NF-κB in Neuroinflammation

A Thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in Systems Biology, in the Faculty of Life Sciences.

2014

Nisha Patel
Contents

Contents ........................................................................................................................................... 2
List of Figures ................................................................................................................................... 10
List of Tables ................................................................................................................................... 14
List of Abbreviations .................................................................................................................. 15
Abstract ......................................................................................................................................... 18
Declaration ...................................................................................................................................... 19
Copyright Statement .................................................................................................................... 19
Acknowledgements ...................................................................................................................... 20
1. Introduction ................................................................................................................................. 21
  1.1. General Considerations .......................................................................................................... 22
    1.1.1. Neuroinflammation .......................................................................................................... 22
    1.1.2. Microglia ......................................................................................................................... 23
    1.1.3. Neural Progenitor Cells ................................................................................................... 25
    1.1.4. Microglial and Neural Progenitor Cell Interactions ....................................................... 26
  1.2. The Nuclear Factor KappaB (NF-κB) Signalling Pathway .................................................. 27
    1.2.1. Overview of NF-κB Signalling ......................................................................................... 27
    1.2.2. Overview of the Structure and Function of NF-κB Pathway Components .......... 29
      1.2.2.1. Rel Proteins ............................................................................................................... 29
      1.2.2.2. Inhibitor KappaB (IκB) Proteins ................................................................................. 30
        1.2.2.2.1. IκBα and IκBε in NF-κB Activation ...................................................................... 31
      1.2.2.3. Inhibitor KappaB Kinase (IKK) Complex ............................................................... 32
        1.2.2.3.1. IKK Complex Components and Formation ....................................................... 32
        1.2.2.3.2. IKK Complex Activation ..................................................................................... 33
      1.2.3. Canonical Activation of NF-κB Pathway ................................................................. 34
        1.2.3.1. TNFα Structure ....................................................................................................... 34
1.2.3.2. TNFR Structure and Function .................................. 35
1.2.3.3. TNFR1 Signalling ................................................. 36
  1.2.3.3.1. Induction of Apoptosis .................................. 37
1.2.3.4. TNFR2 Signalling ................................................. 38
1.2.4. NF-κB Dynamics and Function .................................. 39
1.2.5. Regulation of NF-κB Transcriptional Activity .......... 41
  1.2.5.1. Atypical IκB Proteins .................................... 41
    1.2.5.1.1. B-cell Lymphoma-3 (Bcl-3) .................. 41
    1.2.5.1.2. IκBζ .................................................. 42
    1.2.5.1.3. IκBδ .................................................. 42
    1.2.5.2. IKKα and IKKβ ........................................ 43
  1.2.5.3. Other Factors influencing NF-κB Transcription .... 43
1.2.6. Termination of the NF-κB Response ......................... 44
  1.2.6.1. TNFα-induced Protein 3 (A20) ....................... 44
  1.2.6.2. Cylindromatosis (CYLD) ............................ 45
1.3. NF-κB in the Central Nervous System (CNS) ............... 46
  1.3.1 Overview of NF-κB Components in the CNS .......... 46
  1.3.2. NF-κB Function in the CNS .................................. 47
  1.3.3. NF-κB Activation in Microglia and Neural Progenitor Cells .... 49
1.4. Thesis Aims .......................................................... 50
2. Materials and Methods ................................................. 52
  2.1. Materials ............................................................ 53
    2.1.1. Reagents ...................................................... 53
    2.1.2. Vectors ........................................................ 53
      2.1.2.1 Luciferase Construct .................................. 53
      2.1.2.2 Fluorescent Fusion p65 Constructs .................. 53
2.2. Methods .................................................................................................................. 54
  2.2.1. Propagation of Vectors ...................................................................................... 54
  2.2.2. Cell Culture ..................................................................................................... 54
    2.2.2.1. Cell Lines and Culture Maintenance ......................................................... 54
    2.2.2.2. Long-term Storage of Cell Lines .............................................................. 55
    2.2.2.3. Thawing Cells from Frozen Stocks ......................................................... 55
    2.2.2.4. Cell Enumeration ..................................................................................... 55
    2.2.2.5. Plating Cells for Experiments ................................................................. 55
      2.2.2.5.1. Plating Cells for Live Cell and Endpoint Luminometry .................... 56
      2.2.2.5.2. Plating Cells for Cell Viability and Apoptosis Assays with Inhibitor
      Treatment ........................................................................................................... 57
  2.2.3. Treatment of Cells with TNFα ....................................................................... 57
  2.2.4. Transfections and Transductions .................................................................... 57
    2.2.4.1. Plasmid Transfections ............................................................................ 57
    2.2.4.2. BAC Transfections .................................................................................. 57
    2.2.4.3. Lentiviral Transductions ......................................................................... 57
  2.2.5. Luminometry .................................................................................................... 58
    2.2.5.1. Live Cell Luminometry .......................................................................... 58
    2.2.5.2. Endpoint Luminometry .......................................................................... 58
  2.2.6. Live Cell Imaging ............................................................................................. 58
    2.2.6.1. Analysis of Live Cell Imaging Experiments ............................................ 59
  2.2.7. Western Blotting ............................................................................................... 59
    2.2.7.1. Sample Preparation .................................................................................. 59
    2.2.7.2. Preparation of Gels for SDS Polyacrylamide Gel Electrophoresis (PAGE)
    .............................................................................................................................. 59
    2.2.7.3. Protein Separation by SDS-PAGE ......................................................... 60
    2.2.7.4. Transfer of Protein to Nitrocellulose Membrane .................................... 60
2.2.7.5. Immunostaining of Nitrocellulose Membrane ........................................ 60
2.2.7.6. Relative Quantification of Proteins .......................................................... 61
2.2.8. Cell Viability ................................................................................................. 61
  2.2.8.1. Analysis of Cell Viability Data ................................................................. 62
2.2.9. Apoptosis Assays ......................................................................................... 62
  2.2.9.1. Analysis of Apoptosis Data ................................................................... 63
2.2.10. Flow Cytometry ....................................................................................... 63
  2.2.10.1. Sample Preparation and TNFR1-Labelling .............................................. 63
  2.2.10.2. Flow Cytometry Data Analysis ............................................................... 63
2.2.11. Enzyme-linked Immunosorbent Assay (ELISA) ........................................ 64
  2.2.11.1. Sample Preparation .............................................................................. 64
  2.2.11.2. NanoString Measurements .................................................................. 64
  2.2.11.3. NanoString Data Analysis .................................................................. 64
2.2.12. NanoString ................................................................................................ 65
  2.2.12.1. Sample Preparation .............................................................................. 65
  2.2.12.2. NanoString Measurements .................................................................. 65
  2.2.12.3. NanoString Data Analysis .................................................................. 65
2.2.13. Quantitative-Polymerase Chain Reaction (Q-PCR) ..................................... 66
  2.2.13.1. Sample Preparation .............................................................................. 66
  2.2.13.2. Q-PCR Analysis .................................................................................. 66
  2.2.13.3. Analysis of Q-PCR Results .................................................................. 67
3. Establishing and Characterising a Model System for the Study of NF-κB in C17.2 and
BV.2 Cells ................................................................................................................ 69
  3.1. General Introduction ....................................................................................... 70
  3.2. Results ............................................................................................................ 71
    3.2.1. Analysis of NF-κB Function and Dynamics in a Neural Progenitor Cell Line . 71
      3.2.1.1. Creating C17.2 Reporter Cell Lines for Observation of p65 Dynamics .... 71
      3.2.1.2. Single-cell p65 Dynamics in C17.2 Reporter Cell Lines .................... 71
      3.2.1.3. Population-level NF-κB Activation in C17.2 Cell Lines ..................... 76
3.2.1.3.1. Analysis of NF-κB Transcriptional Activity in the Differentially Transfected C17.2 cells .................................................................76
3.2.1.3.2. TNFα-induced IκBα Degradation Profiles in C17.2 Cell Lines ..........79
3.2.1.3.3. NF-κB-dependent Gene Expression Changes in TNFα Stimulated Cells ..............................................................................................83
3.2.1.3.4. Basal p65 and IκBα Expression in C17.2 Cell Lines ....................86
3.2.2. Investigating NF-κB Dynamics in the BV.2 Cell Line .....................................88
3.2.2.1. Creating BV.2 Reporter Cell Lines for Observation of p65 Dynamics .....88
3.2.2.2. Single-cell p65 Dynamics ..................................................................89
3.2.2.3. Population-level NF-κB Activation in BV.2 Cell Lines .....................90
    3.2.2.3.1. TNFα-induced IκBα Degradation Profiles in BV.2 Cell Lines ......90
    3.2.2.3.2. NF-κB-dependent Gene Expression Changes in TNFα Stimulated BV.2 Cell Lines ..........................................................................92
    3.2.2.3.3. Basal p65 and IκBα Expression in BV.2 Cell Lines ....................94
3.3. Discussion ..................................................................................................95
    3.3.1. NF-κB Activation in C17.2 Cells ...........................................................95
        3.3.1.1. Single-Cell p65 Dynamics in C17.2 Cells ........................................96
        3.3.1.2. NF-κB-dependent Gene Expression ..............................................96
    3.3.2. NF-κB Activation and p65 Dynamics in p65-BAC Stable C17.2 Clones ......97
    3.3.3. NF-κB Activation in BV.2 (UT) Cells ....................................................98
        3.3.3.1. IκBα Degradation in BV.2 (UT) Cells ..........................................98
        3.3.3.2. NF-κB-dependent Gene Expression ..............................................98
    3.3.4. Discussion of Problems with using Lentivirally Transduced Cell Lines ......99
        3.3.4.1. General Problems with p65 Overexpression .....................................99
        3.3.4.2. Choice of C17.2 (lenti) as a Model System and Potential Problems ......100
        3.3.4.3. Choice of BV.2 (lenti) as a Model System and Potential Problems ......101
4. NF-κB Activation and Dynamics in C17.2 and BV.2 Cells in Mono-cultures and Co-cultures .......................................................... 103

4.1. General Introduction .................................................................................................................. 104

4.2. Results ....................................................................................................................................... 104

4.2.1. NF-κB Dynamics and Activation in C17.2 Cells ................................................................. 104

4.2.1.1. Single-cell p65 Dynamics in Mono-cultures and 1:1 Co-cultures ......................... 104

4.2.1.2. NF-κB Activation in Co-cultured Wild-type C17.2 Cells ........................................ 107

4.2.2. Single-cell p65 Dynamics in BV.2 Cells, in Mono-cultures and 1:1 Co-cultures ........................................................................................................................................ 116

4.2.3. TNFα availability in Mono- and Co-cultures ................................................................. 121

4.2.4. TNFR1 Expression in Unstimulated and TNFα-stimulated C17.2 cells, in Mono-cultures and Co-cultures ........................................................................................................................................ 122

4.3 Discussion .................................................................................................................................. 126

4.3.1. NF-κB in Mono-cultured and Co-cultured C17.2 ......................................................... 127

4.3.1.1. TNFα-induced Single-cell p65 Dynamics in C17.2, in Mono-cultures and 1:1 BV.2 Co-cultures ........................................................................................................................................ 127

4.3.1.2. Population-level NF-luc Induction in C17.2, in Mono-cultures and Co-cultures ........................................................................................................................................ 127

4.3.2. Single-cell p65 Dynamics in Mono-cultured and Co-cultured BV.2 ....................... 129

5. Analysis of Co-culture- and TNFα-induced Gene Expression Changes .............................. 130

5.1 General Introduction .................................................................................................................. 131

5.2 Results ....................................................................................................................................... 132

5.2.1. Gene Expression in Untreated C17.2 and BV.2 Mono-cultures ........................................ 133

5.2.2. TNFα-induced Changes in Gene Expression at 230 min Post-TNFα-Stimulation ........................................................................................................................................ 134

5.2.3. TNFα and Co-culture-induced Gene Expression Changes at 230 min Post-TNFα Stimulation ........................................................................................................................................ 136

5.2.4. Co-culture-induced Gene Expression Changes, 28 h after Co-culturing .......... 138
5.2.5. TNFα-induced Changes in Gene Expression at 24 h Post-TNFα-Stimulation 138
5.2.6. TNFα and Co-culture-induced Gene Expression Changes at 24 h Post-TNFα-Stimulation ......................................................... 140
5.2.7. Co-culture-induced Gene Expression Changes, 48 h after Co-culturing .... 142
5.3 Discussion .................................................................................. 143
5.3.1. Potential NF-κB-mediated Interactions between C17.2 and BV.2 ........ 144
5.3.2. Future Experiments ................................................................ 146
6. Effects of TNFα and NF-κB Activity on Neural Progenitor Cells and Microglia .... 148
6.1. General Introduction .................................................................. 149
6.2. Results ....................................................................................... 150
6.2.1. Effects of TNFα on Cell Viability and Apoptosis ......................... 150
6.2.2. Effects of C17.2- BV.2 Interactions on 1:1 Co-culture Viability and Apoptosis ................................................................. 155
6.2.3. Pyrrolidine Dithiocarbamate-induced NF-κB Inhibition ................. 159
6.2.4. Effects of NF-κB Inhibition on Untreated and TNFα-treated Mono-cultures and 1:1 Co-cultures ....................................................... 161
6.2.5. Effects of C17.2-BV.2 Interactions on Cell Viability in 1:1 Co-cultures .... 165
6.3 Discussion .................................................................................. 169
6.3.1. Effects of TNFα on C17.2 and BV.2 Mono-cultures ....................... 170
6.3.2. Effects of NF-κB Inhibition on C17.2 and BV.2 Mono-culture and 1:1 Co-culture Viability ................................................................. 172
6.3.3. C17.2-BV.2 Interactions in 1:1 Co-cultures .................................. 173
7. Discussion .................................................................................... 176
7.1. General Introduction .................................................................. 177
7.2. NF-κB Activation and p65 Dynamics in C17.2 cells ....................... 178
7.3. NF-κB Dynamics to Cell Survival .................................................. 180
7.3.1. BV.2 Prevents TNFα-induced C17.2 Apoptosis ............................ 180
List of Figures

1.1: An overview of the TNFα-induced canonical NF-κB activation pathway ………… 28
1.2: The structure and composition of NF-κB family members. ……………………. 29
1.3: The structure and composition of key IκB family members…………………… 31
1.4: The structure and composition of the main IKK complex constituents………… 33

2.1: TNFR1-phycoerythrin and eGFP fluorescence of lentivirus transduced C17.2 and BV.2 mono-cultures and 1:1 co-cultures……………………………………………….. 64

3.1: Expression of p65 fluorescent fusion proteins in C17.2 cells………………….. 72
3.2: TNFα-induced p65 localisation in C17.2 cells transfected or transduced with different p65 constructs……………………………………………………………………….. 72
3.3: Quantification of nuclear p65 fluorescence in different C17.2 cell populations after TNFα stimulation……………………………………………………………… 74
3.4: TNFα-induced p65 nuclear translocation profiles obtained from C17.2 cell populations……………………………………………………………………………… 75
3.5: Dose-dependent NF-κB activation in differentially transfected / transduced C17.2 cells………………………………………………………………………………… 77
3.6: Comparison of NF-luc induction, after TNFα stimulation, in C17.2 cell populations………………………………………………………………………………………… 78
3.7: Comparison of basal luminescence of NF-luc transfected C17.2 cell populations………………………………………………………………………………………… 78
3.8: Western blot analysis of IκBα levels in wild type C17.2 cells after TNFα stimulation……………………………………………………………………………………….. 80
3.9: Western blot analysis of IκBα levels in lentivirus transduced C17.2 cells after TNFα stimulation…………………………………………………………………………….. 81
3.10: Western blot analysis of IκBα levels in BAC clone A cells after TNFα stimulation………………………………………………………………………………………….. 82
3.11: Western blot analysis of IκBα levels in BAC clone 5 cells after TNFα stimulation………………………………………………………………………………………… 83
3.12: NF-κB-dependent gene expression changes in C17.2 cell populations after TNFα stimulation……………………………………………………………………………… 84
3.13: Basal expression of NF-κB-dependent genes in C17.2 cell populations…………. 86
3.14: Comparison of p65 protein levels in C17.2 cell populations.................................87
3.15: Comparison of IκBα protein levels in unstimulated C17.2 cell populations..........88
3.16: Expression of p65-eGFP in lentivirus transduced BV.2 cells.................................89
3.17: TNFα-induced p65 translocation in lentivirus transduced BV.2 cells.....................89
3.18: Analysis of p65 dynamics in single BV.2 (lenti) cells following TNFα stimulation..........................................................90
3.19: Western blot analysis of IκBα levels in wild type BV.2 cells after TNFα stimulation................................................................................................................91
3.20: Western blot analysis of IκBα levels in lentivirus-transduced BV.2 cells after TNFα stimulation................................................................................................................92
3.21: NF-κB-dependent gene expression changes in wild type vs lentivirus transduced BV.2 cells following TNFα stimulation..........................................................93
3.22: Basal expression of NF-κB-dependent genes in BV.2 cell populations.................94
3.23: Western blot analysis of p65 levels in the wild type BV.2 cells and BV.2 (lenti) cells........................................................................................................................................................................94
3.24: Western blot analysis of IκBα levels in the wild type BV.2 cells and BV.2 (lenti) cells........................................................................................................................................................................95

4.1: Expression of p65-eGFP in C17.2 (lenti) cells, in mono-culture and 1:1 co-culture (with BV.2 cells)..................................................................................................................104
4.2: Quantification of nuclear p65 fluorescence in C17.2 (lenti) cells, after TNFα stimulation, in mono-cultures and 1:1 co-cultures (with BV.2 cells).........................105
4.3: Average peak timing and amplitude of the first p65 nuclear translocation, after TNFα stimulation, in C17.2 (lenti) cells.................................................................106
4.4: TNFα-induced p65 nuclear translocation profiles obtained from C17.2 (lenti) cells in mono-cultures and 1:1 co-cultures (BV.2 cells)..............................................107
4.5: TNFα stimulated NF-luc induction in C17.2 cells, in mono-cultures and co-cultures (BV.2 cells).................................................................................................109
4.6: TNFα stimulated NF-luc induction in C17.2 cells, in mono-cultures and 1:4 co-cultures (with SK-N-AS cells).................................................................110
4.7: TNFα stimulated NF-luc induction in C17.2 cells, in mono-cultures and 1:4 co-cultures (with RAW264.7 cells).................................................................110
4.8: Basal luminescence of NF-luc plasmid-transfected C17.2 cells, in different culture conditions

4.9: Background luminescence from BV.2 cells

4.10: Unstimulated and TNFα stimulated NF-luc induction in mono-cultured C17.2 cells, with and without transwells

4.11: Unstimulated and TNFα stimulated NF-luc induction in C17.2 cells in mono-cultures and 1:4 transwell co-cultures (with BV.2 cells)

4.12: Unstimulated and TNFα stimulated NF-luc induction in C17.2 cells in mono-cultures and 1:8 transwell co-cultures (with BV.2 cells)

4.13: Basal luminescence in NF-luc plasmid transfected C17.2 cells, in different culture conditions

4.14: Expression of p65-eGFP in BV.2 (lenti) cells, in mono-culture and 1:1 co-culture (with C17.2 cells)

4.15: Quantification of nuclear p65 fluorescence in BV.2 (lenti) cells, after TNFα stimulation, in mono-cultures and 1:1 co-cultures (with C17.2 cells)

4.16: Average peak to peak timing of p65 nuclear translocations in mono-cultured and co-cultured BV.2 (lenti) cells, after TNFα stimulation

4.17: Average peak timing and amplitude of the first p65 nuclear translocation, after TNFα stimulation, in BV.2 (lenti) cells, in mono-cultures and 1:1 co-cultures (with C17.2 cells)

4.18: TNFα-induced p65 nuclear translocation profiles obtained from BV.2 (lenti) cells in mono-cultures and 1:1 co-cultures (with C17.2 cells)

4.19: Relative TNFα concentration in mono- and co-cultures, after 2ng/ml TNFα stimulation

4.20: TNFR1-associated fluorescence in unstimulated and TNFα-stimulated wild type C17.2 cells and C17.2 (lenti) cells

4.21: TNFR1-associated fluorescence in unstimulated and TNFα-stimulated C17.2 (lenti) cells

5.1: A comparison of gene expression in unstimulated C17.2 and BV.2 mono-cultures

5.2: Genes upregulated/downregulated, at 230 min post-20ng/ml TNFα-stimulation, in C17.2 and BV.2 mono-cultures and 1:1 co-cultures
5.3: Genes upregulated/downregulated, at 230 min post-20ng/ml TNFα-stimulation, in transwell co-cultures and 1:1 contact-permissive co-cultures.........................................................137
5.4: Genes upregulated/downregulated, at 24h post-20ng/ml TNFα-stimulation, in C17.2 and BV.2 mono-cultures and 1:1 co-cultures.................................................................139
5.5: Genes upregulated/downregulated, at 24h post-20ng/ml TNFα-stimulation, in transwell co-cultures and 1:1 contact-permissive co-cultures..............................................141

6.1: C17.2 viability and apoptosis levels, after 20ng/ml TNFα stimulation.........................152
6.2: BV.2 viability and apoptosis levels, after 20ng/ml TNFα stimulation.........................153
6.3: Viability and apoptosis levels in 1:1 co-cultures (C17.2: BV.2), after 20ng/ml TNFα stimulation...............................................................................................................................154
6.4: Expected versus actual viability in unstimulated 1:1 co-cultures (C17.2: BV.2).................................................................156
6.5: Expected versus actual viability in TNFα-stimulated 1:1 co-cultures (C17.2: BV.2).................................................................................................................................157
6.6: Expected versus actual apoptosis in unstimulated and TNFα-stimulated 1:1 co-cultures (C17.2: BV.2).................................................................................................................................158
6.7: IκBα levels in PDTC-treated cells, after TNFα stimulation........................................160
6.8: TNFα-stimulated NF-luc induction in PDTC- and non-PDTC treated C17.2 cells....161
6.9: C17.2 cell viability after 30 min pre-treatment with water or 0.1mM PDTC and either no stimulation or 20ng/ml TNFα stimulation.............................................................162
6.10: BV.2 cell viability after 30 min pre-treatment with water or 0.1mM PDTC and either no stimulation or 20ng/ml TNFα stimulation.................................................................163
6.11: Viability of 1:1 co-cultures (C17.2: BV.2), after 30 min pre-treatment with water or 0.1mM PDTC and either no stimulation or 20ng/ml TNFα stimulation...............164
6.12: Expected versus actual viability in 1:1 co-cultures (C17.2:BV.2), after either a 30 min treatment with water or 0.1mM PDTC.................................................................166
6.13: Expected versus actual viability in 1:1 co-cultures (C17.2: BV.2) treated for 30 min with either water or 0.1mM PDTC and 20ng/ml TNFα...............................................167
6.14: Apoptosis levels in water- or PDTC-treated C17.2 cells........................................168
6.15: Apoptosis levels in water- or PDTC-treated BV.2 cells........................................169
List of Tables

2.1: Cell numbers, dishes, cell growth area and media volume used in experiments..........................................................56

2.2: Cell numbers, cell growth area and media volume in luminometry experiments..........................................................56

2.3: Primary and secondary antibodies used in Western blots.........................61

2.4: Calculations conducted to assess co-culture-mediated viability changes.........62

2.5.1: Examples of the two different comparisons conducted with NanoString results: Analysis of TNFα-induced gene expression..............................................................66

2.5.2: Examples of the two different comparisons conducted with NanoString results: Analysis of cell-contact-independent co-culture interactions........................................66

2.6: Primers used for Q-PCR..............................................................................67

2.7: Cycling parameters for Q-PCR.................................................................67

5.1: Genes that were differentially expressed between mono-cultures and transwell co-cultures.................................................................142

5.2: A collated list of NF-κB-dependent genes that were differentially regulated, at different time points, by TNFα or co-culture interactions in: C17.2 or BV.2 mono-cultures, transwell co-cultures or 1:1 contact-permissive co-cultures.................................143
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>A20</td>
<td>Tumor necrosis factor alpha-induced protein 3</td>
</tr>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ARD</td>
<td>Ankyrin repeat domain</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>Bcl-3</td>
<td>B-cell lymphoma-3</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CAP-Gly</td>
<td>Cytoskeleton-associated proteins-glycine rich</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>c-IAP</td>
<td>Cellular inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxgenase-2</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>CYLD</td>
<td>Cylindromatosis</td>
</tr>
<tr>
<td>DD</td>
<td>Death domain</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DsRedXP</td>
<td>Discosoma sp. red fluorescent protein</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental allergic encephalomyelitis</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated via death domain protein</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FLIP</td>
<td>FLICE-like inhibitor protein</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
</tbody>
</table>
NF-kappaB in Neuroinflammation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>IkB</td>
<td>Inhibitor kappaB</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor kappaB kinase</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>Itch</td>
<td>Itchy E3 ubiquitin protein ligase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPKAPK2</td>
<td>Mitogen activation protein kinase-activated protein kinase 2</td>
</tr>
<tr>
<td>MCP-1/CCL2</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>mTNFα</td>
<td>Transmembrane tumor necrosis factor alpha</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non-essential amino acids</td>
</tr>
<tr>
<td>NEMO</td>
<td>Nuclear-factor kappaB essential modulator</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear-factor kappaB</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation sequence</td>
</tr>
<tr>
<td>NPC</td>
<td>Neural progenitor cell</td>
</tr>
<tr>
<td>NSC</td>
<td>Neural stem cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDTC</td>
<td>Pyrrolidine dithiocarbamate</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PEST</td>
<td>Proline-, glutamic acid-, serine-, threonine-rich</td>
</tr>
<tr>
<td>PK</td>
<td>Protein kinase</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>Quantitative-polymerase chain reaction</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel homology domain</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor-interacting protein</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>SODD</td>
<td>Silencer of death domains</td>
</tr>
<tr>
<td>STAT6</td>
<td>Signal transducer and activator of transcription-6</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>TACE</td>
<td>Tumor necrosis factor alpha converting enzyme</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TAD</td>
<td>Transcription activation domain</td>
</tr>
<tr>
<td>Tax1BP1</td>
<td>Tax 1 binding protein 1</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TRADD</td>
<td>Tumor necrosis factor receptor type 1-associated death domain</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumor necrosis factor receptor-associated factor</td>
</tr>
<tr>
<td>UT</td>
<td>Untransfected</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
Abstract

The NF-κB stress signalling system has an important role in the control of inflammation, being both stimulated by cytokines and itself regulating cytokine gene expression. NF-κB dynamics have previously been studied in a variety of different cell lines and primary cells. It has been shown, using live-cell microscopy, that NF-κB proteins show complex oscillatory dynamics between the nucleus and cytoplasm at the single-cell level. These dynamics are thought to control gene expression responses.

A key aim of the work described in this thesis was to understand the functional NF-κB-mediated paracrine interactions that occur between different cell types to control tissue-level inflammatory responses. This project aimed to investigate cytokine-mediated interactions between neural progenitor cells (NPCs) and microglia following Tumour Necrosis Factor alpha (TNFα) stimulation. Specifically, the interactions between the C17.2 NPC line and the BV.2 microglial cell line were used as a model system. Understanding the interactions between these cell types might be physiologically important for providing an insight into the regulation of neuroinflammation. A further goal was to establish a better understanding of how single-cell NF-κB dynamics could be related to differential gene expression and cell survival.

Initially, different methods for expressing labelled RelA/p65 (p65) in C17.2 and BV.2 cells were investigated. TNFα-induced NF-κB activation and p65 dynamics were measured in cells that had been transfected or transduced with a range of p65 constructs (plasmid, bacterial artificial chromosome or lentiviral vectors) to express fluorescent p65 fusion proteins. This study resulted in the selection of lentivirally transduced cells, expressing p65-eGFP, as the most appropriate model system for the observation of p65 dynamics in these cells. C17.2 and BV.2 p65 dynamics and C17.2 NF-κB transcriptional activity were measured in mono-cultures and co-cultures. These data indicated that BV.2 cells mediated inhibition of TNFα-induced NF-κB activity and p65 nuclear translocations in the C17.2 NPCs. Preliminary evidence for C17.2 regulation of BV.2 p65 dynamics was also found. However, this project mainly focussed on modulation of the C17.2 cell responses, since it was considered that these were likely to be particularly important in the control of neuroinflammation.

Gene expression was measured in untreated and TNFα-stimulated mono-cultures, co-cultures and transwell co-cultures, in order to assess the functional effects of any intercellular interactions. These studies were also designed to assess the outcome of TNFα treatment and co-culture interactions on cell viability and apoptosis. In addition to this, an NF-κB inhibitor was used to evaluate the NF-κB contribution to changes in cell viability and apoptosis. These results implicated a role for TNFα in C17.2 apoptosis and suggested that the presence of BV.2 cells could prevent this cell fate. Interestingly, NF-κB inhibition was also shown to induce C17.2 apoptosis and increased overall viability. This may suggest promotion of cell proliferation.

Together, these data provide functional evidence for TNFα-induced, co-culture- and NF-κB-mediated interactions between these cell types at the molecular, genetic and cellular level. These interactions had a significant effect on the extent of cell survival in response to TNFα. This observation is relevant to the study of neurodegenerative disease, where high TNFα concentrations are known to mediate tissue damage and NPCs have been shown to provide a therapeutic benefit.
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright Statement

i. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the “Copyright”) and s/he has given The University of Manchester certain rights to use such Copyright, including for administrative purposes.

ii. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

iii. The ownership of certain Copyright, patents, designs, trade marks and other intellectual property (the “Intellectual Property”) and any reproductions of copyright works in the thesis, for example graphs and tables (“Reproductions”), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

iv. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property and/or Reproductions described in it may take place is available in the University IP Policy (see http://documents.manchester.ac.uk/DocuInfo.aspx?DocID=487), in any relevant Thesis restriction declarations deposited in the University Library, The University Library’s regulations (see http://www.manchester.ac.uk/library/aboutus/regulations) and in The University’s policy on Presentation of Theses.
Acknowledgements

Firstly, I would like to thank my supervisors, Prof. M White and Dr. P Paszek, for their help and guidance throughout the course of my PhD.

Also, many thanks go to all members of the Prof. White lab group. Special thanks to Polly Downton for performing a miracle when I needed it, Kathryn Roberts for proofreading (at a remarkable speed) and Angela Pisco for dedicating hours to helping me with formatting (and offering me a bed). Also, thank you to James Bagnall for advice (and harassment) in the lab throughout the years, to Anne McNamara with help troubleshooting (and for showing me funny youtube clips), Connie Lam for helping me with just about everything in the lab (and for listening to me rant) and Dr. Adamson for letting me steal his fine artwork (and the awkward/funny conversations).

I am also grateful to every other member of the group for making the lab and office area a great place to work in. Outside of work, thank you to James Bagnall (again), James Boyd, Will Rowe and Nick Jones for the chuckles and drinks (although, I am not sure the latter has done my liver any good).

I would like to thank my supervisors (again), Prof. M White and Dr. P Paszek, for getting me through this. Special thanks, and eternal gratitude, go to Dr. D Spiller for support throughout, but mainly for pushing me through the final stages- I needed the extra push.

On a personal note, thank you to Miquette Hall for making me laugh when I felt like I was going crazy and Steph Baldwin for being an awesome lab buddy.

Finally, I would like to thank my parents and sister for their continual unconditional love and support, for listening to me complain and giving me hugs when I need them: this really would not have been possible without any of you.
1. Introduction
1.1. General Considerations

Even the most simple of biological processes involves a large number of biomolecules, interactions and emergent properties, with an inherent element of stochasticity. It is commonly accepted that understanding individual components in isolation is not sufficient for understanding biological phenomena: the whole is greater than the sum of its parts.

The challenge of transcending multiple areas of research has been taken up by systems biology. The principle behind this field is the integration of data from multiple levels, e.g. from genes to cells, to achieve a more complete understanding of biological systems.

Neuroinflammation is an exemplar of a complex, disease-related biological process in which there is a gap between molecular knowledge and tissue-level understanding. This process is orchestrated by a wide range of cells, proteins and biomolecules, creating an intricate network of interactions that determine cell fate in neurodegenerative disease. One way to begin to detangle the web is to traverse the various areas/levels of the network, starting with a central node.

1.1.1. Neuroinflammation

Neuroinflammation is a multi-stage defence process launched by the central nervous system (CNS) in response to injury/infection. This process is designed to protect host tissue by destroying noxious stimuli, such as bacterial toxins, and promoting damage repair (Nikbin et al, 2011).

Neuroinflammation involves interactions between a range of different cell types, including astrocytes, neurons, microglia and neural progenitor cells. Inflammatory stimuli trigger cell signalling pathways, leading to the activation of transcription factors, such as nuclear-factor kappaB (NF-κB). Activated NF-κB regulates the expression of many pro- and anti-inflammatory cytokines and chemokines such as tumor necrosis factor α (TNFα; Shakhov et al, 1990), interleukin-1 (IL-1; Mori and Prager, 1996) and monocyte chemotactic protein-1 (MCP-1, also known as chemokine ligand 2; CCL2; Ueda et al, 1997).

Gene expression is modified in a cell-type- and stimulus-specific manner. Cytokines and chemokines, secreted by stimulated cells, create a dynamic cocktail that perpetuates or terminates the inflammatory response, by activating a series of signalling pathways in receptive cells (Nikbin et al, 2011; Ramesh et al, 2013). Many of these factors also activate or repress NF-κB signalling (Pahl, 1999; Gilmore and Herscovitch, 2006). The integration
of these signals determines the fate of cells in inflamed tissue. Due to its intimate relationship with inflammatory factors, NF-κB is a central regulator of inflammatory outcome (Tak and Firestein, 2001).

Neuroinflammation is a major cause of tissue damage sustained in neurodegenerative diseases (Richardson et al., 2005; Klegaris et al., 2007; Frank-Cannon et al., 2009). Prolonged (chronic) inflammation has previously been associated with disease, whereas the acute (transient) inflammatory response was thought to be beneficial (Nikbin et al., 2011).

More recently, Wynn et al (2004) described chronic inflammation as a consequence of dysfunctional microglial activity. In fact, microglia are involved in many CNS diseases (Perry et al., 2010), most notably Alzheimer’s disease (AD; Bader et al., 1994) and experimental allergic encephalomyelitis (EAE; an animal model of stroke; Banati et al., 1995; Bauer et al., 1995).

Due to their ability to regulate inflammation and differentiate to replace damaged cells, neural progenitor cells have emerged as a promising mode of therapy in neurodegenerative diseases (Martino and Pluchino, 2006). Microglial-neural progenitor cell interactions are particularly important governors of neuroinflammatory outcome: neural progenitor cells can promote microglial proliferation and activation (Mosher et al., 2012; Forstreuter et al., 2002; Deierborg et al., 2010) and, in turn, microglia can induce neural progenitor cell apoptosis, thereby preventing tissue regeneration (Guadagno et al., 2013; Liu et al., 2014). The following sections will focus on the role of these cells, and their interactions, in neuroinflammation.

1.1.2. Microglia

Microglia are CNS-specific macrophages that comprise 10-15% of all CNS cells and are strategically localized in the most vulnerable areas of the nervous system (Abbott et al., 1986; Larner et al., 1995). Microglia are unique in that, as well as providing mechanical and structural support, they are the first line of defence against invading pathogens and the main resident mediators of the neuroinflammatory response (Saijo and Glass, 2011; Colton, 2009; Nakajima and Kohsaka, 2001).

Quiescent (non-activated) microglia are guardians of the CNS, actively extending and retracting their processes, patrolling for foreign antigens or apoptotic cells. Upon
encountering an inflammatory stimulus, these cells undergo a morphological and functional transformation; a process referred to as activation. The changes that microglial cells undergo allow them to perform a range of functions including phagocytosis of dead cells/bacteria and promotion of immune cell migration and activation (Saijo and Glass, 2011; Boche et al, 2013; Colton, 2009). Aarum et al (2003) reported that microglia can also promote migration and neuronal differentiation of murine neural precursor cells, suggesting that microglia can play a vital role in regeneration.

Microglial activation state and its functional consequences are determined by the environmental milieu (Saijo and Glass, 2011; Colton, 2009). There are two well described activation states for microglia- M1 (classical) and M2 (often subdivided into alternative and acquired deactivation). Microglial exposure to interferon-γ (IFNγ) triggers the release of cytokines that exaggerate the initial response and lead to classical activation of microglia (via paracrine/autocrine feedback). Classical activation is characterized by phagocytic ability and microglial-release of proinflammatory cytokines, including TNFα, cyclooxygenase-2 (COX-2) and IL-1β (Colton, 2009; Saijo and Glass, 2011). Microglial-derived proinflammatory agents amplify the inflammatory response by triggering the activation of other microglial cells and astrocytes (Saijo and Glass, 2011; Suzumura, 2008).

Classical activation is the first stage of the inflammatory response. The second stage of the response is described as regenerative and requires a termination of the classically-activated phenotype (Nakajima and Kohsaka, 2001). This can involve regulatory proteins that are induced by the initial inflammatory stimulus or constitutively expressed by immune cells. NF-κB inhibition can also resolve the proinflammatory phenotype (Nakajima and Kohsaka, 2001; Colton, 2009).

Classically-activated microglia are a major source of TNFα in inflamed tissue, as well as neurotoxic reactive oxygen species and excitotoxins (Mattson and Camandola, 2001). In AD, TNFα appears to be responsible for much of the amyloid-β-induced neuronal degeneration (Cartier et al, 2005). Microglial-derived TNFα has also been implicated in neural progenitor cell apoptosis (Guadagno et al, 2013).

Alternatively, microglial cells can be switched into a different activation state by other stimuli, produced by the classically activated response. Alternative activation is reportedly induced by exposure to IL-4 or IL-13. This state is defined by the production of anti-
inflammatory cytokines and factors that are intended to promote tissue repair, such as components of the extracellular matrix (Saijo and Glass, 2011; Colton, 2009). The transcription factor, signal transducer and activator of transcription-6 (STAT6), has been implicated in the switch from classical- to alternative-activation (Nguyen and Benveniste, 2000).

