However, culture and differentiation of these cells is time consuming and the yield of cells is variable from one differentiation to the next. Other draw-backs include the artificial drug-induced differentiation involving chemicals and culture conditions which do not necessarily reflect the in vivo environment.

Although differentiation of the NT2 cell line can provide a reasonable model of adult human neurogenesis, the caveats of cell transformation and chemical differentiation still remain. Using primary human cells derived from adult brain tissue is the ultimate in vitro model of adult neurogenesis as these adult human neural progenitor cells (ahNPCs) are tissue specific and are the relevant species and age. However, limited supply of tissue and the time consuming nature of primary cell culture and experiments are limitations of this work.

Based on protocols used to isolate rodent adult NPCs (Rietze et al. 2006), the isolation of human NPCs has been demonstrated from human fetal brain tissue (Ni et al. 2004), postmortem adult human retina (Mayer et al. 2005), and human olfactory organs (Murrell et al. 2008). More recently, the isolation, culture, and neuronal differentiation of ahNPCs from post-mortem and biopsy adult human brain tissue has been demonstrated by several research groups (Coras et al. 2010; Leonard et al. 2009; Park et al. 2012).

Apart from the standard issues of tissue availability and quality, NPC number decreases with age (Heine et al. 2004; Villeda and Wyss-Coray 2012). As a result, the yield of ahNPCs per brain is usually low and highly variable. In addition, NPCs are not easily distinguished from other proliferative cells in culture (Park et al. 2012). Despite this, the field is now at the point of optimising neuronal differentiation and assessing the effect of different factors, such as immune cells and molecules, on neurogenesis (Yu et al. 2009; Zahir et al. 2009). As the vast majority of in vivo and in vitro studies are done in rodents, and rodent and human studies do not always give equivalent results, there is a great need to move this area into the context of the adult human brain (Gonzalez-Perez 2012).
As reviewed in the previous chapter (Chapter 6), immune cells and molecules are integral to the process of neurogenesis (Carpentier and Palmer 2009; Morrens et al. 2012). Microglia are the predominant resident immune cells in the brain and there is evidence to suggest that they can influence the course of neurogenesis (Butovsky et al. 2006; Choi et al. 2008b). Astrocytes have also been shown to influence neurogenesis (Song et al. 2002). Furthermore, blood cells can be recruited from the cerebrospinal fluid into the SVZ (Muzio et al. 2010), and NPCs express receptors for immune molecules such as chemokines and cytokines (Gordon et al. 2009; Krathwohl and Kaiser 2004; Makela et al. 2010).

Microglial conditioned media has been shown to contain various neurogenesis-modifying factors (Butovsky et al. 2006). Use of conditioned media is a way to model the extracellular milieu as it contains all the factors produced by the cells in culture, albeit at a given time point. However, conditioned media studies are complex physiological systems and direct application of soluble immune molecules to the culture media of differentiating NPCs provides the ability to control concentration of the test molecule and has the advantage of testing one factor at a time.

The aim of this study was to investigate the effect of immune molecules and microglia-derived factors on a model of adult human neurogenesis. Initially the protocol of Hill et al. (2008) for NT2N differentiation was modified to obtain a time-efficient model of neurogenesis. ahNPCs were then isolated, differentiated and used as a model of neurogenesis on which to test microglial and immune factors.
7.3. Methods

7.3.1. Differentiation of NT2 precursor cells into NT2Ns

NT2 precursor cells (Stratagene) were grown in DMEM/F-12 (Gibco Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.29 mg/ml L-glutamine (complete DMEM/F-12; all from Gibco Invitrogen). Neuronal differentiation was induced using a method modified from Hill et al. (2008). Resuspended NT2 precursor cells (2×10^6) were plated in 10 ml media into sterile 10 cm diameter non-adherent bacteriological Petri dishes (Greiner) to generate neurospheres. A final concentration of 10 μM all-trans retinoic acid (RA; Sigma) was added to the cells in complete DMEM/F-12 for 1-2 weeks, with re-plating into new dishes with fresh media every 2-3 days. Neurospheres were then plated in 96-well plates (Nunc) in complete DMEM/F-12 (without RA), either 100 μl per well directly from cell suspension in dishes, or diluted 1:1 in fresh media to reduce cell/sphere density. At this point the use of mitotic inhibitors was omitted to shorten the time for differentiation. Neurospheres were maintained in plates for 1 to 2.5 weeks to allow extension of neuronal processes. For electrophysiology, 2 ml of cell suspension containing RA-treated neurospheres were plated into a well of a 6-well plate containing glass coverslips coated with PDL/matrigel.

7.3.2. Isolation of NPCs from adult human brain tissue

Biopsy and autopsy adult human brain tissue was obtained according to ethical guidelines and with informed consent as detailed in Chapter 2 (General Methods). The method for ahNPC isolation is detailed in Chapter 2.

7.3.3. Culture and proliferation of ahNPCs

The NPC cultures were given a half volume media change every 2-3 days. Careful observation under a light microscope allowed identification of NPCs growing as semi-adherent spheres in the flasks. Cells were passaged once many sphere formations were visible (~2-4 weeks). Floating and semi-adherent cells were collected into a falcon tube. Adherent cells were detached using Accutase (Invitrogen) and collected into the falcon tube. Cells were spun and resuspended by gentle trituration in fresh neural progenitor cell proliferation media for counting. Around half of the cells
were plated in a new flask for the next passage, and half of the cells were plated into Poly-D-
Lysine/Laminin (Sigma) coated 96-well plates at 50,000 cells/ml for differentiation.

7.3.4. Differentiation of ahNPCs into neurons and astrocytes

Media was changed to differentiation media with removal of proliferative growth factors (EGF and
FGF-2) and addition of serum and neuronal growth factors Nerve Growth Factor (NGF) and Brain-
Derived Growth Factor (BDNF) (i.e. DMEM:F12 containing 1% FBS, 40 ng/ml NGF and BDNF
(Preprotech)). For conditioned media studies, half of the differentiation media was replaced by
microglial conditioned media. Cells were differentiated for 2-3 weeks, with half media changes every
2-3 days. Cytokines/growth factors IFNγ (1 and 10 ng/ml in PBS with 0.1% BSA), TGFβ1 (10 ng/ml in 1
mM citric acid pH 3 with 0.1 % BSA) and IGF-1 (10 and 100 ng/ml in PBS) were added to
differentiation media with every half media change throughout the differentiation period.
7.3.5. Immunochemistry

