Context dependent cytokine secretion by stem cell derived microglia in vitro.

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Bachelor of Biomedical Science (2014)
Bachelor of Health and Medical Science Honours (2015)

A thesis submitted for the degree of Masters of Philosophy at
Monash University in (2019)
Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences
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Abstract

Thousands of people worldwide suffer from neurodegenerative diseases such as Alzheimer’s Disease (AD) and Parkinson’s Disease (PD). These diseases, characterised by selective breakdown of neurons and inflammation within the central nervous system (CNS), have no known cures, are progressive, terminal, and pose significant economic burden on healthcare systems and severe emotional burden to patients and families.

Research into these diseases has primarily, until recently, focused on neurons. It is now understood that another cell, microglia, play a pivotal role in the development and presentation of neurodegenerative disease. Microglia are haematopoietic, and are considered to be CNS resident macrophages, where they constantly survey their surroundings using their many fine processes. Microglia are capable of phagocytosing neuronal debris, support and maintain neuronal/astrocytic development, secrete cytokines and other communication factors, and migrate in response to damage or infection within the CNS.

This thesis utilised human embryonic stem cells (hESC) and a newly published protocol to generate stem cell derived microglia (SCDmicroglia) to investigate cytokine secretion in the context of in vitro models of inflammation. To allow tracking of SCDmicroglia development, a fluorescent and reporter vector was inserted into CX3CR1 – a microglia unique receptor within the CNS, using CRISPR-Cas9. Correctly targeted hESCs were then differentiated towards microglia using the newly published protocol by Abud et al (2017), and functionally validated using single cell RNA sequencing, immunocytochemistry, phagocytosis, and cytometric bead array assays.

Following functional validation of the differentiation protocol and the fluorescent reporter, an in-depth depth study of secretion of IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, MIP-1α, TNF, IFNγ, VEGF and Fractalkine was performed under a variety of conditions. Cytometric Bead Array was used to determine cytokine concentration via FACs, which was quantified using FCAP array software and analysed in GraphPad Prism 8™. This involved culturing SCDmicroglia, and midbrain neurons (separately and together) while incubating with Vehicle (PBS + 0.01%DMSO), LPS (100ng/mL), IFNγ (20ng/mL), TNF (100ng/mL) or α-synuclein aggregates (2.5µM) over a period of 2-to 48-hours. These experiments revealed that co-cultures secrete significantly greater amounts of cytokines during incubation with inflammatory mediators than SCDmicroglia monocultures, and also that TNF is a more potent stimulus for cytokine secretion than the gold standard LPS.
A preliminary examination into cytokine secretion by SCDmicroglia monocultures in 50% microglia media, and 50% forebrain media was also performed, using Vehicle (PBS + 0.01%DMSO), LPS (100ng/mL), IFNγ (20ng/mL), TNF (100ng/mL) or amyloid-β monomers (60 µM) incubation conditions. Including 50% microglia media in both midbrain and forebrain culture studies allowed investigation into whether media composition affected cytokine secretion by SCDmicroglia. These early experiments have indicated that SCDmicroglia monocultures secrete cytokines in a context-dependent manner, where media composition can and does influence which cytokines are secreted. Additionally, SCDmicroglia in Forebrain media monocultures appear to respond more readily to LPS than TNF, unlike the data obtained from SCDmicroglia monocultures in midbrain media.

This thesis represents the first in-depth examination of SCDmicroglia cytokine secretion during multiple disease modelling conditions in vitro, and presents a novel insight into how culture conditions can affect cytokine secretion.
Declaration

This thesis is an original work of my research and contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature: ....................

Print Name: ....................