A third, less studied, activation state has also been proposed; acquired deactivation is mainly induced by the anti-inflammatory agents IL-10 and transforming growth factor-beta (TGF-β). These microglial cells are specialised to resolve inflammation by uptake of apoptotic cells and immunosuppression (Colton, 2009).

In practice, microglial activation state is not discrete and exists on a continuum comprising classical and alternative activation (Perry et al, 2010; Colton, 2009). Mechanistically, more work is required to fully understand microglial activation and deactivation. It is not known whether microglial cells within a given population can exist in different activation states (Colton, 2009).

In summary, microglial activation status is an important determinant of neural progenitor cell survival and, therefore, neuroinflammatory outcome.

1.1.3. Neural Progenitor Cells

Neural progenitor cells (NPCs) are heterogeneous, multipotent and mitotic cells that differentiate into neural cells during neurogenesis, in developing and adult organisms (Gage, 2000). They are generally believed to reside in only two distinct regions of the adult CNS; the subventricular zone in the lateral ventricles and the subgranular zone of the hippocampus (Gage, 2000; Ivanova et al, 2002; Doetsch, 2003).

It is worth making a point about the nomenclature used in the field of stem cell biology: many researchers use the term ‘neural progenitor cells’ interchangeably with ‘neural stem cells’. Various definitions of stem and progenitor cells have been proposed, in an effort to distinguish between the two (Seaberg and van der Kooy, 2003). However, a commonly-accepted definition has yet to be established, hence, when referring to studies, the term used here reflects the term used by the authors of the study.

In recent years, exogenous NPCs have emerged as a potential candidate for treatment of a range of pathological conditions associated with neuroinflammation, such as stroke (Daadi et al, 2008), Parkinson’s disease (Richardson et al, 2005) and AD (Abdel-Salam, 2011).
The exogenous application of NPCs has demonstrated non-tumorigenic properties, long-term survival and therapeutic benefits in disease (Temple, 2001). Studies have shown that engrafted NPCs migrate into areas of brain injury (Ivanova, 2002; Doetsch, 2003; Merkle et al, 2004) and can differentiate into a range of neural cells including neurons, astrocytes and oligodendrocytes (Park et al, 2006).

Microglial cells are one of the major drivers of NPC migration into sites of neuroinflammation (Aarum et al, 2003). As well as differentiating to replace damaged neurons, exogenous NPCs have been shown to exert immune functions and reportedly create a neuroprotective inflammatory phenotype (Martino and Pluchino, 2006). In order to do this, they must navigate the potentially toxic neuroinflammatory microenvironment. As the main producers of many proinflammatory factors, activated microglia present the biggest threat to survival of NPCs (Pellegatta et al, 2006).

1.1.4. Microglial and Neural Progenitor Cell Interactions

The activation state of microglia contributes to their effect on NPCs. Non-activated microglia have been shown to promote NPC proliferation in vitro (Liu et al, 2013a), whereas culture media from classically activated microglia can cause NPC apoptosis (Guadagno et al, 2013). This latter result was dependent on microglial-derived TNFα, NF-κB activation and downstream Puma expression in NPCs. In another study, culture media from activated microglia reportedly induced proliferation and differentiation of NPCs (Deierborg et al, 2010). This may reflect differences in cell types or microglial activation state: Guadagno et al used lipopolysaccharide (LPS) to activate murine microglia, whereas Deierborg et al used rat microglia that had been activated by a brain injury.

Reciprocally, NPCs can promote microglial proliferation (Liu et al, 2013a) and regulate activation and chemotaxis (Mosher et al, 2012). Microglial proliferation in this latter study was mediated by NPC-derived vascular endothelial growth factor (VEGF); this factor was previously shown to induce microglial chemotaxis and proliferation (Forstreuter et al, 2002). Interestingly, VEGF expression is regulated by NF-κB (Chilov et al, 1997). NF-κB activity has yet to be thoroughly investigated in neural cells. The following chapter explores what is currently known about NF-κB, much of which is derived from non-neural cells.
1.2. The Nuclear Factor KappaB (NF-κB) Signalling Pathway

1.2.1. Overview of NF-κB Signalling

Nuclear-Factor kappaB (NF-κB; also known as Rel) is a family of five proteins, evolutionarily conserved from Cnidaria to humans, which form dimeric eukaryotic transcription factors (Chen and Ghosh, 1999). Almost thirty years ago, NF-κB was discovered as a transcriptional regulator of immunoglobulin light chain genes in B-lymphocytes (Singh et al, 1986). We now know that NF-κB is ubiquitously expressed (Bonizzi and Karin, 2004) and can be activated by a range of factors, e.g. cytokines, viral infection and DNA damage (Brasier, 2006; Gilmore, 2006; Hayden and Ghosh, 2008). As a regulator of hundreds of genes, NF-κB is a central player in many processes such as inflammation, proliferation and apoptosis (Hayden and Ghosh, 2008; Ghosh and Karin, 2002; Bonizzi and Karin, 2004; Gilmore, 2006).

Depending on the stimulus, NF-κB activation can occur via three different pathways: the well-known canonical or non-canonical pathways or the most recently discovered atypical pathway. Each pathway is distinguished by the complement of NF-κB dimers and signalling molecules that are involved (Hoffmann and Baltimore, 2006; Gilmore, 2006). The most prevalent NF-κB dimer, p65:p50, is part of the canonical pathway (Hoffmann and Baltimore, 2006; Hayden and Ghosh, 2008).

Canonical signals converge on a multi-protein kinase complex called inhibitor kappaB kinase (IKK). Downstream of the IKK complex are inactive NF-κB dimers, bound to cytoplasmic inhibitor kappaB (IκB) proteins: this interaction prevents NF-κB nuclear entry. The canonical NF-κB network has to undergo a complex series of events in order to produce transcriptionally activated NF-κB dimers (Hayden and Ghosh, 2008; Gilmore, 2006; Perkins, 2006).

Ligands for the canonical pathway, such as TNFα, trigger the activation of IKK complexes which facilitate degradation of NF-κB-bound IκB proteins. NF-κB is then free to translocate into the nucleus, bind to specific sites on DNA, and modify gene expression (Hoffmann et al, 2002; Ashall et al, 2009). IκB genes are transcriptionally-upregulated and newly-synthesised IκB proteins chaperone NF-κB out of the nucleus and back into the cytoplasm (Hayden and Ghosh, 2008). Due to the pattern of degradation and later resynthesis of IκB proteins, NF-κB can move (oscillate) in and out of the nucleus.
repeatedly in response to a single stimulus (Nelson et al, 2004; Ashall et al, 2009; Turner et al, 2010). The dynamics of NF-κB are described in more detail in section 1.2.4.

With a focus on the canonical pathway, the following sections detail different aspects of the NF-κB network, beginning with NF-κB, IκB and IKK complex proteins (see Figure 1.1). This will be followed by a section on TNFα-induced NF-κB activation, NF-κB dynamics and the regulation of NF-κB transcriptional activity and response.

**Figure 1.1. An overview of the TNFα-induced canonical NF-κB activation pathway.**
This schematic shows the main proteins involved in TNFα-induced canonical activation. TNFR binding to TNFα induces the activation of IKK, via downstream signaling molecules. Activated IKK then phosphorylates (p) IκB proteins, such as IκBα, thereby leading to their ubiquitination (ub) and degradation. This releases NF-κB and allows it to translocate into the nucleus, where it upregulates the expression of IκB genes, as well as other target genes. Newly-synthesised IκB proteins bind to and export nuclear NF-κB, creating a negative feedback loop. Cycles of IκB degradation and resynthesis can lead to multiple NF-κB nuclear translocations (oscillations). Adapted from Dr. A. Adamson.
1.2.2. Overview of the Structure and Function of NF-κB Pathway Components

1.2.2.1. Rel Proteins

In mammals, the NF-κB family is composed of five proteins: p65 (also known as RelA), Rel B, c-Rel, p50 and p52, encoded by RELA, RELB, REL, NFKB1 and NFKB2 genes, respectively (Gilmore, 2006; Hayden and Ghosh, 2008; see Figure 1.2). This family of proteins is defined by an N-terminal Rel Homology Domain (RHD). This domain enables dimerization and allows NF-κB dimers to interact with IκB proteins. When NF-κB is activated, a nuclear localization sequence (NLS) within the RHD permits NF-κB nuclear entry (Gilmore, 2006; Hayden and Ghosh, 2008).

NF-κB proteins can be divided into two groups based on their transcriptional capacity. The p65, c-Rel and Rel B proteins contain transcription activation domains (TADs), which confer transcriptional ability to the NF-κB dimers that these proteins form. The p65:p50 heterodimer is the most prevalent and transcriptionally active NF-κB dimer (Gilmore, 2006; Hayden and Ghosh, 2008). As p50 and p52 lack TADs, they must form dimers with TAD-containing proteins in order to gain transcriptional ability (Hayden and Ghosh, 2008).

Almost all variations of NF-κB homo- and hetero-dimers have been identified (Philips et al, 1996; Liou et al, 1994, Lee et al, 1995; Molitor et al, 1990) with the exception of RelB which only binds to p52 and p50 (Ryseck et al, 1992; Ryseck et al, 1995). Unlike other...
dimers, RelB-containing dimers are constitutively active in B cells and inducible in other cell types investigated (Dobrzanski et al, 1995; Olashaw, 1996).

In unstimulated cells, most NF-κB dimers are cytoplasmic; the exceptions to this are p50 and p52 homodimers, which are readily found in the nucleus. As non-TAD-containing dimers, p50 and p52 homodimers can repress gene transcription by binding to DNA and preventing DNA access to TAD-containing dimers (Zhong et al, 2002; Hayden and Ghosh, 2008; Ghosh et al, 1998).

Nuclear NF-κB binds to κB sites on gene promoter and enhancer sequences. The consensus sequence of κB sites is highly variable: 5’-GGGRN(Y)YYCC-3’ where R= adenine (A) or guanine (G); Y= cytosine (C) or thymine (T) and N= A, G, C or T (Hoffmann and Baltimore, 2006). NF-κB dimer composition determines κB site specificity. For example, p65 homodimers have an affinity for the 5’TTTCC’3 sequence in regions of low GC content: through this interaction p65 homodimers are primarily responsible for NF-κB-dependent IL-8 upregulation (Kunsch and Rosen, 1993). On the other hand, p50 homodimers preferentially bind to GC rich areas, such as the 5’GGGGATTCCC’3 κB site on the major histocompatibility class I gene (Kunsch et al, 1992; Kieran et al, 1990; Grimm and Baeuerle, 1993). The κB site specificity of NF-κB dimers contributes to differential gene regulation: this area of NF-κB activity has yet to be thoroughly investigated. Chromatin immuno-precipitation sequencing data may soon shed more light on this area of NF-κB gene regulation.

1.2.2.2. Inhibitor KappaB (IκB) Proteins

The IκB protein family includes IκBα, IκBβ, IκBδ, IκBε, IκBζ and Bcl-3 (Hayden and Ghosh 2008; Ghosh and Karin, 2002; see Figure 1.3). The role of these proteins in NF-κB activity is largely dependent upon their structure: they all contain central multiple ankyrin repeat domains (ARD), which allow 1:1 association with NF-κB dimers (Hayden and Ghosh, 2008). Cytoplasmic IκBα, IκBδ, IκBε and IκBζ bind to and mask the NF-κB NLS, thereby preventing NF-κB nuclear entry (Beg et al, 1992; Henkel et al, 1992; Zabel et al, 1993). IκBα and IκBβ also have a C-terminal acidic and threonine rich sequence, PEST, which is involved in IκB protein stabilisation (Beauparlant et al, 1996) and disrupting NF-κB-DNA binding (Ernst et al, 1995). Bcl-3 uniquely possesses a TAD, enabling it to endow target NF-κB dimers with transcriptional activity (Bours et al, 1993; Fujita et al, 1993).
IκB proteins have different preferences for NF-κB binding partners. For example, Bcl-3 and IκBδ only associate with p50 and p52 homodimers (Nolan et al, 1993) and RelB:p52 heterodimers (Dobrzanski et al, 1995), respectively. IκBγ is less specific and binds to c-Rel-containing dimers, p65:p50 and p50 homodimers (Inoue et al, 1992). These associations allow most IκB proteins to function in canonical and non-canonical pathways. IκBα, which preferentially binds to the p65:p50 heterodimer, functions primarily as part of the canonical pathway (Hayden and Ghosh, 2008).

IκB proteins are referred to as typical or atypical, and differ according to their cellular localisation, expression and function (Hayden and Ghosh, 2008). Due to their nuclear localisation, the atypical proteins- Bcl-3, IκBδ and IκBζ- function mainly as regulators of differential κB-driven transcription (Bours et al, 1993; Fujita et al, 1993; Dobrzanski et al, 1995). The focus in the remainder of this section will be two of the major regulators of the canonical p65:p50 activation response: IκBα and IκBε (Hayden and Ghosh, 2008).

1.2.2.2.1. IκBα and IκBε in NF-κB Activation

TNFα-stimulation leads to the rapid IKK-mediated degradation of cytoplasmic IκBα and IκBε, thereby releasing NF-κB for nuclear entry (Hayden and Ghosh, 2008; Perkins, 2006). IκBα phosphorylation at serine 32 and serine 36 is required, but not sufficient, for IκBα degradation. Phosphorylation makes IκBα a target for ubiquitination, primarily at lysine 21 and lysine 22, which promotes degradation by the 26S proteasome (Whiteside and Israël,
IκBε is thought to be degraded in a similar ubiquitin-dependent manner (Whiteside and Israël, 1997). Both IκBα and IκBε genes are NF-κB-dependent; their upregulation after NF-κB activation provides a negative feedback loop that is vital in controlling NF-κB response duration (Hayden and Ghosh, 2008). The IκBα gene is an early target of NF-κB, enabling rapid restoration of IκBα protein levels after TNFα stimulation (Moss et al., 2012; Ashall et al., 2009; Hoffmann et al., 2002). Newly-synthesised IκBα translocates into the nucleus, dissociates DNA-NF-κB binding and chaperones NF-κB out of the nucleus and back into the cytoplasm (Hoffmann et al., 2002). The temporal profile of IκBα degradation and resynthesis makes it an effective inhibitor of the early NF-κB response. IκBε degradation and resynthesis profile is slower than that of IκBα, allowing inhibition of later stages of the NF-κB response (Whiteside and Israël, 1997). Together, IκBα and IκBε are vital regulators of the duration of NF-κB activation (Hayden and Ghosh, 2008; Hoffmann et al., 2002; Paszek et al., 2010).

1.2.2.3. Inhibitor KappaB Kinase (IKK) Complex

1.2.2.3.1. IKK Complex Components and Formation

Canonical NF-κB activation signals converge on the multi-unit inhibitor kappaB kinase (IKK) complex (700-900kDa): NF-κB activation is dependent on the kinase activity of this complex (Sun and Ley, 2008). IKK kinase activity is derived from its IKK components, IKKα and IKKβ. These proteins share 50% sequence homology: their N-terminal protein kinase and C-terminal leucine zipper and helix-loop-helix domains are required for protein interactions (Zandi et al., 1997; Hayden and Ghosh, 2008; Li and Lin, 2008).

IKK complex formation and activation is dependent on a non-catalytic regulatory protein called NEMO (NF-κB essential modulator; 48kDa; May et al., 2000; Tegethoff et al., 2000; Li and Verma, 2002). The N-terminal of NEMO binds to the C-terminal hexapeptide NEMO-binding domain (NBD) of IKK subunits (Scheidereit, 2006; Chen, 2012; Laplantine et al., 2009).

The core IKK complex is believed to be composed of two IKKα:IKKβ dimers and a tetrameric NEMO unit (Hayden and Ghosh, 2008; Hacker and Karin, 2006; see Figure 1.4). There is evidence of other IKK components, around the core complex. A homodimer of a kinase chaperone, heat shock protein 90 (Hsp90), is constitutively associated with the IKK complex. Recruitment of Hsp90 to IKK, mediated by Cdc37, is vital in TNFα-induced
IKK activation (Chen et al, 2002). ELKS, a DNA-binding protein, was more recently proposed to act as a regulatory subunit of IKK, although its role in IKK formation and activation has yet to be elucidated (Ducut Sigala et al, 2004).

**Figure 1.4. The structure and composition of the main IKK complex constituents.**

The above schematic shows the structure of the main IKK complex components, with annotations denoting their constituent domains. Alternative names for the proteins are shown in brackets. (Abbreviations: CC, coiled-coil; HLH, helix-loop-helix; LZ, leuzine-zipper; NBD, NF-κB-essential-modulator-binding domain). Schematic taken from Ghosh and Hayden, 2008.

1.2.2.3.2. IKK Complex Activation

IKK kinase activity requires non-degradative ubiquitination and also phosphorylation of two T loop serines of at least one of the IKK subunits: either serine 176 and serine 180 or serine 177 and serine 181 of IKKα and IKKβ, respectively (Mercurio et al, 1997; Delhase et al, 1999; Johnson et al, 1996). These modifications lead to conformational changes and IKK complex activation (Delhase et al, 1999; Johnson et al, 1996).

The exact mechanisms behind IKK complex activation require further investigation. However, evidence from various studies indicates transautophosphorylation as an important driver of IKK activation. The C-terminal of NEMO plays an important role in this process by binding IKK dimers and keeping them in close proximity (Rothwarf and Karin, 1999).

Components of the TNF receptor-1 (TNFR1)-signalosome, upstream of IKK, may play a role in TNFα-induced IKK activation. TNFR-associated factor-1 (TRAF1) recruits IKK into the TNFRI-signalosome, after which receptor-interacting protein kinase (RIP) oligomerization acts as a platform for IKK oligomerisation (Inohara et al, 2000; Devin et al, 2000). This final event is vital in IKK activation, possibly due to its promotion of proximity-induced phosphorylation events.
Once activated, the IKK complex phosphorylates downstream IκB proteins, initiating the first step in their degradation via the 26S proteasome (Chen et al 1996; Whiteside and Israël, 1997). IκBα, particularly NF-κB-bound IκBα, is a major target for activated IKKα and IKKβ. IKKβ phosphorylates IκBα more efficiently than IKKα: it accounts for nearly all of IκBα degradation observed after TNFα stimulation (Hacker and Karin, 2006; Hayden and Ghosh, 2008; Hayden and Ghosh, 2004).

Moreover, it is widely reported that IKKβ kinase activity is required and sufficient for canonical NF-κB activation. However, studies on IKK-deficient mice suggest that both IKKα and IKKβ are non-redundant and differentially effect TNFα-induced NF-κB activation (Takeda et al, 1999; Hu et al, 1999; Li et al, 1999). It has been found that protein phosphatase 2A treatment inactivates IKK, possibly by removing vital T loop serine-associated phosphate groups (DiDonato et al, 1997). The exact mechanisms behind the termination of IKK activation are largely unknown.

1.2.3. Canonical Activation of NF-κB Pathway

Activation of the canonical pathway can be induced by a range of ligands, including TNFα and IL-1. Ligand-receptor interactions on the cell surface trigger the recruitment of intracellular adaptor proteins such as TNFR-associated death domain protein (TRADD) and RIP (Hayden and Ghosh, 2008; Perkins 2006). The resulting intracellular complex recruits and activates IKK. IKK activity is vital for NF-κB activation via the canonical pathway: it phosphorylates IκB proteins, thereby facilitating their degradation and NF-κB release for nuclear entry (Hoffmann et al, 2002; Hayden and Ghosh, 2008). The following section details the TNFα-induced canonical signalling pathway, from ligand to signalling outcome (i.e. cell survival versus apoptosis).

1.2.3.1. TNFα Structure

TNFα is a member of a large cytokine family, the TNF ligand family, which also includes Fas, CD40 ligand and lymphotoxin α. TNFα was initially discovered almost forty years ago as an antitumor agent, capable of inducing apoptosis in tumour cells. It was found to exert therapeutic effect, when combined with chemotherapeutics, in cases of cancer (Carswell et al, 1975; Williamson et al, 1983). Since its initial discovery, the pleotropic nature of TNFα has been revealed: it is an important player in the inflammatory process,

TNFα is synthesised as a monomeric transmembrane protein that requires homotrimerization to become biologically active (Kriegler et al, 1988; Tang et al, 1996). The soluble homotrimers are proteolytically cleaved by TNFα converting enzyme (TACE) to produce two forms of TNFα: soluble 51kDa trimeric TNFα and membrane-anchored 17kDa TNFα protomer (Black et al, 1997). The transmembrane TNFα (mTNFα) has a structure that is typical of members of the TNF ligand family: two anti-parallel beta-pleated sheets with antiparallel beta strands form what is known as a ‘jelly-roll’ structure (Bazan, 1993).

TNFα upregulation is a common feature of neuroinflammatory diseases, most notably AD (Montgomery and Bowers, 2012; Cartier et al, 2005). CNS-specific overexpression of TNFα in mice can lead to astrogliosis, microgliosis and demyelination (Probert et al, 1995). However, TNFα can also be beneficial: the duality of its effects has been attributed to cell-type specificity and TNF receptor engagement (Probert et al, 1995; Fontaine et al, 2002; Kontoyiannis et al, 1999).

**1.2.3.2. TNFR Structure and Function**

TNFα exerts its effects via TNFR1 (also known as CD120a; p55 or p60) and TNFR2 (also known as CD120b; p75 or p80; Tartaglia and Goeddel, 1992). Although both soluble TNFα and mTNFα can interact with TNFR1 and TNFR2, soluble TNFα has a higher affinity for TNFR1 than mTNFα (Grell et al, 1995; Grell et al, 1998). Due to the stability of the TNFR1-TNFα complex, soluble TNFα is thought to primarily signal through TNFR1 (Thoma et al, 1990; Brockhaus et al, 1990) and, conversely, mTNFα preferentially signals through TNFR2 (Eissner et al, 2004; Grell et al, 1995).

Receptor-ligand preferences have been implicated in dual TNFα functionality: TNFR1 and TNFR2 signalling is generally associated with cell damage and cell survival, respectively (Fontaine et al, 2002; Shen et al, 1997; Yang et al, 2002). However, this idea is too simplistic: TNFR2 triggering alone in the CNS can induce cell death (Bigda et al, 1994; Grell et al, 1993; Medvedev et al, 1994). Also, TNFR2 signalling can augment TNFR1-induced apoptosis and cooperation between these receptors reportedly enhances cytotoxicity (Declerc et al, 1998; Vandanabeele et al, 1995; Grell et al, 1999).
The extracellular domain of both receptors contains four cysteine rich repeats that are arranged into elongated shapes, to enable interactions with the lateral grooves of soluble TNFα trimers (Naismith and Sprang, 1998; Banner et al, 1993). Difference in structure is a contributory factor in TNFR1 and TNFR2 signalling outcome. The cytoplasmic portion of TNFR1 contains a death domain (DD), which is essential for the induction of an apoptotic signal (Micheau and Tschopp, 2003; Thorburn, 2004). In contrast, TNFR2 does not contain a DD, but can induce apoptosis by activating the TNFR1 signalling pathway, as TNFR1 has a region which is responsive to TNFR2 activation (Declercq et al, 1998; Vandanabeele et al, 1995).

Like TNFα, TNFR2 undergoes proteolytic cleavage by TACE to release its extracellular domain (Solomon et al, 1999). This domain can bind to soluble TNFα in the extracellular space, thereby reducing TNFα action on cells (Kraft et al, 2009). TNFR1 also undergoes a similar cleavage process; the protease responsible has not yet been identified (Wajant et al, 2003).

TNFR1 is constitutively expressed in most cells, whereas TNFR2 is primarily expressed in immune cells (Wajant et al, 2003). Differential expression of TNFRs may contribute to cell-type specific TNFα effects.

1.2.3.3. TNFR1 Signalling

The formation of TNFR1 trimers is thought to be essential for initiating the response to TNFα (Wajant et al, 2003). This can occur spontaneously in unstimulated cells, as a result of homophilic interactions between receptors, driven by distal cysteine rich domains. Pre-ligand-binding assembly domains prevent this auto-activation and maintain the receptors in an inactive state (Chan et al, 2000).

The death domain of TNFR1 is particularly prone to self-aggregation; a process which is prevented by the binding of silencer of death domain (SODD) protein. SODD dissociates from TNFR1 upon its interaction with TNFα (Jiang et al, 1999). Tyrosine residues, present in the intracellular domain of TNFR1, facilitate rapid internalisation of TNFR1 after binding to TNFα (Kraft et al, 2009; Schutze et al, 1999). This process seems to be required for transduction of the apoptotic signal (Kraft et al, 2009; Hsu et al, 1996a) and also the stimulation of c-Jun N-terminal kinase (JNK) pathways (Wajant et al, 2003). Activation of NF-κB and other pathways, such as extracellular signal-regulated kinases (ERK), is thought to occur independently of receptor internalisation (Wajant et al, 2003).
Within minutes of internalisation, the TNFR1-TNFα complex recruits TRADD which, in turn, initiates a cascade of adaptor protein recruitment (Hsu et al, 1996a). This recruitment cascade is mediated by homophilic interactions between the DDs of adaptor proteins (Schulze-Osthoff et al, 1998). TRADD acts as a platform for recruitment of Fas-associated protein with death domain (FADD); this event is involved in the transduction of apoptotic signals (Hsu et al.1996b; Juo et al, 1998; Yeh et al, 1998). RIP is also recruited to the TNFR1-complex (Hsu et al, 1996a). Wajant et al (2003) speculated that RIP is involved in the stabilization of this complex, via its interactions with TRAF2 (Hsu et al, 1996a).

TRAF 2 polyubiquitinates RIP1 and facilitates the recruitment of IKK to the signalosome, via its Really Interesting New Gene (RING) domain (Inohara et al, 2000; Hayden and Ghosh 2008). Oligomerized TRAF proteins alone are sufficient for recruitment of the IKK complex into the signalosome, whereas RIP1 is a requirement for IKK activation (Devin et al, 2000): RIP1 and 2 oligomerization may provide a platform for IKK complex oligomerization (Inohara et al, 2000).

However, the composition of the signalosome is highly complex and dynamic: over time, members of the signalosome dissociate to form a second complex with other proteins. The balance between the initial and second complex can determine whether or not apoptosis is induced by TNFR1-signalling (Micheau and Tschopp, 2003; Micheau et al, 2001).

1.2.3.3.1. Induction of Apoptosis

Molecular studies have shed light on how elements of the TNFR1 activation pathway can determine how cells respond to TNFα i.e. survival versus apoptosis (Micheau and Tschopp, 2003; Micheau et al, 2001).

Micheau and Tschopp (2003) proposed that TNFR1-induced apoptosis requires the formation of two signalling complexes: the balance between these complexes determines signalling outcome. They utilised wild-type HT1080 cells, modified to express an undegradable form of IκBα: these cells were susceptible to TNFα-induced apoptosis. Wild type HT1080 cells were resistant to TNFα-induced apoptosis. In this study, the composition of the TNFR1 signalling complex was analysed in apoptosis-susceptible and apoptosis-resistant cells.

The plasma-membrane bound complex I, formed rapidly after TNFR1 internalisation, was composed of TRADD, RIP proteins, TRAF2 and cellular inhibitor of apoptosis protein-1
(c-IAP1). This complex signalled to activate NF-κB, via the recruitment and activation of IKK.

Over time, complex I progressively dissociated and adaptor proteins, which included TRADD, RIP1 and TRAF2, formed a second complex (complex II), along with caspase-8, caspase-10 and FADD. Association of these factors became more pronounced over time, reaching a peak at the onset of apoptosis.

It was found that NF-κB activity, initiated by complex I, could prevent complex II-driven apoptosis, by upregulating the expression of anti-apoptotic factors such as TRAF and c-IAP. Caspases-3 and -7 were tagged for proteasomal degradation by the C-terminal RING domain of c-IAP1 and 2 proteins. The NF-κB-dependent caspase-8 inhibitor, FLICE-like inhibitory protein (FLIP) was vital in the prevention of apoptosis: overexpression of FLIP in apoptosis-susceptible cells prevented TNFR1-induced apoptosis (Micheau and Tschopp, 2003; Micheau et al, 2001). Other studies have shown that NF-κB activity regulates the expression of other anti-apoptotic factors, such as immediate early response gene-1L and X-linked inhibitor of apoptosis (Stehlik et al, 1998; Wu et al, 1998). Together, these results highlight the importance of analysing molecular-level data, as well as downstream gene expression, to understand signalling outcomes.

1.2.3.4. TNFR2 Signalling

The TNFR2 signalling pathway is less well understood than TNFR1 signalling. As TNFR2 lacks a DD, its ability to promote apoptosis is reliant on activating TNFR1 signalling: TNFR2 can mediate TNFR1-dependent apoptosis in one of three ways (Wajant et al, 2003). The first involves competitive binding for TRAF2-associated anti-apoptotic proteins, c-IAP1 and 2. TRAF2 affinity for TNFR1 and TNFR2 is comparable (Fotin-Mleczek et al, 2002). Therefore, TNFR2 activation and subsequent recruitment of TRAF2 decreases TRAF2 availability for TNFR1 signalling. Reduction in TRAF2 recruitment can cause apoptosis, possibly via the resulting decrease in c-IAP recruitment to the TNFR1-signalling complex (Weiss et al, 1997; Weiss et al 1998; Duckett and Thompson, 1997; Li et al, 2002; Chan and Lenardo, 2000; Brown et al, 2002; Fotin-Mleczek et al, 2002). Consistent with this idea, TNFR2 stimulation is associated with TNFR1-mediated caspase-8 activation and, thereby, apoptosis (Chan and Lenardo, 2000; Brown et al, 2002; Fotin-Mleczek et al, 2002).
The second method by which TNFR2 could induce TNFR1-driven apoptosis is by promoting c-IAP1-initiated degradation of TRAF2 (Wajant et al, 2003). The consequent TRAF2 depletion would enhance competition between TNFR1 and TNFR2 for recruitment of remaining TRAF2 (Wajant et al, 2003).

Finally, TNFR2-stimulation can promote TNFR2 cleavage to produce mTNFα. This can, in turn, directly activate TNFR1 and, thereby contribute to apoptosis (Grell et al, 1995; Grell et al, 1998).

1.2.4. NF-κB Dynamics and Function

Hoffmann et al (2002) used a mathematical model to predict that the NF-κB system could produce oscillations in certain circumstances. Treating with a 15-minute pulse of TNFα appeared to produce a more transient NF-κB response in MEFs (mouse embryonic fibroblasts). Apparent oscillatory behaviour during continual TNFα treatment was observed using electromobility shift assays (EMSA) for nuclear NF-κB in cells where IκBβ and IκBe were knocked out. Interestingly, Hoffmann et al (2002) reported that different TNFα treatments produced different downstream NF-κB-dependent expression profiles of MCP-1/CCL2 and IP-10/CXCL10 genes.

When NF-κB dynamics were observed for the first time in single-cells (Nelson et al, 2004), cell-type specific NF-κB oscillations were observed, in response to continual TNFα treatment. This was achieved using SK-N-AS (human neuroblastoma) and HeLa (human cervical cancer) cells expressing fluorescent protein-tagged p65 and time lapse microscopy. SK-N-AS cells displayed sustained oscillations for over 20 hours, whereas HeLa cells displayed more transient oscillations, the amplitudes of which dampened over time. Moreover, the oscillatory NF-κB response between cells was highly heterogeneous and asynchronous.

Since these pioneering studies, others have reported TNFα-induced NF-κB oscillations with an average period of around 100 minutes, in a range of cell-types (Ashall et al, 2009; Turner et al, 2010; Paszek et al, 2010; Tay et al, 2010; Sung et al, 2009). Mathematical models have been created to recapitulate the NF-κB dynamics observed. Hypotheses initially generated from mathematical models, and subsequently investigated in the laboratory, have provided vital insights to regulation of NF-κB activation and dynamics (Hoffmann et al, 2002; Ashall et al, 2009; Paszek et al, 2010). This includes models which
proposed that cell-cell heterogeneity is controlled by temporal differences in IkBα and IkBε transcription profiles (Ashall et al, 2009; Paszek et al, 2010).

Heterogeneous NF-κB response within cell populations, and thereby differential gene regulation, has highlighted the importance of studying NF-κB on the single-cell level. Turner et al (2010) revealed that, in SK-N-AS cells, NF-κB heterogeneity increases as TNFα dose decreases. In the inflammatory environment, NF-κB heterogeneity may be a way of preventing the production of toxic levels of cytokines and protecting healthy cells from damage. Cell synchrony in vivo might offer an explanation for the damage caused by inflammation in disease states (Paszek et al, 2010).

Arguably the most important insight from single-cell observation, is on the role of NF-κB dynamics in determining downstream gene expression profile. In vivo, cells in areas of inflammation may release TNFα in a pulsatile manner (Covert et al, 2005; Werner et al, 2005). Ashall et al (2009) studied the effects of pulsatile TNFα-stimulation on NF-κB dynamics in SK-N-AS cells. They found that the amplitude of successive NF-κB nuclear translocations was dependent on the time between TNFα pulses, as a result of IKK activation kinetics. Pulse TNFα treatment at 60 minute- or 100 minute- intervals, led to successively lower NF-κB nuclear translocation amplitudes: this was not observed in cells that were stimulated with 200-minute TNFα pulses. Ashall et al (2009) went on to show differential NF-κB-dependent gene expression in response to different pulsatile treatments. These results strongly suggested that aspects of NF-κB dynamics, such as amplitude of nuclear translocations, could be key to determining gene expression. The amplitude of NF-κB nuclear translocations has more recently been highlighted as a vital determinant of TNFα-induced IL-8, A20 and IkBα mRNA production (Lee et al, 2014).

It has been proposed that the purpose of NF-κB oscillations is to allow continual sensing of an evolving inflammatory environment. NF-κB post-translational modifications over successive nuclear: cytoplasmic translocations may contribute to differential gene regulation and, thereby, allow cells to produce a highly specialised response to inflammatory stimuli (Perkins, 2006; Vermeulen et al, 2002; Saccani et al, 2003). More investigation is required to further delineate the relationship between various aspects of NF-κB dynamics and gene expression.
1.2.5. Regulation of NF-κB Transcriptional Activity

Nuclear NF-κB binds to κB sites on the promoter or enhancer sequences of NF-κB target genes (Hoffmann et al, 2006). However, the presence of nuclear NF-κB alone is not sufficient to induce gene expression: full transactivation of p65 requires a series of post-translational modifications. Phosphorylation of serine residues, mediated by protein kinase A (PKA), is required for most of the transcriptional responses that are driven by p65 (Chen and Green, 2004). Also, phosphorylation of serine 529 is a requirement for transcriptionally-activating p65: this can be done by casein kinase II after TNFα-stimulation (Wang and Baldwin, 1998; Wang et al, 2000). Acetylation of p65 has also been shown to increase transcriptional activity: p65 acetylation leads to higher induction of NF-κB-dependent genes (Chen and Greene, 2004).

NF-κB preference for κB binding sites is conferred by the N-terminus of immunoglobulin-like domains within the RHD (Hayden and Ghosh, 2008). Hence, one source of transcriptional selectivity is NF-κB dimer composition (see section 1.2.2.1). Three other mechanisms which affect the NF-κB transcriptional response are:

1) Post-translation modifications to NF-κB dimers;
2) Recruitment of co-activators and co-repressors of gene transcription and;
3) NF-κB dynamics (see section 1.2.4)

It should be noted that there are many other mechanisms by which NF-κB transcriptional activity is modulated: the above mechanisms were selected here as they have been well studied, in comparison to other mechanisms.

The first two mechanisms above can be intrinsically linked; post-translational modifications determine later recruitment of transcriptional co-factors. Atypical IκB and IKK proteins modify NF-κB-dependent gene expression, via these mechanisms. NF-κB regulation by these proteins, and other important factors are outlined in the following sections.

1.2.5.1. Atypical IκB Proteins

1.2.5.1.1. B-cell Lymphoma-3 (Bcl-3)

Bcl-3 was originally identified as a proto-oncogene in chronic B-lymphocyte leukaemia (Ohno et al, 1990). Of all the atypical IκB proteins, only Bcl-3 contains a TAD and is localized to the nucleus of cells (Nolan et al, 1993; Hayden and Ghosh, 2008). Bcl-3 can
bind to DNA-bound p50 and p52 homodimers and either stabilize the DNA-homodimer complex to repress transcription or trigger the release of homodimers, thereby allowing NF-κB access to κB sites (Hayden and Ghosh, 2008). Stabilisation of DNA-bound repressive complexes mediated by Bcl-3 has been implicated in LPS tolerance (Wessells et al, 2004; Carmody et al, 2007; Ghosh and Hayden, 2008). As this interaction is dependent on the nuclear localisation of Bcl-3, it is mediated by Bcl-3 phosphorylation and ubiquitination (Nolan et al, 1993; Caamano et al, 1996; Bundy and McKeithan, 1997). Cylindromatosis (CYLD) can negatively regulate the activity of Bcl-3 by inhibiting its ubiquitination and thereby preventing nuclear entry (Massoumi et al, 2006).

Phosphorylated Bcl-3 can endow target p50 and p52 homodimers with transcriptional activity (Nolan et al, 1993; Caamano et al, 1996; Bundy and McKeithan, 1997). Rocha et al (2003) showed that reduction in Bcl-3 expression, caused by p53 activation, allows p52 homodimers to repress Cyclin D1 expression by binding to κB sites on the promoter region of the gene. Cyclin D1 has been shown to mediate NF-κB-dependent proliferation of NPCs (Widera et al, 2006a).