Immunocytochemistry and immunohistochemistry were performed as described in Chapter 2.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Catalogue #</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-HLA-DP, DQ, DR</td>
<td>Dako</td>
<td>M0775</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-GFAP</td>
<td>Sigma-Aldrich</td>
<td>G3893</td>
<td>1:5000</td>
</tr>
<tr>
<td>Rabbit anti-GFAP8</td>
<td>Abcam</td>
<td>ab28926</td>
<td>1:5000</td>
</tr>
<tr>
<td>Mouse anti-βIII-tubulin</td>
<td>Sigma-Aldrich</td>
<td>055k4771</td>
<td>1:1000</td>
</tr>
<tr>
<td>Mouse anti-MAP2</td>
<td>Sigma-Aldrich</td>
<td>M4403</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-GAP-43</td>
<td>Abcam</td>
<td>Ab7462</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-PSA-NCAM</td>
<td>Chemicon</td>
<td>MAB5324</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-Synaptophysin</td>
<td>Dako</td>
<td>A0010</td>
<td>1:1000</td>
</tr>
<tr>
<td>Mouse anti-Sox2</td>
<td>R&amp;D</td>
<td>MAB 2018</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-Nestin</td>
<td>Chemicon</td>
<td>MAB5326</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-Vimentin</td>
<td>Abcam</td>
<td>ab15248</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-Ki67</td>
<td>Dako</td>
<td>A0047</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-BrdU</td>
<td>Roche</td>
<td>11170376001</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG Alexa Fluor® 594</td>
<td>Invitrogen</td>
<td>A11012</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-mouse IgG Alexa Fluor® 488</td>
<td>Invitrogen</td>
<td>A11001</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG Alexa Fluor® 594</td>
<td>Invitrogen</td>
<td>A11005</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG Alexa Fluor® 488</td>
<td>Invitrogen</td>
<td>A11008</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-rabbit biotinylated</td>
<td>Sigma-Aldrich</td>
<td>B7389</td>
<td>1:500</td>
</tr>
<tr>
<td>Goat anti-mouse biotinylated</td>
<td>Sigma-Aldrich</td>
<td>B7264</td>
<td>1:500</td>
</tr>
<tr>
<td>ExtrAvidin-HRP</td>
<td>Sigma-Aldrich</td>
<td>E2886</td>
<td>1:500</td>
</tr>
</tbody>
</table>

Table 7.1: Antibodies used for immunochemistry.
7.3.6. Quantitative image analysis

High-throughput fluorescence imaging and image analysis (with Discovery-1 automated fluorescence microscope and MetaMorph image analysis software) was used to quantify cell number, protein expression and number of cell processes. The *Neurite Outgrowth* journal in MetaMorph was used to quantify number of cell processes.

Where it was not possible to analyse images automatically, such as where cells were in clumps or had particularly complex morphology, cell number was counted by eye.

7.3.7. Statistical analysis

Representative data are displayed as mean ± standard error of the mean (SEM). Experiments were replicated at least 3 times. Statistical analysis was carried out using ANOVA. P values of <0.05 were considered statistically significant differences.
7.4. Results

7.4.1. The NT2 cell model of neurogenesis

The NT2 precursor cell line expresses proteins characteristic of stem cells including nestin (an intermediate filament protein expressed during development and in undifferentiated cells; (Wiese et al. 2004)) and Oct4 (a stem cell transcription factor; (Wernig et al. 2007)) (Figure 7.1A and B). NT2 precursor cells are highly proliferative and incorporate BrdU into their DNA during cell division (Figure 7.1C). They express low levels of Microtubule Associated Protein 2 (MAP2; a ‘later’/mature neuronal protein; (Chamak et al. 1987)) and only diffuse amounts of βIII-tubulin (a microtubule component expressed by ‘early’/immature neurons; (Roskams et al. 1998)) (Figure 7.1 D and E).

Using a method shortened from the protocols of Paquet-Durand et al. (2003) and Hill et al. (2008), NT2 precursor cells were differentiated into NT2 Neurons (NT2Ns) using the neurosphere method. When plated into non-adherent petri dishes and treated with retinoic acid (RA), NT2 precursor cells grow as spheres (Figure 7.1F). Cells were initially treated with RA for 2 weeks and then seeded into 96-well plates for neurite development. Over time (up to 2.5 weeks) in 96-well plates, markers of stem cells (NT2 precursors) and of astrocytes (not selected for by this protocol) decrease with
increased time of differentiation. Figure 7.2 shows reduced expression of the stem cell marker nestin and the astrocytic marker vimentin (an intermediate filament protein expressed by astrocytes; (Janeczko 1993)) following neuronal differentiation. With increased time of differentiation in 96-well plates, expression of neuronal proteins βIII-tubulin and MAP2 is simultaneously increased (Figure 7.2E-H).

Figure 7.2: Neuronal differentiation of NT2 cells is increased with time in 96-well plates following 2 weeks RA treatment.

A) High levels of nestin are still expressed in RA-treated NT2 cells but are reduced (B) with increasing differentiation time in plates. C) NT2 cells express vimentin astrocytic protein but this is reduced (D) with increased differentiation time in plates. Neuronal proteins βIII-tubulin (E and F) and MAP2 (G and H) expression are increased with time throughout the neuronal differentiation protocol. Scale bar = 50 µm.

To determine the minimum time needed for RA treatment, a comparison was made between cells differentiated with 1, 1.5 or 2 weeks RA. As shown in Figure 7.3, 1-1.5 weeks of RA treatment is sufficient for NT2N expression of markers βIII-tubulin, MAP2 and Growth Associated Protein 43 (GAP-43; a presynaptic protein involved in axonal development). Nevertheless, expression of these proteins is increased from 1-2 weeks treatment with RA (Figure 7.3). This shorter differentiation
protocol (1-1.5 weeks RA) also gave rise to immunocytochemically mature neurons which express Polysialylated Neural Cell Adhesion Molecule (PSA-NCAM; a molecule involved in migration of newly born neurons; (Kam et al. 2009)) and synaptophysin (a presynaptic vesicle protein) (Figure 7.4). However, different repeats of differentiation gave rise to variable numbers of NT2N cells and also to slightly variable expression of neuronal markers.

Figure 7.3: NT2N expression of neuronal markers βIII-tubulin, MAP2 and GAP-43 is increased by longer RA treatment times.

A-C) NT2Ns express high levels of βIII-tubulin after 2 weeks differentiation in 96-well plates and this expression is increased by longer prior RA exposure. D) Distinct MAP2 protein expression is evident in NT2Ns from as little as 1 week RA treatment and more MAP2-immunopositive NT2Ns are present when RA treatment is increased to 2 weeks (E and F). G-I) GAP-43 neuronal protein expression is similarly increased in NT2Ns from 1-2 weeks RA treatment. Scale bar = 50 µm.
NT2Ns predominantly grow out from neurospheres and neuronal differentiation markers are rarely found in isolation from other cells. NT2Ns have small, round cell bodies and long processes extending up to several hundred µm in length (Figure 7.4C and D). This observation is comparable to what has been described in the literature and distinct from NT2-derived astrocytes which are flat, have large nuclei and lack processes (Lim et al. 2007).

To test for physiological maturity of the NT2Ns produced by this method, electrophysiology was performed on NT2Ns produced by 1.5 weeks RA treatment and 2 weeks differentiation in plates (electrophysiology was kindly performed by Dr Thomas Park). Cells were selected for electrophysiology based on morphological properties of NT2Ns – small, round cell bodies and long, fine processes (Figure 7.5A and B). A fluorescent dye injected into the cell after recording shows its morphology with fine processes extending far from the cell body (Figure 7.5B). Figure 7.5 shows an example of an NT2N cell with immature neurophysiological properties. This cell fired an immature (slow) action potential and had a resting membrane potential of -40 mV (resting potential is typically
-60 to -65 mV for mature neurons; Figure 7.5C and E). When held at -60 mV (to assess whether it expressed the molecular machinery to fire mature action potentials) a more mature action potential was produced (Figure 7.5D and F). Thus, some NT2Ns have the ability to fire action potentials after this short differentiation protocol. However, not all cells tested had the ability to fire action potentials. For example, a cell with a slightly flatter and less round cell body displayed electrical characteristics more similar to that of an astrocyte, despite having multiple processes (data not shown).