Date: .............................
Publications during enrolment

Posters
- Generation of Fluorescent and Enzymatic H9 Reporter Lines for Microglial Differentiation – Presented at International Society for Stem Cell Research, 2018, Melbourne

Published Manuscripts
- “Mouse and human microglial phenotypes in Alzheimer’s disease are controlled by amyloid plaque phagocytosis through Hif1α”
  Dr. Alexandra Grubman, Xin Yi Choo, Mr. Gabriel Chew, Dr. John Ouyang, Ms. Guizhi Sun, Dr. Nathan Croft, Dr. Fernando Rossello, Rebecca Simmons, Dr. Sam Buckberry, Dulce Vargas Landin, Dr. Jahnvi Pflueger, Teresa Vandekolk, Zehra Abay, Jonathan Chan, John Haynes, Dr. Sarah Williams, Dr. Siew Chai, Dr. Trevor Wilson, Prof. Ryan Lister, Prof. Colin Pouton, Prof. Anthony Purcell, Dr. Owen Rackham, Dr. Enrico Petretto
  Submitted to Nature Neuroscience, July 2018

Submitted Manuscript
“A CX3CR1 reporter hESC line facilitates integrative analysis of in vitro derived microglia and reveals improvement of microglia identity upon neuron-astrocyte co-culture”
Alexandra Grubman, Teresa H Vandekolk, Guizhi Sun, Jan Schroder, Cameron Hunt, John M Haynes, Colin W Pouton, Jose M Polo
Manuscript in progress. Co-first Author with Alexandra Grubman.
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I would like to thank my supervisors, Dr John Haynes, and Prof. Colin Pouton. A Ph.D is difficult, and fitting three quarters of a Ph.D degree into a Masters thesis would not have been possible without your assistance. Thank you for respecting and supporting my choice to submit for a Master’s instead of a Ph.D. It was an enormous decision, and I am very grateful for your help during this time.

Next, I would particularly like to thank John, Erica, Stewart and Brad for their feedback and critiques of my (many) thesis chapters. I doubt my thesis would be either coherent or legible without your help.

This thesis would not have been completed without the substantial support I have been fortunate enough to receive.

Josh, thank you for the many cups of tea, blankets and chocolate during the late nights and weekends spent working on my thesis. Thank you for being so patient and helpful during my many, many weekends in the lab, and for helping to keep our house from descending into chaos. I absolutely could not have gotten through this without you and Reba to come home to every day. You are my rock.

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Emma, thank you for the laughs and your friendship. I needed them.

To Megan and Deb. Thank you for the many cups of tea and hugs during my thesis. Your friendship and support mean the world to me. I am lucky to call you my sisters in law.
## Glossary

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This document will address the specific examiner’s comments for the thesis titled “Context dependent cytokine secretion by stem cell derived microglia in vitro”, submitted by Teresa Vandekolk in fulfillment of the requirements for the degree of Masters of Philosophy.

Examiners comments will be in **bold** throughout this document, and the author’s responses will be *italicised*. Comments identifying specific issues will be addressed, and general comments will be addressed if possible to do so. The author has made every effort to address all comments.
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The thesis makes a significant contribution to knowledge and understanding of the field of research.
Examiner’s Report

Masters of Philosophy Thesis Examination for Teresa Helena Vandekolk (9th December 2019)

In this thesis Teresa details her research converting human embryonic stem cells into microglia with the protein expression and behavioural traits of primary microglia. She also details her research showing their response to and secretion of cytokines in vitro. It is my opinion that the thesis contains sufficient original research data for the candidate to be awarded a Masters of Philosophy. However, the unusual layout of the thesis, numerous typographical and referencing errors, and the high level of repetition of findings / content across thesis sections, means that the student needs to carry out additional text editing to ensure this thesis meets the scientific standards expected for this degree.

My specific comments follow:

1) Please edit the thesis to ensure the text is not constantly broken by half pages lacking text. For example, on page 9, half of the page has no text. I assume this is because the student does not know how to manage Figure and Legend insertion. It makes the thesis appear sloppy and unnecessarily long. This is also true of pages 14, 15, 19, 20, 48, 51, 68, 71, 74, 81, 82, 86, 89, 91, 92 etc. Where possible, the thesis text has been formatted to remove half pages lacking text, except where figure details make this not possible.