1.2.5.1.2. IκBζ

IκBζ expression can be induced by NF-κB signalling in a ligand-specific manner (Yamazaki et al, 2005). Although it lacks a well-defined TAD, IκBζ is more homologous to Bcl-3 than other IκB proteins (Hayden and Ghosh, 2008). Like Bcl-3, IκBζ binding to p50 homodimers may confer transcriptional activity (Yamamoto et al, 2004).

The loss of IκBζ has been reported to result in the loss of expression of a subset of NF-κB target genes (Yamamoto et al, 2004). Of these, IκBζ has a particularly profound effect on IL-6 expression: induction of IL-6 is abrogated in IκBζ-deficient mice. IκBζ binds to p50 homodimers located on κB sites within the IL-6 promoter: it is thought that this interaction is vital for IL-6 expression (Yamamoto et al, 2004).

1.2.5.1.3. IκBδ

IκBδ was initially identified in T-cells undergoing negative selection (Fiorini et al, 2002). The exact role of IκBδ in regulating NF-κB transcriptional activity is largely unknown. IκBδ preferentially binds to p50 and its overexpression has an inhibitory effect on NF-κB reporter gene expression; together, this suggests that IκBδ may promote the formation of repressive NF-κB-DNA complexes (Fiorini et al, 2002).
1.2.5.2. IKKα and IKKβ

IKKα has a dual function in NF-κB transcriptional activity, as it is able to promote and inhibit the expression of NF-κB target genes (Scheidereit, 2006; Chen and Greene, 2004; Hayden and Ghosh, 2008). IKKα-deficient macrophages express higher levels of NF-κB target pro-inflammatory genes than wild-type cells (Li et al., 2005). On the other hand, IKKα-mediated phosphorylation events can promote chromatin opening and, thereby, facilitate the binding of NF-κB to κB sites (Hoberg et al., 2004). Phosphorylation of SMRT complexes induces the release of histone deacetylase 3 from these complexes which can, in turn, increase NF-κB transcriptional activity by increasing p65 acetylation. This effect requires the removal of a histone acetyltransferase complex (Hoberg et al., 2006; Chen and Greene, 2004).

IKKα can also facilitate p65 binding to the transcriptional co-activator CREB-binding protein (CBP), by CBP phosphorylation at serine 1382 and serine 1386 (Huang et al., 2007).

1.2.5.3. Other Factors influencing NF-κB Transcription.

One of the first kinases implicated as a regulator of p65 transcriptional activity was protein kinase C zeta (PKCζ). PKCζ-driven phosphorylation of p65 at serine 311 is required for CBP recruitment to transcriptionally active p65-containing NF-κB dimers (Zhong et al., 1998; Duran et al., 2003).

Another kinase, MAP kinase-activated protein kinase 2 (MAPKAPK2), regulates p65 phosphorylation via its target substrate, Hsp27 (Gorska et al., 2007). MAPKAPK2-deficient mice display lower induction of NF-κB-dependent pro-inflammatory cytokines, compared to wild-type mice. Deficiencies in MAPKAPK2 or Hsp27 cause an increase in p65 phosphorylation at serine 276: this finding is associated with increases in IκBα transcription and earlier p65 nuclear exit (Gorska et al., 2007).

MAPKAPK2 is also involved in p38-mediated regulation of NF-κB dependent transcripts. The p38-MAPKAPK2 pathway promotes translation of TNFα mRNA, produced downstream of NF-κB activation (Kontoyiannis, et al., 1999; Neininger et al., 2002). Also, p38-mediated phosphorylation of histone 3 at serine 10 can increase p65 binding to IL12p40 and IL-6 promoters (Saccani et al., 2002).
As well as phosphorylation, ubiquitination regulates NF-κB transcriptional activity (Tanaka et al, 2007). PDZ and LIM domain protein 2 (PDLIM2), a nuclear ligase, ubiquitinates p65 and transports it to nuclear compartments called PML nuclear bodies. This promotes degradation of p65-containing NF-κB dimers and, thereby, transcriptional silencing (Ghosh and Hayden, 2008; Tanaka et al, 2007).

1.2.6. Termination of the NF-κB Response

IκBα and IκBε are vital in the inhibition of NF-κB during the early stages of NF-κB activation: these proteins bind to and export nuclear NF-κB dimers (Hayden and Ghosh, 2008; Perkins 2006). Other proteins, such as A20 and CYLD, exert their action on NF-κB at later stages of the NF-κB response and thereby determine the persistence of NF-κB activation (Lee et al, 2000; Brummelkamp et al, 2003). Some mechanisms of this inhibition will be outlined here.

1.2.6.1. TNFα-induced Protein 3 (A20)

Discovered in 1990, the zinc finger protein TNFα-induced protein 3 (A20) is an ubiquitin-editing enzyme (Opipari et al, 1990; Jaattela et al, 1996; He and Ting, 2002). A20-deficient MEFs display prolonged TNFα-induced NF-κB activation and NF-κB-DNA binding (Lee et al, 2000). Deficiency of A20 in mice causes severe inflammation and premature death, highlighting A20 as an important factor in the resolution of inflammation (Lee et al, 2000).

Unlike IκB proteins, A20 does not directly interact with NF-κB, but instead targets components of the IKK complex. A20 targets include IKKα and IKKβ (Zhang et al, 2000), as well as upstream molecules, TNFR1, RIP1 and TRAF6 (Song et al, 1996; Heyninck et al, 1999; Heyninck and Beyaert, 1999).

A20 directly modifies RIP1 via its ubiquitin-editing domains: an N-terminal ovarian tumour deubiquitinating domain and 7 carboxyterminal zinc finger domains (Wertz et al, 2004; Shembade et al, 2008). These domains each have different targets: the N-terminal domain preferentially deubiquitinates K63-linked ubiquitin chains from RIP1, whereas the C-terminal domain catalyzes the polyubiquitination of K48 on RIP1. This process of site-specific ubiquitination and deubiquitination triggers the proteasomal degradation of RIP1 (Wertz et al, 2004; Shembade et al, 2008).

A20 interactions with the proteins Tax1 binding protein 1 (Tax1BP1), Ring finger protein 11 and Itchy E3 ubiquitin protein ligase (Itch) are required to terminate TNFα-induced NF-
κB activation (Shembade et al., 2007; Shembade et al., 2008; Shembade et al., 2009). Deficiencies in Tax1BP1 and Itch lead to a prolonged TNFα-induced NF-κB response: as observed in A20-deficient MEFs. In these Tax1BP1-deficient and Itch-deficient MEFs, enriched K63-linked ubiquitination of TRAF6 and RIP1 was observed, respectively (Iha et al., 2008; Shembade et al., 2007).

The mechanisms behind the formation of the A20 ubiquitin-editing complex are yet unknown. IKKα-mediated phosphorylation of serine residues on Tax1BP1 induces formation of the A20 complex, by unknown mechanisms (Shembade et al., 2011). In turn, A20 can promote the phosphorylation of IKKα and IKKβ within the IKK complex (Zhang et al., 2000). Phosphorylated IKKβ can undergo autophosphorylation, resulting in decreased kinase activity (Delhase et al., 1999): it has been speculated that A20 can stabilise inactive, phosphorylated IKKβ (Wajant et al., 2003). Together, this creates a closed loop: IKKα-drives activation of the A20 complex which, in turn, feeds back to deactivate IKKα, as well as IKKβ. From this, it has been postulated that assembly of the A20 complex may be dependent on signalling from activated receptors, such as TNFR1 (Pujari et al., 2013).

1.2.6.2. Cylindromatosis (CYLD)

Cylindromatosis (CYLD) is an evolutionarily conserved deubiquitinating enzyme that was originally identified in tumour development (Bignell et al., 2000). The C-terminal of CYLD contains three cytoskeleton-associated proteins-glycine (CAP-Gly) motifs and a domain which comprises the catalytic pocket-forming active histidine and cysteine residues (Courtois, 2008).

The first evidence of CYLD involvement in NF-κB inhibition stemmed from a shRNA screening study (Brummelkamp et al., 2003). CYLD-targeting shRNA could prolong TNFα-induced NF-κB activation in HeLa cells. NF-κB activation was also inhibited by CYLD overexpression in IL-1 and TNFα-stimulated cells (Kovalenko et al., 2003; Brummelkamp et al., 2003; Trompouki et al., 2003; Yoshida et al., 2005; Wright et al., 2007). Conversely, CYLD knockdown results in
increased ubiquitination of these proteins (Zhang et al, 2006; Reiley et al, 2007; Wright et al, 2007; Reiley et al, 2005; Jin et al, 2008). Together, this suggests that CYLD constitutively prevents IKK activation and, thereby, NF-κB activation.

As yet, all the mechanisms by which CYLD inhibits NF-κB are not fully understood. It has been suggested that CYLD can exert its effects via physical interaction with target molecules: the N-terminal proline-rich area of NEMO directly associates with a CYLD CAP-Gly motif (Kovalenko et al, 2003; Trompouki et al, 2003; Saito et al, 2004). It has been speculated that this association may allow CYLD to interact with NEMO-bound RIP1 and TRAF2 (Kovalenko et al, 2003). However, it appears CYLD target specificity is heavily regulated by its association with adaptor proteins (Courtois, 2008). The adaptor protein p62 mediates interactions between CYLD and TRAF6 (Jin et al, 2008; Wooten et al, 2008). The consequences of these interactions require further investigation.

Interestingly, CYLD mRNA expression was reduced in TNFα-treated, IKKα-, IKKβ- and NEMO-knockout MEFs (Jono et al, 2004). These findings suggest that, similar to A20, CYLD expression may be regulated by NF-κB activation. As well as this, there is evidence to suggest that CYLD activation is driven by NEMO-mediated phosphorylation events (Reiley et al, 2005). Although the outcome of these phosphorylation events is unknown, it appears that stimulus-induced NEMO activation may initiate CYLD negative feedback. The studies discussed here suggest that CYLD has multiple roles in preventing IKK activation.

1.3. NF-κB in the Central Nervous System (CNS)

Much of what is known about NF-κB has been derived from non-neural cells. Here, the role of NF-κB in the CNS is outlined, with a particular focus on the central mediators of neuroinflammation; microglia and NPCs.

1.3.1 Overview of NF-κB Components in the CNS

NF-κB pathway components are present throughout the nervous system. As in non-neural cells, the most ubiquitously expressed are RelA, p50 and IκBα- although NF-κB expression levels are cell-type specific (O’Neill and Kaltschmidt, 1997). Dimers of p65:p50 have been found in astrocytes (Sparacio et al, 1992), neurons (Kaltschmidt et al,
1993; Schmidt-Ullrich et al, 1996), microglia (Nakajima and Kohsaka, 1998) and neural stem cells (Denis-Donini et al, 2005).

The NF-κB pathway has a CNS-specific NF-κB subunit, called neuronal κB factor (Moerman, 1999), and a set of CNS-specific stimuli which includes nerve growth factor (NGF; Carter et al, 1996), brain-derived neurotrophic factor (BDNF; Gavalda et al, 2009) and amyloid precursor protein (Barger and Mattson, 1996). In turn, NF-κB regulates the expression of nervous system-specific factors, including BDNF (Kairisalo et al, 2009; Saha et al, 2006), and NGF (Heese et al, 2006).

1.3.2. NF-κB Function in the CNS

Most NF-κB knockouts are embryonic lethal due to defects unrelated to the nervous system; this provides a limited time window for observing the role of NF-κB in the developing CNS (Beg et al, 1995). Transient NF-κB activation is observed during development in the spinal cord and certain brain areas (Schmidt-Ullrich et al, 1996). RelB knockout mice show no CNS developmental defects (Weih et al, 1995), but RelA, p50 and c-Rel have been implicated in CNS development, mainly through experiments with knockout mice (Middleton et al, 2000; Nickols et al, 2003; Pizzi et al, 2002; Köntgen et al, 1995). RelA knockout mice display reduced neuronal survival in response to ciliary neurotrophic factor (Middleton et al, 2000) and p50 knockout mice develop normally, but later show increased age-related degeneration and apoptosis (Grumont et al, 1998; Lu et al, 2006). Another study showed that p50 knockout mice exhibit greater tissue damage after excitotoxic stimulation, suggesting that p50-containing dimers can serve a neuroprotective function (Yu et al, 1999). Also, survival of neurons in c-Rel knockout mice is severely impaired.

In the adult CNS, NF-κB is important in synaptic plasticity, learning and memory formation (Kaltschmidt et al, 2006a). Knockout of c-Rel impairs memory formation, by impairing synaptic-plasticity (Ahn et al, 2008). Also, NF-κB inhibition in hippocampal neurons diminishes neurite extension and branching, thereby impairing the formation of memory-associated neuronal connections (Sanchez-Ponce et al, 2011). TNFR1 and p65 double knockout mice have a reduced capacity for spatial memory formation (Meffert et al, 2003) and short term memory formation is impaired in p50 knockout mice (Denis-Donini et al, 2008). Together, these observations implicate the canonical NF-κB activation pathway in memory formation. The mechanisms by which NF-κB regulates memory
NF-κB activation corresponds to apoptosis and disease onset in mouse models of AD (Niu et al, 2010). Also, NF-κB can induce the expression of amyloid precursor protein, the amyloid-β precursor (Grilli et al, 1996), as well as being activated by it (Pahl, 1999). This positive feedback motif might result in NF-κB-driven runaway inflammation, perpetuating the proinflammatory environment and, consequently, tissue damage. It could be speculated that the same feedback motif may also be created by TNFα in neurodegenerative diseases, such as AD (Niu et al, 2010; Cartier et al, 2005).

As in non-neural cells, NF-κB is a major controller of cell survival, via its regulation of pro- and anti-apoptotic factors, which include Bcl-2, Bax and c-IAPs (Tamatani et al, 1999; Baud and Karin, 2001; Shou et al, 2002). NF-κB-dependent expression of Cyclin D1 is associated with apoptosis of cortical and striatal neurons (Liang et al, 2007). In contrast, NF-κB inhibition in neurons reportedly increases their vulnerability to toxins like amyloid-β. Prostrate apoptosis response-4 (a protein implicated in AD) also causes neuron death, partly via NF-κB inhibition (Guo et al, 1998; Mattson and Camandola, 2001). Interestingly, NF-κB levels are reportedly elevated in the forebrains of AD patients, particularly around senile plaques (Kaltschmidt et al, 1997; Kaltschmidt et al, 1999). Moreover, microglial NF-κB inhibition prevents amyloid β-induced cell death (Chen et al, 2005). This highlights the importance of studying NF-κB at the single-cell level, particularly when studying mixed cell populations.

Mattson and Camandola (2001) proposed that, as observed in non-neural cells, stimuli induce different NF-κB-dependent gene expression profiles, according to cell type and
receptor engagement in the nervous system. They speculated that TNFR2 signalling in neurons causes the upregulation of anti-apoptotic genes, whereas TNFR1 activation in microglia promotes the production of toxic agents. This idea is supported by studies that report TNFα-induced neuronal toxicity only in the presence of glial cells (Mir et al, 2008; Taylor et al, 2005; Tolosa et al, 2011). Microglial-specific inhibition of NF-κB has been suggested as a form of therapy for AD (Chen et al, 2005).

In summary, NF-κB activity is central in a network that determines cell survival in neuroinflammation. A better understanding of the complex profiles of cell-type specific NF-κB activation and downstream gene expression will help to design therapies that can manipulate the neuroinflammatory process. The next sections will outline current knowledge on NF-κB activation in vital cellular components of the neuroinflammatory network: microglia and NPCs.

1.3.3. NF-κB Activation in Microglia and Neural Progenitor Cells

NF-κB activation in microglia is essential for their exertion of neuroprotective properties: microglial NF-κB activation results in clearance of amyloid-β deposits in animal models of AD (Li and Schmidt, 1997; Herber et al, 2007). In rat microglia, LPS exposure also causes an NF-κB-dependent increase in NGF release (Heese et al, 1998). These observations suggest that microglial NF-κB is involved in both phagocytosis and neuroprotection.

Microglial NF-κB activation plays a multidimensional role in neuroinflammation; NF-κB-dependent gene expression is implicated in classical and alternative activation, as well as acquired deactivation (Colton, 2009).

Microglia control the fate of NPCs through the release of NF-κB-dependent factors such as nitric oxide (NO), COX-2 and TNFα (Das and Basu, 2008). Microglial-derived TNFα induces apoptosis of NPCs, via NPC NF-κB activation (Guadagno et al, 2013). Interestingly, non-activated microglia reportedly promote NPC proliferation (Liu et al, 2013a), whereas activated microglia induce NPC apoptosis (Guadagno et al, 2013; Liu et al, 2014; Liu et al, 2013b). In turn, NPCs promote activation and proliferation of microglia (Forstreuter et al, 2002; Mosher et al, 2012; Liu et al, 2013a).

The therapeutic effect of NPCs, observed in many disease states, is dependent on NPC migration into sites of injury, proliferation and survival (Martino and Pluchino, 2006). In
particular, NF-κB-mediated proliferation and apoptosis have been well studied in NPCs (Widera et al, 2006a; Widera et al, 2006b; Piotrowska et al, 2006; Guadagno et al, 2013).

Although most insults in the CNS lead to NPC proliferation (Parent, 2003), opposing effects have also been observed. Proliferation of NPCs from the subventricular zone is promoted by NF-κB activity: this effect is mediated by the downstream expression of NF-κB-dependent genes, c-myc and Cyclin D1 (Widera et al, 2006a). It is generally believed that TNFR1 activation suppresses NPC proliferation, whereas TNFR2 promotes NPC proliferation and survival (Das and Basu, 2008). We can conjecture that differences in single-cell NF-κB dynamics may be central to determining NPC fate.

To date, NF-κB activation in microglia and NPCs has been measured using population-level biochemical assays including EMSA and p65 Western blots; single-cell observations have been made via p65 immunostaining in fixed cells (Sheppard et al, 2011; Widera et al, 2006a; Piotrowska et al, 2006). Due to its dynamic and pleotropic nature, simply measuring NF-κB activation status is not an accurate indicator of downstream effects: NF-κB activation has been reported in classical and alternative activation of microglia (Colton, 2009), as well as proliferation and apoptosis of NPCs (Widera et al, 2006a; Widera et al, 2006b). In order to understand the consequences of NF-κB activation, it has to be studied in greater depth, from observing single-cell dynamics to measuring downstream gene expression, and consequent cell fate decisions.

1.4. Thesis Aims

The studies outlined in this chapter provide evidence for vital NF-κB mediated interactions between microglia and NPCs: these interactions have yet to be thoroughly investigated. TNFα, a potent NF-κB activator, is upregulated in many disease states, most notably AD. Single-cell NF-κB dynamics contributes to gene expression and, thereby, cell survival/apoptosis: these life/death decisions govern neuroinflammatory outcome.

Given the importance of microglia and NPCs in these neuroinflammatory states, the main aim of this thesis was to investigate NF-κB-mediated interactions between these cells, the downstream gene expression profiles and the consequent effects on microglial and NPC fate. TNFα stimulation could be used to mimic neuroinflammatory conditions in vivo: these results would inform on the role of NF-κB in neuroinflammation. As TNFα is a
potent activator of the canonical NF-κB pathway, the p65:p50 heterodimer was the primary focus of this project.
2. Materials and Methods
2.1. Materials

2.1.1. Reagents

Standard laboratory chemicals were obtained from Sigma-Aldrich (USA) or Fisher Scientific (UK), unless otherwise stated. Tissue culture media, trypsin, non-essential amino acids (NEAA), bovine serum albumin (BSA) and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich. Foetal bovine serum (FBS) was purchased from Life Technologies (USA). Murine recombinant TNFα was obtained from Merck Millipore (Germany). D-luciferin was obtained from Biosynth AG (Switzerland). Primers for quantitative polymerase chain reactions (Q-PCR) were obtained from Sigma-Aldrich. All dishes, plates, transwells and flasks used in experiments were obtained from Corning (USA), with the exception of microscopy dishes, which were obtained from Greiner Bio-One (Germany).

2.1.2. Vectors

2.1.2.1 Luciferase Construct

The NF-κB-responsive luciferase construct (NF-luc) contains five κB site repeats upstream of the TATA box controlling the expression of a firefly luciferase gene (Stratagene, USA).

2.1.2.2 Fluorescent Fusion p65 Constructs

In the course of this project, a fluorescent fusion p65 plasmid, bacterial artificial chromosome (BAC) and lentiviral construct have been used. These constructs were made by members of the laboratory group and validated in HeLa, SK-N-AS and mouse embryonic fibroblast cells (Nelson et al, 2004 and unpublished data). The p65 reporter plasmid expresses p65 fused at the C-terminus to Discosoma sp.-Red-Express (DsRedXP) under the control of the CMV promoter. The p65 BAC reporter construct contains a 108 kb fragment of the human genome encompassing the p65 locus, modified to express p65 fused at the C-terminus to DsRedXP. The lentiviral reporter plasmid expresses p65 fused at the C-terminus to enhanced green fluorescent protein (eGFP) under the control of the ubiquitin ligase C promoter.
2.2. Methods

2.2.1. Propagation of Vectors

Plasmid DNA was purified using the GenElute plasmid miniprep kit (Sigma-Aldrich) or the PureLink HiPure plasmid maxiprep kit (Life Technologies), according to the manufacturer’s instructions. BAC DNA was purified using the NucleoBond BAC 100 kit (Macherey-Nagel, Germany), according to the manufacturer’s instructions. Purified DNA was the kind gift of members of the White laboratory group.

2.2.2. Cell Culture

2.2.2.1. Cell Lines and Culture Maintenance

Murine microglia and neural progenitor cell lines, C17.2 and BV.2, were selected for use in this investigation due to their reported roles in neuroinflammatory processes.

C17.2 is a multipotent murine neural progenitor cell line that has demonstrated therapeutic potential in many disease models (Teng et al, 2002; Park et al, 2002; Ourednik et al, 2002). These cells are non-tumourigenic and capable of differentiation into a variety of neural cell types, including neurons, glia and oligodendrocytes (Ryder et al, 1990).

BV.2 cells express many properties of primary microglial cells, including changes in activation status, physical characteristics, ion channel expression and relationship with astrocytes. In response to LPS, they display anti-microbial activity, phagocytic behaviour and proteomic changes that are comparable to primary microglia (Bocchini et al, 1992; Blasi et al 1990; Henn et al, 2009). Also, classically-activated microglial cells can activate nearby astrocytes; an action which is reproduced by BV.2 cells in culture (Henn et al, 2009).

C17.2 and BV.2 cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% (v/v) FBS and maintained at 37°C and 5% CO₂ in a humidified New Brunswick Galaxy 170S incubator (Eppendorf, Germany). Cells were split at a ratio of 1:10, every 2-3 days. Cell cultures were discarded upon reaching passage 30.

RAW267.4 cells, a murine peripheral macrophagic cell line, were maintained in the same conditions as C17.2 and BV.2 cells. SK-N-AS cells, a human neuroblastoma cell line, were
cultured in Minimal Essential Medium (MEM) supplemented with 10% (v/v) FBS and 1% (v/v) NEAA.

2.2.2.2. Long-term Storage of Cell Lines

Cell stocks were diluted to 2x10^6 cells per ml in DMEM containing 10% (v/v) FBS solution and 10% (v/v) dimethyl sulfoxide. This cell mixture was aliquoted into cryovials (1 ml/vial) and stored at -80°C overnight, before transfer to liquid nitrogen storage.

2.2.2.3. Thawing Cells from Frozen Stocks

To grow new cell cultures, cryovials were removed from liquid nitrogen storage, defrosted at 37°C in a water bath for one minute and poured into 9 ml of warm DMEM containing 10% (v/v) FCS. This solution was then centrifuged at 160 x g for 5 minutes in an Eppendorf 5804 centrifuge (Eppendorf). The supernatant was removed and the cell pellet was resuspended in fresh, warm culture medium. Cells were transferred into a sterile tissue culture flask and placed at 37°C in a 5% CO₂ incubator.

2.2.2.4. Cell Enumeration

Cell counts were determined using either a Bio-Rad TC10 cell counter (Bio-Rad, USA) or a Coulter Z2 Counter (Beckman Coulter, USA), according to the manufacturer’s instructions.

2.2.2.5. Plating Cells for Experiments

For most experiments, cells were plated directly into the final experimental dish/plate (see sections 2.2.2.5.1. and 2.2.2.5.2 for exceptions). Table 2.1 shows the dishes and the cell densities used for different types of experiments. With the exception of NanoString experiments, all experiments were performed in triplicate.
### Table 2.1: Cell numbers, dishes, cell growth area and media volume used in experiments.

<table>
<thead>
<tr>
<th>Experiment Conducted</th>
<th>Total Number of Cells</th>
<th>Dish/Plate</th>
<th>Growth Area</th>
<th>Media Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live Cell Imaging</td>
<td>5x10^4 cells</td>
<td>35 mm Greiner glass bottomed dishes</td>
<td>8.7 cm²</td>
<td>3 ml</td>
</tr>
<tr>
<td>Western blot</td>
<td>5x10^5 cells</td>
<td>60 mm dishes</td>
<td>21 cm²</td>
<td>3 ml</td>
</tr>
<tr>
<td>Cell Viability Assays (without PDTC)</td>
<td>1000 and 3000 cells per well</td>
<td>96-well plate</td>
<td>0.32 cm²</td>
<td>100 µl</td>
</tr>
<tr>
<td>Apoptosis Assays (without PDTC)</td>
<td>1000 and 3000 cells per well</td>
<td>96-well plate</td>
<td>0.32 cm²</td>
<td>100 µl</td>
</tr>
<tr>
<td>Flow Cytometry</td>
<td>1x10^6 cells</td>
<td>60 mm dishes</td>
<td>21 cm²</td>
<td>3 ml</td>
</tr>
<tr>
<td>ELISA</td>
<td>2x10^5 cells</td>
<td>60 mm dishes</td>
<td>21 cm²</td>
<td>3 ml</td>
</tr>
<tr>
<td>NanoString</td>
<td>1x10^5 cells</td>
<td>60 mm dishes</td>
<td>21 cm²</td>
<td>3 ml</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>5x10^5 cells</td>
<td>60 mm dishes</td>
<td>21 cm²</td>
<td>3 ml</td>
</tr>
<tr>
<td>Transwell Experiments/Flow Cytometry/Nanostring</td>
<td>See specific experiments for cell number</td>
<td>Target cell type was seeded in a 6-well plate. Non-target cell type was seeded in transwell inserts, which were placed in the wells.</td>
<td>Well surface area = 8.96 cm² Transwell insert area = 4.7 cm²</td>
<td>4 ml</td>
</tr>
</tbody>
</table>

### 2.2.2.5.1. Plating Cells for Live Cell and Endpoint Luminometry

C17.2 cells were trypsinised and 5x10^5 cells were seeded in a 100 mm dish. The following day, cells were transfected with the NF-luc construct (see section 2.2.4.1). Approximately 24 hours after transfection, C17.2 cells were trypsinised and seeded in a 24-well plate, according to details in Table 2.2. For co-culture luminometry, untransfected BV.2/SK-N-AS/RAW267.4 cells were seeded directly into the 24-well plate with transfected C17.2 cells at an appropriate ratio.

### Table 2.2: Cell numbers, cell growth area and media volume in luminometry experiments

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Total Number of Cells (Per Well)</th>
<th>Growth Area</th>
<th>Media Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>C17.2</td>
<td>2x10^4</td>
<td>1.9 cm²</td>
<td>1 ml per well</td>
</tr>
<tr>
<td>BV.2, SK-N-AS or RAW267.4</td>
<td>Ratio of 1:2= 4x10^4 cells</td>
<td>1.9 cm²</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ratio of 1:4= 8x10^4 cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ratio of 1:8=1.6x10^6 cells</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2.2.5.2. Plating Cells for Cell Viability and Apoptosis Assays with Inhibitor Treatment

Pyrrolidine dithiocarbamate (PDTC) is an NF-κB inhibitor. For experiments with PDTC treatment, 5x10^5 cells were initially plated in a 60 mm dish. The day after plating, cells were incubated with 0.1 mM PDTC for 30 minutes at 37°C. Cells were then washed with PBS, to remove PDTC, and seeded into 96-well plates, according to details in Table 2.1, for viability and apoptosis readings.

2.2.3. Treatment of Cells with TNFα

Murine recombinant TNFα (10 µg) was resuspended in 1 ml PBS containing 0.1% (w/v) BSA to make a working stock solution with a concentration of 10 ng/µl. Aliquots were stored at -80°C until use. Unless otherwise stated, 20ng/ml TNFα was used to stimulate cells in experiments.

2.2.4. Transfections and Transductions

2.2.4.1. Plasmid Transfections

Cells were transfected with the p65 plasmid using ExGen 500 (Thermo Fisher Scientific, USA) at a reagent: DNA ratio of 1: 8 (e.g. 1 µg DNA to 8 µl ExGen 500). Cells were incubated with the transfection mixture for at least 24 hours before imaging or splitting cells for experiments.

2.2.4.2. BAC Transfections

Cells were transfected with the p65 BAC using ExGen 500 (Thermo Fisher Scientific, USA) at a reagent: DNA ratio of 1: 10. Cells were grown in selection medium (DMEM supplemented with 10% (v/v) FCS and 1400 µg/ml zeocin (Thermo Fisher Scientific, USA) until they were suitable for ring cloning. Cells were cultured in selection medium for approximately 2 months to ensure the production of BAC-stable populations. BAC-stable clones were labelled with letters or numbers e.g. clone A, clone 3, clone 5.

2.2.4.3. Lentiviral Transductions

Lentivirus was prepared for transduction of C17.2 and BV.2 cells by J. Bagnall (University of Manchester). HEK293T cells were transfected with third generation packaging vectors and a lentivirus-compatible p65-eGFP vector. Virus was then harvested and subsequently
concentrated by ultracentrifugation. Concentrated virus was used to transduce C17.2 and BV.2 cells.

2.2.5. Luminometry

2.2.5.1. Live Cell Luminometry

Cells were transfected with NF-\(\text{luc}\) and/or seeded according to section 2.2.2.5.1. Approximately 24 hours after seeding in 24-well luminometry plates, D-luciferin was added to each well to a final concentration of 1 mM and left to incubate for at least 3 hours. Cells were then stimulated with different doses of TNF\(\alpha\) (control cells were left unstimulated), a breathable membrane was used to seal the plate and it was placed in a FLUOstar Omega microplate reader running Omega software v3.00 (BMG Labtech, Germany). Luminescence readings were taken every 15 minutes for at least 5 hours per experiment, using an integration time of 18 seconds. Experiments were performed in triplicate, with three technical replicates per plate. Data was exported from MARS Data Analysis software v2.40 (BMG Labtech). One-way ANOVAs were performed to assess significant differences in NF-luc induction between culture conditions.

2.2.5.2. Endpoint Luminometry

Cells were transfected with NF-luc and/or seeded according to section 2.2.2.5.1. C17.2 mono-cultures and C17.2 co-cultures (with BV.2 cells seeded in transwell inserts) were investigated. Approximately 24 hours after seeding, some cells were left unstimulated and others were stimulated with different doses of TNF\(\alpha\). At an appropriate time point after treatment, cells were washed with PBS and lysed in 200 \(\mu\)l luminometry lysis buffer (0.025% (w/v) dithiothreitol, 1% (w/v) BSA, 25 mM Tris PO\(_3\) pH 7.75, 1% (v/v) Triton X-100, 15% (v/v) glycerol, 0.1 mM EDTA, 8 mM MgCl\(_2\)). Plates were shaken at room temperature for 15 minutes. For analysis, ATP was added to each sample to a final concentration of 1 mM and 80 \(\mu\)l duplicate samples were transferred to a 96-well luminometry plate. Experiments were performed in triplicate. Unpaired t-tests were used to assess significant differences.

2.2.6. Live Cell Imaging

Cells were imaged using a LSM 5 Exciter Axiovert microscope (Zeiss, Germany), with a 20X objective. During these experiments, cells were maintained in a humidified chamber,
at 37°C and 5% CO₂. An argon ion 488 nm laser was used to excite eGFP and the emitted light was detected using a bandpass filter of 505-530 nm. A green helium ion 543 nm laser was used to excite DsRedXP; emission was detected using a 560 nm long-pass filter.

Data acquisition was performed at a resolution of 512 x 512 pixels, using LSM software AIM (version 4.1; Zeiss). Acquisition was started after selection of 10 to 12 spatial locations per imaging dish. Time taken to image cells across all locations did not exceed 7 minutes in any experiment. Unstimulated cells were imaged for at least 2 hours before TNFα was added to the dish.

All microscopy experiments were performed in triplicate, with the exception of co-cultured lentivirus transduced BV.2 cells: only one microscopy experiment was performed with this co-culture.

2.2.6.1. Analysis of Live Cell Imaging Experiments

Cell Tracker software (Perkins, 2007) was used to obtain fluorescence intensity readings for cell nuclei. Pre-stimulatory nuclear intensities were averaged for each cell; post-stimulatory nuclear fluorescence was normalised to these mean values. These values were then interpolated to allow data from replicate experiments to be collated and plotted together.

2.2.7. Western Blotting

2.2.7.1. Sample Preparation

Cells were plated according to section 2.2.2.5. The day after plating, cells were stimulated with TNFα (section 2.2.3.). At the appropriate timepoint after treatment, cells were washed with cold PBS and lysed by the addition of 200 μl lysis buffer (1% (w/v) sodium dodecyl sulphate (SDS), 10% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 40 mM tris HCl pH 6.8, 0.001% (w/v) bromophenol blue). Cell lysates were stored at -20°C until use. Experiments were performed in triplicate. Unstimulated protein samples were used in Western blots for measuring p65 and IκBα protein levels across cell lines: one Western blot was performed per cell line.

2.2.7.2. Preparation of Gels for SDS Polyacrylamide Gel Electrophoresis (PAGE)

Gels for SDS-PAGE were cast using the Mini-Protean 3 system (Bio-Rad). After set-up of the casting apparatus, the resolving gel (10% acrylamide, 125 mM Tris HCl pH 6.8, 0.1%
NF-kappaB in Neuroinflammation

(w/v) SDS, 0.064% (w/v) ammonium persulphate (APS), 0.064% (v/v) N,N,N’,N’-tetramethylethylenediamine (TEMED)) was poured between the glass plates and left to solidify. The resolving gel was topped with stacking gel (4% acrylamide, 375 mM Tris HCl pH 8.8, 0.1% (w/v) SDS, 0.06% (w/v) APS, 0.12% (v/v) TEMED) and wells made by insertion of a comb into the unset gel. Once set, gels were used immediately for protein separation by SDS-PAGE.

2.2.7.3. Protein Separation by SDS-PAGE

Gels were loaded into a Mini-Protean gel tank and connected to a PowerPac Basic power supply unit (both Bio-Rad). The tank was filled with SDS running buffer (25 mM Tris, 192 mM glycine, 3.4 mM SDS). Protein samples were heated at 100°C for 10 minutes prior to loading onto the gel. ColorPlus prestained protein ladder (New England Biolabs, USA) and horseradish peroxidase (HRP)-linked protein ladder (Cell Signalling Technology, USA) were loaded for identification of protein size. Protein samples were separated at 100 volts for 20 minutes, and then at 160 volts for 30 to 90 minutes, until markers appeared sufficiently separated.

2.2.7.4. Transfer of Protein to Nitrocellulose Membrane

Proteins were transferred from the SDS-PAGE gel to BA83 Protran nitrocellulose membrane (GE Healthcare, UK) using a Mini Trans-Blot Cell (Bio-Rad). Transfer was made by wet blotting at 300 mA in chilled transfer buffer (20% methanol, 25 mM Tris, 200 mM glycine), for approximately 1-2 hours.

2.2.7.5. Immunostaining of Nitrocellulose Membrane

Nitrocellulose membranes were incubated in blocking buffer (5% (w/v) skimmed milk powder, 20 mM Tris HCl pH 7.8, 140 mM NaCl, 0.1% (v/v) Tween 20) for 1 hour and then incubated with the primary antibody in blocking buffer at 4°C overnight (see Table 2.3 for antibodies used). Cyclophilin A (CycA) and α-tubulin antibodies were used as loading controls.
Membranes were washed three times for five minutes with wash buffer (20 mM Tris HCl pH 7.8, 140 mM NaCl, 0.1% (v/v) Tween 20). After washing, membranes were incubated with the secondary antibody in blocking buffer at room temperature for one hour. Membranes were washed three times for five minutes with wash buffer, and then incubated with enhanced chemiluminescent substrate (ECL; Thermo Fisher Scientific) for approximately five minutes. The membrane was washed and photographic films (Kodak BioMax MR film, Sigma-Aldrich) were exposed to membranes for varying amounts of time (depending on the strength of the light signal). Exposed films were developed in an automated processor.

### 2.2.7.6. Relative Quantification of Proteins

In order to quantify changes in IκBα levels after TNFα stimulation, densitometry readings were taken using AQM Version 6 software. To account for differences in protein concentration or loading between samples, all IκBα levels were normalised using CycA or α-tubulin bands. IκBα values were then normalised to pre-stimulatory IκBα.

### 2.2.8. Cell Viability

Cells were plated according to methods described in sections 2.2.2.5. (without PDTC treatment) and 2.2.2.5.2 (with PDTC treatment). Cells were either plated in normal medium or medium containing 20ng/ml TNFα. The CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Kit (Promega, USA) was used to determine cell viability. This kit utilises a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTS). MTS is reduced to a soluble formazan product by live cells; absorbance of formazan can be measured at 490 nm as a measure of viable cells. Cells in the 96-well plate were incubated for 3 hours with
MTS mix, made according to manufacturer’s instructions. Absorbance was then measured at 490 nm at an appropriate time point (24, 48 or 72 hours) after treatment, using a FLUOstar Omega microplate reader. Experiments were performed in triplicate, with three technical replicates per plate.