These results present a model paradigm of adult human neurogenesis which can be used to test the effect of molecules/drugs on the process of neurogenesis.

**Figure 7.5:** NT2Ns derived from a shortened differentiation protocol are electrophysiologically active.

A) Bright field image of differentiated NT2Ns during whole-cell patch-clamp recordings. B) The patched cell was injected with dye to visualise the cell morphology after patching. C-F) Electrophysiological responses to single (C and D) and multiple (E and F) current steps in current clamp conditions. This cell had a resting membrane potential of -40 mV and fired an immature action potential (AP) in response to depolarizing current (C and E). When held at -60 mV (the resting membrane potential of mature neurons) the same cell generated a more rapid and mature action potential (D and F).
7.4.2. Characterisation of adult human neural progenitor cells

Our laboratory has previously characterised the cultures of ahNPCs isolated from biopsy and autopsy adult human brain tissue, and distinguished these cells from the other proliferative cell type in the primary human brain cell cultures – the fibroblast-like cells (Chapter 5). The ahNPCs used in this study have been similarly characterised as described by Park et al. (2012).

ahNPCs grow as spheres and as occasional monolayers in proliferative conditions (Figure 7.6). Cells are maintained for 1-3 months post-isolation and monitored carefully for formation of spheres which could contain NPCs. To assess the phenotype of these cells, spheres and monolayer cells were plated into 96-well plates for immunocytochemistry. NPCs have the ability to divide, and thus express the cell division protein Ki67 (Figure 7.7A) and incorporate BrdU during cell division. These cells also express characteristic ‘stem/progenitor cell’ proteins nestin, Sox2 (a stem cell transcription factor; (Wernig et al. 2007)) and GFAP (expressed by NPCs as well as mature astrocytes; (Matyash and Kettenmann 2010; Quiñones-Hinojosa et al. 2006; Van Den Berge et al. 2010) (Figure 7.7).

Figure 7.6: ahNPCs form neurospheres and monolayers in proliferative culture media.

Following isolation from brain tissue, ahNPCs are cultured in T25 flasks with ‘proliferation media’ (see Methods). A) A floating neurosphere derived from ahNPCs isolated from adult human brain tissue. B) A semi-adherent sphere of progenitor cells. C) Cells from ahNPC isolations also adhere to the culture flasks and grow as monolayers. Scale bar = 50 µm.

A limited number of cells expressing markers of ‘differentiated’ cells are seen in the initial isolation and proliferation stage of NPC culture. Predominantly GFAP (as both NPCs and parenchymal astrocytes express GFAP) and some βIII-tubulin positive cells are present (Figure 7.7C). However, very little MAP2 expression is present in proliferating conditions. The GFAP immunopositive cells are similar to the mixed glial cultures which contain a small percentage of GFAP immunopositive astrocytes (Chapters 3 and 5). The amount of GFAP and βIII-tubulin expression in proliferative conditions (Figure 7.7) is minimal compared to after the differentiation procedure (Figure 7.11 below).
7.4.3. Differentiation of ahNPCs into neurons and astrocytes

Once the ahNPCs had divided to produce sufficient cells and spheres in proliferative culture conditions, cells were seeded in 96-well plates and switched to differentiation media with serum and the growth factors BDNF and NGF. Following differentiating conditions, markers of immature (βIII-tubulin) and mature (MAP2) neurons were used. Astrocytic differentiation was also assessed using anti-GFAP antibodies, as astrogenesis is a prominent and important feature of NPC proliferation and differentiation (Alvarez-Buylla et al. 2001).

As markers of astrocytes and neurons are virtually not detected in cultures isolated from human adult post-mortem brain tissue (compared to biopsy tissue), these cultures provide valuable confirmation of de novo differentiation of mature neurons and astrocytes from the NPC cultures. As shown in Figure 7.8, differentiated neurons and astrocytes were obtained from post-mortem cultures. These ahNPCs have previously been shown to differentiate into electrophysiologically active neurons in vitro (Park et al. 2012).

---

**Figure 7.7: ahNPCs express progenitor cell markers.**

A) Sox2 transcription factor (green) is expressed by ahNPCs, some of which also express cell division markers such as Ki67 (red). B) Nestin (green) is expressed by cells in adult human NPC cultures. GFAP (red) is also a marker of NPCs and some cells co-express nestin and GFAP. C) Mature brain cells are also sometimes present in NPC cultures. However βIII-tubulin (green) and GFAP (red) expression is minimal compared to differentiated cultures (see Figure 7.11). Scale bar = 50 µm.
Figure 7.8: ahNPCs differentiate into neurons and astrocytes.

This example of ahNPC differentiation is from a neurologically normal post-mortem brain. As neurons and astrocytes do not survive from cultures of post-mortem tissue, the neuronal and astrocytic cells seen after differentiation have formed de novo from NPCs. ahNPCs differentiate into neurons with multiple processes expressing βIII-tubulin (A) and MAP2 (B). ahNPCs also differentiate into GFAP-expressing astrocytes (C). Scale bar = 50 µm.

7.4.4. Differentiation of ahNPCs in mixed glial cell conditioned media

As reviewed in the previous chapter, microglia are present in the neurogenic regions of the adult human brain and in the tissue from which ahNPCs are cultured. Figure 7.9 shows a brain tissue section of the adult human SVZ with microglia and putative NPCs in close proximity. Parenchymal astrocytes present in the adult human neurogenic niche are another glial cell type with immune functionality (Curtis et al. 2005b; Quiñones-Hinojosa et al. 2006).

Figure 7.9: Microglia are in close proximity to NPCs in the adult human SVZ.

A section of adult human brain tissue containing the wall of the lateral ventricle has been double-labeled for microglia (using HLA-DP, DQ, DR) and neural progenitor cells (using GFAPδ). The ventricle is indicated by an asterisk. Microglia (blue arrows, brown staining) are found adjacent to NPCs (red arrows, black staining) in the adult human SVZ. Scale bar = 50 µm.
To directly examine the effect of immune molecules produced by microglia and astrocytes on the process of neurogenesis, a conditioned media paradigm was used to assess whether microglia and astrocytes secreted factors into their extracellular environment which could act on NPCs to influence their differentiation course. Initially, conditioned media was collected from flasks containing mixed glial cell isolations. The mixed glial cultures were maintained through each passage by seeding a new T75 flask with cells. After 3 passages there is a limited number of microglia and astrocytes remaining, as they do not divide, and the culture consists almost entirely of proliferative fibroblast-like cells (Chapter 5, (Gibbons et al. 2007)). The conditioned media from these later passage flasks can thus serve as a negative control for first passage mixed glial conditioned media containing factors produced by microglia and astrocytes.

The glial conditioned media was mixed half-half with standard differentiation media and compared to 100% of the standard differentiation media as a control for ahNPC differentiation potential. An additional control for conditioned media was fresh glial growth media (DMEM/F12 + 10% FBS + 1% PSG) which was also mixed with standard differentiation media. Cells were differentiated in these media for 2 weeks and assessed for expression of neuronal and astrocytic differentiation markers. No consistent significant differences in either the number or nature of neuronal or astrocytic differentiation were observed using the markers βIII-tubulin, MAP2 and GFAP (Figure 7.10).
Figure 7.10: Adult human glial conditioned media does not influence ahNPC differentiation.