2) FGF2 should be defined when first used on page 10. It should also be written as FGF2 for the rest of the thesis. The 2 should NOT be subscript. The candidate often writes FGF2 (see pages 10 and 43). This is not standard nomenclature. Please correct. Similarly, stick with hESCs and MEFs and do not swap to hESC$ and MEF$ (see page 49 where this happens). FGF2 has been corrected to FGF2 throughout the thesis, and has been defined. hESCs and MEFs have been corrected.

3) The candidate often has subheadings that have no more than 1 paragraph of information included in that subheading. For example, the Heading “what are stem cells?” could still contain the information that is placed under “Maintaining pluripotency in stem cells” as that information still relates to a key stem cell property. Similarly, on page 28 “Migration towards sites of damage” and “Synaptic pruning” can surely come under a shared heading of “Microglial behaviour”. The heading on page 29 “Key microglial genes” is sufficient to include all of the information that follows about CX3CR1, TMEM119 etc – each paragraph on each gene is not a subheading, but information that belongs together under a unifying subheading. Combine everything up to and including TREM2. The Heading “Maintaining pluripotency in stem cells” has been removed, and these paragraphs have been included under the heading of “What are stem cells?”. The Heading “Functional roles and behavior of Microglia” now covers the sections on “CNS Surveillance”, “Context Dependent Responses to Stimuli”, “Migration towards sites of Damage”, and “Synaptic pruning”, and these sub-headings no longer appear. Finally, the sub-headings for CX3CR1, TMEM119, CSF1R, AIF1 and TREM2 have been removed, and all paragraphs are included under the heading “Key Microglial Genes”.

4) On page 14 “for what has been described as an inappropriate and unnecessary ..” Fixed.

5) On page 14 “there is clearly a need for a more thorough grasp of genome ..” Fixed.

6) On page 15 “adult central and peripheral nervous systems”.
7) On page 15 “white matter is from the deeper regions of the brain” makes no sense – do you mean is located within the deeper regions of the brain? 
This has been fixed. The sentence is now “…while white matter tissue is observed within the deeper regions of the brain…”

8) On page 16 the candidate provides information about the CNS, but does not indicate what species this is relevant to. For example, which brain has 67-85 billion neurons? It is critical in scientific writing to be precise. 
Fixed. The sentence is now “…it is estimated that there are between 67-85 billion neurons contained within the human brain…”.

9) On page 16 the candidate indicates that neurons are classified in a number of different ways but fails to indicate a key method of subtype identification, which is electrophysiology. 
Fixed. The sentence is now “Neurons are classified into subtypes based on a variety of factors, including their size, electrophysiology, the types and shape of spines present on their dendrites, types of receptors, neurotransmitter release, and location.”

10) On page 16, the candidate indicates that neurons are “relatively delicate”. This is a strange way to describe neurons as relative to what? 
The sentence: “Finally, neurons are relatively delicate – once they reach maturity, there is limited capacity for repair, replication, or regeneration in the event of damage or death” was not intended as a comparison of neuron delicacy relative to other cells types or tissues. It was intended to highlight the generally accepted inability of neurons to regenerate or recover in the event of damage due to illness or cellular trauma. In light of this comment, this sentence has been amended by removing the word “relatively” to remove confusion.

11) On page 18, please correct the way that you reference papers in text. Please use the format “Louveau et al. (YEAR) and Negi and Das (YEAR)”. Similarly, on page 27 “Kettenham et al. (YEAR) and Kierdorf and Prinz (YEAR)”.
Fixed.

12) On page 26, please correct “functions, including well as phagocytosis and autophagy”. 
Fixed. The sentence is now “…functions, including phagocytosis and autophagy.”