2.2.8.1. Analysis of Cell Viability Data

Negative control values were subtracted from cell viability values to eliminate background signal. Data was collated across replicate experiments and tested for significant differences, against unstimulated culture readings, using Mann-Whitney U tests. Data was also analysed for co-culture mediated viability changes. In order to do this, expected co-culture mean and standard deviation values were calculated from mono-culture data (as shown in Table 2.4). These values were then tested for significance differences (compared to actual co-culture data), using z-tests. The same analysis was conducted for TNFα-treated, PDTC-treated and PDTC + TNFα-treated cultures.

<table>
<thead>
<tr>
<th>Table 2.4: Calculations conducted to assess co-culture-mediated viability changes. Expected co-culture means and standard deviations were tested against actual co-culture data, using z-tests.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Basal C17.2</td>
</tr>
<tr>
<td>Average or Standard Deviation (S.D.) of Viability Readings</td>
</tr>
</tbody>
</table>

2.2.9. Apoptosis Assays

As with cell viability assays, cells (both untreated and PDTC-treated) were plated in normal medium or medium containing 20 ng/ml TNFα, according to methods and cell density described in sections 2.2.2.5. and 2.2.2.5.2. The Caspase-Glo® 3/7 Kit (Promega) was used to determine apoptosis rates in different conditions. A proluminescent caspase3/7 substrate and luciferase mix was made according to manufacturer’s instructions. The substrate is cleaved by caspase3/7 to create aminoluciferin, which produces a luminescent signal in the presence of luciferase. Cells were incubated for 1 hour with the substrate mix. Luminescence was then measured at the appropriate timepoint (24, 48 or 72 hours) using a
FLUOstar Omega microplate reader, and an integration time of 0.1 seconds. Experiments were performed in triplicate, with two technical replicates per plate.

2.2.9.1. Analysis of Apoptosis Data

Apoptosis data was analysed according to the methods described in section 2.2.8.1 and Table 2.4.

2.2.10. Flow Cytometry

2.2.10.1. Sample Preparation and TNFR1-Labelling

The day after plating (as described in Table 2.1), cells were either left unstimulated or treated with 20ng/ml TNFα. Two hours after treatment, cells were washed with PBS, trypsinised and counted. For each sample, 1x10^6 cells were incubated with 2 μg/ml phycoerythrin (PE) conjugated- TNFR1 antibody mix (BioLegend, UK) in the dark for 45 minutes. Cells were washed with PBS, to ensure removal of the antibody mix, and resuspended in 500 μl PBS for analysis. Experiments were performed in triplicate. Each sample was analyzed in a BD FACS Calibur machine (Becton Dickinson, USA), using a 488 nm laser to excite eGFP and PE. Fluorescence from eGFP and PE were detected using 514-545nm and 564-601nm filters, respectively. Mono-culture experiments were conducted with lentivirus-transduced C17.2 cells (expressing p65-eGFP) and untransfected C17.2 and BV.2 cells. Co-culture experiments were performed with lentivirus transduced C17.2 cells and untransfected BV.2 cells, to enable separation of cell types during data analysis (see sections 2.2.4.2 and 2.2.10.2.).

2.2.10.2. Flow Cytometry Data Analysis

Mean fluorescence intensities of cell populations were obtained using FlowJo software version 7.6.5. Mann Whitney U tests were used to assess differences in TNFR1 expression between groups. For the analysis of TNFR1-expression on co-cultured cells, eGFP expression was used to differentiate between lentivirus transduced C17.2 cells (expressing eGFP) and untransfected BV.2 cells. Figure 2.1 shows how cell populations were separated along PE and eGFP expression axes.
2.2.11. Enzyme-linked Immunosorbent Assay (ELISA)

The day after seeding (see Table 2.1), cells were treated with 2ng/ml TNFα, after which 100 µl culture medium was removed at the appropriate time points (i.e. before treatment, 2 hours, 4 hours and 6 hours after TNFα-treatment). Experiments were performed in triplicate. Samples were frozen at -80°C, until use in an ELISA. Mouse TNFα was quantified using the BD OptEIA mouse TNFα ELISA set (Becton Dickinson), according to the manufacturer’s instructions. Duplicate readings were taken for each sample.
Differences between culture conditions were assessed using Kruskal-Wallis ANOVA tests. Data across replicates was collated, averaged and normalised to time 0 values for display.

### 2.2.12. NanoString

#### 2.2.12.1. Sample Preparation

Cells were plated as described in section 2.2.2.5. RNA was extracted from mono-cultures and 1:1 co-cultures (from a mixture of C17.2 and BV.2 cells). In addition, extracts were taken from cells in transwell co-cultures. For example, RNA was extracted from C17.2 cells that had been cultured with BV.2 cells seeded in transwell inserts; these cultures were referred to as ‘C17.2 transwell co-culture’. Likewise, BV.2 cells that had been cultured with C17.2 cells in transwells were labelled ‘BV.2 transwell co-culture’. Experiments were performed in duplicate or triplicate.

One day after plating, cells were either left untreated or stimulated with 20ng/ml TNFα. RNA was extracted from cells 230 minutes or 24 hours after treatment, using the High Pure RNA Isolation Kit (Roche, UK) according to the manufacturer’s instructions. RNA samples were quantified using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific).

#### 2.2.12.2. NanoString Measurements

The quality of each undiluted RNA sample was determined using a 2200 TapeStation system (Agilent Technologies, USA). Prepared RNA samples were submitted for analysis by the Genomic Technologies Core Facility of the Faculty of Life Sciences, University of Manchester. Samples were assayed using the nCounter Mouse Inflammation Kit v1 (NanoString Technologies, USA), which contains a CodeSet of capture and reporter probes for 185 genes, including cytokines, chemokines, inflammatory receptors and transcription factors. For each sample, 125ng RNA was used per CodeSet hybridisation reaction.

#### 2.2.12.3. NanoString Data Analysis

NanoString results were analysed using nSolver analysis software v1.1 (NanoString Technologies). All results were normalised to the level of house-keeping genes and positive controls incorporated into the CodeSet. Negative control values were subtracted from the resulting data. Biological replicates were averaged and averages across different groups were compared. Genes that exhibited fold differences in expression that were greater than or equal to two were considered to be differentially regulated.
Two different comparisons between conditions and treatments were made: Tables 2.5.1 and 2.5.2 show an example of each. Analyses in Tables 2.5.1-2.5.2 were conducted with unstimulated and TNFα-treated cultures.

<table>
<thead>
<tr>
<th>Two different comparisons conducted with NanoString results</th>
<th>Table 2.5.1</th>
<th>Basal C17.2 cells</th>
<th>24 hour TNFα-treated C17.2 cells</th>
<th>Fold Change in Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Analysis of TNFα-induced gene expression</td>
<td>300</td>
<td>900</td>
<td>(= \frac{900}{300}) = 3</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2.5.2</th>
<th>Basal C17.2 cells</th>
<th>Basal C17.2 cells (seeded with transwell BV.2 cells)</th>
<th>Fold Change in Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>2) Analysis of cell-contact-independent co-culture interactions</td>
<td>500</td>
<td>250</td>
<td>(= \frac{250}{500}) = 0.5</td>
</tr>
</tbody>
</table>

2.2.13. Quantitative-Polymerase Chain Reaction (Q-PCR)

2.2.13.1. Sample Preparation

Cells were stimulated with 20ng/ml TNFα and RNA was extracted at time 0, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours and 8 hours post-stimulation. Experiments were performed in triplicate. RNA was extracted using the High Pure RNA Isolation kit (Roche, Switzerland), according to the manufacturer’s instructions. RNA samples were quantified using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific). Samples were converted to complementary DNA (cDNA) using the SuperScript VILO cDNA Synthesis Kit (Life Technologies), according to the manufacturer’s instructions. Amplification steps were performed in a C1000 thermal cycler (Bio-Rad), using 1 μg RNA as starting material. The resulting cDNA samples were diluted 1 in 20 with RNase-free, DEPC-treated water and stored at -20°C.

2.2.13.2. Q-PCR Analysis

cDNA samples were analysed by Q-PCR to assess relative expression levels of IkBα, IkBε, TNFα and A20 genes. Primer sequences are shown in Table 2.6. These primers have been validated by members of the laboratory, using murine cell lines.
Each reaction was performed in one well of an opaque 96-well plate, with 1 μl of cDNA, 10 μl SYBR-Green Master Mix (Roche), 1.5 μl each of forward and reverse primers (final concentration 0.25 μM each) and 6 μl of DEPC-treated water. Each reaction was performed with three technical replicates per plate. Plates were covered with sealing foil, spun briefly, and then analysed in a LightCycler 480 machine (Roche). Cycling parameters are described in Table 2.7.

### Table 2.7: Cycling parameters for Q-PCR

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Initial denaturation</th>
<th>Amplification</th>
<th>Melt curve</th>
<th>Cooling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycles</td>
<td>1</td>
<td>45</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Target temperature</td>
<td>95°C</td>
<td>60°C</td>
<td>95°C</td>
<td>40°C</td>
</tr>
<tr>
<td>Time</td>
<td>5 minutes</td>
<td>10 s</td>
<td>5 s</td>
<td>10 s</td>
</tr>
<tr>
<td>Ramp speed</td>
<td>4.4 °C/s</td>
<td>2.2 °C/s</td>
<td>4.4 °C/s</td>
<td>1.5 °C/s</td>
</tr>
<tr>
<td>Acquisition mode</td>
<td>None</td>
<td>Once per cycle</td>
<td>Ten per °C</td>
<td>None</td>
</tr>
</tbody>
</table>

### 2.2.13.3. Analysis of Q-PCR Results

Q-PCR data was analysed using LightCycler 480 software release 1.5.0. Samples were annotated according to treatment, time points and primer content (reference versus target gene). Relative gene expression levels were then calculated using the automated relative quantification method in the LightCycler 480 software. Gene expression data was normalised to the reference gene CycA, then expressed relative to time 0. These values were calculated for each cell population and Kruskal-Wallis ANOVA tests were performed.
on the resulting dataset to assess significant differences in gene expression. This analysis was performed on unstimulated expression levels of IkBa, IkBe, A20 and TNFα in untransfected cells, lentivirally transduced cells, clone A, clone 3 and clone 5.
3. Establishing and Characterising a Model System for the Study of NF-κB in C17.2 and BV.2 Cells
3.1. General Introduction

The initial aim in this project was to investigate NF-κB dynamics and activation (i.e. p65 translocation and a surrogate for NF-κB activation) in individual neural progenitor cells in response to stimulation. NF-κB single cell dynamics had never previously been studied in this cell type and therefore it was important to observe the response of these cells to stimulation. The C17.2 cell line was chosen for this purpose as these cells have been widely used as a model system for neural progenitor cell function (Bakshi et al, 2006; Lu et al, 2003). Relevant published data revealed strong nuclear p65 presence in murine stem cells with p65 immunostaining, 30 min after TNFα stimulation (Widera et al, 2006a).

The most obvious way to study p65 dynamics in these cells was to make stable cell lines. Initially, it was thought that the optimal construct to use would be a human BAC expressing p65-DsRedXP fusion protein, as it includes the endogenous promoter and regulatory sequences for the p65 gene. This means that the fusion protein would be expressed at a similar level to an endogenous p65 allele. However, obtaining stable clones containing the integrated BAC required a lengthy process of culture from single cells, ring cloning and subsequent expansion. Also available were plasmid vectors, expressing p65-DsRedXP, and a lentivirus construct that could be used to transduce cells so they would produce p65-eGFP. The plasmid could be used for transient transfection of neural progenitor cells to observe the dynamics of p65 fusion proteins. The particular advantage of the lentivirus was that it could be used to rapidly produce a population of cells stably expressing the fusion protein, without the requirement for extensive cell cloning.

Since the main aim of the work in this thesis was to investigate neural progenitor cell – microgliial cross-talk in the control of neuroinflammation (see Chapter 1), it was also important to choose an appropriate microgliial cell line. To this end, the BV.2 cell line was chosen as it accurately reproduces key characteristics of primary microgliial cells (Henn et al, 2009). Similar to the work with C17.2, it was important to develop a BV.2 cell population expressing p65 fusion protein that could be used to investigate NF-κB dynamics in these cells. No such study had previously been performed, although some population-level biochemical studies of the NF-κB pathway had been performed in BV.2 cells (Sheppard et al, 2011; Magni et al, 2012; Wang et al, 2011).

In conclusion, this chapter describes the single-cell imaging of p65, population-level NF-κB activation and gene expression analyses of BAC stable clonal, plasmid-transfected and
lentivirus-transduced cells, derived from C17.2 and BV.2, in order to develop model cell systems suitable for use in the remainder of the project.

3.2. Results

3.2.1. Analysis of NF-κB Function and Dynamics in a Neural Progenitor Cell Line

3.2.1.1. Creating C17.2 Reporter Cell Lines for Observation of p65 Dynamics

The C17.2 neural progenitor cell line was chosen as a model system for studying NF-κB function. As discussed above, the first aim was to express a p65 fluorescent fusion protein in C17.2 cells, to allow observation of p65 dynamics within individual living cells. In order to observe p65 in the C17.2 cell line, plasmid and Bacterial Artificial Chromosome (BAC) constructs, encoding p65-DsRedXP, and a lentiviral construct, expressing p65-eGFP, was used.

Wild-type C17.2 cells were transiently transfected with the p65-DsRedXP plasmid and a lentiviral transduction was performed to create cells expressing p65-eGFP. C17.2 cells were also stably-transfected with the p65-BAC. One transfection was performed and resulting BAC positive cells were ring cloned to isolate a set of six p65-DsRedXP expressing clones: three of which were chosen for further investigation, due to their display of a growth rate that was comparable to wild-type C17.2 cells. These cell lines are referred to as BAC stable clones A, 3 and 5, the lentivirus transduced cells are called C17.2 (lenti), plasmid-transfected cells are called ‘C17.2 (transient)’ and untransfected cells are labelled with the suffix ‘(UT)’.

Transfection/transduction of these constructs into cells successfully led to the expression of fluorescent-p65, which was observable under a confocal microscope (Figure 3.1). The next step was to determine which cell line(s) best represented characteristics of wild-type C17.2 cells. To do this, reporter cell lines were characterised and compared to wild-type C17.2 cells, using a variety of different methods.

3.2.1.2. Single-cell p65 Dynamics in C17.2 Reporter Cell Lines

In order to observe and compare p65 dynamics between the differently transfected cell populations, confocal microscopy was used to take time-lapse images of cells from 0 to 400 min after 20ng/ml TNFα stimulation.
NF-kappaB in Neuroinflammation

**Figure 3.1. Expression of p65 fluorescent fusion proteins in C17.2 cells.**
C17.2 cells were transfected or transduced with different p65-fluorescent protein constructs. Confocal microscopy images were taken of (A) Lentivirus transduced cells, (B) Transiently (plasmid) transfected cells, (C) BAC stable clone 3, (D) BAC stable clone 5 and; (E) BAC stable clone A. Scale bar = 50µm.

Figure 3.2 displays microscopy images showing the localization of fluorescent p65 in representative cells at different times after TNFα stimulation. The BAC clonal lines were found to respond in a similar manner to each other and, therefore, one representative clonal cell line is displayed in Figure 3.2.

**Figure 3.2. TNFα-induced p65 localisation in C17.2 cells transfected or transduced with different p65 constructs.**
Cells were stimulated with 20 ng/ml TNFα at time 0 and visualised by confocal microscopy at the different time points after TNFα stimulation. (A) Lentivirus transduced cells, (B) Transiently-transfected cells, (C) BAC clone 5. Scale bar = 50µm.

C17.2 (lenti) cells appeared to respond with stronger p65 nuclear movements than the BAC clones and the transiently transfected cells. There also appeared to be a greater proportion of responsive cells in the C17.2 (lenti) culture than the other cell populations. In order to
quantify these apparent differences, nuclear p65 fluorescence intensity was analysed using Cell Tracker software (Perkins, 2007). Nuclear fluorescence intensity, post-TNFα stimulation, was normalised to the mean pre-stimulation nuclear fluorescence. The total number of cells analysed for the plasmid transfected, lentiviral transduced and BAC clones 3, 5 and A was 22, 32, 22, 66 and 47, respectively. Except for some clone 5 cells, all cell data obtained is displayed in Figure 3.3: representative clone 5 cells were selected to avoid presenting too much information in one graph.

All of the C17.2 (lenti) cells exhibited a clear, rapid nuclear p65-eGFP movement (>7 fold increase in nuclear p65), in response to TNFα. On average, the initial peak of nuclear p65 occupancy occurred 20 min after stimulation and many cells displayed subsequent nuclear p65 movements - as signified by the average p65 nuclear fluorescence being sustained at a higher level (>2 fold), for over 200 min. BAC clones 5, A and the plasmid-transfected C17.2 cells exhibited smaller changes in nuclear p65 and delayed peaks in nuclear p65 translocation (Figure 3.3F). Their responses were also more transient; average nuclear p65 fluorescence returned to pre-stimulatory levels within 200 min of TNFα treatment. In contrast to this, the BAC clone 3 cells were largely unresponsive, displaying no change/very low amplitude changes in nuclear p65, after TNFα stimulation.

To further characterize the differences in p65 response observed across reporter cell lines, the single-cell data displayed in Figure 3.3 was clustered using a k-means algorithm. Initially, clustering was conducted using different cluster numbers (results not shown). It was concluded that a cluster number of 4 was the most suitable for representing the p65 dynamic profiles observed in cells. This algorithm stratified cells into 4 different p65 response categories (Figure 3.4A-D). Figures 3.4E and 3.4F show the distribution of different cell lines across categories of p65 response.

Categorisation of cells by the k-means algorithm reflected differences in amplitude and persistence of p65 response to TNFα. Cluster A accommodated cells that did not exhibit any clear nuclear p65 translocations (95% of clone 3 cells were associated with this category, see Figure 3.4E). Cells that displayed small nuclear p65 movements were sorted into cluster B: this included over 50% of C17.2 (transient), clone 5 and clone A cells. The cells that displayed higher amplitude p65 movements were classified into clusters C or D, depending on whether they exhibited a single nuclear p65 translocation only (cluster D) or a more persistent response (cluster C).
Figure 3.3. Quantification of nuclear p65 fluorescence in different C17.2 cell populations after TNFα stimulation.

Nuclear p65 fluorescence of cells was recorded between 0 and 400 min after TNFα stimulation. Readings were normalised to mean pre-stimulatory nuclear p65 fluorescence. Each graph shows data from one experiment; each line represents analysis of a single cell and the thick black lines show the average of all of the cells. (A) Transiently-transfected cells. (B) Lentivirus transduced cells. (C) BAC clone 3. (D) BAC clone 5. (E) BAC clone A. (F) Average initial nuclear p65 peak amplitudes and timings. Graphs A-E display data for 22, 32, 22, 55 and 47 cells respectively.
To further investigate differences between the cell lines, data in Figure 3.4E was used to determine the distribution of cells across low and high amplitude cluster groups (Figure 3.4F). Over 92% of all BAC clonal cells fitted into the low amplitude response group. This group was also comprised of 68% of C17.2 (transient) cells. In contrast, over 90% of C17.2 (lenti) cells were placed in the high amplitude category, which was composed of clusters C and D.
3.2.1.3. Population-level NF-κB Activation in C17.2 Cell Lines

Having established differences in TNFα-induced p65 dynamics, the next aim was to investigate population-level NF-κB activation in the different C17.2 cell populations, beginning with NF-κB-regulated transcriptional response. It was decided to exclude clone 3 from these experiments due to the lack of a visible p65 response.

3.2.1.3.1. Analysis of NF-κB Transcriptional Activity in the Differentially Transfected C17.2 cells

A useful method for studying transcription responses to NF-κB activation is to measure luciferase production from the NF-luc reporter plasmid. This contains a minimal promoter with 5 copies of a consensus NF-κB response motif. To measure NF-luc induction, the cells were transiently transfected with an NF-luc plasmid and stimulated with different doses of TNFα. Luminescence readings were taken every 15 min, from 0 to 5 h after TNFα stimulation. The results of these experiments can be seen in Figure 3.5.

Experiments were performed in triplicate for each cell population; one experiment for each was extended to take luminescence readings for 12 h after TNFα stimulation. This allowed the determination of the time of peak luciferase expression. No further increases in luciferase expression were seen later than 5 h post-stimulation. Therefore, only data up to 5 h is displayed here.

In the wild type C17.2 cells, doses under 100pg/ml did not induce NF-luc, as signified by the results of a one-way ANOVA. Peak NF-luc induction was observed after 1ng/ml TNFα stimulation. Peak induction occurred at 3 h and 4-5 h after stimulation with 100pg/ml TNFα and doses ≥1ng/ml TNFα, respectively. The C17.2 (lenti) cells exhibited a similar temporal and dose-dependent response, except that they showed lower peak amplitudes. In the BAC clone 5 cells, the peak activation levels exhibited were reduced at all doses. Clone A cells were even less responsive: only doses of ≥10ng/ml TNFα gave significant NF-luc upregulation. The significance of the observed differences between the cells was investigated further. Peak NF-luc induction levels, after stimulation with 100pg/ml and ≥1ng/ml TNFα, were calculated and results are displayed in Figure 3.6. Kruskal-Wallis ANOVA test results indicated that the peak induction level of atleast one cell population was significantly different to that of the other cell populations (p-value<0.05): Kruskal-Wallis ANOVA tests do not inform on exactly which groups within a dataset are significantly different.
Overall, the C17.2 (lenti) cells best displayed the key features of NF-luc induction that were observed in wild-type cells. The BAC clones, on the other hand, displayed dampened NF-luc induction profiles. There were a number of possible explanations for this observation. These include the possibility that basal NF-κB activity was higher in the clones than in the wild-type cells.

Experiments were performed with each cell line individually, making comparison of basal luminescence data problematic. Assuming similar transfection efficiency across the cells, basal luminescence can be taken as a measure of basal NF-κB activity. To this end, a comparison was conducted of basal luminescence values from wild-type C17.2, clone A and clone 5 cells (see Figure 3.7).
Figure 3.6. Comparison of NF-\textit{luc} induction, after TNF\textalpha{} stimulation, in C17.2 cell populations. Each cell population was transfected with the NF-\textit{luc} plasmid and stimulated with varying doses of TNF\textalpha{}. Luciferin was added to the cells and luminescence was measured every 15 min from 0 to 300 min after TNF\textalpha{} stimulation. Experiments were performed in triplicate, with three biological replicates per experiment. Mean maximum fold change in luminescence was calculated 3 hours after 100pg/ml stimulation and 5 hours after stimulation with higher doses. Bars represent ± S.D. and asterisks represent significant Kruskal-Wallis ANOVA test results (*=p-value<0.05).

Figure 3.7. Comparison of basal luminescence of NF-\textit{luc} transfected C17.2 cell populations. Basal luminescence of different NF-\textit{luc}-transfected cell populations was measured. Experiments were performed in triplicate with three technical replicates per experiment. Data was averaged and normalised to the C17.2 (wild-type) average, for display. Bars represent ± S.D. and asterisks represent significant Mann Whitney U test results (*=p-value<0.05).

The basal luminescence values from clone A and clone 5 cells were lower than that of wild-type C17.2 cells. Mann Whitney U test results suggested that the difference between clone 5 and wild-type C17.2 cells was significant (p-value<0.05). No transfection control
was included in these experiments and so it is not possible to make strong conclusions from this comparison.

3.2.1.3.2. TNFα-induced IκBα Degradation Profiles in C17.2 Cell Lines

The degradation and resynthesis of IκB proteins, such as IκBα, allows the shuttling of NF-κB between the nucleus and cytoplasm. Without IκBα degradation, NF-κB is generally believed to be retained in the cytoplasm (reviewed by Hoffmann et al, 2006). For this reason, it was important to characterize the TNFα-induced IκBα degradation and resynthesis profiles in wild-type cells and C17.2 reporter cell lines.

To achieve this goal, protein extracts were taken from cells at 15 min intervals, from 0 to 120 min, after 20ng/ml TNFα stimulation. These experiments were performed in triplicate. Figure 3.8 shows the TNFα-induced IκBα degradation profile obtained from wild-type C17.2 cells.

Within 15 min, there was ≥85% decrease in IκBα, which was then rapidly resynthesized between 30 and 45 min. In two out of three replicates, IκBα levels were comparable to their pre-stimulatory state within 2 h of TNFα treatment. C17.2 (lenti) cells showed a similar temporal IκBα degradation and resynthesis profile (Figure 3.9).

Once again, the profiles from clone A and clone 5 were distinct from wild-type and C17.2 (lenti) cells, but were similar to each other (see Figures 3.10 and 3.11). With the exception of one replicate from each dataset, the clonal cells maintained stable IκBα levels after TNFα stimulation. It was noted that, additional IκBα bands were observed in the Western blots; these bands were not obtained with extracts from the wild type or C17.2 (lenti) cells.
Figure 3.8 Western blot analysis of IκBα levels in wild type C17.2 cells after TNFα stimulation.

Protein extracts were taken from C17.2 cells at 15 min intervals from 0 to 120 min after stimulation with 20ng/ml TNFα. Western blots for IκBα were performed with the protein extracts. Experiments were performed in triplicate. (A) Representative Western blot. (B) Densitometry analysis of triplicate experiments (Black line = blot shown in A).
**Figure 3.9 Western blot analysis of IκB-α levels in lentivirus transduced C17.2 cells after TNFα stimulation.**

Protein extracts were taken from lentivirus transduced C17.2 cells at 15 min intervals from 0 to 120 min after stimulation with 20ng/ml TNFα. Western blots for IκB-α were performed with the protein extracts. Experiments were performed in triplicate. (A) Representative Western blot. (B) Densitometry analysis of triplicate experiments (Blue line = blot shown in A).
Figure 3.10 Western blot analysis of IκBα levels in BAC clone A cells after TNFα stimulation.

Protein extracts were taken from BAC clone A cells at 15 min intervals from 0 to 120 min after stimulation with 20ng/ml TNFα. Western blots for IκBα were performed with the protein extracts. Experiments were performed in triplicate. (A) Representative Western blot. (B) Densitometry analysis of triplicate experiments (Blue line = blot shown in A).
NF-kappaB in Neuroinflammation

Figure 3.11 Western blot analysis of IκBα levels in BAC clone 5 cells after TNFα stimulation. Protein extracts were taken from BAC clone 5 cells at 15 min intervals from 0 to 120 min after stimulation with 20ng/ml TNFα. Western blots for IκBα were performed with the protein extracts. Experiments were performed in triplicate. (A) Representative Western blot. (B) Densitometry analysis of triplicate experiments (Black line = blot shown in A).

3.2.1.3.3. NF-κB-dependent Gene Expression Changes in TNFα Stimulated Cells

Experiments discussed so far have explored p65 localization and NF-κB activation. In these experiments, C17.2 (lenti) cells provided an accurate representation of wild-type cells. The next aim was to further investigate whether NF-κB target gene transcriptional activity in the fluorescent cell lines was similar to that in wild-type cells.

RNA was extracted from cells at 0, 15, 30, 60, 120, 240, 360 and 480 min after 20ng/ml TNFα stimulation. RT-PCR was conducted to produce cDNA and Q-PCR was performed with primers for prototypical NF-κB-dependent genes: IκBa, IκBe, A20 and TNFα. Q-PCR results are displayed as fold changes in mRNA expression in Figure 3.12.
All cell lines showed induction of the target NF-κB genes. Given that TNFα treated clones showed little to no nuclear p65 translocations, NF-κB-dependent gene expression changes were unexpected. Expression of the target genes IκBα and IκBε are believed to be critical in determining NF-κB subcellular localisation: therefore, the ideal C17.2 reporter cell line
would display the same temporal profile and fold changes as those observed in wild-type C17.2 cells (Figure 3.12A). In wild-type cells, IκBα, A20 and TNFα mRNA levels peaked 1 h post-TNFα stimulation and then dropped to a plateau after 4 h. IκBε expression peaked at 2 h post-stimulation and fell to a plateau after 6 h. The relative increase in expression was similar between IκBα, IκBε and TNFα (8.5-10 fold), whereas A20 was higher (26 fold).

The timing of expression of IκBα and A20 in clone A cells was similar to that of the wild-type cells (Figure 3.12B). Peak IκBε expression occurred 2 h post-stimulation, which was consistent with results from wild-type C17.2. However, unlike wild-type cells, IκBε expression showed a greater increase (19 fold) and the levels of expression remained elevated, even at 8 h post-stimulation. TNFα expression also remained elevated for longer than observed in wild-type cells.

The expression profiles obtained from clone A were similar to that of clone 5 (see Figures 3.12B- D), with the exception of the TNFα gene expression profile. Similar to wild-type cells, TNFα expression in clone 5 peaked at 1 hour post-stimulation. However, the upregulation of TNFα expression was higher than observed in wild-type cells (69 fold).

The relative activation of IκBα and IκBε gene expression in the C17.2 (lenti) cells was comparable to that seen in wild-type cells. However, as with clone 5 cells, the C17.2 (lenti) cells displayed a larger activation of TNFα expression (187 fold). Also in contrast to the wild-type C17.2 results, A20 expression in these cells exhibited an apparent oscillatory profile, peaking at 1 h and 4 h post-TNFα stimulation.

This data suggested differences in the gene expression activation profiles, in response to TNFα, between the different cell populations. It is important to note that these were examined in terms of relative fold activation, following TNFα stimulation. One important factor in considering this data is whether there were differences in basal expression of NF-κB-dependent genes, across C17.2 cell populations. To elucidate differences in basal gene expression, average C_T values of the target gene were subtracted by the average C_T values of the control gene (see Chapter 2 for methods). These calculations were conducted using data obtained from three Q-PCRs per cell line. A Kruskal-Wallis ANOVA was performed using the values obtained and the datasets were averaged for display (Figure 3.13).
The Kruskal-Wallis ANOVA results indicated differences in the basal expression of IκBα and A20; at least one cell line had a significantly different expression level to the other cell lines (p-value<0.05). The biggest differences observed were between wild-type and C17.2 (lenti) and clone A.

So far, the work described in this chapter has suggested differences between the differently labelled C17.2 cell populations in terms of: TNFα-induced p65 dynamics, NF-κB reporter gene activation, IκBα degradation and NF-κB-dependent gene expression changes. Many of these factors might be influenced by the levels of p65 and negative feedback proteins (e.g. IκBα, IκBε and A20) in the cells. Therefore the next aim was to quantify and compare p65 and IκBα levels in the C17.2 cell populations.

3.2.1.3.4 Basal p65 and IκBα Expression in C17.2 Cell Lines

The next aim was to measure the levels of p65 and IκBα protein in the different cell populations by Western blotting. Western blots for p65 are shown in Figures 3.14A and B. In all of the cell populations, a band that corresponded to endogenous p65 was observed. As expected the C17.2 (lenti) cells showed a single higher molecular weight band that corresponded to p65-fluorescent fusion proteins. However, multiple intermediate molecular weight bands were seen in the BAC clones. Clone 5 also had an even higher molecular weight band. It was not possible to obtain control α-tubulin bands for the
Western blot conducted with C17.2 (lenti) cells (Figure 3.14A), therefore it is difficult to draw any conclusions on the relative levels of p65 across C17.2 cell populations.

IκBα levels were analysed by Western blotting and this is shown in Figure 3.15. As observed with p65, multiple unexplained (lower molecular weight) bands were bound by the IκBα antibody in the BAC clone cells. Again, it is difficult to make conclusions based on these results: more p65 and IκBα Western blots should be conducted in the future to fully characterise these cell lines.

From the results outlined in this chapter, it would seem that the C17.2 (lenti) cells represented the most appropriate model system for study of NF-κB dynamics in C17.2 cells. In contrast to the BAC clone cells, they had similar levels of TNFα-stimulated NF-luc induction and IκBα degradation to wild-type C17.2. The data obtained from single cell imaging of fluorescently-tagged p65 dynamics in the C17.2 (lenti) and C17.2 (transient) cells was more consistent with data from the literature than the results from BAC clonal cells (Widera et al, 2006a). Transiently-transfected cells were excluded for use as a model system, due to the inherent variability and time-consuming nature of transfections.
3.2.2. Investigating NF-κB Dynamics in the BV.2 Cell Line

Population-level data on TNFα-induced NF-κB activation has previously been reported in the BV.2 cell line (Sheppard et al, 2011). In this study free (activated) NF-κB levels were measured using ELISA. Peak NF-κB activation was observed at 25 min post-10ng/ml TNFα stimulation. To date, there has been no description in the literature of the time-lapse analysis of p65 dynamics in single living BV.2 cells.

3.2.2.1. Creating BV.2 Reporter Cell Lines for Observation of p65 Dynamics

BV.2 cells are difficult to transfect and therefore the production of stable cell lines using transfections was not possible. (Many unsuccessful attempts were made to transfect the cells with the p65 plasmid; results not shown). Lentiviral transduction was therefore employed to produce a BV.2 (lenti) cell line expressing p65-eGFP (Figure 3.16).
3.2.2.2. Single-cell p65 Dynamics

To investigate NF-κB response, BV.2 (lenti) cells were stimulated with 20ng/ml TNFα and the dynamics of p65-eGFP localization were observed with a confocal microscope (see Figure 3.17 for images and Figure 3.18 for Cell Tracker quantification). The cells exhibited a heterogeneous and asynchronous NF-κB response with multiple p65-eGFP oscillations being observed in single cells. On average, the initial peak in nuclear p65 fluorescence occurred at 30 min post-TNFα stimulation. This was consistent with results reported by Sheppard et al (2011).

![Image of single-cell p65 dynamics](image)

**Figure 3.17. TNFα-induced p65 translocation in lentivirus transduced BV.2 cells.** Cells were stimulated with 20 ng/ml TNFα at time 0 and visualised by confocal microscopy at the different time points after TNFα stimulation. Scale bar = 20µm.
3.2.2.3. Population-level NF-κB Activation in BV.2 Cell Lines

The next step in the characterisation of the BV.2 (lenti) cells was to compare the NF-κB response in these cells to that of wild-type BV.2 cells. This involved analysis of IκBα levels over time and gene expression analysis at the cell population level.

3.2.2.3.1. TNFα-induced IκBα Degradation Profiles in BV.2 Cell Lines

Western blot analysis of IκBα levels before and after TNFα stimulation were performed in triplicate with the wild type and BV.2 (lenti) cells. IκBα bands were quantified using densitometry and normalised to α-tubulin bands and pre-stimulation IκBα levels (Figures 3.19 and 3.20).
Both cell lines displayed a >60% decrease in IκBα within 15 min post-TNFα stimulation. IκBα returned to, and was maintained at, pre-stimulatory levels within 60 min post stimulation (with the exception of replicate 2 in Figure 3.19). It was noted that the wild type cells might show a slower recovery in IκBα levels as indicated by the level of newly-synthesized IκBα observed at 30 min. (In 2 out of 3 replicates, IκBα was ≥ 48% and <3% of the pre-stimulatory level in BV.2 (lenti) and BV.2 (UT) cells, respectively.) This difference in timing might be reflected by differences in NF-κB transcriptional activity between the cell populations.
3.2.2.3.2. NF-κB-dependent Gene Expression Changes in TNFα Stimulated BV.2 Cell Lines

To observe transcriptional NF-κB activity in BV.2 cell lines, NF-κB-dependent gene expression changes were measured over a timecourse after TNFα stimulation (Figure 3.21).
The different cell populations showed similar expression profiles for A20 and TNFα, with similar timing and amplitude of peak changes. The IkBα expression profile of the BV.2 (lenti) cells had a similar temporal profile to the wild type cells, but had a lower maximum fold induction (4 fold versus 10 fold).

The IkBε gene expression profiles between the two cell populations were noticeably different. In the BV.2 (lenti) cells, this gene showed an oscillatory expression profile peaking at 2 and 6 h post-stimulation whereas, in wild-type cells, expression peaked at 1 h post-stimulation. IκBε expression in the wild-type cells stayed elevated throughout the remainder of the time course.

Basal expression of NF-κB-dependent genes was compared across BV.2 cell lines (results are shown in Figure 3.22). The results of Mann Whitney U tests indicated that there were no significant differences in basal gene expression between the cell populations. Therefore, basal gene expression levels do not play a role in the gene expression differences seen in Figure 3.21.
To complete characterisation of the BV.2 cell lines, p65 and IκBα levels were measured in these cell lines.

3.2.2.3. Basal p65 and IκBα Expression in BV.2 Cell Lines

Western blots were conducted to analyse the levels of p65 and IκBα in the wild-type and BV.2 (lenti) cells (see Figures 3.23 and 3.24).

![Western blot analysis of p65 levels in the wild-type BV.2 cells and BV.2 (lenti) cells. Protein extracts from unstimulated BV.2 cells and lentivirus transduced BV.2 cells were analysed by Western blotting with a p65 antibody.](image)
Levels of endogenous p65 appeared to be comparable between the cell lines. Total p65 expression (endogenous plus p65-eGFP fusion protein) in the BV.2 (lenti) cells appeared to be higher than in the wild type BV.2 cells. Western blot analysis of IκBα levels showed similar results: BV.2 (lenti) cells appeared to express more IκBα than wild-type cells. However, these Western blots did not contain control α-tubulin bands, which preclude comparison of p65 and IκBα levels across cell populations.

3.3. Discussion

Analysis of TNFα-induced NF-κB activation has previously been reported at the cell population level in neural progenitor/stem cells (Widera et al, 2006a) and microglia (Sheppard et al, 2011). These studies might have missed some important characteristics of the NF-κB response in cells, as they did not make single-cell level observations. To this end, the first goal of the work described in this chapter was to create neural progenitor and microglial cell lines, which could be readily used for p65 observation within individual living cells. The next goal was to systematically characterise NF-κB activation, in wild-type and reporter cell lines, to determine the best model system for use in future experiments. The major findings of this characterisation procedure will be described in the following sections.