Cultured in standard differentiation media, ahNPCs differentiate into GFAP-positive astrocytes and βIII-tubulin-positive neurons (A and E). This neuronal and astrocytic differentiation is not significantly altered by culturing the ahNPCs in conditioned media from mixed glial cultures containing microglia and astrocytes (C and E). Nor is ahNPC differentiation significantly affected by negative control standard glial media or later passage conditioned media (without microglia or astrocytes) (B, D and E). Scale bar = 100 µm.
7.4.5. Effects of microglia-specific conditioned media on ahNPC differentiation

Next the effect of microglia only on ahNPC differentiation was investigated. The microglial population in mixed glial cultures was specifically targeted using an siRNA against a microglia-specific transcription factor – PU.1 (Chapter 3). As astrocytes and fibroblast-like cells in mixed glial cultures do not express PU.1, any differences found between conditioned media from control siRNA and PU.1-specific siRNA treated cells would be due to factors in the conditioned media produced by microglia alone.

Microglia in mixed glial cultures were transfected as detailed previously in Chapter 3. 4-7 days following transfection, conditioned media was collected from mixed glial cultures and stored at -80 °C until needed. When the ahNPCs were ready for differentiation, their standard differentiation media was compared to additions of either negative (control) siRNA conditioned media or PU.1-specific siRNA conditioned media. The cells were differentiated for 2 weeks in the presence of these different media.

No differences in neurogenic or astrocytic differentiation between different media conditions were observed by immunocytochemistry (Figure 7.11A-F). βIII-tubulin, MAP2 and GFAP expression did not qualitatively or quantitatively differ between control and PU.1 siRNA conditioned media (Figure 7.11). Thus, media produced by normal, PU.1-expressing microglia had no observably different effect on ahNPC differentiation than media produced by microglia lacking the transcription factor PU.1.
Figure 7.11: Factors produced by microglia were not identified as having an effect on ahNPC differentiation.

Differentiation of ahNPCs was compared in the presence of conditioned media from control transfected glia and conditioned media from PU.1 siRNA transfected glia (with the PU.1 target expressed only by microglia in mixed glial cultures). ahNPCs differentiate into βIII-tubulin expressing neurons (A-C) and GFAP expressing astrocytes (D-F) whether they are exposed to control siRNA conditioned media or PU.1 siRNA conditioned media. G) Differentiation into GFAP, βIII-tubulin and MAP2-positive cells is quantifiably similar in both conditioned media conditions and standard differentiation media. Scale bar = 50 µm.
7.4.6. IFNy increases astrocytic differentiation of ahNPCs

As no changes could be detected in differentiation induced by conditioned media derived from glial cells, it was tested whether direct application of immune molecules (cytokines and growth factors) would influence the process of ahNPC differentiation. During the 2 weeks differentiation stage in 96-well plates, IFNy (1 and 10 ng/ml), TGFβ1 (10 ng/ml) or IGF-1 (10 and 100 ng/ml) were added to the cells in standard differentiation media.

Immunocytochemistry for ‘early’ neurons (βIII-tubulin) and astrocytes (GFAP) did not reveal any differences in neurogenesis or astrogenesis with TGFβ1 or IGF-1 treatment (data not shown). However, IFNy did have an effect on ahNPC differentiation. A greater amount of GFAP expression was observed in wells treated with IFNy compared to vehicle (Figure 7.12A-C). There was a greater number of GFAP immunopositive cells, and a significant increase in GFAP processes, with IFNy treatment. This effect was seen for 1 ng/ml as well as 10 ng/ml IFNy. As seen in Figure 7.12, IFNy produced a greater number of thin, fine GFAP-positive processes. However, there was no observable effect of IFNy on βIII-tubulin expression.

Figure 7.12: Astrocytic differentiation of ahNPCs is increased by IFNy.

A) GFAP-positive astrocytic differentiation is evident from ahNPC cultures. B and C) IFNy increases the number of GFAP-positive cells, and their branching/process extension, in ahNPC differentiation cultures. D) IFNy increases the number of GFAP-positive cells which differentiate from ahNPC cultures, though not to significance. E) A significantly greater amount of GFAP-positive cell processes are formed with IFNy treatment. Scale bar = 250 µm.
As seen previously for astrocytes in mixed glial cultures from cortical tissue (Chapter 5), IFNγ treatment also increased HLA expression by differentiated ahNPCs with astrocytic morphology (Figure 7.13A-C). IFNγ slightly but significantly reduced cell viability (assessed by AlamarBlue viability assay) but did not significantly reduce total cell number (Figure 7.13D and E).

Figure 7.13: IFNγ induces HLA expression in ahNPC differentiation cultures and slightly reduces their viability.

A) Little HLA (green) is expressed by cells in ahNPC differentiation conditions. B and C) 1 and 10 ng/ml IFNγ induces HLA expression in these cultures. HLA-immunopositive cells with astrocytic morphology are indicated by arrows. D) IFNγ has a slight negative effect on viability of ahNPC differentiation cultures as assessed by AlamarBlue viability assay. However, total cell number is not significantly affected by IFNγ treatment (E). Scale bar = 100 µm.
7.5. Discussion

The results of this chapter present two in vitro models of adult human neurogenesis – 1) Differentiation of NT2 precursor cells into NT2Ns, 2) Isolation and differentiation of ahNPCs.

7.5.1. The NT2 cell model of neurogenesis

The constant availability and human origin of NT2 precursor cells led themselves to be an attractive option to use as a model of neurogenesis. The P19 rodent cell line can similarly be differentiated into functional neurons with a shorter RA induction time (4 days) (Monzo et al. 2012). However these cells were not used due to potential differences between species. The relatively short NT2N differentiation protocol produced here, derived from Hill et al. (2008), was favourable as it did not require a mitotic inhibitor step, and it was substantially shorter (less than 2 weeks RA and 2 weeks differentiation in 96-well plates) than the standard 7-8 week NT2 neuronal differentiation protocol. The neurosphere method of neuronal differentiation may indeed be more representative of the in vivo scenario than a monolayer of cells and may account for their increased differentiation.

Although it was important to prove that the cells assessed here had the potential to convert into mature functioning neurons, fully differentiated neurons were not required for this study as the process of neurogenesis involves many steps and can be assessed at many different stages from initial proliferation of a NPC, to functional integration in a neuronal circuit. Nevertheless, NT2N expression of several neuronal proteins is demonstrated including the ‘early’ neuronal markers βIII-tubulin and PSA-NCAM, as well as the more mature neuronal markers MAP2, GAP-43 and synaptophysin (Figures 7.3 and 7.4).

To assess the functional maturity of these NT2Ns produced in a short time frame, they were assessed for their electrophysiological potential as a measure of the ability of the NT2Ns to communicate with each other via synapses. It was found that some cells had neuronal firing patterns, although the cultures at this point in differentiation were at a relatively immature electrophysiological stage (Figure 7.5). However, the mature action potentials fired when the cell was held at physiologically mature resting membrane potential (-60 mV) demonstrate that NT2Ns are in the process of fully maturing. As NT2Ns usually grow in clusters from neurospheres and neuronal differentiation markers are rarely found in isolation from other cells it appears that NT2Ns are dependent on each other for support, perhaps through trophic molecules, or to form synapses.
Thus a model of neurogenesis was produced, which takes a relatively short time period to differentiate immunocytochemically mature and electrophysiologically active neurons (Figures 7.3-7.5). This model of neurogenesis can be used to test the effect of different factors on neurogenesis. However, several disadvantages of using a cell line still remain and it is not known to what extent the teratogenic origin of NT2 precursor cells prevents them from representing the *in vivo* adult neurogenic environment.

### 7.5.2. Adult human neural progenitor cells as a model of neurogenesis

Due to the above disadvantages of the NT2 cell line and the availability of adult human brain tissue and primary ahNPCs, the method of ahNPC differentiation was chosen as the neurogenesis model with which to test immune molecules. Despite case to case variability and the limited source of cells compared to immortal cell lines, primary cells have the advantage of better reflecting a range of individuals and are from the relevant age and species. A robust protocol has been developed in our laboratory for isolation, culture, and differentiation of ahNPCs into neurons and astrocytes (Figures 7.6-7.8; (Park et al. 2012)).

Although *de novo* differentiation of NPCs into neuronal cell types is a more definitive step towards the production of new neurons, the proliferation of NPCs can lead to production of beneficial factors for pre-existing brain cells (Pluchino et al. 2009; Wu et al. 2008). It may even be more physiologically relevant to assess NPC proliferation as an end-point (Balasubramaniam et al. 2009), as there is evidence to suggest that proliferating progenitor cells secrete neuroprotective molecules which help neurons survive, and this may play an even bigger role in regeneration than differentiation and functional integration of new neurons (Pluchino et al. 2009; Pluchino et al. 2005). Furthermore, NPCs release immune molecules which can act on and influence immune cells and immunological processes (Mosher et al. 2012). Recent evidence has also emerged of transplanted NPCs contacting spinal cord monocytic cells via cellular-junctional coupling, and regulating immune cell gene expression *in vivo* (Cusimano et al. 2012).

### 7.5.3. No effect of glial conditioned media was observed on ahNPC differentiation

Despite no observable difference in ahNPC differentiation in the presence of different glial conditioned media (Figures 7.10 and 7.11) it is still possible that microglia and astrocytes release factors extracellularly which influence NPC differentiation. This has been demonstrated with rodent
cells using a method similar to that used here (Aarum et al. 2003; Butovsky et al. 2006; Nakanishi et al. 2007; Walton et al. 2006) and for adult human retinal NPCs (Balasubramaniam et al. 2009). Possible reasons for not detecting an effect of glial conditioned media on neurogenesis with human adult cells include high variability within treatment groups, inadequate concentration/activity of immune molecules in conditioned media, and inappropriate time-frame of exposure to immune molecules or the chosen end-point. There is much evidence in the literature to suggest that immune factors can indeed affect neurogenesis (Morrens et al. 2012), and the cytokine/chemokine screens performed in Chapters 3 and 5 demonstrate that mixed glial cultures secrete immune molecules. The immune system provides support for all aspects of neurogenesis from progenitor self-renewal to differentiation (Deierborg et al. 2010). The adaptive as well as innate immune system plays a major role in monitoring and influencing neurogenesis (Ziv et al. 2006a; Ziv et al. 2006b). However, the effect of a particular factor on neurogenesis will be context and time dependent, reflecting the combinations of factors and cell types present (Cacci et al. 2008; McPherson et al. 2011).

Further investigation to increase our understanding of this topic includes optimisation of neurogenic differentiation of ahNPCs, and continuing to analyse the factors produced by glial cells under different conditions (e.g. Cytometric Bead Array cytokine quantification, Chapters 3 and 5). Another protocol to assess the effect of the immune system on the process of neurogenesis is to directly coculture microglia or astrocytes with NPCs. However, due to the limited availability of both primary human glia and ahNPCs, and the extensive optimisation required for co-cultures of different cell types, this option was not feasible in the time-frame of this thesis. Therefore the effect of individual immune cytokines and growth factors on neurogenesis was tested in a more controlled environment.

### 7.5.4. IFNy increases astrocytic differentiation of ahNPCs

The results show that IFNy influences ahNPC differentiation when present in the differentiating media over a course of 2 weeks (Figure 7.12). GFAP immunocytochemistry revealed a greater number of astrocytic cells and more astrocytic processes after IFNy treatment compared to vehicle (Figure 7.12). βIII-tubulin expression on the other hand was not observed to differ with IFNy treatment compared to vehicle.

It is possible that IFNy is having an effect on astrocytic differentiation and morphology via other molecules produced by cells in culture (including astrocytes themselves) when exposed to IFNy. For example, as IFNy treatment of mixed glial cells produces a massive increase in IP-10 release, does IP-
10 have an effect on ahNPC differentiation? As in Chapter 5, IFNy increases HLA expression in ahNPC differentiation cultures (Figure 7.13). It is interesting to note that the IFNy-induced increase in HLA is both rapid (after 4 days, Chapter 5) and sustained (2 weeks exposure to IFNy, Figure 7.13). In contrast, no significant change in GFAP-positive cell number, or obvious change in GFAP-positive processes, was found in mixed glial cultures with IFNy treatment for 96 h (Chapter 5). It may be that newly differentiated astrocytes respond differently to IFNy than fully mature astrocytes, or that a long exposure to IFNy is necessary to produce changes to astrocytic morphology or number.

This effect of IFNy on astrocytic differentiation was surprising given the findings of previous studies which indicate IFNy as a pro-neurogenic factor. IFNy administered in vivo or to differentiating NPCs in vitro resulted in increased neurogenesis (Baron et al. 2008; Zahir et al. 2009). In addition, conditioned media from IFNy-activated microglia promoted neurogenic differentiation of mouse adult NPCs (Butovsky et al. 2006). Astrocytic differentiation was negligible and unchanged in these rodent studies (Butovsky et al. 2006; Zahir et al. 2009).

No effect was observed for either TGFβ1 or IGF-1 in this model of adult human neurogenesis. Nevertheless, a pro-neurogenic role for TGFβ1 has been previously demonstrated in rodent models of adult neurogenesis (Battista et al. 2006; Graciarena et al. 2010), and microglial-produced IGF-1 has been similarly shown to promote neurogenic differentiation (Butovsky et al. 2006; Thored et al. 2009). These contrasting results could indicate that the neuronal-inducing potential of immune molecules is inconsistent across species as well as potentially cell origin (age and location), and number of passages in vitro (Zahir et al. 2009). These discrepancies highlight the need to use multiple models, and in particular to include human cells, when studying complex biological phenomena such as neurogenesis.

Progenitor cells, including NPCs, are in a very plastic state and their lineage development can be manipulated relatively easily (Forsberg et al. 2010). Therefore, a future challenge is to determine the extent to which in vitro models of neurogenesis reflect the in vivo physiological process (Conti and Cattaneo 2010). The further study of factors which influence endogenous neurogenesis in the adult human brain is warranted as this physiological process holds great promise for brain regeneration.
Chapter 8.
General Discussion

The brain is under constant surveillance by the immune system throughout health and disease. Neuroinflammation and inappropriate immune activity are hallmarks of neurological disorders and normal ageing processes. Microglia, the resident immune cells of the brain, are key players in neurophysiology as they integrate immune signals into the brain environment and are important mediators between the peripheral immune system and the central nervous system (CNS). Microglia have diverse functions in the CNS and this thesis explores the important microglial functions of phagocytosis, cytokine and chemokine secretion, migration, and expression of antigen presentation cell surface receptors. Enhancing our understanding of the mechanisms of microglial activation will identify new targets for treatment of neurological disorders.