3.3.1. NF-κB Activation in C17.2 Cells

NF-κB activation was measured using a consensus NF-κB-luciferase reporter. Overall the response was similar to that reported for other cell types (Cheong et al, 2006; Khabar et al, 1995; Turner et al, 2010).
The lowest TNFα dose required to achieve maximal induction of the NF-luc reporter was between 100pg/ml and 1ng/ml (Figure 3.5). Peak induction occurred at 5 h post-stimulation with doses ≥1ng/ml TNFα; 1 h later than previously observed in SK-N-AS cells (Turner et al, 2010). Conversely, with doses ≤100pg/ml, peak NF-luc induction was observed earlier in C17.2 compared to SK-N-AS cells (3 h versus 6 h post-stimulation). This latter result cannot be explained by higher basal TNFR expression in C17.2 cells, as these cells were less sensitive to lower doses of TNFα than SK-N-AS cells: the lowest TNFα dose that led to NF-luc induction was 100pg/ml in C17.2, versus 10pg/ml in SK-N-AS cells (Turner et al, 2010).

3.3.1.1. Single-Cell p65 Dynamics in C17.2 Cells

Nuclear localisation of p65 in C17.2 cells, 30 min after TNFα stimulation, has previously been reported in fixed cells, via p65 immunostaining (Widera et al, 2006a). Results from transiently-transfected and C17.2 (lenti) cells were consistent with this observation.

In previous studies of p65 dynamics, cells that were highly overexpressing p65 were eliminated from the analysis (Nelson et al, 2004; Ashall et al, 2009; Turner et al, 2010). Here, all C17.2 (transient) cells were included in the analysis: the consequent variations in p65 overexpression may explain the differences between C17.2 (transient) and C17.2 (lenti) cells: lower amplitude p65 nuclear translocations were observed in transiently-transfected cells, compared to C17.2 (lenti) cells (Figure 3.3).

To minimise perturbation of the NF-κB response, it is important to only analyse transfected cells which express relatively low levels of the fusion protein. Combined with low transfection efficiency, this cell selection procedure reduces the amount of data that can be acquired from each transfection/microscopy experiment. Stable transfection with the p65 plasmid was not possible as it would have required geneticin selection, an antibiotic to which all C17.2 cells are immune. Moreover, lentiviral transductions usually result in integration of low copy number of genes across cells in a population, making them more reliable as reporter systems. For this reason, C17.2 (lenti) cells were selected as the best model system for representing untransfected C17.2 cells in future experiments.

3.3.1.2. NF-κB-dependent Gene Expression

Strong induction of NF-κB-dependent genes was observed after TNFα-stimulation (Figure 3.12). Consistent with data from other cell lines, IκBε induction in C17.2 cells was delayed compared to IκBα. A forty-five minute delay, observed in MEFs, is thought to be optimum
for maximising asynchronicity of NF-κB response between cells (Ashall et al., 2009; Paszek et al., 2010). The delay in C17.2 cells cannot be established to the same degree of accuracy from timepoints observed here, but expression profiles of IκBα and IκBε were similar to that observed in MEFs (Paszek et al., 2010). This would suggest that the NF-κB response in C17.2 cells is asynchronous, as reported with other cell lines (Nelson et al., 2004; Ashall et al., 2009; Paszek et al., 2010).

IκBε expression is important for the termination of the NF-κB response (Hoffmann et al., 2002). Unlike IκBα, the IκBε profile in C17.2 cells was similar to that observed in SK-N-AS (Ashall et al., 2009). This might suggest a similar duration of NF-κB response. Also, a peak in TNFα gene expression observed here at 1 h may have contributed to a later resurgence of NF-κB activation in C17.2 cells; TNFα-induced upregulation of TNFα could create a feed forward loop (Covert et al., 2005).

3.3.2. NF-κB Activation and p65 Dynamics in p65-BAC Stable C17.2 Clones

TNFα invoked a strong activation of NF-κB in wild-type C17.2, as measured by NF-κB-dependent increases in luciferase expression (see Figure 3.5). However, TNFα stimulation of clones A and 5 induced very low levels of NF-κB activation and did not mirror the dose-dependent profile generated by wild-type cells. IκBα levels in clones reflected this observation: levels were maintained for up to 2 h post-20ng/ml TNFα stimulation.

Single-cell data with the BAC clones was consistent with the population-level results: clones A and 5 exhibited very small to no p65 nuclear translocations, compared to C17.2 (lenti) and transiently transfected cells (Figure 3.3). Although low TNFα-induced NF-κB activation was observed in these cells, strong induction of NF-κB-dependent gene expression was observed after 20ng/ml TNFα stimulation (Figure 3.12). These differences may have been caused by the single cell cloning process.

Ryder et al. (1990) suggested that significant changes in culture conditions, such as cell-cell contact and composition of culture media, could incline C17.2 cells towards a more neuronal phenotype. This suggests that BAC clonal cells were more differentiated than wild-type C17.2 cells. Support for this idea comes from a study by Listwak et al. (2013). They obtained similar, seemingly contradictory, TNFα-induced NF-κB activation results from murine cortical neurons. In this study, IκBα level, NF-κB-dependant gene expression and nuclear p65 was measured after 100ng/ml TNFα stimulation. Very little to no nuclear
p65 presence was reported at 30 minutes and IκBα levels were maintained up to 1 hour post-stimulation. However, NF-κB-dependant gene expression changes were observed. Mixed brain and microglial cell cultures were also investigated in this study; these cells all displayed strong TNFα-induced nuclear p65 and IκBα degradation. Together with the results of this study, the present study suggests that during the process of cloning, the p65-BAC stable cells may have adopted a more neuronal phenotype.

3.3.3. NF-κB Activation in BV.2 (UT) Cells

NF-κB activation has previously been reported in BV.2 cells, in response to TNFα using population-level assays (Sheppard et al, 2011). Peaks in nuclear p65 were reported at 25 min and 105 min after 10ng/ml TNFα stimulation. Here, average nuclear p65 peaks were observed around 30 and 105 min after TNFα stimulation: this provides support for the use of BV.2 (lenti) as a representative for p65 in untransfected BV.2 cells (Figure 3.18).

3.3.3.1. IκBα Degradation in BV.2 (UT) Cells

In the BV.2 cultures, over 60% of IκBα was degraded within 15 min of TNFα stimulation (Figure 3.19), which was comparable to that observed in other cell types (e.g. 70% in HepG2 cells (Moss et al, 2012) and ≥85% in C17.2 cells). Pre-stimulatory levels of IκBα were reached within 60 min of TNFα treatment, compared to 65 +/- 8% of the pre-stimulatory level after 137 +/- 5 min in HepG2s (Moss et al, 2012). Assuming similar basal nuclear p65 presence in these cells, variations in IκBα degradation could manifest in different amplitudes of p65 nuclear translocation.

3.3.3.2. NF-κB-dependent Gene Expression

TNFα-induced changes in NF-κB-dependant gene expression were measured in BV.2 cells (Figure 3.21). Peak IκBα induction was lower in BV.2 cells than SK-N-AS (~10 fold versus >20 fold; Ashall et al, 2009). This may reflect smaller TNFα-induced changes in nuclear p65. The timings of IκBα gene induction were similar between these cells; that might be mirrored in the timings of the initial nuclear p65 translocations. Unlike in SK-N-AS cells, IκBα upregulation decreases between 1-2 h. SK-N-AS cells exhibit sustained nuclear p65 oscillations in response to TNFα, which allows IκBα gene expression to remain upregulated for >7 h (Ashall et al, 2009). Differences in the upregulation of IκBα expression indicate a more transient NF-κB response in BV.2 cells.
IκBε gene induction in BV.2 was comparable to SK-N-AS cells in terms of peak induction and timings. Interestingly, IκBε peak induction occurred simultaneously to IκBα. Delayed induction of the IκBε gene in relation to IκBα is believed to be responsible for asynchronous NF-κB responses between cells in a population (Ashall et al, 2009; Paszek et al, 2010). The lack of a delay in BV.2 cells may manifest in more synchronous NF-κB dynamics in microglial populations. However, Sheppard et al (2011) produced a mathematical model to reproduce population level data on p65 response in BV.2 cells and they found that parameters relating to IκBε had little to no effect on NF-κB activity.

3.3.4. Discussion of Problems with using Lentivirally Transduced Cell Lines

NF-κB activation data from C17.2 and BV.2 reporter cell lines was compared to data from the respective wild-type cell line. It was concluded that lentivirally transduced cell lines were the best model systems for further use. However, there are potential problems with using these lentivirally transduced cell lines that will be addressed here. These problems include those that are specific to a cell line and lentiviral transductions, as well as generic issues regarding the use of fusion proteins.

3.3.4.1. General Problems with p65 Overexpression

Many studies have used EMSA, bioluminescence and Western blots to observe population-level NF-κB activation (Sheppard et al, 2011; Magni et al, 2012). Single-cell p65 localisation is commonly observed in fixed cells using immunostaining (Widera et al, 2006a). These methods do not offer any insights into single-cell p65 dynamics: an important determinant of gene expression (Ashall et al, 2009; Lee et al, 2014). From this, it can be conjectured that single-cell dynamics are vital determinants of cell fate. In order to visualise p65 in living cells, many studies have taken advantage of constructs that enable cells to express fluorescently-tagged p65 (Nelson et al, 2004; Ashall et al, 2009; Turner et al, 2010). These studies have revealed many aspects of NF-κB activation that were not detected on a population-level, including asynchronicity and heterogeneity of p65 response within cell populations.

An unavoidable consequence of genetically modifying cells is increasing p65 levels. It has been argued that overexpression of p65 within cells alters NF-κB localisation (Scott et al, 1993; Beg et al, 1992; Zabel et al, 1993). This argument is countered with the observation that physiological levels of fluorescent p65 were expressed in modified cell lines (Ashall et
al, 2009). Also, it has been shown that the NF-κB system is subject to autoregulation by p65-induced upregulation of IκBα (Scott et al, 1993; Brown et al, 1993; Sun et al, 1993). Although literature suggests that moderate levels of p65 overexpression do not affect NF-κB dynamics, this topic is discussed in more depth in relation to C17.2 and BV.2 reporter cell lines.

3.3.4.2. Choice of C17.2 (lenti) as a Model System and Potential Problems

From the data collected in the characterisation of C17.2 cell lines, it was concluded that the C17.2 (lenti) cell line was the best model system for observation of p65 dynamics. This was based on a few key characteristics. Firstly, TNFα-stimulated NF-luc induction in C17.2 (lenti) showed a time- and dose-dependent profile similar to that observed in wild-type cells (Figure 3.5). Secondly, the IκBα degradation profile in C17.2 (lenti) cells strongly resembled that reported in wild-type cells, whereas clonal cells did not exhibit TNFα-induced IκBα-degradation (Figure 3.8-3.11). Finally, TNFα-induced p65 translocations were observed in C17.2 (lenti) cells; this is consistent with the limited data available on p65 localisation in NPCs (Figure 3.3). Again, BAC clonal cells responded very differently, exhibiting little to no nuclear p65 translocations.

Although the C17.2 (lenti) cell line was selected as the best model system for use, some differences were observed between the wild-type and C17.2 (lenti) cells: these differences, and their potential consequences, will be discussed here.

A major concern when working with progenitor/stem cells is ensuring that they remain in an undifferentiated state. Blits et al (2005) reported no change in NPC expression of stem, glial and neuronal cell markers after lentiviral transduction with different constructs. Some studies have employed transduction to overexpress transcription factors and, thereby, induce differentiation (Maire et al, 2009; Falk et al, 2002; Stock 2010). There is currently no data to suggest that NF-κB expression can be used to differentiate cells in the same manner.

NF-κB-driven gene expression, as measured by NF-luc, was lower in C17.2 (lenti) than wild-type cells after stimulation with doses ≥100pg/ml TNFα (Figure 3.5). However, the same dose- and time-dependent profile was preserved: this was not the case with the BAC clones investigated.
Two out of three replicates from wild-type C17.2 cells exhibited complete degradation of IκBα within 15 minutes following TNFα treatment (Figure 3.8). However, IκBα degradation in the C17.2 (lenti) was incomplete (Figure 3.9). When combined with the above observations made on activation of the luciferase reporter, these results might suggest smaller amplitude NF-κB nuclear translocations in the C17.2 (lenti) cells.

IκBα in the C17.2 (lenti) cells returned to pre-stimulatory levels within 45 min post-TNFα treatment, compared to 60 min in wild-type C17.2 (Figures 3.8 and 3.9). Also, rapid reduction in IκBα level was observed in C17.2 (lenti) at 105 min after TNFα stimulation: this was not observed in wild-type cells. These results are surprising and no evidence of increased NF-κB activity was seen in the luciferase reporter assay experiments conducted with the C17.2 (lenti) cells (Figure 3.5).

Finally, the expression of key NF-κB-dependent genes, IκBα, IκBε and A20, was observed in wild-type and C17.2 (lenti) cells: overall, the cells displayed similar expression profiles. C17.2 (lenti) cells exhibited the same delay in IκBε gene induction, compared to IκBα, as observed in wild-type C17.2 cells and non-neural cell lines (Paszek et al, 2010). However, TNFα gene induction was markedly stronger in C17.2 (lenti) cells, compared to wild-type C17.2: peak fold induction was >180 at 1 h post-TNFα stimulation (versus ~9 fold in wild-type cells). TNFα can contribute to a feed-forward loop and could lead to later, secondary NF-κB activation in cells (Covert et al, 2005). This may have contributed to IκBα degradation observed at 105 min post-TNFα stimulation (Figure 3.9).

3.3.4.3. Choice of BV.2 (lenti) as a Model System and Potential Problems

To ensure that the lentiviral transduction procedure did not change the activation status of BV.2 cells, TNFα expression can be compared between BV.2 (UT) and BV.2 (lenti): TNFα expression is commonly used as an indicator of BV.2 activation status (Kobayashi et al, 2013; Liu et al, 2013b). There was no difference in the basal expression of TNFα between wild-type and BV.2 (lenti) cells (Figure 3.21). Moreover, the same was true for IκBα, IκBε and A20 gene expression. This indicates that transduction did not alter the activation status of microglia or basal NF-κB transcriptional activity.

However, TNFα-induced IκBα gene induction was weaker in BV.2 (lenti) cells compared to wild-type cells. This was not reflected in the resynthesis of IκBα: 30 min post-TNF-α stimulation, IκBα levels were ≥48% of pre-stimulatory levels in BV.2 (lenti) cells, compared to 20% on average in wild-type cells (Figures 3.19 and 3.20). It is difficult to
draw any definitive conclusions from this, but differences in IkBα resynthesis time may affect NF-κB nuclear export times. IkBα degradation and the time taken to restore IkBα to pre-stimulatory levels were similar between the cell populations.

Activation of IkBε was higher in BV.2 (lenti) cells than BV.2 (UT). Sheppard et al (2010) have previously suggested that the effects of IkBε on TNFα-induced NF-κB activity are negligible. However, this conclusion originated from a mathematical model (the Ashall et al (2009) model) that was adapted to fit population-level data. Population-level data has previously failed to provide insights on the various mechanisms for fine-tuning NF-κB activity and dynamics, whereas single-cell data has filled this niche (Nelson et al, 2004; Ashall et al, 2009; Turner et al, 2010; Paszek et al, 2010). For this reason, it must be noted that differences in IkBε levels may cause premature termination of NF-κB activity in BV.2 (lenti), compared to wild-type BV.2.

Cells have an ability to compensate for small increases in p65, either through an inherent availability of excess, or an induced upregulation of IkBα (Scott et al, 1993; Brown et al, 1993; Sun et al, 1993). However, large increases in p65 can disrupt normal NF-κB activity and localisation (Beg et al, 1992; Zabel et al, 1993). Preliminary Western blots showed that p65 expression in BV.2 (lenti) cells was comparable to endogenous p65 expression in the wild-type cells. Also, IkBα levels in BV.2 (lenti) cells appeared to be upregulated (Figures 3.23 and 3.24). As observed in cells that overexpress p65, this may be a compensatory method to minimise perturbation to the NF-κB network (Scott et al, 1993).
4. NF-κB Activation and Dynamics in C17.2 and BV.2 Cells in Mono-cultures and Co-cultures
4.1. General Introduction

Having established lentivirally transduced cell lines, C17.2 (lenti) and BV.2 (lenti), as suitable model systems for the study of NF-κB in C17.2 and BV.2 cells, the project could progress onto the next stage: investigation of NF-κB-mediated interactions between cells. This chapter details NF-κB activation profiles in mono-cultures and 1:1 co-cultures, after TNFα-stimulation.

4.2. Results

4.2.1. NF-κB Dynamics and Activation in C17.2 Cells

4.2.1.1. Single-cell p65 Dynamics in Mono-cultures and 1:1 Co-cultures

C17.2 cells were seeded in mono-cultures or 1:1 co-cultures with BV.2 in glass-bottom dishes. Figures 4.1A and B show C17.2 (lenti) cells in mono-culture and co-culture, respectively. Dishes were put on a confocal microscope, cells were stimulated with 20ng/ml TNFα and time-lapse images were taken from 0 to 300 min after stimulation. Microscopy experiments were performed in triplicate.

Nuclear p65 was quantified, using CellTracker software (Perkins, 2007). A complete cell tracking time line was impossible for cells that moved out of the field of view, moved out of focus, died or divided during the course of the experiment. Post-TNFα stimulation data
was normalised to mean pre-stimulation nuclear p65 levels to show fold changes in nuclear p65 (Figures 4.2A and B). Data is displayed as heat maps in Figures 4.2C and D. Higher nuclear p65 fold changes are represented by darker colours in these heat maps.

![Heat maps and graphs showing nuclear p65 fluorescence intensity over time after stimulation.](image)

**Figure 4.2. Quantification of nuclear p65 fluorescence in C17.2 (lenti) cells, after TNFα stimulation, in mono-cultures and 1:1 co-cultures (with BV.2 cells).**

Nuclear p65 fluorescence was recorded between 0 and 300 min after TNFα stimulation. Readings were normalised to mean pre-stimulation nuclear p65 fluorescence. (A) and (B) Single cell traces for 63 mono-cultured cells and 56 co-cultured cells, respectively. Thick black lines show the average of all of the cells in each population. (C) and (D) Heat maps representing fold changes in nuclear p65 fluorescence in mono-cultured and co-cultured cells, respectively. Each row displays data from a single-cell; darker colours represent higher amplitude changes in nuclear p65 fluorescence. Single-cell data was collated from microscopy experiments performed in triplicate.

All cells in mono-culture displayed a clear initial p65 nuclear translocation (as reported previously in Chapter 3). Some cells displayed a more sustained nuclear p65 presence,
after the initial p65 translocation. In contrast, C17.2 (lenti) cells in co-culture displayed an initial translocation, of lower amplitude, and no subsequent translocations.

Timing and amplitude of initial nuclear p65 translocations were quantified for each cell in mono-culture and co-culture. Unpaired t-tests were conducted and averaged data is displayed in Figure 4.3. On average, the amplitude of the initial p65 translocation was significantly lower in co-cultured C17.2 (lenti) cells than mono-cultured cells (p-value≤0.01; Figure 4.3B). There was no significant difference in the initial nuclear p65 peak timing between culture conditions (Figure 4.3A).

To further characterize the dynamic profiles seen in Figure 4.2, single-cell data was clustered using a k-means algorithm and a cluster number of 4. Different cluster numbers were investigated initially (results not shown): clustering into 4 groups produced results that best represented the heterogeneity of the p65 response. The k-means algorithm sorted cells into 4 different p65 response categories (Figure 4.4A-D). Figures 4.4E and 4.4F show the distribution of cells across p65 response categories.

Cells were sorted into clusters according to two different aspects of p65 response, predominantly: 1) amplitude of initial nuclear p65 translocation and 2) response duration. Amplitude of nuclear p65 translocation decreases going from Figure 4.4A through to 4.4D. Clusters A and B accommodated cells that displayed sustained nuclear p65 occupancy: on average, nuclear p65 returned to pre-stimulatory levels at ≥150 min after TNFα-
stimulation. Only cells that were tracked for at least 300 min were included in this analysis (47 and 45 mono-cultured and co-cultured C17.2 (lenti) cells, respectively).

4.2.1.2. NF-κB Activation in Co-cultured Wild-type C17.2 Cells

C17.2 (lenti) cells that were co-cultured with BV.2 displayed smaller and fewer p65 nuclear translocations in response to TNFα, compared to mono-cultured cells. In an effort...
to exaggerate co-culture effects and measure transcriptional output from the NF-κB system, live cell luminometry was utilised (see Chapter 2 for methods).

Wild-type C17.2 cells were transiently transfected with an NF-luc plasmid then seeded in 24-well plates as mono-cultures or with BV.2 cells. Cultures were stimulated with different doses of TNFα and luminescence measurements were taken every 15 min from 0 to 5 h after stimulation. Experiments were performed in triplicate, with three technical replicates per plate. Data from mono-cultures and co-cultures was normalized to the corresponding unstimulated group. Figure 4.5 shows averaged data and the results of a one-way ANOVA, comparing NF-κB response across mono- and co-culture groups.

The first set of experiments were conducted using 1:2 (C17.2:BV.2) co-cultures and doses ≤20ng/ml TNFα (1ng/ml, 10ng/ml and 20ng/ml; Figure 4.5A). Under these conditions, NF-luc induction in co-cultured C17.2 cells was comparable to that in mono-cultures. Continuing with the effort to enhance co-culture effects on C17.2 NF-κB response, higher C17.2:BV.2 co-culture ratios were investigated (1:4 and 1:8; Figures 4.5B and C, respectively) and TNFα dose was lowered (500pg/ml, 1ng/ml or 10ng/ml TNFα). One-way ANOVA test results showed that peak C17.2 NF-luc induction was significantly lower in 1:4 co-cultures than in mono-cultures, at all doses investigated (p-value≤0.01; Figure 4.5B). Interestingly, this effect is not seen in 1:8 co-cultures.

To eliminate the possibility of confluency-dependent changes in NF-luc induction, luminometry experiments were conducted using 1:4 co-cultures with SK-N-AS (human neuroblastoma) cells instead of BV.2 (Figure 4.6). SK-N-AS cells were selected for use as they are neural cells that, unlike BV.2 cells, are not part of the immune system. NF-luc induction in these co-cultured C17.2 cells was comparable to that in mono-cultured C17.2. Therefore, the effects displayed in Figure 4.5B were BV.2-specific.

In order to investigate whether the ability to inhibit C17.2 NF-luc induction was unique to BV.2 or common to macrophages, luminometry experiments were conducted using a murine peripheral macrophage cell line, RAW 264.7 (Figure 4.7). Significantly lower NF-luc induction was observed in co-cultured C17.2 compared to mono-cultured cells (p-value≤0.01), thus both BV.2 and RAW 264.7 inhibited TNFα-stimulated NF-luc induction in C17.2.
Figure 4.5. TNF-α-stimulated NF-luc induction in C17.2 cells, in mono-cultures and co-cultures (with BV.2 cells).

Cells were transfected with the NF-luc plasmid, and stimulated with varying doses of TNF-α. Luminescence was measured every 15 min from 0 to 300 min after TNF-α stimulation. Each graph represents NF-luc induction in mono-cultured cells and: (A) 1:2 co-cultured cells, (B) 1:4 co-cultured cells and (C) 1:8 co-cultured cells. All experiments were performed in triplicate. Bars represent ±S.D. and asterisks represent significant one way ANOVA test results (** = p-value < 0.01, versus corresponding mono-culture group).
Figure 4.6. TNFα-stimulated NF-luc induction in C17.2 cells, in mono-cultures and 1:4 co-cultures (with SK-N-AS cells). C17.2 cells were transfected with an NF-luc plasmid, then seeded in mono-cultures and 1:4 co-cultures with SK-N-AS cells. Luminescence was measured every 15 min from 0 to 300 min after TNFα stimulation. All experiments were performed in triplicate. Bars represent ± S.D. and asterisks represent significant one-way ANOVA test results (**=p-value≤0.01, versus corresponding mono-culture group).

Figure 4.7. TNFα-stimulated NF-luc induction in C17.2 cells, in mono-cultures and 1:4 co-cultures (with RAW 264.7 cells). C17.2 cells were transfected with an NF-luc plasmid, then seeded in mono-cultures and 1:4 co-cultures with RAW 264.7 cells. Luminescence was measured every 15 min from 0 to 300 min after TNFα stimulation. All experiments were performed in triplicate. Bars represent ± S.D. and asterisks represent significant one-way ANOVA test results (**=p-value≤0.01, versus corresponding mono-culture group).
Due to the method of analysing luminometry data, co-culture effects displayed in Figures 4.5-4.7 may have been a result of differences in basal NF-luc expression. After normalising luminescence data to the unstimulated control, TNFα-induced fold changes in luciferase expression were calculated relative to unstimulated luciferase expression levels. For example, if after normalisation, basal and 5 hours post-TNFα stimulated luminescence was 300 and 1500 then the fold change would have been 5 (i.e. 1500/300). This means that a high fold change would be obtained with a low basal luminescence and vice versa: higher basal luciferase expression may have contributed to the lower fold changes in NF-luc induction in co-cultures (versus mono-cultures).

To investigate this possibility, basal luminescence values across culture conditions were compared. Each co-culture ratio was investigated in parallel with mono-cultures, so basal luminescence of co-cultures could be compared directly to corresponding mono-cultures. However, each co-culture ratio was investigated independently, in separate experimental plates, therefore, basal luminescence of different co-cultures could not be compared directly e.g. basal luminescence of 1:2 co-cultures cannot be compared to that of 1:4 co-cultures. Basal luminescence values from mono-cultures and co-cultures were evaluated for significant differences using unpaired t-tests. Values were then averaged, and normalised to the corresponding mono-culture average, for display in Figure 4.8.

No difference in basal luminescence was detected between mono-cultures and SK-N-AS co-cultures (Figure 4.8B). In contrast, average basal luminescence was significantly higher in RAW 264.7 (p-value≤0.01; Figure 4.8C), 1:4 BV.2 (p-value≤0.05), and 1:8 BV.2 co-cultures (p-value≤0.01; Figure 4.8A), compared to mono-cultures. Basal luminescence values from untransfected BV.2 cells were measured to ensure that the observed differences in luminescence could not be attributed to increasing background from cells. The background luminescence from BV.2 remained the same across increasing cell densities (moving from left to right in Figure 4.9). This indicates that BV.2, as well as RAW267.4, increased basal NF-luc expression in co-cultured C17.2.
Having established that co-culture of BV.2 cells increased basal luminescence but significantly reduced TNFα-induced NF-luc induction in C17.2 cells, luminometry experiments were conducted to ascertain whether or not these effects were cell-contact dependent. In these experiments, transwell membranes were used to prevent contact between BV.2 and C17.2 in co-cultures, whilst allowing the passage of secreted biomolecules. To avoid the possibility of transwell interference with luminescence detection, endpoint luminometry was used in preference to live cell luminometry.
First, control experiments were performed to observe any effects that transwells might exert on TNFα-induced NF-luc induction in C17.2. Cells were transfected with an NF-luc plasmid and seeded in mono-cultures either with or without empty transwells. These cells were then stimulated with 500pg/ml, 1ng/ml or 10ng/ml TNFα and lysed 5 h later. Luminescence readings were taken from these lysates. Experiments were performed in triplicate with three technical replicates per plate. Data was collated from replicates, averaged and normalised to the corresponding unstimulated mono-culture (Figure 4.10A). Also, basal luminescence readings were normalised to readings from mono-cultures without transwells (Figure 4.10B).

As conducted earlier with data in Figures 4.5-4.7 (see Figure 4.8), unpaired t-tests were used to compare basal NF-luc expression and TNFα-induced NF-luc induction in C17.2 cells seeded with and without transwells. For example, basal luminescence of unstimulated mono-cultures without transwells was compared to that of unstimulated mono-cultures with transwells. This same analysis was performed with TNFα-stimulated mono-cultures with and without transwells; each TNFα-treated culture was compared to the corresponding unstimulated culture. Results showed that transwells did not affect basal NF-luc expression or NF-luc induction after TNFα-stimulation. Having established this, the same luminometry protocol was used to investigate the effects of BV.2 cells on NF-luc
induction in C17.2 cells, in transwell co-cultures. BV.2 cells were seeded in transwell inserts, separated from C17.2, at co-culture ratios of 1:4 and 1:8 (C17.2:BV.2).

Results for 1:4 transwell co-cultures can be seen in Figure 4.11. TNFα-stimulated NF-luc induction and basal NF-luc expression were compared across culture conditions using unpaired t-tests (Figures 4.11A and B, respectively). NF-luc induction was significantly lower in transwell co-cultures after stimulation with ≥1ng/ml TNFα (p-value≤0.05). Stimulating transwell co-cultured C17.2 with 1ng/ml and 10ng/ml TNFα resulted in 42% and 39% lower NF-luc induction, respectively (compared to mono-cultured C17.2). No differences were observed in basal luminescence across culture conditions. Therefore, increased basal NF-luc expression in co-cultured C17.2 (previously observed in Figure 4.8) was dependent on physical contact with BV.2.

In contrast to results from 1:4 transwell co-cultures, NF-luc induction in NPCs in 1:8 transwell co-cultures was not significantly different to that of NPCs in mono-culture. (Figure 4.12B). This suggests that TNFα-treated BV.2 cells are producing an NF-κB activating agent that, in high ratio co-cultures, can counteract the NF-κB inhibition observed in 1:4 co-cultures.
In order to compare the effects of transwells, BV.2 cell-contact and increasing BV.2 cell numbers, NF-luc transfected C17.2 were seeded in different conditions, in the same 24-well plate. Some co-cultures were made using transwells to prevent C17.2-BV.2 cell contact ('transwell co-culture' groups). Other co-cultures were seeded to enable contact between cells ('w/empty transwell'). Cells were left overnight then lysed for basal
luminescence readings. Experiments were performed in triplicate. Luminescence data from the ‘mono-culture with empty transwell’ groups was compared to that from all other groups, using unpaired t-tests. All data was averaged and normalised to the ‘mono-culture w/empty transwell’ group (Figure 4.13).

4.2.2. Single-cell p65 Dynamics in BV.2 Cells, in Mono-cultures and 1:1 Co-cultures

BV.2 cells inhibited TNFα-stimulated NF-luc induction in co-cultured C17.2 (in contact-permissive 1:4 co-cultures). On a single-cell level, nuclear p65 translocations were inhibited in C17.2 (lenti) by BV.2 (in 1:1 co-cultures). The directionality of co-culture effects was studied by observation of p65 dynamics in co-cultured BV.2 cells.
with C17.2 (UT) cells, in glass-bottom dishes (Figure 4.14A). Time lapse images were taken from 0 to 400 min after TNFα-stimulation. Microscopy experiments were performed in triplicate with mono-cultures. One co-culture microscopy experiment was performed and analysed for discussion here.

Nuclear p65 fluorescence was quantified, using CellTracker software (Perkins, 2007). Post-stimulation nuclear p65 fluorescence was normalised to mean pre-stimulation nuclear p65 for each individual cell analysed (Figures 4.15A and B). This data is displayed as heat maps in Figures 4.15C and D. Higher nuclear p65 fold changes are represented by darker colours in these heat maps.

All BV.2 (lenti) cells exhibited at least one nuclear p65 translocation in response to TNFα, in mono-cultures and co-cultures, respectively. Unlike C17.2 (lenti), many BV.2 (lenti) cells displayed subsequent strong nuclear p65 translocations, with some degree of regularity. These oscillatory dynamics have previously been reported in many different cell lines (Nelson et al, 2004; Ashall et al, 2009; Turner et al, 2010). Peak to peak timing of nuclear p65 translocations was calculated for every cell that displayed oscillations (i.e. ≥2 nuclear p65 translocations). These values were averaged for each cell to give a mean oscillation period, represented by the blue dots in Figure 4.16. Mean values were averaged and standard deviations were calculated: this data is represented by the black horizontal and vertical lines in Figure 4.16, respectively.
Figure 4.15. Quantification of nuclear p65 fluorescence in BV.2 (lenti) cells, after TNFα stimulation, in mono-cultures and 1:1 co-cultures (with C17.2 cells).

Nuclear p65 fluorescence was recorded between 0 and 400 min after TNFα stimulation. Readings were normalised to mean pre-stimulation nuclear p65 fluorescence. (A) and (B) Single cell traces for 57 mono-cultured cells and 20 co-cultured cells, respectively. Thick black lines show the average of all of the cells in each population. (C) and (D) Heat maps representing fold changes in nuclear p65 fluorescence in mono-cultured and co-cultured cells, respectively. Each row displays data from a single-cell: darker colours represent higher amplitude changes in nuclear p65 fluorescence. Mono-culture data was collated from microscopy experiments performed in triplicate; co-culture data was obtained from one microscopy experiment.

The mean period for cells in mono-culture and co-culture was evaluated for significant differences using an unpaired t-test, the results of which can be seen in Figure 4.16. The average period of oscillations in co-cultured cells was 63 min, which was significantly faster than the period observed in mono-cultured BV.2 (lenti) cells (108 min; p-value≤0.01).
Figure 4.16. Average peak to peak timing of p65 nuclear translocations in mono-cultured and co-cultured BV.2 (lenti) cells, after TNFα stimulation.

Nuclear p65 fluorescence readings were taken from 0 to 400 min after TNFα stimulation in BV.2 (lenti) cells, in mono-cultures and 1:1 co-cultures (with C17.2 cells). Fluorescence readings were used to calculate peak to peak timing of p65 nuclear translocations in cells. Average peak to peak timing (period) of each cell is represented by a blue dot. 53 mono-cultured and 20 co-cultured BV.2 cells are represented here. Averages and standard deviations are represented by the black horizontal and vertical bars, respectively. Asterisks signify the results of a Mann Whitney U test (**=p-value≤0.01).

Peak timings and amplitudes of initial nuclear p65 translocations were quantified, unpaired t-tests were conducted and data was averaged for display in Figure 4.17. Initial nuclear p65 translocation peaks occurred significantly earlier in co-cultured BV.2, compared to mono-cultured cells (p-value≤0.05). No difference was found in the average amplitude of this response between different cultures.

Single-cell data was clustered using a k-means algorithm and a cluster number of 2: this cluster number was selected, out of a variety of cluster numbers tested (results not shown), as it best represented the p65 dynamic profiles obtained here. Some cells were excluded from this analysis, as it was not possible to track them for 400 min: the analysis was conducted with 32 and 13 mono-cultured and co-cultured cells, respectively. Dynamic profiles of nuclear p65 generated are displayed in Figures 4.18A and B. Figure 4.18C represents the distribution of mono-cultured and co-cultured BV.2, across clusters A and B.

All of the cells analysed exhibited p65 oscillations. Cells that displayed lower amplitude oscillations, with a noticeable dampening of amplitude over time, were stratified into cluster B; the remaining cells, in cluster A, exhibited only a slight dampening over time.
92% of co-cultured BV.2 (lenti) cells were sorted into cluster B, compared to 41% of mono-cultured cells.

In conclusion, BV.2 cells inhibited TNFα-stimulated NF-luc induction and p65 nuclear translocations in C17.2. Conversely, TNFα-induced BV.2 p65 dynamics were altered by C17.2: later initial peak timings and faster oscillations were observed in co-cultured BV.2, compared to mono-cultured cells. The observations on BV.2 cells originated from only one co-culture microscopy experiment, whereas results on C17.2 involved collation of data from triplicate experiments. For these reasons, NF-κB in C17.2 became the primary focus of further experiments.

To elucidate the mechanisms that may have been involved in BV.2 effects on C17.2 NF-κB, the TNFα signalling pathway was targeted for further investigation.
4.2.3. TNFα availability in Mono- and Co-cultures.

TNFα-stimulated NF-luc induction in C17.2 was inhibited by BV.2. On a single-cell level, average TNFα-induced p65 nuclear translocations were of lower amplitude in co-cultured C17.2 than in mono-cultured cells. Simple explanations for differences in p65 dynamics would be: lower TNFα availability in co-cultures and lower TNFR expression in co-cultured C17.2, compared to mono-cultured C17.2. First, TNFα availability in mono-cultures and co-cultures was investigated, using TNFα ELISA.

C17.2 (UT) and BV.2 (UT) mono-cultures and 1:1 co-cultures were stimulated with 2ng/ml TNFα: this dose was selected based on sensitivity of the subsequent ELISA that was conducted. Media from cultures was extracted at 0, 2, 4 and 6 h after stimulation. Experiments were performed in triplicate and one ELISA was used to determine TNFα concentration in all extracts simultaneously. This data was averaged and normalised to concentration at time 0. Results are displayed in Figure 4.19.
Kruskal-Wallis ANOVA was used to test for significant differences in relative TNFα concentrations at different time points, across culture conditions: no significant differences were found. This suggests that TNFα availability was not responsible for the differences in p65 response observed in co-cultured C17.2 (compared to mono-cultured C17.2). Following on from this, TNFR expression was studied in mono-cultured and co-cultured C17.2.

4.2.4. TNFR1 Expression in Unstimulated and TNFα-stimulated C17.2 cells, in Mono-cultures and Co-cultures.

Having ruled out TNFα availability as a causative factor in C17.2 NF-κB inhibition, TNFR expression became the next candidate for investigation. There are two receptors for TNFα: TNFR1 and TNFR2, also known as p55 and p75, respectively (Smith et al, 1994). TNFR1 and TNFR2 expression has been reported on NPC and microglial cell lines (Cacci et al, 2005). The response to soluble TNFα is predominantly driven by TNFR1 interaction (Grell et al, 1998). Therefore, focus was placed on analysis of TNFR1 expression in C17.2.