The overall aims of this research were to explore the nature of adult human microglia and learn more about their control mechanisms and functions. Specifically, the role of the transcription factor PU.1 was investigated and the influence of immune growth factors and cytokines on microglial phenotype and functions was assessed. In addition, microglia from different brain regions were compared and the effect of immune cells and molecules on the process of neurogenesis was investigated.

In order to do this, mixed glial cell cultures containing microglia, astrocytes and fibroblast-like cells were isolated from adult human brain tissue. The results show human-specific microglial effects; diverse microglial phenotypes in response to different activating factors; and brain region-specific microglial phenotypes. The most important outcome of this research is the demonstration that microglia from human brain tissue can be cultured from a range of neurological disease states and used for pharmacological and genetic manipulation to investigate their basic biology and to identify mechanisms for therapeutic targeting.
8.1. Summary of major findings

Initial investigations identified the key myeloid transcription factor PU.1 in adult human microglia. PU.1 was shown to be expressed by microglia in the adult human brain and to play a critical role in adult human microglial function (Chapter 3). Adult human microglia can be transfected with short interfering RNA (siRNA) with high efficiency to assess protein function. The ease of transfection of primary adult human microglia is unprecedented. It is generally considered technically challenging to transfet primary cells, let alone primary human adult cells (de Fougerolles et al. 2007; Whitehead et al. 2009). Nevertheless, with minimal optimisation, a high level of PU.1 knock-down was achieved using siRNA. siRNA is a powerful tool for protein function analysis and has great potential as a tool to investigate the biology of human adult microglia.

PU.1 siRNA was used to investigate the role of PU.1 in microglial phagocytosis. Phagocytosis is a key function of microglia and is integral to numerous physiological and pathological brain processes. Understanding the mechanisms of phagocytic control may enable direct manipulation of this process for therapeutic intervention. For example, in Alzheimer’s disease there is an increasing body of literature that supports the idea that phagocytosis is beneficial for clearing amyloid-beta and preventing the formation of amyloid-beta plaques (Rogers et al. 2002). The subsequent chapter showed that Macrophage Colony-Stimulating Factor (M-CSF) increases microglial phagocytosis, concurrent with an increase in PU.1 expression. The M-CSF-PU.1 pathway is a candidate for further investigation for manipulation of this important process in the context of brain disorders.

M-CSF is shown to be a major determinant of adult human microglial phenotype (Chapter 4). M-CSF not only influenced microglial phagocytic function, but also their rate of proliferation and expression of multiple proteins. In addition, microglia from different brain regions were found to be differentially responsive to M-CSF. A less activated microglial phenotype, assessed by morphology and Human Leukocyte Antigen (HLA) expression, is brought about by M-CSF treatment. This deserves further investigation as over-activated microglia with amoeboid morphology and increased HLA expression are observed in disease and injury processes and are thus thought to exacerbate neuropathology. The ability of microglia to undergo phagocytosis, without being activated to produce pro-inflammatory cytokines, could be particularly useful following cell death, such as after stroke or trauma. In these situations microglia are required to help clear up debris in order to limit inflammatory responses, but excessive microglial activation would further hinder regeneration.

Such a pro-inflammatory phenotype was found to be induced in microglia by Interferon (IFN)-γ, as well as in astrocytes and fibroblast-like cells. Primary human mixed glial cultures were induced to
express the antigen presentation molecule HLA and secrete pro-inflammatory cytokines and chemokines (Chapter 5). These three cell types were then found to have differential responses to M-CSF and Transforming Growth Factor (TGF)-β growth factors, where M-CSF reduced microglial HLA expression and TGFβ1 reduced fibroblast-like cell HLA expression. A significant species difference was also found between rodent and human microglia when investigating the effects of different cytokines and growth factors on expression of the antigen presenting protein HLA. Human adult microglia did not respond to TGFβ1 as rodent microglia have been reported to do (O’Keefe et al. 1999; Pazmany and Tomasi 2006). This finding has important implications for neuroinflammation and targeting of brain inflammation to treat disease. Identification of the TGFβ1 effect on brain derived fibroblast-like cells marks these cells as distinctive immune players in the brain environment. Their further response to TGFβ1 in scar formation discovered another differential effect from rodent models. While TGFβ1 has been reported to promote scar formation in rodent studies, the opposite was found to be true for these human brain cells.

The different responses of human microglia, and other cell types, to various immune molecules (M-CSF, IFNγ and TGFβ1) demonstrate the complexity of neuroinflammatory processes. Understanding the balance of pro- and anti-inflammatory microglial activities and phenotypes is central to understanding brain disease mechanisms. The realisation that such key molecules as TGFβ1 (whose anti-inflammatory properties have been studied for decades) can have strikingly different microglial effects in rodents and humans is a reminder of the importance of using relevant experimental models.

Adult human microglia cultured from the neurogenic regions of the hippocampus and wall of the lateral ventricle (Alvarez-Buylla et al. 2002) not only had a greater proliferative response to M-CSF but were also basally more proliferative than cortical region microglia (Chapter 6). This is the first detailed study of neurogenic region microglia from adult human brain tissue. It reveals that microglia exhibit different phenotypes depending on their surrounding microenvironment and emphasises the importance of the growth factor M-CSF. This study also confirms the results of previous rodent studies that microglia in neurogenic regions have unique proliferative capability. The finding that this phenomenon is still evident in the adult brain highlights the continued presence of neurogenesis in adult humans. Microglia of neurogenic regions may have an important role to play in future endogenous or exogenous cell replacement therapies.

The exciting discovery that microglia in neurogenic regions of the adult human brain have unique properties of proliferation and response to M-CSF not only demonstrates that microglia have diverse phenotypes, but also provides the opportunity to specifically target a microglial population in one
region of the brain over another. This discovery provides basis for increased specificity of microglia-targeting therapeutics. As demonstrated throughout this thesis, microglial phenotype is context dependent and therapies aiming to limit the detrimental effects of microglia will need to be tailored to specific situations.

The final results chapter shifts focus to the process of neurogenesis itself (Chapter 7). This study, which is the first of its kind, was designed to assess the effect of immune molecules on adult human neural progenitor cells (ahNPCs). This specialised cell type was isolated and cultured from adult human brain tissue. The neurogenic and astrocytic differentiation potential of ahNPCs was then assessed in various immune environments. Conditioned media from mixed glial cells was unable to produce an observable change in ahNPC differentiation. It is likely that with more sensitive techniques and/or more robust treatment protocols the influence of microglia and astrocytes on ahNPC differentiation could be measured. Interestingly, the immune cytokine IFNy was found to direct adult human neurogenesis towards an astrocytic fate. This is again not the expected result based on reports of rodent studies which find IFNy to promote neurogenic differentiation (Butovsky et al. 2006).

A large amount of research is being performed in the fields of neurogenesis and regenerative medicine focusing on endogenous neurogenic processes (Vukovic et al. 2011). Immunomodulation is likely to have significant impact on neurogenic outcomes and further research in this area is bound to reveal exciting results. This study shows that future experiments performed with human NPCs are necessary to identify the molecular targets best suited for therapeutic investigation.

8.2. Microglia: species differences matter

Previous work in our laboratory has revealed differential responses of rodent and human microglia to the anti-epileptic drug valproic acid (VPA). Primary adult human microglia exhibited reduced phagocytosis with VPA treatment, whereas rodent microglia had enhanced phagocytosis and also underwent apoptosis in response to VPA (Gibbons et al. 2011; Smith et al. 2010). In the case of VPA, a drug modifier of the epigenome, there are likely to be multiple differences in rodent and human gene expression which lead to the functional differences in viability and phagocytosis.

This thesis found further species differences in microglia. The role of TGFβ1 as an anti-inflammatory factor capable of mitigating IFNy-induced inflammatory responses in rodent microglia, was not confirmed in primary human microglia. Instead, no response in HLA expression was evident with
TGFβ1 treatment (Chapter 5). These intriguing results of species differences with regards to cytokine regulation of cell surface receptor expression demonstrate fundamental underlying differences between rodent and human gene regulation mechanisms. In the final results chapter focusing on neurogenesis there were again differences found in the response of adult human cells to cytokines compared to their rodent counterparts. While IFNγ has been reported to have a neurogenesis-promoting effect on rodent NPCs, it induced increased astrogenesis of ahNPCs (Chapter 7).

65 million years of evolutionary divergence is bound to produce significant differences in many immunological and neurological mechanisms between rodents and humans (Church et al. 2009; Mestas and Hughes 2004). As well as differences in genetic information between these two species, it is possible that differences between human and murine immune responses reflect the immunologic imprint of our respective microbiomes in addition to inherent differences in immunity (Virgin et al. 2009). Whilst most experimental animals are kept in relatively sterile environments, humans are in contact with a host of microbes and carry many chronic viral infections which influence our immune systems (Gevers et al. 2012; Virgin et al. 2009).

While rodent models have a place in biomedical research, my project further demonstrates the incompleteness of the translatability of this model to humans. This thesis adds evidence to the idea that human cells and model systems are essential tools to allow translation of neuroscience and immunological research to the clinic.

8.3. Limitations of primary adult human mixed glial cultures

There are advantages and disadvantages to culturing microglia in a mixed glial population. The strengths of mixed glial cultures as a model of the adult human brain include their human tissue source and their ease of isolation and use. Compared to cell lines, primary human adult cells have limited availability and inconsistent supply. However, this thesis demonstrates the feasibility of using primary human microglia for large-scale studies and shows that in fact their use alone can generate a large amount of data.

A mixed cell population requires more caution when assessing microglial-specific effects due to the potential for indirect actions of other cell types present. This point has been addressed throughout this dissertation by using immunocytochemical methods to distinguish the different cell types in culture, and also by testing treatments on cultures of fibroblast-like cells only (without microglia and astrocytes). Furthermore, the proportions of different cell types in culture will change over time as
the culture contains proliferating fibroblast-like cells and virtually non-proliferating microglia and astrocytes. Nevertheless, a mixture of cell types more accurately models the intact brain. Similar microglia yields per gram of tissue have been reported using a density gradient separation technique and selection of microglia with magnetic microbeads (Melief et al. 2012). The separation of microglia from other cell types in mixed glial cell isolations has its merits, for example limitation of bystander effects, but is also further removed from the brains’ in vivo environment. Further addition of neurons and oligodendrocytes (as could for example be generated using induced pluripotent stem cell techniques) into the mixed glial culture would increase the applicability of this model to the adult human brain as a whole. As a model of the adult human brain, mixed glial cultures would also be improved by 3-dimensional, instead of 2-dimensional, culture environments. The development of more sophisticated culture techniques which mimic the in vivo environment are needed to better perform long-term pharmacological and genetic experiments using primary cell cultures.

A potential limitation of dissociated mixed glial cultures is that the tissue dissociation process used to isolate cells may alter the activation state of microglia. As microglia are so sensitive to changes in their environment, it is likely that phenotypic changes occur to microglia in culture. For example, increased CD14 expression has been observed on primary human microglia within a few days of culture (Melief et al. 2012). Nevertheless, the present experiments show that adult human microglia in culture are able to be further activated by treatment with various factors, for example upregulation of HLA in response to IFNy (Chapter 5), demonstrating that microglia are not at maximal activation capacity after isolation from tissue and culture in vitro. Thus there is a balance between minimising effects of in vitro culture, and allowing microglia to recover from isolation procedures, to determine the optimal experimentation time-frame. It is possible that the presence of other cell types in mixed glial cultures (astrocytes and fibroblast-like cells) more closely mimics in vivo tissue structure and helps to maintain cells in an in situ state.

These primary adult human microglia have similar characteristics to those reported by other lab groups (Durafourt et al. 2012; Klegeris et al. 2005). Furthermore, adult human microglia express the same markers in culture as they do in situ in adult human brain tissue. Nevertheless, there are other possible sources of macrophage-like cells which would potentially express the same markers as microglia. These include monocytes in the blood, and macrophages from the leptomeninges, subarachnoid space, or perivascular regions. While they share many characteristics and functions, peripheral macrophages and brain microglia also have several phenotypic differences. These differences are likely to be, in part, due to brain-specific environmental cues. Distinct differences
have been found between peripheral monocytes/macrophages and brain microglia in terms of phenotypic marker expression and functions. However there is as yet no general consensus on a defining marker or function which distinguishes these two myeloid populations (Durafourt et al. 2012; Guillemin and Brew 2004; Lambert et al. 2010; Melief et al. 2012). Therefore, several important questions remain unanswered: Are the microglial populations of the adult human brain self-renewing, or are they replenished by peripheral monocytic cells? What are the fundamental differences between peripheral monocytes/macrophages and CNS microglia, and are these populations distinguishable in vivo or in vitro? Nevertheless, break down of the blood-brain barrier in neurological diseases makes the study of both cell types relevant (Shlosberg et al. 2010). Thus despite the current uncertainty, we can combine knowledge from microglial and peripheral blood studies to investigate, and possibly treat, immune aspects of neurological disease.

Observation of microglia in situ in human brain tissue was used to complement in vitro functional studies. This thesis demonstrates microglial detection in human brain tissue with antibodies to several microglial proteins, confirming widespread presence of microglia in tissue and also demonstrating similarities between microglia in situ and in vitro. The studies performed here show that combining adult human brain immunohistochemistry with in vitro brain cell cultures is a powerful way of investigating microglial phenotype and function.

The use of primary human cultures brings with it inevitable variability between cases produced by differences in age, post-mortem or post-biopsy delay, and composition of the mixed glial cultures. Therefore data was collected and analysed for each case separately, rather than aggregated and analysed across the population. Cells from each individual culture had variable basal levels of protein expression (e.g. HLA protein; Chapter 5) and function (e.g. percentage of microglia undergoing phagocytosis; Chapters 3 and 4). Thus, pooling data from different cases would often be inappropriate. The results presented in this thesis represent consistent trends seen across a number of cases (minimum of 3). The variability in these experiments from case to case is an advantage when it comes to modelling the effect of treatments at a population level. When a treatment effect is observed across a number of cases there is increased chance of it being applicable to the population as a whole.

The methods described here for adult human microglia culture can be put to many uses in neuroscience research and drug discovery. I believe that their characteristics of primary human origin, ease of use, and sufficient supply provide ample reason to use them in conjunction with in
vivo pre-clinical studies. Culturing microglia in 96-well plates, or smaller, is ideal for high-throughput screening and drug development purposes.