TNFR1 expression was measured with the use of a phycoerythrin-associated anti-TNFR1 antibody. After antibody labelling, fluorescence from phycoerythrin excitation was
measured via flow cytometry (see Chapter 2). All experiments were performed in triplicate: each replicate involved collecting fluorescence measurements from 10,000 cells. TNFR1 expression was measured in unstimulated and 20ng/ml TNFα-stimulated wild-type C17.2 and C17.2 (lenti) cells, 2 h after stimulation. At this time point, little to no nuclear p65 was detected in co-cultured C17.2 (lenti) cells, whereas some cells in mono-culture were still exhibiting nuclear p65 translocations (see Figure 4.2). Mean TNFR1-associated fluorescence was calculated and compared across C17.2 cell lines (Figure 4.20). Mean fluorescence was also normalised to corresponding unstimulated fluorescence to represent TNFα-induced changes in TNFR1 expression (Figure 4.21). Mann Whitney U tests were performed to determine statistical significance. Representative flow cytometry profiles are shown alongside each individual comparison.

Figure 4.20. TNFR1-associated fluorescence in unstimulated and TNFα-stimulated wild-type C17.2 cells and C17.2 (lenti) cells.

TNFR1 expression was measured in wild-type C17.2 and C17.2 (lenti) cells, using an anti-TNFR1 antibody. Labelling was conducted in unstimulated and 20ng/ml TNFα-stimulated cultures (2 h after stimulation). Flow cytometry was used to measure TNFR1-associated fluorescence; all experiments were performed in triplicate. (A) and (B) Mean fluorescence of cells in unstimulated cultures and representative flow cytometry profile, respectively. (C) and (D) Mean fluorescence of cells in TNFα-treated cultures and representative flow cytometry profile, respectively. (E) and (F) TNFα-induced changes in fluorescence in wild-type C17.2 cells and representative flow cytometry profile, respectively. (G) and (H) TNFα-induced changes in fluorescence in C17.2 (lenti) cells and representative flow cytometry profile, respectively. Bars represent ± S.D. Mann Whitney U tests were performed on this data: no significant differences were found.
Figures 4.20A and C show mean fluorescence in unstimulated and TNFα-stimulated C17.2 cell lines, respectively. Statistical analysis showed that differences in fluorescence were not significant, suggesting comparable TNFR1 expression between C17.2 cell lines, in unstimulated and TNFα-stimulated conditions. TNFα-induced changes in mean fluorescence in wild-type C17.2 and C17.2 (lenti) cells are displayed in Figures 4.20E and 4.20G, respectively. No significant differences were detected within cell lines (unstimulated versus TNFα-stimulated TNFR1 expression). These results supported use of the C17.2 (lenti) cell line as representative of TNFR1 expression in wild-type C17.2. This was important for co-culture experiments: C17.2 (lenti) could be separated from untransfected BV.2 cells, according to eGFP fluorescence.

Figure 4.21 displays the mean fluorescence of unstimulated and TNFα-treated C17.2 (lenti) cells in mono- and co-cultures. No difference was observed in TNFR1-associated fluorescence between mono-cultured and co-cultured C17.2 (lenti) cells in unstimulated and TNFα-treated conditions (Figures 4.21A and C, respectively). A key question was whether these cells displayed greater decreases in TNFR1 expression in co-culture, compared to mono-cultures. On average, mono-cultured C17.2 (lenti) cells exhibited a 17% decrease in TNFR1-associated fluorescence, after 2h TNFα-stimulation (Figure 4.20): this was similar to the decrease exhibited by co-cultured C17.2 (lenti) cells (15%; see Figure 4.21). These results strongly suggest that variations in TNFR1 expression levels are not a causative factor in the differences in C17.2 p65 response, across culture conditions (seen in Figure 4.2).
NF-kappaB in Neuroinflammation

Figure 4.21. TNFR1-associated fluorescence in unstimulated and TNFα-stimulated C17.2 (lenti) cells.

TNFR1 expression was measured in C17.2 (lenti) cells, in mono-cultures and 1:1 co-cultures (with BV.2 cells), using an anti-TNFR1 antibody. Labelling was conducted in unstimulated and 20ng/ml TNFα-stimulated cultures (2 h after stimulation). Flow cytometry was used to measure TNFR1-associated fluorescence; all experiments were performed in triplicate. (A) and (B) Mean fluorescence of cells in unstimulated cells and representative flow cytometry profile, respectively. (C) and (D) Mean fluorescence of cells in TNFα-treated cultures and representative flow cytometry profile, respectively. (E) and (F) TNFα-induced changes in fluorescence in co-cultured C17.2 cells and representative flow cytometry profile, respectively. Bars represent ± S.D. Mann Whitney U tests were performed on this data; no significant differences were found.
4.3 Discussion

Most studies on NF-κB activation and p65 dynamics have been conducted in non-neural cell lines (Nelson et al, 2004; Ashall et al, 2009; Turner et al, 2010; Paszek et al, 2010) and paracrine effects on p65 dynamics have yet to be investigated in mixed cell cultures. Together, NPCs and microglia are vital governors of neuroinflammatory outcome: NPCs regulate microglial proliferation and activation (Forstreuter et al, 2002; Mosher et al, 2012) and, in turn, microglia regulate NPC survival, proliferation and differentiation (Liu et al, 2014; Liu et al, 2013a; Liu et al, 2013b; Guadagno et al, 2013). Other studies have shown that NF-κB activation can determine NSC fate (Widera et al, 2006a; Widera et al, 2006b; Piotrowska et al, 2006), as well as microglial activation status (Saijo and Glass, 2011; Colton, 2009). Taken together, these observations provide a strong argument for the study of NF-κB in NPCs and microglia, in mono-cultures and co-cultures.

Here, for the first time, p65 dynamics in the neural progenitor and microglial cell lines, C17.2 and BV.2 respectively, have been characterised in mono-cultures and co-cultures. To complement this data, transcriptional output of NF-κB in C17.2 was measured and compared across culture conditions. The major findings of this investigation are as follows:

1) Mono-cultured and co-cultured C17.2 mostly displayed single nuclear p65 translocations, in response to TNFα;

2) BV.2 inhibited TNFα-induced p65 nuclear translocations in C17.2 (in 1:1 co-cultures);

3) BV.2 and RAW267.4 both inhibited TNFα-stimulated NF-luc induction in 1:4 co-cultured C17.2: the effect of BV.2 cells was independent of cell-contact;

4) Basal NF-luc expression was induced by BV.2 cells in C17.2, in a cell-contact-dependent manner (in 1:4 and 1:8 BV.2 co-cultures);

5) Mono-cultured and co-cultured BV.2 exhibited oscillatory p65 dynamics, in response to TNFα.

6) Co-cultured BV.2 displayed later initial nuclear p65 peaks and faster oscillatory p65 dynamics, compared to mono-cultured cells (in 1:1 co-cultures).

The dynamics of p65 in C17.2 and BV.2 cultures will be compared to previous observations in other cell lines. Co-culture findings will be used to formulate new hypotheses on the mechanisms of paracrine interactions between C17.2 and BV.2 and, thereby, NPCs and microglia.
4.3.1. NF-κB in Mono-cultured and Co-cultured C17.2

4.3.1.1. TNFα-induced Single-cell p65 Dynamics in C17.2, in Mono-cultures and 1:1 BV.2 Co-cultures

In contrast to most cell lines, which show oscillatory p65 dynamics in response to TNFα, C17.2 cells displayed a single nuclear translocation of p65 (Ashall et al, 2009; Turner et al, 2010). Some of these cells exhibited sustained and fluctuating nuclear p65 presence. Co-cultured C17.2 exhibited, on average, lower amplitude initial p65 nuclear translocations and a more transient nuclear p65 presence (Figures 4.2-4.3).

TNFα availability was initially investigated as a possible explanation for the differences observed across co-cultures conditions: TNFα ELISA results showed no difference in TNFα availability between mono-cultures and co-cultures (see Figure 4.19). Next, TNFα-stimulated TNFR1 expression was measured in mono-cultured and co-cultured C17.2: TNFR1 signalling dominantly drives the response to soluble TNFα (Grell et al, 1998). TNFR1 expression analysis concluded with no significant differences being observed across culture conditions (see Figures 4.20-4.21).

An important point to note is that differences in TNFR1 expression in a few C17.2 cells may have been masked in the population-level analysis conducted here. To improve on this analysis, future experiments should focus on TNFR1 expression in 1:4 co-cultured C17.2, where differences in TNFR1 may be more pronounced. Alternatively, TNFR1 expression could be observed on a single-cell level, ideally in live cells so that TNFR1 expression can be directly correlated with p65 dynamics. The potential involvement of other factors, such as TNFR2 and non-TNFα related signals, is discussed in the following section.

4.3.1.2. Population-level NF-luc Induction in C17.2, in Mono-cultures and Co-cultures

To investigate differences observed in the single-cell C17.2 p65 response to TNFα, NF-luc induction was characterised in co-cultured C17.2. NF-luc transfected C17.2 cells were cultured with BV.2 in 1:2, 1:4 and 1:8 (C17.2:BV.2) co-cultures. It was thought that increasing the co-culture ratio would enhance the inhibition of TNFα-induced p65 nuclear translocations in C17.2. This inhibition would be represented by decreases in C17.2 NF-luc induction. C17.2 in 1:4 BV.2 co-cultures displayed lower peak NF-luc induction levels, compared to mono-cultured cells. C17.2 in 1:4 RAW267.4 co-cultures also displayed the same dampened induction levels, indicating that inhibition of C17.2 NF-luc induction is a trait that is shared by BV.2 and RAW267.4.
Interestingly, BV.2 did not exert an inhibitory effect on C17.2 NF-luc induction in 1:2 or 1:8 (C17.2:BV.2) co-cultures. Basal C17.2 NF-luc expression was higher in 1:4 BV.2, 1:8 BV.2 and 1:4 RAW267.4 co-cultures: this effect was cell-contact dependent in BV.2 co-cultures. On the other hand, inhibition of TNFα-stimulated NF-luc induction was independent of cell-contact. Co-culture effects will now be discussed in two parts: 1) cell-contact-dependent factors which may be involved in basal NF-luc induction and; 2) soluble factors that may contribute to inhibition of TNFα-driven NF-luc induction and nuclear p65 translocations.

TNFR1 expression was ruled out as a player in co-culture effects; this leaves the possibility of TNFR2 involvement in cell-contact dependent effects. Compared to TNFR1, TNFR2 has a low affinity for soluble TNFα. Instead, TNFR2 is preferentially activated by the transmembrane form of TNFα (mTNFα; Grell et al, 1998), which is expressed on microglia (Zhou et al, 2010). Cell-contact dependent increases in basal NF-luc expression may have been due to interactions between C17.2 TNFR2 and BV.2 mTNFα. This would be an interesting area to study in the future, as mTNFα can also act as a receptor and reverse signal to cells (Xin et al, 2006).

Paracrine or cell contact-dependent interactions between microglia and NPCs are largely unknown. However, Liu et al (2013) found a role for CD200 in mediating NPC-microglial interactions. CD200 is a cell-surface ligand, expressed throughout the cerebellum, the same area from which the C17.2 cell line originates (Ryder et al, 1990). The receptor for CD200 (CD200R) is expressed on microglia (Liu et al, 2013a) and can signal to induce IL-4 and IL-10 expression (Gorczynski, 2001), as well as inhibit classical activation of microglia (Broderick et al, 2002). CD200 and CD200R expression is reportedly upregulated in co-cultured NPCs and microglia, respectively (Liu et al, 2013a).

As CD200 expression is NF-κB-dependent (Pahl, 1999), it could be hypothesised that C17.2 expression of CD200, induced by TNFα, interacts with CD200R on BV.2 cells in co-culture. This may cause the induction of IL-4 and/or IL-10 expression, which feeds back into the C17.2 NF-κB signalling pathway and inhibits p65 nuclear translocations. However, this explanation does not account for inhibition of C17.2 NF-luc induction, seen in transwell co-cultures (Figure 4.11); considering the network complexity, it is likely that multiple different mechanisms are converging to produce the co-culture effects observed.
4.3.2. Single-cell p65 Dynamics in Mono-cultured and Co-cultured BV.2

Consistent with observations in other cell lines, 93% and 100% of mono-cultured and co-cultured BV.2 cells, respectively, exhibited oscillatory p65 dynamics in response to TNFα. Co-cultured BV.2 displayed faster oscillations on average (63 min versus 108 min observed in mono-cultured BV.2).

Reverse signalling is largely uninvestigated in microglia, but it may be play a role in the effects of C17.2 on BV.2 p65 dynamics. Reverse signalling has been shown to influence the expression of important inflammatory genes, such as IL-2, IL-6, IL-10 and TNFα (Xin et al, 2006). It is thought that TNFR1 may be cleaved in response to TNFα stimulation to produce soluble TNFR1 (Wajant et al, 2003). Activation of mTNFα, using soluble TNFR1, enhances later soluble-TNFα driven IκBα degradation in a human lymphocytic cell line (Xin et al, 2006). Using this data, it could be hypothesised that C17.2-derived soluble TNFR1 abrogates the influence of inhibitory agents in the culture media and modifies BV.2 NF-κB activity. The effects of C17.2-BV.2 contact can be investigated in future experiments with the use of NF-luc transduced BV.2 cells.
5. Analysis of Co-culture- andTNFα-induced Gene Expression Changes
5.1 General Introduction

The main aim of this work was to better understand the role of neural progenitor cell-microglial cross-talk in neuroinflammation. On the molecular level, NF-κB-mediated interactions were observed in C17.2 and BV.2 reporter cell lines, after TNFα stimulation in 1:1 co-cultures. Notably, co-cultured C17.2 cells displayed dampened κB-driven transcription activity and lower amplitude nuclear p65 translocations, compared to monocoloncultured cells. Also, there was preliminary evidence of C17.2-driven changes in TNFα-induced BV.2 p65 dynamics.

Having established co-culture effects on TNFα-induced NF-κB dynamics, the next goal of this work was to investigate downstream TNFα-induced changes in gene expression profiles. This work was conducted with a particular focus on associating differential NF-κB dynamics with distinct gene expression profiles. In order to simultaneously quantify the expression of hundreds of immune genes, NanoStringTechnology was utilised (Geiss et al, 2008). This involves a multiplexed probe library that contains two-sequence specific probes for each target RNA molecule: a capture probe and a reporter probe. The capture probes also contain a common sequence bound to an affinity tag e.g. biotin, and the reporter probe is coupled to a colour-coded tag. Probe hybridization to target RNA sequences results in a tripartite structure, which can be purified using the affinity tag in the capture probe and the appropriate capture reagent e.g. streptavidin. Colour-coded tags on the capture probe enable identification and absolute quantification of target RNA sequences (Geiss et al, 2008).

The nCounter Mouse Inflammation Kit v1 was selected to assess mRNA expression of 185 inflammatory genes, including transcription factors, chemokines and pro- and anti-inflammatory cytokines.

RNA from untreated and TNFα-stimulated cultures could be analyzed to see how gene expression in mono-cultures and 1:1 co-cultures was affected by TNFα. Transwell inserts would create a physical barrier between cell types, in order to observe the effects of paracrine factors on gene expression changes. Comparison of mono-cultures with transwell co-cultures would potentially identify gene expression changes driven by C17.2-BV.2 interactions.

This chapter describes gene expression changes at two different time points after TNFα-stimulation: 230 min and 24 h. The 24 h time point was selected for analysis to observe
long-term effects of TNFα stimulation on gene expression. The 230 min time point was selected for two reasons. Firstly, previous and current members of the Prof. White laboratory group have constructed a comprehensive microarray database of TNFα-induced gene expression in SK-N-AS cells, using a range of time points (unpublished data): this data shows that the 230 min time point is optimum for observing TNFα-induced gene expression changes in early, middle and late responding genes (Dr. W. Rowe, personal communication).

Secondly, the suitability of this time point was established by results shown in Chapter 4: p65 localisation within mono-cultured C17.2 was different to that of co-cultured C17.2, after TNFα-stimulation (Figure 4.2). More specifically, TNFα-induced p65 dynamics were inhibited, between 0 and 230 min after stimulation, in co-cultured C17.2 (compared to mono-cultured C17.2). Moreover, some C17.2 cells in mono-cultures exhibited nuclear p65 localisation at 230 min post-stimulation, whereas nuclear p65 in co-cultured C17.2 had returned to pre-stimulation levels before this time point. These findings strongly suggested that different NF-κB-dependent gene expression profiles would be obtained from mono-cultured and co-cultured C17.2 cells at 230 min post-TNFα stimulation.

In summary, this chapter contains a preliminary analysis of TNFα- and co-culture-induced gene expression changes, in C17.2 and BV.2 mono-cultures and 1:1 co-cultures, with and without transwells; NF-κB-dependent genes are the focus of this analysis.

5.2 Results

Cells were seeded in mono-cultures and 1:1 co-cultures, with and without transwells i.e. BV.2 cells were seeded with transwell inserts containing C17.2 cells and, conversely, C17.2 cells were seeded with transwells containing BV.2: these cultures are referred to as ‘BV.2 transwell co-cultures’ and ‘C17.2 transwell co-cultures’, respectively. These transwell co-cultures prevented contact between C17.2 and BV.2, but allowed paracrine communication via permeable transwell membranes. Contact between C17.2 and BV.2 was permitted in 1:1 co-cultures that were seeded without transwells.

The day after seeding, these cultures were left untreated or stimulated with 20ng/ml TNFα. At the appropriate time point (i.e. 230 min or 24 h after stimulation), RNA was extracted from all cultures: in transwell co-cultures, RNA was extracted from the cell type that was
seeded directly into wells (and not the cell type seeded into transwells). Each experiment was performed in duplicate or triplicate.

For each condition, 125ng of extracted RNA was analysed for mRNA expression level using NanoString Technology. Gene expression was normalised to internal house-keeping genes and positive controls. The resulting expression levels were corrected by subtracting negative controls, after which data across replicates was averaged. All data presented in this chapter was derived from these average values.

It should be noted that some genes were not highly expressed so subtraction by negative control values resulted in gene expression values below one. These values were all corrected to one, in order to enable ease of analysis.

5.2.1. Gene Expression in Untreated C17.2 and BV.2 Mono-cultures

Throughout this chapter, fold differences are calculated between different sets of averages: statistical analysis was not conducted, due to the low number of replicates. A fold threshold of 2 was selected as a filter to account for natural variability in gene expression and, thereby, avoid false positive results.

For example, say C17.2 average expression of gene A was 2 in unstimulated mono-cultures and 10, after 230 min TNFα-stimulation. This would equate to a fold change of 5 (i.e. 10/2); therefore, gene A would be of interest here. However, differences in gene expression levels between C17.2 and BV.2 may mask upregulation of gene A in co-culture. Following the previous example, if BV.2 average expression of gene A was 2000, then unstimulated 1:1 co-culture gene A expression would be 1001 (i.e. (2000 + 2)/2). In these co-cultures, a 5-fold upregulation of gene A in C17.2 cells would lead to an average co-culture expression of 1006 (i.e. 1001 + (1*5)): this would equate to a fold change of 1.005, leading to gene A being discounted as a gene of interest.

To ensure awareness of potential false negatives in this analysis, a comparison of expression levels was performed between C17.2 and BV.2 in unstimulated mono-cultures. The average expression levels of genes were plotted, with C17.2 expression level along the x-axis and BV.2 expression level along the y-axis (Figure 5.1).

Figure 5.1 shows differences in gene expression between C17.2 and BV.2, which may result in false negative results. More specifically, some genes that were highly expressed in
BV.2 mono-cultures were expressed at low levels in C17.2 cultures. This disadvantage is noted here and improvement on future techniques is discussed later.

Figure 5.1. A comparison of gene expression in unstimulated C17.2 and BV.2 mono-cultures.
RNA was extracted from unstimulated C17.2 and BV.2 cells. Samples were analysed for the mRNA level of 185 genes. Experiments were performed in duplicate or triplicate and average mRNA levels are displayed here (on a logarithmic scale). Each gene is represented by a single point.

5.2.2. TNFα-induced Changes in Gene Expression at 230 min Post-TNFα-Stimulation
A major goal of this chapter was to observe changes in gene expression, induced by 230 min TNFα-stimulation, in C17.2 and BV.2 mono-cultures and 1:1 co-cultures. To do this, fold differences in average expression of each gene was calculated between untreated cultures and TNFα-treated cultures: TNFα-induced upregulation of a gene would result in a positive fold change, whereas downregulation would produce a negative fold change. A fold threshold of two was selected to filter out any false positive results. Genes that exhibited a fold change ≥2 or ≤-2 are displayed in Figure 5.2A, and the corresponding fold changes are displayed in Figure 5.2B.
Figure 5.2. Genes upregulated/downregulated, at 230 min post-20ng/ml TNFα-stimulation, in C17.2 and BV.2 mono-cultures and 1:1 co-cultures.

RNA was extracted from C17.2 and BV.2 mono-cultures and 1:1 co-cultures, at 230 min post-20ng/ml TNFα stimulation. Each experiment was performed in duplicate or triplicate and mRNA levels were detected using NanoString technology. Fold differences in mRNA expression between unstimulated and TNFα-stimulated cultures was calculated. (A) Genes induced by a fold change of ≥2 or ≤-2 (genes in blue and red were downregulated and upregulated, respectively). (B) Fold changes of mRNA levels of genes in A.
Figure 5.2A shows that C1qb, Csfl, Il23r and Ripk2 genes were differentially regulated by TNFα in C17.2 mono-cultures and not in 1:1 co-cultures. Moreover, Ripk2 is an NF-κB-dependent signalling molecule in the canonical NF-κB activation pathway (Matsuda et al, 2003; Devin et al, 2000). Together, this suggests that Ripk2 upregulation may be dependent on aspects of p65 dynamics that were only observed in mono-cultured C17.2 cells. Alternatively, given the low fold change (2.2), it is likely that C17.2 upregulation of Ripk2 was masked in 1:1 co-cultures.

BV.2 cells upregulated the expression of pro-inflammatory factors and, notably, NF-κB activators (e.g. Cd40, Il1a, Il1b and Tnf; Pahl, 1999). All genes that were differentially regulated by TNFα in co-cultures were also similarly altered in one or both mono-cultures e.g. Ccl5 was upregulated in 1:1 co-cultures and also C17.2 mono-cultures, suggesting that Ccl5 upregulation in co-cultures can be attributed to C17.2 cells.

5.2.3. TNFα and Co-culture-induced Gene Expression Changes at 230 min Post-TNFα Stimulation

Having established TNFα-dependent gene expression changes, the combined effects of TNFα and paracrine interactions were investigated using transwell co-cultures. C17.2 and BV.2 cells were seeded with BV.2 and C17.2 cells in transwell inserts, respectively (at a 1:1 ratio). RNA was extracted, 230 min after TNFα-stimulation, from one cell population in these transwell co-cultures i.e. RNA from C17.2 cells in transwell co-cultures is referred to as ‘C17.2 transwell co-cultures’. Average fold changes in gene expression were calculated between untreated and TNFα-treated transwell co-cultures: results are displayed in Figure 5.3, along with genes that were differentially regulated by TNFα in contact-permissive 1:1 co-cultures.

Comparison of Figures 5.2 and 5.3 shows evidence of TNFα- and co-culture-dependent gene expression changes. For example, Ccl17 is upregulated in TNFα-treated C17.2 transwell co-cultures but not in C17.2 mono-cultures. Therefore, Ccl17 upregulation is dependent on TNFα-induced paracrine interactions with BV.2.

Interestingly, TNFα-induced Il1a upregulation was seen in BV.2 mono-cultures, but not in BV.2 transwell co-cultures: this suggests that C17.2 can mediate inhibition of BV.2 pro-inflammatory gene expression (discussed later in this chapter).
NF-kappaB in Neuroinflammation

**Figure 5.3. Genes upregulated/downregulated at 230 min post-20ng/ml TNFα-stimulation, in transwell co-cultures and 1:1 contact-permissive co-cultures.**

C17.2 and BV.2 cells were seeded with transwell inserts containing BV.2 and C17.2 cells, respectively. Cells were also seeded in 1:1 contact-permissive co-cultures. Cultures were stimulated with 20ng/ml TNFα and RNA was extracted 230 min after stimulation. Each experiment was performed in duplicate and mRNA levels were detected using NanoString technology. Fold differences in mRNA expression between unstimulated and TNFα-stimulated cultures was calculated. (A) Genes induced by a fold change of ≥2 or ≤-2 (genes in blue and red were downregulated and upregulated, respectively). (B) Fold changes of mRNA levels of genes in A.
5.2.4. Co-culture-induced Gene Expression Changes, 28 h after Co-culturing

The previous analysis performed highlighted gene expression changes that were dependent on TNFα and co-culture-interactions (at 230 min post-TNFα-stimulation, which was approximately 28 h after seeding cultures). To investigate gene expression changes that were induced as a result of co-culturing cells, fold differences in gene expression were calculated between untreated mono-cultures and corresponding transwell co-cultures. For example, untreated gene expression in C17.2 mono-cultures was compared to that of untreated C17.2 transwell co-cultures: this would highlight genes that were differentially regulated by paracrine interactions with BV.2. Accordingly, the same analysis was conducted with untreated BV.2 mono-cultures and transwell co-cultures.

This analysis highlighted 3 genes that were differentially regulated by paracrine interactions between C17.2 and BV.2: C1qb, Il15 and Tlr3. C1qb was inhibited by 39.6 fold in untreated C17.2 transwell co-cultures, compared to untreated C17.2 mono-cultures. In untreated BV.2 transwell co-cultures, Il15 was upregulated and Tlr3 was inhibited, compared to the corresponding mono-cultures. It should be noted that the fold changes of Il15 and Tlr3 expression were close to the 2-fold threshold set for filtering out false positives: these results are therefore less reliable than the results obtained on C1qb.

5.2.5. TNFα-induced Changes in Gene Expression at 24 h Post-TNFα-Stimulation

To assess long-term effects of TNFα treatment on cells, RNA samples were extracted after 24 h 20ng/ml TNFα-stimulation. As with gene expression data from 230 min RNA samples, TNFα-induced gene expression changes were analysed: average gene expression in untreated cultures was compared to that of the corresponding TNFα-stimulated cultures. Again, genes that exhibited a fold change ≥2 or ≤-2 are displayed in Figure 5.4A, and the corresponding fold changes are displayed in Figure 5.4B.

The gene set displayed in Figure 5.4 is dominated by chemokines, such as Ccl2, Ccl5 and Ccl7, as well as NF-κB dependent genes and NF-κB activators e.g. Nos2, Il1a, Il1b and Tnf (Pahl, 1999).

Il1a, Il1b and Tnf were upregulated in BV.2 mono-cultures, and not in C17.2 mono-cultures, which suggests that BV.2 were also responsible for the upregulation of these genes in 1:1 co-cultures. Moreover, upregulation of Il1a, Il1b and Tnf genes was observed in the same culture conditions at 230 min post-TNFα stimulation (see Figure 5.2A): this is
consistent with the role of microglia in propagating neuroinflammatory signals (Nakajima and Kohsaka, 2001; Henn et al, 2009).

**Figure 5.4. Genes upregulated/downregulated, at 24 h post-20ng/ml TNFα-stimulation, in C17.2 and BV.2 mono-cultures and 1:1 co-cultures.**

RNA was extracted from C17.2 and BV.2 mono-cultures and 1:1 co-cultures, at 24 h post-20ng/ml TNFα stimulation. Each experiment was performed in duplicate or triplicate and mRNA levels were detected using NanoString technology. Fold differences in mRNA expression between unstimulated and TNFα-stimulated cultures was calculated. (A) Genes induced by a fold change of ≥2 or ≤-2 (genes in blue and red were downregulated and upregulated, respectively). (B) Fold changes of mRNA levels of genes in A.
More genes were differentially regulated by TNFα in BV.2 mono-cultures and 1:1 co-cultures at 24 h- than at 230 min-post-TNFα stimulation (compare Figures 5.2A and 5.4A). Together, this suggests that TNFα-stimulation has a persistent and ongoing effect on BV.2 gene expression. In contrast, the same number of genes were differentially regulated in C17.2 mono-cultures at both time points.

Another similarity between results from 230 min and 24 h post TNFα-stimulation was in the regulation of Ccl5: this gene was more strongly upregulated than other genes at both time points, primarily by C17.2 cells. Ccl5 is an NF-κB dependent gene, involved in attracting immune cells to sites of inflammation (Wickremasinghe et al, 2004).

5.2.6. TNFα and Co-culture-induced Gene Expression Changes at 24 h Post-TNFα-Stimulation

Analysis of TNFα- and co-culture- induced gene expression changes were conducted using C17.2 transwell, BV.2 transwell and 1:1 contact-permissive co-cultures: fold changes in gene expression were calculated between untreated cultures and corresponding TNFα-stimulated cultures. Genes that exhibited a fold change ≥2 or ≤-2, after 24 h TNFα-treatment, are displayed in Figure 5.5A, and the corresponding fold changes are displayed in Figure 5.5B.

C17.2 transwell co-cultures and mono-cultures both downregulated Fos expression after TNFα-stimulation (Figures 5.4A and 5.5A), whereas Il15 and Tlr1 were differentially regulated only in C17.2 transwell co-cultures. Cd40 upregulation, observed in C17.2 mono-cultures, was prevented in C17.2 transwell co-cultures. Together, this provides strong evidence for TNFα- and paracrine interaction-dependent changes in C17.2 gene expression.

BV.2 cells also displayed co-culture- and TNFα-induced changes in gene expression: Nos2 was upregulated in BV.2 transwell co-cultures, and not in mono-cultured cells, after TNFα-stimulation. Therefore, C17.2- BV.2 interactions are bidirectional and result in differential gene regulation by both cell types, after TNFα-stimulation.
NF-kappaB in Neuroinflammation

Figure 5.5. Genes upregulated/downregulated at 24 h post-20ng/ml TNFα-stimulation, in transwell co-cultures and 1:1 contact-permissive co-cultures.

C17.2 and BV.2 cells were seeded with transwell inserts containing BV.2 and C17.2 cells, respectively. Cells were also seeded in 1:1 contact-permissive co-cultures. Cultures were stimulated with 20ng/ml TNFα and RNA was extracted 24 h after stimulation. Each experiment was performed in duplicate and mRNA levels were detected using NanoString technology. Fold differences in mRNA expression between unstimulated and TNFα-stimulated cultures was calculated. (A) Genes induced by a fold change of ≥2 or ≤-2 (genes in blue and red were downregulated and upregulated, respectively). (B) Fold changes of mRNA levels of genes in A.
5.2.7. Co-culture-induced Gene Expression Changes, 48 h after Co-culturing

Previous analysis performed highlighted differences in gene expression between untreated mono-cultures and the corresponding untreated transwell cultures. This analysis was conducted on RNA samples extracted from cells which had been growing in transwell co-cultures for approximately 28 h. Here, we look at RNA samples that were extracted approximately 48 h after co-cultures were seeded.

Fold differences were calculated between average gene expression in untreated mono-cultures and the corresponding untreated transwell co-cultures e.g. gene expression in untreated C17.2 mono-cultures was compared to that of untreated C17.2 transwell co-cultures. The same analysis was conducted for BV.2 mono-cultures and transwell co-cultures (results are shown in Table 5.1).

| Table 5.1: Genes that were differentially expressed between mono-cultures and transwell co-cultures. Fold differences in gene expression were calculated between untreated C17.2/BV.2 mono-cultures and the corresponding transwell co-cultures (after co-culturing for 48 h). |
|-----------------|--------|-----------------|
|                 | C17.2  | BV.2            |
| C3              | -2.6   |                 |
| Ccr1            |        | 3.9             |
| Creb1           | -2.3   |                 |
| Fos             | 2.6    |                 |
| Hras1           | -34.5  |                 |
| Il1b            |        | 3.2             |
| Il1r1           |        | 7.5             |
| Il8rb           |        | 2.3             |
| Myc             |        | 2.4             |
| Nos2            |        | 36.3            |
| Ppp1r12b        | -2.3   |                 |

Fos expression was higher in C17.2 transwell co-cultures than C17.2 mono-cultures. Fos upregulation has been related to increases in Cyclin D1, D2 and D3, resulting in NPC proliferation (Adepoju et al, 2014). Also, Myc was upregulated in BV.2 transwell cultures: the transcription factor, Myc, is responsible for controlling the expression of proliferation genes (Bretones et al, 2014). This suggests that paracrine interactions between C17.2 and BV.2 may mediate changes in the proliferation profile of these cells.

In summary, many inflammatory genes were differentially regulated according to treatment, time, and co-culture interactions between C17.2 and BV.2 cells. In particular,
many NF-κB dependent genes and activators were highlighted by the analysis conducted here: these include Tnf, Il1a, Il1b, Ccl5 and Nos 2 (Pahl, 1999). To assess NF-κB contribution to gene regulation in these analyses, Table 5.2 was compiled to show a list of the NF-κB-dependent genes that were differentially regulated (seen in Figures 5.2-5.5).

**Table 5.2: A collated list of NF-κB-dependent genes that were differentially regulated, at different time points, by TNFα or co-culture interactions in: C17.2 or BV.2 mono-cultures, transwell co-cultures or 1:1 contact-permissive co-cultures (seen in Figures 5.2-5.5).** (Reference: http://www.bu.edu/NF-κB/gene-resources/target-genes/, visited on 24/09/14).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Time point</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3</td>
<td>24 h</td>
</tr>
<tr>
<td>Ccl2</td>
<td>230 min and 24 h</td>
</tr>
<tr>
<td>Ccl5</td>
<td>230 min and 24 h</td>
</tr>
<tr>
<td>Ccl17</td>
<td>230 min</td>
</tr>
<tr>
<td>Cxcl1</td>
<td>230 min and 24 h</td>
</tr>
<tr>
<td>Cxcl10</td>
<td>230 min and 24 h</td>
</tr>
<tr>
<td>Cd40</td>
<td>230 min and 24 h</td>
</tr>
<tr>
<td>Csf1</td>
<td>230 min</td>
</tr>
<tr>
<td>Fos</td>
<td>24 h</td>
</tr>
<tr>
<td>Il1a</td>
<td>230 min and 24 h</td>
</tr>
<tr>
<td>Il1b</td>
<td>230 min and 24 h</td>
</tr>
<tr>
<td>Il15</td>
<td>230 min and 24 h</td>
</tr>
<tr>
<td>Il23a</td>
<td>24 h</td>
</tr>
<tr>
<td>Myc</td>
<td>24 h</td>
</tr>
<tr>
<td>Nos</td>
<td>24 h</td>
</tr>
<tr>
<td>Ripk2</td>
<td>230 min</td>
</tr>
<tr>
<td>Tlr-2</td>
<td>230 min and 24 h</td>
</tr>
<tr>
<td>Tnf</td>
<td>230 min and 24 h</td>
</tr>
</tbody>
</table>

As can be seen in Table 5.2, many NF-κB-dependent genes were differentially regulated at 230 min and 24 h after TNFα-stimulation (Figures 5.2-5.5), as well as in untreated transwell co-cultures (compared to mono-cultures; Table 5.1). Together, these results suggest that NF-κB plays an important role in TNFα- and co-culture induced gene expression changes in C17.2 and BV.2: these results require further investigation in the future.

### 5.3 Discussion

In this chapter, NanoString technology was used to analyse the expression of 185 inflammatory genes in different C17.2 and BV.2 cultures. As this was a preliminary
screening, findings will be discussed only briefly. The goal of this discussion will be to generate hypotheses on NF-κB-mediated interactions in co-cultures and, via a critique of the methods used in this chapter, design future experiments to test new hypotheses.

5.3.1. Potential NF-κB-mediated Interactions between C17.2 and BV.2

Analysis of gene expression highlighted differential gene regulation by TNFα in C17.2 and BV.2 mono-cultures and 1:1 co-cultures, as well as transwell co-cultures. BV.2 cells consistently upregulated the expression of proinflammatory cytokines. This finding is supported by data in the literature which reports microglial upregulation of pro-inflammatory cytokines, in response to inflammatory stimuli (Guadagno et al, 2013; Colton, 2009; Saijo and Glass, 2011).

More specifically, BV.2 consistently upregulated the expression of NF-κB-dependent pro-inflammatory cytokines, Il1a, Il1b and Tnf, after TNFα treatment (Pahl, 1999). These genes can be used as markers for microglial classical activation (Colton, 1999; Saijo and Glass, 2011). Interestingly, C17.2 cells in transwell inserts prevented BV.2 upregulation of Il1a at 230 min post-TNFα-stimulation; this effect was not reproduced in contact-permissive 1:1 co-cultures (see Figures 5.2 and 5.3). Together, this suggests cell-contact-dependent or juxtacrine regulation of IL-1 expression in BV.2.

Upstream of IL-1 expression, these results could relate to differences in BV.2 p65 dynamics (reported in Chapter 4; Figure 4.15). TNFα stimulation induced p65 oscillations in both mono-cultured and co-cultured BV.2 cells, however, the average period of these oscillations differed between culture conditions (108 min versus 63 min, respectively). As Il1a expression is a marker of classical activation, this suggests that differential p65 dynamics determines microglial activation status. From this, it could be hypothesised that C17.2 inhibits BV.2 activation by modulating BV.2 p65 dynamics. This hypothesis contradicts findings that report NPC-driven microglial classical activation (Mosher et al, 2012).

However, microglial activation status is not discrete and, therefore, the use of one or two markers of activation is not sufficient for determining microglial behaviour. Moreover, Nos2 expression was upregulated after 24 h TNFα-stimulation in BV.2 transwells, and not in BV.2 mono-cultures. This finding suggests that C17.2 promote BV.2 classical activation. These seemingly contradictory findings draw attention to one of the advantages
of NanoString Technology: the simultaneously quantification of hundreds of mRNA molecules. Use of this technology in the future will shed more light on the spectrum of microglial activation status and, therefore, their effects in neuroinflammatory tissue.

As well as C17.2 effects on BV.2, NanoString analysis conducted in this chapter provided evidence of bidirectional paracrine interactions. An example of this was differential regulation of Cd40 in C17.2 cultures: Cd40 was upregulated in C17.2 mono-cultures (11.5 fold), after 24 h TNFα-stimulation. This upregulation was prevented by BV.2 cells in C17.2 transwell co-cultures. As Cd40 is an NF-κB activator (Hayden and Ghosh, 2004; Bonizzi and Karin, 2004), these results may relate to differences in C17.2 p65 dynamics that were reported in Chapter 4 (Figure 4.2). In response to TNFα-stimulation, p65 in mono-cultured C17.2 displayed a rapid, high amplitude nuclear translocation, followed by some irregular nuclear movements. Co-cultured C17.2, on the other hand, displayed an inhibited p65 response to TNFα-stimulation. It could be conjectured that differential p65 dynamics led to the differences observed in Cd40 expression. However, single-cell dynamics were observed from 0-300min after TNFα-stimulation and the effects reported here were measured some time later, at 24 h post-stimulation. Also, microscopy experiments were conducted with co-cultures that were seeded approximately 24 h before stimulation, whereas the results obtained on Cd40 expression were derived from C17.2 cells that had been co-cultured with BV.2 for 48 h: data outlined in this chapter provides evidence for time-dependent co-culture interactions. Moreover, C17.2-BV.2 cell-contact was enabled in microscopy experiments and not in the transwell co-cultures described here.

In summary, NanoString data outlined in this chapter has provided preliminary evidence for TNFα- and co-culture-mediated interactions between C17.2 and BV.2 cells, albeit inconclusive. More importantly, there is preliminary evidence to suggest that co-culture-induced p65 dynamics (reported in Chapter 4), can contribute to differential gene regulation in C17.2 and BV.2 cells.

The following section will critique the experimental design described in this chapter and discuss more thorough experiments to be performed in the future.
5.3.2. Future Experiments

As well as measuring TNFα-induced gene expression, the goal of this chapter was to observe differential gene expression that may be associated with differential p65 dynamics in mono-cultures and co-cultures (described in Chapter 4). One of the main findings described in Chapter 4 was the inhibition of C17.2 p65 dynamics in 1:1 contact-permissive co-cultures, after TNFα-stimulation. Certain aspects of NF-κB dynamics can determine downstream gene expression: IL-8, A20 and IkBα gene expression in single cells is regulated by the amplitude of p65 nuclear translocations (Lee et al, 2014). Also, pulsatile TNFα stimulation has been used to force p65 nuclear translocations, thereby modulating NF-κB-dependent gene expression (Ashall et al, 2009). Therefore, it was hypothesised that the differences in p65 dynamics observed in C17.2 cells might correspond to different NF-κB-dependent gene expression profiles.

In order to investigate this thoroughly in the future, RNA extracts should be taken from co-cultures that reproduce the conditions of the microscopy experiments performed (see Chapter 4). This entails seeding cells at the same density, and co-culturing cells for the same amount of time, before stimulating with TNFα. Most importantly though, 1:1 contact-permissive co-cultures should be used: transcriptional activity of NF-κB in C17.2 cells was higher in untreated contact-permissive co-cultures than in transwell co-cultures. Differences in NF-κB activity may affect TNFα-induced gene expression changes.

A major disadvantage of the NanoString technology is the inability to separate different cell populations in co-cultures, therefore this type of population-level analysis masks cell-cell heterogeneity. Instead, gene expression observations on a single-cell level would allow cell types to be distinguished in co-culture and also enable direct association of p65 dynamics with gene expression. A disadvantage to this method is that the expression of only a few genes can be observed in parallel in single-cell experiments. Lee et al (2014) observed TNFα expression only in fixed cells, which would not capture the dynamic nature of single-cell gene expression.

Recently, a microfluidics procedure has been developed to obtain high-throughput data on the expression of up to 96 genes and, in parallel, single-cell p65 dynamics (Kellogg et al, 2014). Multiple time points can be used with this technique to obtain more dynamic gene expression data. This technique is not suited to allow direct correlation of single-cell p65 dynamics with gene expression but it might be informative in certain culture conditions.
Luminometry results in Chapter 4 (Figure 4.5) showed that BV.2 inhibition of κB-driven transcription in C17.2 cells can be enhanced by increasing co-culture ratio i.e. 1:4 (C17.2: BV.2), instead of the 1:1 co-cultures utilised in microscopy experiments. By enhancing co-culture effects, NF-κB-dependent differential gene expression may be observable on a population-level, therefore, 1:4 co-culture experiments could be conducted with microfluidics in the future.
6. Effects of TNFα and NF-κB Activity on Neural Progenitor Cells and Microglia
6.1. General Introduction

Results in Chapter 4 detailed NF-κB-mediated interactions between C17.2 and BV.2. Specifically, BV.2 cells inhibited TNFα-induced p65 nuclear translocations in C17.2 (lenti) cells, in 1:1 co-cultures (C17.2:BV.2). On a population-level, in 1:4 co-cultures (C17.2:BV.2), C17.2 cells displayed increased, and inhibited, basal and TNFα-induced NF-κB transcriptional activity, respectively. Endpoint luminometry experiments with transwells showed that basal NF-luc induction was dependent on physical contact between C17.2 and BV.2 cells; TNFα-induced NF-luc inhibition occurred independent to this contact.

Having established these differences in NF-κB activation and dynamics, gene expression changes downstream of TNFα were investigated (Chapter 5). Expression of inflammatory genes was measured in C17.2 (UT) and BV.2 (UT) mono-cultures and 1:1 co-cultures, with and without transwells. The goal was to observe differential gene regulation that may be a consequence of changes in TNFα-induced NF-κB dynamics. Without the use of an NF-κB inhibitor in these experiments, it is difficult to attribute any gene expression changes directly with NF-κB. However, evidence of NF-κB-mediated differential gene regulation was reported: the expression of many NF-κB-dependent genes was altered in C17.2 and BV.2 cells in different culture conditions (see Table 5.2).

Here, the outcome of differential NF-κB dynamics and TNFα-induced gene expression is investigated in the context of cell fate decisions. The effects of TNFα on cell viability and apoptosis is established with the use of MTS and caspase-3/7-associated assays, respectively. To complement data detailed in previous chapters, assays were conducted with mono-cultures and 1:1 co-cultures, and combined with the use of pyrrolidine dithiocarbamate (PDTC; an NF-κB inhibitor).

In brief, the goals of this chapter are to assess TNFα effects on C17.2 and BV.2 mono-cultures and 1:1 co-cultures, and investigate the role that NF-κB activity plays in exerting these effects.


6.2. Results

6.2.1. Effects of TNFα on Cell Viability and Apoptosis

In order to observe potential TNFα-induced changes in cell viability, MTS assays were performed with C17.2 and BV.2 cultures. These assays utilised a tetrazolium compound, MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), which is reduced (depending on the cellular metabolic activity, due to NAD(P)H flux) by cells into a formazan product. The absorbance of formazan, measured at 490nm, is proportional to the number of living cells in culture. Experiments were conducted using mono-cultures and 1:1 co-cultures, seeded at two different cell densities (1000 or 3000 cells per cell-type). So, for example, lower cell density mono-cultures were composed of either 1000 C17.2 or 1000 BV.2: co-cultures were composed of 1000 C17.2 and 1000 BV.2 cells. Apoptosis assays were conducted separately using caspase3/7-associated luminescence reactions. These assays were carried out at the aforementioned cell densities for comparability between datasets. For both viability and apoptosis assays, cells were seeded in 100µl of either normal medium or 20ng/ml TNFα-medium.

Readings for viability/apoptosis were taken 24, 48 and 72 h after seeding. All experiments were performed in triplicate on 96-well plates, with two and three technical replicates per plate for viability and apoptosis assays, respectively. Viability graphs display averaged data collated from the replicates. There was a large difference between the lowest and highest apoptosis readings obtained; therefore, apoptosis data was normalised to data from unstimulated groups at each time point.

Figures 6.1, 6.2 and 6.3 show the results of viability and apoptosis assays in C17.2 mono-cultures, BV.2 mono-cultures and 1:1 co-cultures, respectively. Mann Whitney U tests were performed on data to evaluate changes in viability/apoptosis. Significantly lower cell viability was observed in all TNFα-treated C17.2 cultures, except at 72 h in the higher cell density cultures (p-value≤0.05; Figures 6.1A and B). Accordingly, significant TNFα-induced increases in apoptosis were seen at the same time points (p-value≤0.05; Figures 6.1C and D).

Viability and apoptosis readings were normalised to readings obtained at 24 h to measure growth or apoptosis rates between 24 h and 72 h (Figures 6.1E and F). Culture growth and apoptosis rates were assessed for significant differences using Kruskal-Wallis ANOVA
tests. This enabled discrimination between long-term effects of early TNFα-induced apoptosis and ongoing apoptosis induction. Analysis revealed that between 24 and 72 h, there were no significant differences in culture growth or apoptosis rates between unstimulated and TNFα-treated groups. This suggests that later decreases in viability were a long-term effect of apoptosis at 24 h and not an ongoing TNFα effect on cell viability.

However, more rapid increases in apoptosis levels were observed in TNFα-stimulated groups, at both cell densities between 48 and 72 h (see Figures 6.1E and F; compare blue and grey lines). Together, this suggests the possibility of TNFα-induced increases in apoptosis between 48 and 72 h. Increases in proliferation rate could account for increases in apoptosis, resulting in a culture that exhibits growth rates that are identical to unstimulated cultures.

In contrast to C17.2 cultures, TNFα-induced decreases in BV.2 viability were observed only at 72 h, at the higher cell density (p-value≤0.01; Figures 6.2A and B). Levels of apoptosis in BV.2 cultures were unaffected by TNFα. This suggests that the significant difference in viability may have been due to a confluency-dependent saturation of the MTS signal or a difference in culture growth rate, between 48 and 72 h. No differences in growth rates were highlighted by statistical analysis, although there is a marked reduction between 48 and 72 h in TNFα-stimulated cultures (compare black and red lines in Figure 6.2F). These observations are mirrored in the viability and apoptosis results for co-cultures: TNFα caused a decrease in viability at 72 h (p-value≤0.05; Figures 6.3B) that could be attributed to a decrease in growth rate between 48 and 72 h (Figure 6.3F) or saturation of the MTS signal.

Both cell types displayed cell density-dependent changes in growth rate between 24-48 h and 48-72h. Comparison of C17.2 and BV.2 growth rates (seen in Figures 6.1E-F and Figures 6.2E-F, respectively) revealed that BV.2 cultures grow at a faster rate than C17.2 cultures. In lower density unstimulated cultures, between 24 and 72 h, cell number in C17.2 and BV.2 cultures increased by a fold change of 4.5 and 10.7, respectively. In high density cultures, these fold changes were 3.1 and 7.8, respectively. Therefore, BV.2 population growth occurred at >2 times the rate of C17.2 culture growth, independent of cell density.
Figure 6.1. C17.2 viability and apoptosis levels, after 20ng/ml TNFα stimulation.

C17.2 cells were seeded, at two different cell densities, in 96-well plates in either normal medium ('Unstimulated' group) or medium + 20ng/ml TNFα ('TNFα' group). Viability/apoptosis measurements were taken 24, 48 and 72 h after plating: (A) Viability readings in cells seeded at 1000 cells/well; (B) Viability readings in cells seeded at 3000 cells/well; (C) Apoptosis readings in cells seeded at 1000 cells/well; (D) Apoptosis readings in cells seeded at 3000 cells/well; (E) Measurements in A & C, normalised to corresponding 24 h readings and (F) Measurements in B & D, normalised to corresponding 24 h readings. Graphs E and F represent TNFα-induced effects that occurred between 24 and 72 h; each line is colour-matched with the same condition in graphs A & C and graphs B & D, respectively (viability readings and apoptosis readings are plotted on the left and right y-axis, respectively). Experiments were performed in triplicate. Averaged data is displayed, bars represent ± S.D. and asterisks represent significant Mann Whitney U test results (**p-value≤0.01, ***p-value≤0.001). Kruskal-Wallis ANOVA tests were performed on data in graphs E and F: no significant differences were found.
Figure 6.2. BV.2 viability and apoptosis levels, after 20ng/ml TNFα stimulation.

BV.2 cells were seeded, at two different cell densities, in 96-well plates in either normal medium ('Unstimulated' group) or medium + 20ng/ml TNFα ('TNFα' group). Viability/apoptosis measurements were taken 24, 48 and 72 h after plating: (A) Viability readings in cells seeded at 1000 cells/well; (B) Viability readings in cells seeded at 3000 cells/well; (C) Apoptosis readings in cells seeded at 1000 cells/well; (D) Apoptosis readings in cells seeded at 3000 cells/well; (E) Measurements in A & C, normalised to corresponding 24 h readings and (F) Measurements in B & D, normalised to corresponding 24 h readings (viability readings and apoptosis readings are plotted on the left and right y-axis, respectively). Graphs E and F represent TNFα-induced effects that occurred between 24 and 72 h; each line is colour-matched with the same condition in graphs A & C and graphs B & D, respectively. Experiments were performed in triplicate. Averaged data is displayed, bars represent ± S.D. and asterisks represent significant Mann Whitney U test results (**=p-value≤0.01). Kruskal-Wallis ANOVA tests were performed on data in graphs E and F: no significant differences were found.
NF-kappaB in Neuroinflammation

Figure 6.3. Viability and apoptosis levels in 1:1 co-cultures (C17.2: BV.2), after 20ng/ml TNFα stimulation.

C17.2 and BV.2 cells were seeded, at two different cell densities, in 96-well plates in either normal medium (‘Unstimulated’ group) or medium + 20ng/ml TNFα (‘TNFα’ group). Viability/apoptosis measurements were taken 24, 48 and 72 h after plating: (A) Viability readings in cells seeded at 1000 cells/well; (B) Viability readings in cells seeded at 3000 cells/well; (C) Apoptosis readings in cells seeded at 1000 cells/well; (D) Apoptosis readings in cells seeded at 3000 cells/well; (E) Measurements in A & C, normalised to corresponding 24 h readings and (F) Measurements in B & D, normalised to corresponding 24 h readings (viability readings and apoptosis readings are plotted on the left and right y-axis, respectively). Graphs E and F represent TNFα-induced effects that occurred between 24 and 72 h; each line is colour-matched with the same condition in graphs A & C and graphs B & D, respectively. Experiments were performed in triplicate. Averaged data is displayed, bars represent ± S.D. and asterisks represent significant Mann Whitney U test results (*=p-value≤0.05). Kruskal-Wallis ANOVA tests were performed on data in graphs E and F: no significant differences were found.
6.2.2. Effects of C17.2-BV.2 Interactions on 1:1 Co-culture Viability and Apoptosis

Using the data displayed in Figures 6.1-6.3, co-culture-induced changes in cell viability and apoptosis were investigated. Mono-culture viability and apoptosis results were used to calculate expected co-culture results; these are the results that would be obtained if viability and apoptosis in co-cultures was unaffected by C17.2-BV.2 interactions. An example of the calculations conducted is in Table 2.4, Chapter 2. Analysis was conducted separately for unstimulated and TNFα-treated cultures. The expected results obtained were compared to the actual results observed, which can be seen in Figure 6.3. Although, previous analysis of viability/apoptosis data has utilised non-parametric statistical tests, parametric z-tests were selected for use here.

The parametric z-test assumes a normal distribution, which cannot be reliably assigned to a dataset composed of 3 replicate values, as was the case here. However, the non-parametric alternative to the z-test was a chi-squared test which required discrete data. Therefore, it was decided to proceed with use of the z-test, whilst acknowledging the risk of obtaining false positives. To identify any false positive results, the magnitude of any significant differences will be taken into account.

As a guideline, significant differences in Figures 6.1-6.3 were revisited: significance was assigned with high confidence in these figures, due to use of the non-parametric Mann Whitney U test. A difference of 24% was the smallest that was assigned statistical significance (see 24 h time point in Figure 6.1B). This difference will be used to evaluate the validity of significance assigned by z-tests in Figures 6.4-6.6.

Viability was significantly lower than expected in unstimulated higher density co-cultures at 72 h (14% lower than expected; p-value≤0.01; Figure 6.4B). This result mirrors observations in BV.2 cultures (see Figure 6.2). Assuming that there are little or no co-culture-associated changes in C17.2 or BV.2 growth rate, co-culture readings would be dominated by BV.2 cells. Expected and actual co-culture readings displayed similar growth profiles between 24 and 72 h (Figure 6.4C and D). Taking this into consideration, it is possible that the significance assigned in this dataset is a consequence of confluency-dependent saturation of MTS signal. The assay used in this study is reportedly a reliable indicator of viability for cultures of up to 2.5x10^5 cells: the possibility of saturation is noted here and discussed later in this chapter (see section 6.3).
No significant differences were reported between expected and actual TNFα-induced coculture viability and growth rates (Figures 6.5C and D).

![Graph showing relative absorbance at 490 nm for expected and actual viability in unstimulated 1:1 cocultures](image)

**Figure 6.4. Expected versus actual viability in unstimulated 1:1 co-cultures (C17.2:BV.2).**

Mono-culture viability results (displayed in Figures 6.1A-B and 6.2A-B) were used to calculate expected viability in unstimulated co-cultures. (A) and (B) Expected & actual viability in 1000 cells/well/cell-type and 3000 cells/well/cell-type cultures, respectively. Z-tests were used to determine whether actual co-culture viability results were significantly different to expected results. Viability readings in A and B were normalised to 24 h measurements, to represent culture growth between 24 and 72 h; this normalised data is displayed in (C) and (D), respectively. Averages are displayed, bars represent ± S.D. and asterisks represent significant z-test results (** p-value ≤ 0.01). Expected apoptosis was calculated and compared to actual apoptosis results, in basal and TNFα-treated co-cultures (Figure 6.6A-D). The rate of apoptosis between 24 and 72 h was similar across groups; a high degree of overlap can be seen in Figure 6.6E-F. However, z-tests highlighted higher than expected apoptosis levels at 48 h in all conditions, except for lower density unstimulated cultures (p-value ≤ 0.01; Figure 6.6B-D). Average increases of 105%, 49% and 61% were observed in TNFα-stimulated lower cell density (Figure 6.6C), unstimulated higher cell density (Figure 6.6B) and TNFα-stimulated higher cell density (Figure 6.6D) cultures, respectively. These findings were not reflected by changes in coculture viability (see Figures 6.4 and 6.5), suggesting that increases in proliferation rate may have accounted for increases in apoptosis.
In summary, co-culture interactions may have increased apoptosis and proliferation rate of cells. Increases in apoptosis appeared to be counteracted by increases in proliferation, thus co-culture interactions did not affect overall viability of cell populations.

TNFα triggered apoptosis in C17.2 mono-cultures, but not in 1:1 co-cultures. This suggests the possibility of BV.2 inhibition of TNFα-induced C17.2 apoptosis, which could be linked to differences in NF-κB dynamics that were discussed in Chapter 4 (refer to Figure 4.2). An alternative possibility is that, since BV.2 growth rate was higher than that of C17.2 cells, co-culture readings were dominated by BV.2 cells and, thereby, apoptosis of C17.2 was masked (this is debated in section 6.3).

Having established the effects of TNFα and co-culture interactions on viability and apoptosis, the next goal was to investigate NF-κB contribution to these effects.
Figure 6.6. Expected versus actual apoptosis in unstimulated and TNFα-stimulated 1:1 co-cultures (C17.2:BV.2).

Mono-culture apoptosis results (displayed in Figures 6.1C-D and 6.2C-D) were used to calculate expected apoptosis in unstimulated and TNFα-stimulated co-cultures. (A) and (B) Expected & actual apoptosis in 1000 cells/well/cell-type and 3000 cells/well/cell-type unstimulated cultures, respectively. (C) and (D) Expected & actual apoptosis in lower cell density and higher cell density TNFα-stimulated cultures, respectively. Z-tests were used to compare expected and actual co-culture apoptosis results. Apoptosis readings in A & C, and B & D, were normalised to 24 h readings, to represent culture growth between 24 and 72 h; this normalised data is displayed in (E) and (F), respectively. Graphs E and F represent co-culture-induced effects that occurred between 24 and 72 h; each line is colour-matched with the same condition in graphs A & C and graphs B & D, respectively. Averages are displayed and bars represent ± S.D. Mann Whitney U tests were performed; no significant difference was found between expected and actual co-culture viability.
6.2.3. Pyrrolidine Dithiocarbamate-induced NF-κB Inhibition

In order to investigate NF-κB contribution to changes in viability and apoptosis, pyrrolidine dithiocarbamate (PDTC) was identified as a candidate to inhibit NF-κB activation. PDTC inhibits IκB phosphorylation and, thereby, p65 nuclear translocation and NF-κB DNA-binding activity (Zhang et al, 2011). It has previously been used on neural stem cells to show that NF-κB activation is necessary for TNFα-induced increases in apoptosis and proliferation (Widera et al, 2006a).

To test PDTC efficacy, C17.2 and BV.2 cells were pre-treated with 0.1mM PDTC for 30 min, then PDTC was washed away and cells were stimulated with 20ng/ml TNFα. Protein extracts were taken at 15 min intervals from 0 to 120 min post-TNFα stimulation. Western blots were conducted with these extracts: IκBα levels were used as a measure of NF-κB activation. IκBα levels were quantified using densitometry and normalised to pre-stimulation level, for display in Figure 6.7.

Strong TNFα-induced IκBα degradation (without PDTC treatment) was observed in these cell lines and described in Chapter 3 (refer to Figures 3.8 and 3.19). Here, IκBα in both cell lines was maintained at pre-stimulation levels for 2 h after TNFα stimulation (Figure 6.7). This suggests that TNFα-induced NF-κB activation was strongly inhibited and/or delayed by pre-treatment with PDTC.

To investigate the duration of PDTC-dependent NF-κB inhibition, NF-luc induction was measured in luminometry experiments. C17.2 cells were transfected with an NF-luc plasmid and seeded as mono-cultures and 1:1 co-cultures with BV.2 cells. Cultures were pre-treated for 30 min with water or 0.1mM PDTC, then washed and stimulated with 20ng/ml TNFα, as before. Luminescence measurements were taken at 15 min intervals from 0 to 15 h after TNFα stimulation. Results from TNFα-treated conditions were normalised to the corresponding unstimulated controls (Figure 6.8).

One-way ANOVA tests were performed to assess significant differences between groups. Significant increases in NF-luc induction was observed in all TNFα-treated groups, compared to unstimulated controls (these significant differences are not represent in Figure 6.8). Figure 6.8 displays significant differences in NF-luc induction, between water + TNFα groups and PDTC + TNFα groups.
Figures 6.8A-B show that NF-luc induction was inhibited in PDTC + TNFα treated C17.2 cells, compared to water + TNFα treated cells, in mono-cultures and co-cultures (p-value≤0.01). Slower increases in luciferase expression were observed in PDTC-treated cultures; curves representing non-PDTC-treated groups show a steeper increase in luciferase expression after TNFα stimulation.

Each luminometry experiment was conducted with mono-cultures and co-cultures, seeded with the same batch of NF-luc-transfected C17.2 cells. This made the results from mono-cultures and co-cultures comparable. Significant differences between these groups can be seen in Figure 6.8C. No difference in NF-luc induction was observed between PDTC + TNFα-treated mono-cultures and co-cultures.

In summary, pre-treatment with 0.1mM PDTC inhibited, not prevented, TNFα-induced NF-κB activation in C17.2 cells. IκBα level after TNFα-stimulation indicated that PDTC also strongly inhibited/prevented NF-κB activation in BV.2 cells. For these reasons, PDTC was deemed suitable for use to assess NF-κB contribution to TNFα-induced changes in cell viability and apoptosis.
6.2.4. Effects of NF-κB Inhibition on Untreated and TNFα-treated Mono-cultures and 1:1 Co-cultures

Previously outlined data described TNFα-induced changes in cell viability and apoptosis in mono-cultures and co-cultures (Figures 6.1-6.3). To learn more about the role that NF-κB played in these changes, viability readings were taken from water/0.1mM PDTC-pretreated cells that were washed and then left unstimulated or stimulated with 20ng/ml TNFα. These experiments were conducted at two different cell densities with C17.2 and BV.2 mono-cultures (Figures 6.9 and 6.10, respectively), as well as 1:1 co-cultures (Figure 6.11). Experiments were performed in triplicate, with three technical replicates per assay. One-
way ANOVA tests were performed to evaluate differences between groups and data was averaged for display. Kruskal-Wallis ANOVA tests were used to assess differences in growth rates across culture conditions.

PDTC-treated C17.2 cultures were significantly more viable than TNFα-treated cultures at the lower cell density (p-value≤0.01; Figure 6.9A). Moreover, it should be noted that PDTC and PDTC + TNFα-treated cultures were consistently more viable than water and water + TNFα-treated groups, although these results were not highlighted by statistical analysis. The growth rate of cultures between 24 and 72 h was comparable, indicating that early TNFα-induced decreases in viability (at 24 h) are responsible for the viability differences observed at later time points: this is consistent with the conclusions drawn from the previous set of viability experiments (see Figure 6.1).
Earlier analysis of viability data from unstimulated and TNFα-stimulated C17.2 cultures highlighted significant differences between the two groups (see Figures 6.1A-B): this was not observed in Figure 6.9. This discrepancy may be due to the use of different statistical tests; the non-parametric Mann Whitney U test is more apt for analysis of small datasets than the one-way ANOVA. The one-way ANOVA was employed here due to the large number of groups in this analysis. Although, not assigned statistical significance, TNFα-treated cultures were consistently less viable than water-treated cultures.

Unlike C17.2 observations, one way ANOVA of BV.2 viability results highlighted many significant differences (p-value≤0.05; Figures 6.10A-B). As previously observed in Figure
6.2, MTS signal appears saturated in higher density cultures at 48 and 72 h: this is seen in Figure 6.10D as a plateau in growth rate. For clarity, focus will be placed on two consistent findings across culture conditions. Firstly, PDTC + TNFα treated groups were more viable than TNFα-treated cultures, at both cell densities. This difference was assigned significance in higher cell density cultures only. Secondly, viability of PDTC-treated cultures was significantly higher than that of TNFα-stimulated cultures at 72 h, across cell densities, and at 48 h in higher cell density cultures only.

As with C17.2, growth rates of different culture conditions were comparable. Therefore, significant differences observed at 72 h, at higher cell densities, was most likely due to the effects of PDTC observed at 24 h.
Overall, PDTC-treatment was found to promote viability of both C17.2 and BV.2 monocultures. Next, the effects of PDTC were investigated in 1:1 co-cultures (Figure 6.11). As with BV.2 data, one-way ANOVA tests highlighted many significant differences between culture conditions (p-value≤0.05). One consistent finding mirrors observations made in C17.2 and BV.2 monocultures: PDTC-treated cultures were significantly more viable than TNFα-treated cultures at 48 and 72 h at both cell densities. Differences at 72 h may be the effect of significant differences in growth rate observed between 24 and 48 h (compare blue and red lines in Figures 6.11C and D) or MTS signal saturation, as previously noted in BV.2 cultures (Figures 6.2 and 6.10). The significant difference in growth rates cannot be assigned to any particular groups: Kruskal-Wallis ANOVA results only reveal that at least one group is significantly different to the others. Considering the plateau in growth rate between 48 and 72 h in high cell density cultures, MTS saturated signals could be masking the effects of PDTC treatment at later time points. Hence, results from low cell density cultures are likely to be more reflective of treatment-induced changes in viability at later time points.

### 6.2.5. Effects of C17.2-BV.2 Interactions on Cell Viability in 1:1 Co-cultures

In order to establish whether C17.2- BV.2 interactions contributed to co-culture viability results obtained, expected co-culture results were calculated from mono-culture data (see Table 2.4 in Chapter 2 for methods). These calculations were performed separately for water only, water + TNFα, PDTC only and PDTC + TNFα-treated groups. Z-tests were used to determine significant differences between expected results and actual co-culture results obtained (which are displayed in Figure 6.11). As mentioned earlier in this chapter, ideally the non-parametric Chi-squared test would have been used but, due to the continuous nature of this dataset, this was not possible. Therefore, caution will be exercised in the interpretation of significance assigned by z-tests.

Water-treated co-culture viability results were lower than expected at 24 h in lower cell density cultures (p-value≤0.01; Figure 6.12A). This is inconsistent with results shown in Figure 6.4. The effects of saturation could be seen in higher cell density co-cultures: significance assigned at 72 h can be attributed to this (p-value≤0.01).

PDTC-treated co-culture viability was lower at the same time points and cell densities as observed in unstimulated co-cultures (p-value≤0.01; Figure 6.12A). Again, significance
assigned at 72 h, in lower cell density cultures, and at 48 h and 72 h in higher cell density cultures is most likely an artefact of confluency-dependent saturation of absorbance.

Growth of cultures that contained BV.2 cells consistently plateaued after reaching absorbance readings around 10-15 arbitrary units, independent of treatment and initial cell density (see Figures 6.2, 6.3, 6.10 and 6.11). The plateau effect may be due to confluency-dependent changes in culture growth rate or saturation of the MTS signal.

Consistent with previous observations, growth of water + TNFα-stimulated cultures plateaued at higher cell densities (Figure 6.13C-D), hence lower cell density results will be the focus here. Viability of water + TNFα-treated co-cultures was significantly lower than...
expected, at 48 h after TNFα-stimulation (p-value≤0.05; Figure 6.13). This observation is inconsistent with data in Figure 6.5, where no difference was found between expected and actual co-culture viability. However, it should be noted that the difference observed here is small and that this dataset is not entirely suited to statistical analysis with z-tests.

![Graphs showing co-culture viability data](image)

**Figure 6.13. Expected versus actual viability in 1:1 co-cultures (C17.2:BV.2), after either a 30 min pre-treatment with water or 0.1mM PDTC and 20ng/ml TNFα.**

Mono-culture viability results (displayed in Figures 6.9A-B and 6.10A-B) were used to calculate expected viability in water treated co-cultures. (A) and (B) Expected & actual viability in 1000cells/well/cell-type and 3000cells/well/cell-type co-cultures, respectively. Z-tests were used to determine whether actual co-culture viability results were significantly different to expected results. Viability readings in A and B were normalised to 24 h measurements, to represent culture growth between 24 and 72 h; this normalised data is displayed in (C) and (D), respectively. Averages are displayed, bars represent ± S.D. and asterisks represent significant z-test results (**=p-value≤0.01).

TNFα stimulation, preceded by PDTC-treatment, led to significantly lower co-culture viability, across time points (p-value≤0.01; Figure 6.13). Considering potential effects of saturation, credence will be paid to results at earlier time points i.e. 24 h and 48 h in low cell density cultures and 24 h in high cell density cultures. Expected and actual co-culture growth curves were similar between 24 h and 72 h, which suggest that earlier changes in
viability (at 24 h) were propagated over time. Therefore, it can be concluded that coculture viability was lower than expected at 24 h after PDTC + TNFα treatment.

Overall, PDTC treatment increased viability of C17.2 and BV.2 cells in mono-cultures and 1:1 co-cultures, compared to TNFα-treated cells. Due to time constraints, it was not possible to study the effects of PDTC on proliferation and apoptosis rates. Instead, preliminary apoptosis assays were conducted to observe any PDTC-induced effects that may have contributed to results outlined in this chapter.

C17.2 and BV.2 cells were treated for 30 min with either 0.1mM PDTC or water, then washed and seeded in 96-well plates in mono-cultures at two different cell densities (1000 and 3000 cells per well). Apoptosis readings were measured 24 h after treatment. Experiments were performed in triplicate, with three technical replicates per plate, and Mann Whitney U tests were conducted on collated datasets. Averaged data is displayed in Figures 6.14 and 6.15.

**Figure 6.14. Apoptosis levels in water- or PDTC-treated C17.2 cells.**

C17.2 cells were washed and seeded at two different cell densities, after 30 min treatment with either water (Unstimulated) or 0.1mM PDTC (PDTC-treated). Apoptosis levels were measured 24 h after treatment. Experiments were performed in triplicate. Averaged data is displayed, bars represent ± S.D. and asterisks represent significant Mann Whitney U test results (**=p-value≤0.01).

PDTC-treated C17.2 mono-cultures were more apoptotic than control cultures, at both cell densities investigated (p-value≤0.01; Figure 6.14). No change in BV.2 apoptosis rate was observed after PDTC-treatment (Figure 6.15). Increased viability in PDTC-treated cultures (versus TNFα-treated cultures; seen in Figures 6.9-6.11) must have been due to increased proliferation of both C17.2 and BV.2 cells. The results outlined here strongly suggest that
NF-kB inhibition increased viability and proliferation of C17.2 and BV.2 cells and also increased apoptosis in C17.2 cells only.

**Figure 6.15. Apoptosis levels in water- or PDTC-treated BV.2 cells.**

BV.2 cells were washed and seeded at two different cell densities, after 30 min treatment with either water (‘Unstimulated’) or 0.1 mM PDTC (‘PDTC-treated’). Apoptosis levels were measured 24 h after treatment. Experiments were performed in triplicate. Averaged data is displayed, and bars represent ± S.D. Mann Whitney U test results indicate no significant difference between apoptosis levels in unstimulated and PDTC-treated groups.

### 6.3 Discussion

Numerous studies have been reported on the effects of TNFα on NPCs/NSCs (Ben-hur et al, 2003; Wong et al, 2004; Widera et al, 2006a; Widera et al, 2006b; Wang et al, 2014). The findings from these studies are somewhat contradictory: TNFα was found to inhibit proliferation and promote proliferation of NPCs by separate studies (Wong et al, 2004; Widera et al, 2006a). In general, TNFα effects on apoptosis are more consistent: findings show that TNFα can promote apoptosis (Wang et al, 2014; Widera et al, 2006a).

The effects of TNFα on NPCs are reported to be dose-dependent: Bernardino et al (2008) reported NPC proliferation in response to 1ng/ml TNFα and apoptosis after stimulation with doses of ≥10ng/ml TNFα. The outcome of TNFα treatment can also be determined by TNFR engagement by TNFα: TNFR1 and TNFR2 interactions are generally associated with apoptosis and cell survival, respectively (MacEwan, 2002), although this picture is too simplistic and TNFR2 signaling can contribute to apoptosis (Wajant et al, 2003). TNFR1 is thought to be the major NF-κB activating TNFR in NPCs (Kolesnick and Golde,
NF-κB activity may be a negative regulator of proliferation. Many factors converge to determine the outcome of TNFα stimulation: this complexity may explain the seemingly contradictory observations of TNFα effects on NPCs. In contrast to NPCs, there is a deficiency in studies on the effects of TNFα on microglia. It has been reported that TNFα leads to an increase in viability of microglial cultures (Kuno et al, 2005).

None of the studies mentioned above were conducted with the cell lines used in this study. Here, with the use of MTS and caspase3/7 assays, viability and apoptosis was investigated in C17.2 and BV.2 mono-cultures, as well as 1:1 co-cultures, after 20ng/ml TNFα stimulation. NF-κB contribution to TNFα effects was also studied with the use of PDTC (an NF-κB inhibitor). The main findings of this investigation are as follows:

1) TNFα induced apoptosis and decreased cell viability in C17.2 mono-cultures: this effect was prevented by BV.2 cells in 1:1 co-cultures;
2) TNFα did not affect BV.2 viability or apoptosis rates;
3) NF-κB inhibition caused apoptosis in C17.2 cultures and increased viability in C17.2 and BV.2 mono-cultures and 1:1 co-cultures.

TNFα-induced changes in cell survival will be considered in relation to observations made in other microglial and NPC lines. The goal of this discussion is to formulate hypotheses that may explain the above findings.

6.3.1. Effects of TNFα on C17.2 and BV.2 Mono-cultures

TNFα treatment decreased the viability of C17.2 mono-cultures. This effect was at least partially mediated by increased caspase3/7-dependent apoptosis: proliferation rates cannot be ruled out as a contributory factor. As many studies report on simultaneous TNFα-induced changes in proliferation and apoptosis; future proliferation assays would provide a more thorough understanding of TNFα effects on this cell line. TNFα reportedly increased proliferation and apoptosis in NSCs via an NF-κB-dependent mechanism (Widera et al, 2006a). From this, it was predicted that inhibition of NF-κB activation, dominantly via TNFR1, would prevent TNFα-induced apoptosis in C17.2 mono-cultures.
Contrary to observations on C17.2 cultures, 20ng/ml TNFα had no effect on BV.2 viability or apoptosis levels, up to 72 h post-treatment: this contradicts observations from the Kuno et al study (2005) where an increase in cell viability was reported after TNFα treatment. However, this increase was measured at 6 days after treatment- later than the observations made here.

The decrease in cell viability detected at 72 h in higher cell density BV.2 cultures was most likely due to saturation of the MTS signal, caused by high cell confluency (see Figure 6.2). Strong evidence of saturation was observed with some consistency in other results on BV.2-containing cultures. This was a surprising observation, as it has been reported that the MTS assay employed here is a reliable indicator of cell viability for a population of up to 2.5x10^5 cells (manual by kit supplier, see Chapter 2). The same problem with saturation was not observed with C17.2 cultures: this reflects the differences in proliferation rate of these cell lines. BV.2 cell proliferation rate was at least 2-fold higher than C17.2 proliferation rate.

Due to the problems associated with a saturated MTS signal, the results from lower cell densities will be the primary focus for discussion here, as they are more reliable markers of changes in viability.

Co-culture viability results reflected observations made in BV.2 mono-cultures, rather than the decreases in viability and increases in apoptosis measured in C17.2 mono-cultures. This suggests that BV.2 cells can prevent TNFα-induced NPC apoptosis. However, since BV.2 growth rates were higher than those of C17.2, the viability readings from co-cultures will be dominated by BV.2 cell fate. As BV.2 viability does not decrease in response to TNFα, BV.2 cell viability may have been masking C17.2 apoptosis.