8.4. The potential of fibroblast-like cells

Another note-worthy cell population in primary adult human mixed glial cultures is the fibroblast-like cells. This important but previously over-looked cell type was found here to have immune qualities. Although they were not the main cell type of interest for this thesis, they were found to respond in interesting ways to immune cytokines and growth factors. Specifically, they were found to express HLA in response to IFNy (as for astrocytes and microglia) and secrete pro-inflammatory cytokines and chemokines, particularly IP-10 (Chapter 5). Whereas microglia lacked the predicted response to TGFβ1, fibroblast-like cells were sensitive to its effect and their inflammatory responses of IFNγ-induced HLA expression and scratch injury were abolished by TGFβ1. This cell type thus presents a potential target to treat neuroinflammatory processes. In particular, the TGFβ1 pathway could be harnessed for anti-inflammatory effect. The equivalent cell type in vivo is presently unclear but is likely to reside in a meningeal or perivascular location (Park et al. 2012; Paul et al. 2012). Previous studies have reported a role for meningeal cells and pericytes in scar formation, multiple sclerosis and migraine (Goritz et al. 2011; Kawano et al. 2012; Magliozzi et al. 2010; Reuter et al. 2001). Possible uses for this brain cell type include drug development and screening for neuroprotective agents. These cells are easily obtained from different individuals with a variety of neurological disease states. Investigating the basic biology of the cell types in close proximity to neurons and glia will advance our understanding of physiological brain processes. Finally, the fibroblast-like qualities of these cells suggests that they may be amenable to re-programming and provides the exciting possibility that they could be used for regeneration and cell replacement (Karow et al. 2012).

8.5. Future directions

As with all scientific inquiry, this thesis generates more questions than it answers. The next step in the PU.1 transcription factor story is to perform a microarray comparing microglia with PU.1 expression and microglia with siRNA-reduced PU.1 expression. It is hoped that this screening technique will allow us to identify new microglial gene expression pathways that are under PU.1 control.
A limitation of a relatively small cell number and a heterogeneous population of cells in mixed glial cultures is that treatment effects can be diluted. Single-cell techniques, such as single-cell polymerase chain reaction, can extract a far greater amount of information per cell than cell population techniques. Differences in gene expression between PU.1-expressing microglia and microglia with PU.1 knocked-down could be investigated in this way. Furthermore, as cells can be selected based on morphology, the gene expression of round and elongated microglia could be compared. This would greatly advance the understanding of different microglial morphologies in relation to function.

The diverse microglial phenotypes explored in this thesis provide intriguing clues as to the dynamic nature of microglia in vivo. To further explore the active behaviour of microglia in real-time, live cell imaging could be performed to observe the responses of microglia to different agents over time. As imaging technologies develop it is hoped that microglia and immune activity can be monitored in live patients (Politis et al. 2012). This would greatly aid testing of anti-inflammatory compounds and microglia-targeting treatments to link in vivo and in vitro human observations.

The underlying question that remains is: How can we target microglia to treat neurological disorders? Context dependent microglial activity is likely to be of major importance, as this thesis highlights the diversity of microglial phenotype according to their surrounding microenvironment. This dissertation explores various different ways of manipulating microglial phenotype. Chapter 3 demonstrates for the first time the proof-of-principle that adult human microglia can be targeted with siRNA and their protein expression and phenotype altered. siRNA is being developed as a technique to be used in the clinic and it may not be long before we can modulate microglial function in humans using this method (Burnett et al. 2011). Previous microglial findings should be validated in human models to determine which aspects of microglial function to target for beneficial clinical effects.

8.6. Conclusions

In conclusion, the results of this thesis indicate that primary adult human microglia are an invaluable tool for biomedical research. This thesis extends the current literature of microglial biology and expands our knowledge of adult human microglia regulation and function. This thesis adds a greater depth of understanding to the effects of M-CSF, IFNγ and TGFβ1 on microglial function, and recognises PU.1 as a critical microglial regulator. There are many aspects of microglial function that
are amenable to modulation by targeting of regulatory factors such as the PU.1 transcription factor, or by treatment with soluble molecules such as growth factor or cytokine small molecule mimics.

This study establishes a firm basis for the use of primary adult human microglia in neuroscience and neuroimmunology research. Reliable methods are now fully established for high-throughput isolation, culture and experimentation of these cells. With convincing evidence that neuroimmunological effects can differ in rodent and human cells, there is good reason to believe that adult human microglia will play a major role in biomedical drug-discovery for neurological treatments in years to come.


Park T-H, Monzo H, Mee EW, Bergin PS, Teoh HH, Montgomery JM, Faull RLM, Curtis MA, Dragunow M. 2012. Adult human brain neural progenitor cells (NPCs) and fibroblast-like cells have similar properties in vitro but only NPCs differentiate into neurons. PLoS ONE 7(6):e37742.


Piskurich J, Linhoff M, Wang Y, Ting J. 1999. Two distinct gamma interferon-inducible promoters of the major histocompatibility complex class II transactivator gene are differentially regulated


Prolonged Expression of Interferon-Inducible Protein-10 in Ischemic Cortex After Permanent 
Occlusion of the Middle Cerebral Artery in Rat. Journal of Neurochemistry 71(3):1194-1204.

favoring microglia migration via HIF-1alpha activation. Biochemical and Biophysical Research 

Wang X, Scott E, Sawyers CL, Friedman AD. 1999. C/EBPalpha Bypasses Granulocyte Colony- 
Stimulating Factor Signals to Rapidly Induce PU.1 Gene Expression, Stimulate Granulocytic 

Weigelt K, Carvalho LA, Drexhage RC, Wijkhuys A, de Wit H, van Beveren NJM, Birkenhager TK, 
Bergink V, Drexhage HA. 2011. TREM-1 and DAP12 expression in monocytes of patients with 
severe psychiatric disorders. EGR3, ATF3 and PU.1 as important transcription factors. Brain 
Behavior and Immunity 25(6):1162-1169.

Dap12 expression in activated microglia from retinoschisin-deficient retina and its PU.1- 

Weigelt K, Lichtinger M, Rehli M, Langmann T. 2009. Transcrip tomic profiling identifies a PU.1 
regulatory network in macrophages. Biochemical and Biophysical Research Communications 
380(2):308-312.

Weiss R, Lifshitz V, Frenkel D. 2011. TGF-b1 affects endothelial cell interaction with macrophages 
and T cells leading to the development of cerebrovascular amyloidosis. Brain, Behavior, and 

Springer MS, DeMartino JA. 1998. Binding and Functional Properties of Recombinant and 
Endogenous CXCR3 Chemokine Receptors. Journal of Biological Chemistry 273(29):18288- 
18291.

Werner K, Bitsch A, Bunkowski S, Hemmerlein B, Brück W. 2002. The relative number of 
macrophages/microglia expressing macrophage colony-stimulating factor and its receptor 

Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R. 
448(7151):318-324.


Wierzba-Bobrowicz T, Gwiazda E, Kosno-Kruszewska E, Lewandowska E, Lechowicz W, Bertrand E, 
Szpak GM, Schmidt-Sidor B. 2002. Morphological analysis of active microglia - rod and 
ramified microglia in human brains affected by some neurological diseases (SSPE, 


Wilkinson NC, Navarro J. 1999. PU.1 regulates the CXCR1 promoter. Journal of Biological Chemistry 