Although BV.2 cells may have dominated co-culture readings, there are two important factors that should be considered here: 1) it is unknown whether C17.2-BV.2 interactions influenced the growth rate of these cells and; 2) assuming no co-culture-related changes in growth rate of either cell line, C17.2 cells would have composed approximately one-third of co-cultures. Elaborating on the first point, it is possible that increased growth rate of one cell line would have been counteracted by a decrease in growth rate of the other cell line, so that no change was observed in overall culture viability. On the second point, assuming that C17.2 cells continue to proliferate as usual in co-cultures, it is unlikely that C17.2 numbers were too small to allow detection of apoptosis. This is particularly true for later
time points, when small differences caused by early C17.2 apoptosis would have been propagated to create lower viability readings (compared to unstimulated co-cultures).

Microglial-derived TNFα can induce NPC apoptosis in an NF-κB-dependent manner (Guadagno et al, 2013). BV.2-mediated prevention of TNFα-induced C17.2 apoptosis may be related to inhibition of p65 nuclear translocations (reported in co-cultured C17.2 cells in Chapter 4; see Figure 4.2). This idea will be discussed further in Chapter 7.

6.3.2. Effects of NF-κB Inhibition on C17.2 and BV.2 Mono-culture and 1:1 Co-culture Viability

From data described in this chapter, it was predicted that NF-κB inhibition would block TNFα-dependent decreases in C17.2 mono-culture viability. The results of MTS assays performed with TNFα and PDTC (an NF-κB inhibitor) supported this idea. With the exception of one data point (in Figure 6.10A), all groups pre-treated with PDTC gave a stronger MTS signal than the corresponding untreated or TNFα-stimulated groups. Some of the differences in viability were not statistically significant.

An important factor to consider was the efficacy of NF-κB inhibition by PDTC. PDTC pre-treatment delayed and partially inhibited TNFα-induced NF-κB activation, as measured by NF-luc activity in C17.2 cells and by Western blots for IκBα degradation in both cell types (see Figures 6.7-6.8). Complete inhibition of NF-κB may have led to more pronounced, statistically significant, differences between PDTC and non-PDTC treated groups. However, even partial inhibition led to an observable effect on C17.2 viability: consistency across the dataset provides a convincing argument for a PDTC-dependent increase in cell viability. Future experiments with other NF-κB inhibitors could be used to further investigate NF-κB-mediated effects of TNFα.

Widera et al, (2006a) used a combination of NF-κB inhibitors, including PDTC and a super repressor IκB construct. They reported that TNFα-induced NF-κB activation increased both proliferation and, to a lesser degree, apoptosis in NSCs. The differences between their observations and those made here might be explained by methodology. They used NSCs isolated from the subventricular zone of adult rats and 10ng/ml TNFα, whereas murine cells and 20ng/ml TNFα was used here. Low and high doses of TNFα have been associated with cell survival and apoptosis, respectively (Bernardino et al, 2008): this may also contribute to differences between published results and those seen here. Another
contributory factor could be TNFR2 expression levels. TNFR2 is expressed on NPCs and has a high affinity for mTNFα, a form of TNFα that is expressed on the membranes of microglial cells (Zhou et al, 2010). Signalling through TNFR2 may be responsible for prevention of TNFα-induced C17.2 apoptosis, observed in co-cultures (see Figure 6.3), although TNFR2 signaling can also enhance TNFR1-driven apoptosis (Wajant et al, 2003). TNFR1 versus TNFR2 signaling, and downstream NF-κB dynamics, may be an interesting avenue of investigation in the future.

From the results seen in Figures 6.9-6.11, it was hypothesised that PDTC-treatment might increase culture viability by decreasing apoptosis or increasing proliferation rate. To address these possibilities, preliminary apoptosis assays were conducted on PDTC-treated C17.2 and BV.2 mono-cultures (see Figures 6.16-6.17). Surprisingly, these results showed that PDTC increased C17.2 apoptosis and had no effect on BV.2 apoptosis rates (at 24 h post-treatment). Combined with the viability results obtained from PDTC-treated cultures, this strongly suggests that PDTC treatment increases proliferation of cells, which accounts for increased apoptosis, and results in increased culture viability. (This is discussed further in Chapter 7).

6.3.3. C17.2-BV.2 Interactions in 1:1 Co-cultures

Previous studies have reported changes in co-culture viability and apoptosis that occur as a result of interactions between microglia and NPCs (Guadagno et al, 2013; Mosher et al, 2012; Liu et al, 2013a; Liu et al, 2014; Deierborg et al, 2010). Many of these involved the exchange of culture media, thereby showing that soluble microglial or neural progenitor cell-derived factors drive the effects observed (Guadagno et al, 2013; Deierborg et al, 2010). These factors include NPC-derived VEGF: this factor was shown to promote microglial proliferation in co-cultures (Mosher et al, 2012) and its expression is regulated by NF-κB (Chilov et al, 1997).

Mosher et al (2012) observed NPC and microglial response in separate cultures, thereby preventing cell-contact. However, microglia are densely populated in neurogenic niches in vivo and ideally located to have physical contact with NPCs (Mosher et al, 2012). Also, results in Chapter 4 show that basal NF-κB activity in C17.2 is increased by physical contact with BV.2 (Figure 4.13). Together, this suggests that contact-permissive co-cultures are the ideal model systems to use for accurate representation of NPC-microglial interactions in vivo.
It has been reported that microglia promote NPC proliferation and induce apoptosis (Guadagno et al, 2013; Liu et al, 2013b; Liu et al, 2014). Microglial effects on NPCs are determined by activation status: ‘resting’ and classically activated microglia have been found to increase and decrease NPC number, respectively (Cacci et al, 2005). Moreover, NPC apoptosis was induced by microglial-derived TNFα (Guadagno et al, 2013; Liu et al, 2013b). In turn, NPCs increase proliferation and promote activation of microglia (Mosher et al, 2012; Forstreuter et al, 2002). It should be noted that co-culture ratios used in these studies were unclear and may play an important role in determining the outcome of co-culture interactions.

Here, seemingly contradictory findings were observed in co-culture. The second set of viability experiments conducted resulted in lower than expected co-culture viability, at 24 h, in unstimulated co-cultures (see Figure 6.12A). Also, lower co-culture viability was observed in TNFα-treated co-cultures, at 48 h and 72 h (see Figure 6.13A and B). Co-culture-induced decreases in cell viability were small, compared to TNFα-induced changes (see Figure 6.1-6.3).

No significant differences between expected and actual co-culture viability were observed from the first analysis conducted (see Figures 6.4 and 6.5), although unstimulated co-culture viability was consistently lower than expected. Together, this suggests that interactions between C17.2 and BV.2 cells could lead to an overall decrease in cell viability. These decreases were observed in PDTC + TNFα and water + TNFα treated groups, indicating that co-culture interactions appear to be treatment-dependent (see Figures 6.14-6.15). The requirement for TNFα in these effects suggests that BV.2 activation state, and co-culture specific NF-κB activity, may be involved in determining co-culture viability. Results from literature suggest that decreases in co-culture viability may be due to C17.2 apoptosis, driven by microglial-derived factors. These factors could include microglial-TNFα, previously reported to induce NPC apoptosis (Guadagno et al, 2013).

In summary, TNFα and PDTC treatment exerted different effects on C17.2 and BV.2 viability and apoptosis, strongly implicating NF-κB activity in the determination of cell fate. Some of the results in this chapter were difficult to interpret as a time 0 reading was not taken in viability and apoptosis assays: this time point should be included in future experiments. Also, MTS signal saturation was observed in some high cell density BV.2-containing cultures: this made some results unreliable. Results from lower cell density
cultures provided reliable readings for viability and apoptosis here, but, in future, it would be informative to calibrate cell number against MTS signal in BV.2 cultures.
7. Discussion
7.1. General Introduction

This project aimed to investigate NF-κB-mediated interactions between C17.2 and BV.2 cells in TNFα-treated cultures, as a model system for neural progenitor cell-microglial interactions in neuroinflammation. The ultimate goal was to get a better understanding of how single-cell NF-κB dynamics, can be related to differential gene expression and thereby, cell survival.

Although many studies report on NF-κB on a population-level, single-cell level analysis was selected as the better method for studying NF-κB, as this level of analysis has provided valuable insight into the NF-κB system that have eluded population-level studies. Arguably, one of the most important insights is the observation that specific aspects of NF-κB dynamics within individual cells, such as amplitude and frequency of nuclear translocations (Lee et al, 2014 and Ashall et al, 2009, respectively), determine downstream gene expression profiles.

Initially, different methods of p65 observation in C17.2 and BV.2 cells were investigated (Chapter 3). The investigation then progressed onto analysis of C17.2 and BV.2 p65 dynamics and C17.2 NF-κB transcriptional activity, in mono-cultures and co-cultures (Chapter 4). Moving down the TNFα signalling network, mRNA expression was measured in TNFα-stimulated mono-cultures and co-cultures: this analysis showed TNFα- and co-culture-induced modulation of gene expression (Chapter 5). Finally, the outcome of TNFα treatment and co-culture interactions on cell viability and apoptosis, with and without an NF-κB inhibitor, was measured (Chapter 6).

Lentivirally transduced cells, expressing p65-eGFP, as the best model systems for observation of p65 dynamics. Results showed BV.2-mediated inhibition of TNFα-induced NF-κB activity and p65 nuclear translocations in C17.2 cells. Preliminary evidence for C17.2 regulation of BV.2 p65 dynamics was also found; more emphasis has been placed on observations made in C17.2 as a more complete analysis was conducted with these cells.

Viability and apoptosis results showed that TNFα induced C17.2 apoptosis and suggested BV.2-mediated prevention of this TNFα-stimulated apoptosis. Interestingly, NF-κB inhibition was also shown to induce C17.2 apoptosis and increase overall viability, which may suggest promotion of cell proliferation.
These data provided strong evidence for TNFα-induced, co-culture- and NF-κB-mediated interactions between C17.2 and BV.2 cells occurring at the molecular, genetic and cellular levels. These interactions were vital in determining the cell survival in response to TNFα, an observation which is relevant to the study of neurodegenerative disease, where high TNFα concentrations are known to mediate tissue damage (Cartier et al, 2005) and NPCs have been shown to provide a therapeutic benefit (Park et al, 2002; Park et al, 2006; Teng et al, 2002).

The results obtained from Chapters 3-6 will be discussed together here, in the context of relevant literature, taking into consideration the advantages and problems with techniques used in this thesis. This chapter will be concluded with some suggestions for relevant future experiments.

7.2. NF-κB Activation and p65 Dynamics in C17.2 cells

The C17.2 cell line was selected as a model system to represent NPCs. These murine cells display key defining characteristics of NPCs, including multipotency and high proliferative capacity (Ryder et al, 1990). A trait that is more unique to C17.2 cells are their ability to exert therapeutic effects when transplanted into disease models (Teng et al, 2002; Park et al, 2002; Ourednik et al, 2002), via long-term immune regulation, as well as differentiation to regenerate neuronal circuitry (Snyder et al, 1992; Park et al, 2006). For these reasons, the C17.2 cell line is considered to be a good model system for NPCs and also a useful tool for studying beneficial immune regulation.

Although C17.2 cells are commonly referred to as NPCs, researchers have argued that these cells are more ‘stem-like’. There is no common agreement on the definition of neural progenitor cells and neural stem cells and consequently the two terms have been used interchangeably in the literature (Seaberg and van der Kooy, 2003). For this reason, literature on NPCs and neural stem cells (NSCs) has been referenced throughout this thesis: it is recognised that C17.2 cells may share characteristics of both, depending on the definitions adopted, as well as displaying their own unique features.

Despite their importance in regulating inflammation, studies that have observed NF-κB activity in C17.2 cells have been few in number. Those that have reported on TNFα-induced NF-κB activity in NPCs/NSCs have commonly done so via p65 immunostaining methods. Using these methods, increased nuclear p65 presence in stimulated NSCs has
been observed, 30 min after TNFα stimulation, compared to unstimulated cells (Widera et al, 2006a).

Here, NF-κB activation was measured in C17.2 cells: NF-luc plasmid-transfected cells were stimulated with TNFα doses of 10pg/ml up to 50ng/ml TNFα (Figure 3.5A). Peak NF-luc induction was reached at ~3 h and 5 h after stimulation with doses ≤100pg/ml TNFα and ≥1ng/ml TNFα, respectively. Turner et al (2010) reported peak NF-luc induction times of 6 h with 10pg/ml and 4 h with 100pg/ml and 10ng/ml TNFα in SK-N-AS cells. NF-luc induction with 10pg/ml TNFα was not observed in C17.2, suggesting that these cells are less sensitive to low dose TNFα compared to SK-N-AS cells.

NF-luc induction is a good reporter for NF-κB activation over a period of hours. However, due to the stability of luciferase, it cannot provide high temporal resolution data. To get more information on the temporal profile of NF-κB activation, IκBα levels were measured after 20ng/ml TNFα stimulation. Western blots showed almost complete degradation and resynthesis of IκBα within 15 min and 60 min of TNFα stimulation, respectively (Figure 3.8). In HepG2 cells, ~70% of IκBα is degraded in response to 10ng/ml TNFα and newly synthesised IκBα levels peak at 65 +/- 8% of the pre-stimulatory level after 137 +/- 5 minutes (Moss et al, 2012). IκBα degradation in C17.2 cells appears to be comparable to that seen in HepG2 cells. However, return to pre-stimulation IκBα levels occurs more rapidly in C17.2 cells than HepG2 cells. Use of a higher TNFα dose on C17.2 cells (20ng/ml versus 10ng/ml) might explain the difference observed in IκBα resynthesis times: Moss et al (2012) found that increasing TNFα dose increased IκBα resynthesis kinetics. An alternative explanation is that C17.2 cells basally express lower levels of IκBα than HepG2 cells.

Having established NF-κB activation in C17.2 cells in response to TNFα, corresponding p65 dynamics were observed using C17.2 (lenti) cells (expressing p65-eGFP; Figure 3.1A). In mono-cultures, these cells exhibited high amplitude initial nuclear p65 translocations, which peaked on average at 26 min after TNFα stimulation (Figures 4.2A and 4.3A). In contrast to observations in other cell lines, few cells displayed subsequent p65 nuclear translocations (Ashall et al, 2009; Turner et al, 2010; Paszek et al, 2010).

C17.2 cells were also stably-transfected with a p65-BAC to create clonal cell populations expressing p65-DsRedXP: these clonal cell lines were evaluated as a model system for studying NF-κB in the C17.2 cell line. Observation of single-cell p65 dynamics in these
cells after TNFα stimulation revealed very little nuclear movement of NF-κB, as oppose to the lentivirally-transduced C17.2 cells. Consistent with single-cell data, clonal cells displayed dampened NF-luc induction, as well as maintenance of IκBα levels, after TNFα stimulation. These results are similar to those obtained from murine cortical neuronal cells in mixed cell cultures: neuronal cells display sustained levels of IκBα and little nuclear movement of NF-κB, after TNFα stimulation (Listwak et al, 2013). This suggests that the C17.2 p65-BAC stable cells endured a process of neuronal differentiation during the cloning procedure: for this reason, work with these cells was discontinued.

Following mono-culture experiments, C17.2 (lenti) cells were seeded in 1:1 co-cultures with untransfected BV.2 cells, in order to observe co-culture effects on C17.2 p65 dynamics. The following sections detail the results of co-culture microscopy experiments, along with co-culture-induced changes in C17.2 viability.

7.3. NF-κB Dynamics to Cell Survival

7.3.1. BV.2 Prevents TNFα-induced C17.2 Apoptosis

Confocal microscopy experiments were performed with C17.2 (lenti) cells that were co-cultured with BV.2 cells (at a ratio of 1:1; Figure 4.1). After TNFα-stimulation, co-cultured C17.2 (lenti) cells exhibited a single phase of p65 nuclear translocation (Figure 4.2A). On average, these translocations were of lower amplitude than those observed in mono-cultured C17.2 (lenti) cells (3.3 versus 4.8 fold change in average nuclear NF-κB; Figures 4.2-4.3). Moreover, p65 nuclear translocations were not observed after the initial phase of translocation in co-cultured C17.2 (lenti) cells, whereas mono-cultured cells displayed lower amplitude subsequent p65 nuclear movements (Figure 4.2).

Higher amplitude p65 nuclear translocations in individual C17.2 cells after TNFα treatment (Figure 4.2), were associated with increased apoptosis and decreased cell viability 24h post-stimulation (Figure 6.1). Conversely, lower amplitude p65 responses in co-cultured C17.2 cells (Figure 4.2) corresponded to levels of viability and apoptosis that were similar to unstimulated controls (Figure 6.3).

In summary, co-cultured C17.2 (lenti) p65 dynamics may be functionally associated with prevention of TNFα-induced apoptosis (Figures 4.2, 6.1 and 6.3). Direct links have yet to be made between single-cell NF-κB dynamics and cell fate. However, population-level
studies may elucidate the potential mechanism of cell fate regulation by NF-κB. Previous studies conducted to observe differences in NF-κB localisation in TNFα-susceptible and TNFα-resistant cell lines are particularly relevant and will be compared to data obtained in this project.

Park et al (1996) modified the TNFα-resistant HepG2 cell line to create a cell line that was sensitive to TNFα-induced apoptosis (HepG2 2.15). These cells were stimulated with TNFα, nuclear NF-κB was measured (using EMSA) and downstream gene expression was observed. The TNFα-induced NF-κB response in HepG2 cells resulted in gradual up-regulation in the expression of NF-κB-dependent anti-apoptotic genes, Bcl-Xl and FLIP between 0-120 min after TNFα-stimulation: downregulation of these genes was observed in Hep 2.15 over the same time frame (Park et al, 1996). Expression of these genes was associated with survival in HepG2 cells and apoptosis in HepG2 2.15, in response to TNFα. In turn, cell fate was associated with differences in nuclear NF-κB: lower amplitude changes in nuclear NF-κB were reported in HepG2 2.15, compared to HepG2 (~1.5 versus ~5; relative to pre-stimulation nuclear NF-κB). These differences in amplitude were mirrored by mono-cultured and co-cultured C17.2 (lenti) cells: higher amplitude changes in nuclear p65 were observed, after TNFα stimulation, in mono-cultured C17.2 (lenti), compared to co-cultured cells.

Together, these data suggest that lower amplitude NF-κB nuclear translocations lead to down-regulation of NF-κB-dependent anti-apoptotic factors, leading to apoptosis. NF-κB activity also regulates the expression of many TNFR1-signalosome components that play a role in apoptosis, including caspases, TRAFs and c-IAPs (Micheau and Tschopp, 2003; Micheau et al, 2001). Future experiments in this area should focus on measurement of NF-κB-dependent gene expression, with a focus on apoptotic and anti-apoptotic genes, at different time points. Determining whether the C17.2 (lenti) p65 dynamics observed, and the prevention of C17.2 apoptosis in co-culture, is dependent on C17.2-BV.2 cell contact would also shed light on the molecular mechanisms involved in NF-κB-dependent cell survival decisions. Analysis of microscopy experiments conducted here showed that some C17.2 cells make physical contact with BV.2 in co-cultures. Interestingly, cell-contact dependent increases in NF-luc expression were observed in 1:1 co-culture luminometry experiments (Figure 4.13). The nature of this physical contact was not investigated (Figure 4.1). Previous literature has reported that NPCs and microglia can communicate via cell surface receptor and ligand interactions, although focus has been placed on NPC ligand-
microglial receptor interactions (Liu et al, 2013a; Wang et al, 2007). These ligand-receptor molecules include CD200 and its receptor CD200R, both of which are upregulated by NPCs and microglia, respectively, in co-cultures (Liu et al, 2013). CD200 is an NF-κB-dependent and TNFα-responsive gene, suggesting the possibility of its upregulation in mono-culture studies conducted here. As yet, there are no studies reporting NF-κB activation in response to CD200-CD200R interactions, however, there are reports that this interaction prevents tissue-damaging activation of macrophages (Nathan and Muller, 2001). This is consistent with the idea that microglia increase NPC survival in co-culture after TNFα stimulation (Figure 6.3).

Also, there is evidence to suggest that TGF-β may play a role in NPC-microglia crosstalk: higher levels of TGF-β2 were found in co-cultures, compared to NPC and microglial mono-cultures (Liu et al, 2013). Furthermore, the proportion of TGF-β1 expressing NPCs was larger in co-cultures than in NPC mono-cultures (Liu et al, 2013). TGF-β1 has been reported to decrease NF-κB activity, by upregulating IκBα expression, in unstimulated immature B cells (Arsura et al, 1996). This offers another possible mechanism for the inhibition of NF-κB in NPCs in co-culture after TNFα stimulation: microglia may be promoting NPC expression of TGF-β1 which acts in an autocrine manner to inhibit NF-κB nuclear translocation. Although, it should be noted that NanoString analysis conducted showed that TGF-β1 was not upregulated in co-cultures after TNFα stimulation (Figures 5.2-5.5).

In summary, there are a few candidates offered by current literature as to how microglial cells can modulate NF-κB activity in NPCs but, as yet, there are no thorough and conclusive studies on this subject. It seems likely that the outcome of NPC-microglia interactions is determined by the balance between cell-contact dependent mechanisms (e.g. CD200-CD200R interactions) and cell-contact-independent mechanisms (e.g. secreted molecules such as TGF). Transwell experiments showed elevated basal NF-κB activity in co-cultured NPCs: this effect was dependent on contact between NPCs and microglia (Figure 4.13). NanoString data showed that NPCs upregulate the expression of chemokines such as Ccl2, Ccl5 and Ccl7, in response to TNFα (Figures 5.2-5.5), suggesting that in the neuroinflammatory environment they can attract microglia and alter the balance between cell-contact dependent and cell-contact-independent actions on NF-κB activity. This may lead to differences in downstream cell fate i.e. cell survival vs. apoptosis. The ability to attract microglia would also affect the neural progenitor:microglial cell ratio. As such,
there is a deficiency in quantitative data on NPCs and microglia in vivo, but the results
displayed in this thesis (Figures 4.2, 4.5 and 4.8) highlight the importance of considering
cell ratios in vivo in healthy tissue, as well as inflamed tissue. This area should be
investigated in the future, in relation to findings reported here.

7.3.2. NF-κB Inhibition in Mono-cultures Induces C17.2 Apoptosis and Increases
Viability

Viability assays conducted with the use of PDTC (an NF-κB inhibitor) revealed that NF-
κB activity has a pleotropic role in C17.2. C17.2 mono-cultures and 1:1 co-cultures with
BV.2 cells were pre-treated with PDTC or water for 30 min, before stimulation with TNFα.
The viability readings from both PDTC- and PDTC + TNFα-treated cultures were
consistently higher than those from water + TNFα treated cultures (Figure 6.9). These
observations were consistent with data on p65 dynamics and corresponding viability and
apoptosis results (Figures 4.2 and 6.1). Surprisingly, preliminary apoptosis assays showed
that PDTC treatment increased apoptosis in C17.2 mono-cultures (Figure 6.14). Together,
these results strongly suggest that NF-κB inhibition in C17.2 mono-cultures induces
apoptosis and, markedly increases proliferation, to lead to an overall increase in culture
viability (Figures 6.9 and 6.16). In contrast with this observation, microglia may prevent
NPC apoptosis in 1:1 co-cultures: this result was associated with microglial-driven NF-κB
inhibition in NPCs, after TNFα stimulation. These contradictory findings can be explained
in a number of different ways, one of which involves interpretation of the viability results
obtained in this thesis (Figures 6.1-6.3 and 6.9). BV.2 cell growth was more rapid than
C17.2 growth in mono-cultures (see Figures 6.1 and 6.2) and viability results obtained
from BV.2 mono-cultures were similar to those obtained from 1:1 co-cultures (see Figure
6.3): these observations suggest that BV.2 cells dominated the 1:1 co-cultures and
therefore the lower apoptosis levels observed in these cultures may have been due to very
small C17.2 cell numbers.

Alternatively, the temporal profile of NF-κB inhibition after TNFα may contribute to the
differential effects in 1:1 co-cultures and PDTC-treated C17.2 mono-cultures. Western blot
analysis and NF-luc induction data from PDTC-treated C17.2 cultures show that PDTC
treatment may prevent early NF-κB activation (<1 h post-stimulation), whereas single-cell
analysis shows that microglial cells prevent later NF-κB activation (≥2 h post-stimulation)
and only dampen initial NF-κB nuclear translocations.
Previous analyses of NF-κB activity have been described using NSCs pretreated with PDTC. These studies contradict the observations made here: basal and TNFα-induced NF-κB activity promoted NSC proliferation (Widera et al, 2006a; Piotrowska et al, 2006). These results may emphasise the importance of studying NF-κB dynamics at the single-cell level, in order to understand cell fate decisions. Here, the population-level effects of PDTC pre-treatment on TNFα-induced NF-κB activation was measured using Western blot analysis of IκBα protein levels. There was no evidence of NF-κB activation in C17.2 cells up to 2 h post-TNFα stimulation (Figure 6.7). However, luminometry experiments conducted with NF-luc plasmid transfected cells showed delayed NF-luc induction in PDTC + TNFα treated cells, compared to control TNFα-simulated cultures (Figure 6.8).

In summary, NF-κB inhibition was shown to induce apoptosis, but was also associated with the prevention of apoptosis in co-cultured C17.2 cells (see Figures 6.3 and 6.16). This duality in functional effect highlights the need for observations of NF-κB dynamics at the single cell level. A key experiment for the future would be the observation of TNFα-induced p65 dynamics in cells that have been pre-treated with PDTC. Comparing this data with co-cultured C17.2 (lenti) p65 dynamics would inform on the relationship between p65 dynamic profiles and cell fate. Single-cell observations on the expression of NF-κB-dependent apoptotic and anti-apoptotic factors would also greatly enhance progress in this field of research. Particularly interesting results might arise from apoptosis assays with PDTC-treated 1:1 co-cultures. The apoptotic effect of TNFα on C17.2 cells was negated by BV.2 cells in 1:1 co-cultures (Figures 6.1 and 6.3). One could hypothesise that BV.2 cells might prevent PDTC-induced apoptosis (Figure 6.14), possibly by altering p65 response in C17.2 cells.

7.3.3. TNFα-induced BV.2 p65 Dynamics

The BV.2 cell line shows many properties of primary microglial cells. Classically-activated BV.2 display anti-microbial activity, phagocytic behaviour and proteomic changes which are comparable to primary microglia (Bocchini et al, 1992; Blasi et al 1990; Henn et al, 2009). Also, classically-activated BV.2 cells can activate nearby astrocytes; an action which is also performed by primary microglia (Henn et al, 2009). For these reasons, BV.2 was selected as a suitable cell line representing microglia for studies of NPC-microglial interactions. Data acquired on BV.2 cells will be discussed in relation to current data available on primary microglia and other microglial cell lines.
The single-cell p65 dynamics of BV.2 cells have been described for the first time in the present study. One previous study has reported population-level analysis of activated NF-κB levels in 10ng/ml-TNFα stimulated BV.2 (Sheppard et al, 2010). This analysis reported that NF-κB activation reached a peak at 25 min and 105 min post-TNFα stimulation. Here, average peaks in nuclear p65 were observed at 30 min and 105 min after TNFα stimulation. These data provide support for the use of BV.2 (lenti) to represent wild-type BV.2 p65 dynamics.

On the single-cell level, BV.2 (lenti) exhibited p65 oscillations in response to TNFα, in both mono-cultures and co-cultures. The average period of these oscillations was 108 min and 63 min, respectively (Figures 4.15-4.16). Interestingly, the average timing of initial peaks in nuclear p65 was earlier in co-cultures, compared to mono-cultures (22 min compared to 30 min; Figure 4.17). In contrast to C17.2, these differences in p65 dynamics were not correlated with differences in cell survival: BV.2 mono-cultures and co-cultures displayed no changes in viability or apoptosis in response to TNFα (see Figures 6.10-6.11). This shows that NF-κB regulation of cell fate is cell-type specific between these cell lines.

TNFα is known to induce p65 oscillations, with periods of around 100 min, in non-neural cell lines (Ashall et al, 2009; Turner et al, 2010; Paszek et al, 2010). The faster oscillations seen in BV.2 (lenti) co-culture (63 min; see Figures 4.15-4.16) have interesting implications for C17.2 regulation of BV.2 activation status, as NF-κB activity had been implicated in microglial activation (Colton, 2009; Saijo and Glass, 2011; Liu et al, 2013b). However, co-culture data observing p65 in BV.2 (lenti) cells was only obtained from a single microscopy experiment. More replicates would be required before any conclusions could be reached on the C17.2 regulation of BV.2 p65 dynamics. Factors that might influence oscillation period and p65 peak timings will be discussed briefly next, before progressing on to a plan for future experiments.

Sheppard et al (2010) modified a mathematical model, which was published by Ashall et al (2009), to reproduce TNFα-induced changes in population level NF-κB activation in BV.2. They concluded that NF-κB activation timing was strongly influenced by IKK-dependent degradation of IκBα and, thereby, factors that regulate IKK activation. This could suggest that the expression of the TNFR1-signalosome components or A20-mediated inhibition of IKK activation might be involved in the delay in nuclear p65 peak timing observed in co-cultured BV.2 (lenti) cells.
The parameters that influence NF-κB oscillation period have been investigated using mathematical models, many of which incorporated single-cell data on non-neural cell lines. Nelson et al (2004) conducted sensitivity analysis of the parameters in a mathematical model created by Hoffmann et al (2002). It was found that two parameters were particularly important in determining oscillation dynamics: IKK deactivation rate and the rate of IκBα nuclear import. Decreasing the rate of IKK deactivation and increasing the rate of IκBα nuclear import could shorten the period. The Hoffmann et al (2002) mathematical model was based on results from IκBα protein-deficient cells, so caution should be exercised when applying this model to BV.2, although Sung et al (2009) highlighted the same parameters from their mathematical model which was based on MEFs.

7.4. Future Experiments

Results obtained in this thesis provide evidence for: the role of differential NF-κB dynamics in regulating C17.2 cell fate and; BV.2 regulation of C17.2 cell fate, possibly through modulation of the C17.2 NF-κB response. However, associations between the two were indirect and there were issues with the experimental design and techniques used. These will be discussed here and used to suggest improved future experiments to advance the data described in this thesis.

One issue with the apoptosis and viability assays conducted here was the lack of a time 0 reading (Figures 6.1-6.3 and 6.9-6.11). In retrospect, initial apoptosis/viability of the cells should have been measured when they were seeded in the experimental dish. This would have provided extra data on culture growth between 0 and 24 h. Also, any changes in co-culture apoptosis/viability over this time period would have been captured. In theory, co-culture-induced changes in viability or apoptosis may have occurred, independent to TNFα-treatment, between 0 and 24 h. Since, viability/apoptosis readings from TNFα-stimulated cultures were compared to readings from unstimulated cultures, differences caused by co-culturing cells would have been masked. Time 0 readings in future experiments will highlight any cell fate-determining co-culture interactions.

Another problem with viability results was saturation of the MTS signal in higher cell density cultures. This was observed at 48 h and 72 h time points in experiments with BV.2 cells, either in mono-cultures or co-cultures (Figures 6.2, 6.3, 6.10 and 6.11). These results were unexpected, as the manual for the viability kit (obtained from the supplier; see
Chapter 2), claimed that the procedure was suitable for use with up to $2.5 \times 10^5$ cells per well. As 3000 BV.2 cells per well were seeded for the assays conducted in the present study, it is unlikely that cell numbers exceeded $2.5 \times 10^5$ cells per well, even considering the rapid proliferation rate of BV.2 cells. This highlights the importance of calibrating BV.2 cell number against MTS absorbance readings, to ensure that signal saturation is not a problem in future viability experiments.

As well as viability and apoptosis assays, proliferation assays are required to investigate some results obtained with PDTC. NF-κB inhibition by PDTC-treatment led to increases in viability across all culture conditions (Figure 6.10-6.11), but most notably, in C17.2 monocultures (Figures 6.9). Surprisingly, PDTC treatment also increased C17.2 apoptosis, measured at 24 h post-treatment (Figure 6.14). This suggests that NF-κB activity inhibits C17.2 proliferation.

Also, in future experiments co-culture ratios should be optimised to maximise co-culture effects, making them easily detectable. Live-cell luminometry experiments in this project have set a foundation for this work. These experiments were performed using NF-luc plasmid-transfected C17.2 cells, seeded in different co-culture ratios (Figure 4.5). BV.2 inhibition of TNFα-induced NF-κB transcriptional activity was not observed in 1:2 (C17.2:BV.2) co-cultures, but this effect was observed in 1:4 co-cultures. Also, lower TNFα doses (500pg/ml, 1ng/ml and 10ng/ml) were used in these experiments, to enhance co-culture-induced decreases in NF-κB transcriptional activity (compared to 20ng/ml TNFα in microscopy experiments). Considering these results, microscopy experiments in future could use high ratio co-cultures (1:4) and lower TNFα doses: this could enhance co-culture effects on C17.2 viability/apoptosis. Combining these experiments with NF-κB inhibitors would allow assessment of NF-κB contribution to C17.2 cell fate.

Endpoint luminometry experiments with transwells showed that C17.2 NF-luc inhibition by BV.2 occurred independently of cell contact (Figure 4.11). From this result, it was concluded that BV.2 inhibition of TNFα-induced NF-luc expression was due to soluble autocrine/paracrine factors in co-cultures. Multiplex ELISA assays in future would help to determine potential candidates for mediating C17.2-BV.2 communication and modulating NF-κB dynamics. These experiments could be combined with single-cell imaging of fluorescent protein tagged-Annexin V labelling (Moulding et al, 2000) and p65 dynamics, thereby enabling NF-κB dynamics to be correlated with cytokine secretions and cell survival/apoptosis.
In parallel to the above experiment, a recently developed microfluidics procedure could be used to measure the expression of up to 96 genes, in one sample, after TNFα stimulation (Kellogg et al, 2014). This analysis would not be conducted on live cells, but with the selection of a few key time points, it would complement the results from Annexin-V experiments. The NanoString assay conducted during the course of this project (Chapter 5) was focussed on the expression of inflammatory factors. It might be more informative to focus on the expression of apoptotic and anti-apoptotic factors in future experiments.

An important goal of future microscopy experiments should be the simultaneous observation of NF-κB dynamics in co-cultured BV.2 (lenti) and C17.2 (lenti) cells. This would halve the number of microscopy experiments required for full datasets and, therefore, save time. Since both of the lentivirally transduced cell lines expressed p65-eGFP, it was not possible to confidently distinguish between them in co-cultures. Therefore, co-culture microscopy experiments detailed in this thesis were conducted with one p65-labelled cell-type and another untransfected cell population (e.g. BV.2 (lenti) + untransfected C17.2; Figures 4.1 and 4.14). This was due to the limited availability of other fluorescently-tagged p65 constructs, a problem which has since been remedied in the laboratory. Now, with a whole range of p65 constructs available, BV.2 or C17.2 cells can be lentivirally transduced with a non-eGFP construct which would enable them to be observed simultaneously.

Much of the focus in this chapter has been on C17.2 p65 dynamics. This is due to the preliminary nature of the data obtained on BV.2 (lenti) p65 dynamics. More specifically, BV.2 (lenti) cells displayed faster oscillations in co-culture, than in mono-cultures, but these results were obtained from only a single microscopy experiment (Figure 4.15-4.16). Further co-culture experiments should be conducted with BV.2 (lenti) to either discount or confirm the preliminary findings reported here.

7.5. Concluding Remarks

In summary, the results detailed in this thesis have provided a solid foundation for establishing NF-κB-mediated interactions between C17.2 and BV.2 cells. Evidence of these interactions was found at the molecular, genetic and cellular level. Combining data from these different levels of analysis has led to new hypotheses on NF-κB dynamics in these cells: 1) differential NF-κB dynamics in C17.2 is involved in cell fate decisions and;
2) BV.2 cells inhibit TNFα-induced C17.2 p65 dynamics, via paracrine factors, and thereby influence C17.2 fate.

The findings described are the first of their kind: paracrine interactions in the field of NF-κB dynamics are largely uninvestigated. These findings highlight the importance of studying NPC-microglial interactions. These results are particularly important given the role that these cells play in the neuroinflammatory process and, thereby, in diseases such as Alzheimer’s disease. Future experiments using confocal microscopy techniques, microfluidic Q-PCR, multiplex ELISA and single-cell apoptosis imaging will help to test the hypotheses proposed above and advance the field of NF-κB-mediated paracrine interactions.

A long term objective would be to understand these interactions and their dynamics in primary NPC and microglia. Our lab now has p65-DsRed and IκBα-eGFP-expressing transgenic mouse lines that are proving to be useful tools for studies of NF-κB dynamics in a range of cells and tissues. Therefore, in the future, it may be possible to use the mouse lines to move onto studying neuroinflammatory interactions in primary cells. In addition, the transgenic mouse lines may allow in vivo physiological studies of the role of NF-κB in neuroinflammation.
References


Ben-Hur, T., O. Ben-Menachem, et al. (2003). "Effects of proinflammatory cytokines on


NF-kappaB in Neuroinflammation


Grilli, M., F. Goffi, et al. (1996). "Interleukin-1beta and glutamate activate the NF-


Neurol 181(2): 115-129.


NF-kappaB in Neuroinflammation

31359-31370.


Ryder, E. F., E. Y. Snyder, et al. (1990). "Establishment and characterization of


Tanaka, T., M. J. Grusby, et al. (2007). "PDLIM2-mediated termination of transcription


NF-kappaB in Neuroinflammation


Weiss, T., M. Grell, et al. (1998). "TNFR80-dependent enhancement of TNFR60-induced cell death is mediated by TNFR-associated factor 2 and is specific for TNFR60." J


Wright, A., W. W. Reiley, et al. (2007). "Regulation of early wave of germ cell apoptosis and spermatogenesis by deubiquitinating enzyme CYLD." Dev Cell 13(5): 705-
NF-kappaB in Neuroinflammation

716.