13) On page 29, please correct typographical errors within “A very recent study as used single cell” and “grey matter compared the hippocampus”. On page 30, the candidate refers to the phenotype of the CX3CR1 receptor knockout mice, but does not provide any information as to whether the phenotype is the result of a loss of CX3CR1 from microglia or other cell types. Does the literature provide any insight into the cell type/s primarily responsible for the phenotype? Please add this information in. 
These issues have been fixed. The first sentence now reads “…recent study has used single cell...”. The second sentence now reads “…occipital grey matter, when compared to the hippocampus...”. Additionally, this section now describes the secretion of IL-18 by microglia as a consequence of disruption to the CX3CL1-CX3CR1 axis through knock-out of the CX3CR1 receptor in microglia, and includes references.

14) On page 32 the candidate states “There are many genetic, phenotypic and functional
differences between human and animal microglia (308-311)”. This is a key reason for the current project, and yet is glossed over in this simple statement. It would be good if the candidate could pull out some key examples of species-specific differences. The examples that follow the statement relate to various sources of human cells to explain why stem cell-derived microglia are used, but don’t get into the species differences.

This section has been edited to discuss species specific differences in microglia gene profiles between human and mice, and to highlight previous sections which do discuss some differences between human and murine microglia.

15) On page 35, delete the extra space between “last” and “three” in the first sentence of “Stem cell derived microglia”. Please also combine paragraphs 1 and 2 in this section.
       Fixed.

16) On page 36, “this thesis aims to fill the gap”.
       Fixed.

17) On page 43 the candidate needs to be careful to use appropriate written scientific nomenclature rather than lab “slang”. For example, when they say “band on the gel” I assume they mean the size of the amplified DNA product?
       Fixed. “…band on the gel” has been re-written as “…The PCR amplicon, visualised as a band on the agarose gel…”.

18) On page 44, “On the day of lysis”.
       Fixed.

19) On page 45 “Following incubation, 4.2mL of 70% ethanol was added to each tube, which were centrifuged”. Please edit this sentence so that you don’t switch from was to were incorrectly.
       Fixed. This sentence now reads “…4.2mL of 70% ethanol was added to each tube, which was then centrifuged…”.

20) On page 46 the thesis methods have an unnecessary and unconventional level of detail e.g. “Bubbles were smoothed out of the Whatman 3MM paper using a roller, and the gel was carefully placed …”. There are many instances in this thesis where the protocols are not written as they would be in a scientific paper, but are written as they would be in a very detailed laboratory protocol.
       This section of the thesis detailing the transferal and binding of genomic DNA to a membrane has been edited to a more formal tone. The methods chapter has been edited where possible to reduce the feeling of a detailed laboratory protocol.

21) On page 52 the candidate states that “FACs resulted in large volumes of cell death”. Please rephrase this to indicate that a high proportion of the cells underwent cell death, as cell death does not occur in volumes.
       Fixed. This sentence now reads “…FACs resulted in large numbers of dead cells.”

22) When monocultures were compared with co-cultures in this thesis, were the monocultured cells grown in the same neuronal culture medium? Monoculture microglial medium is described as is the co-culture medium for midbrain and forebrain neurons, however, for experimental comparisons please make it very clear whether you are comparing cells grown in the same medium as well as in a different configuration or in a different medium and a different configuration and discuss the implications of your choice.
Chapter 2.5 – Co-culture methodology for neurons and microglia – describes microglia being cultured with microglia media up to Day 35 of differentiation, at which point, depending on the co-culture requirements, microglia are incubated in media containing 50% Terminal Differentiation Media and 50% Midbrain (if co-cultured with midbrain neurons) OR Forebrain media (if co-cultured with forebrain neurons). Additionally, at the beginnings of Chapters Four and Five, the media composition for the experiments within the specific chapter are clearly outlined.

23) On page 58 the numbering starts with 2.3 then goes to 3.1 then the next subheading has no number. Please ensure you have appropriately formatted your thesis. These subheadings have been amended to be correctly formatted.

24) On page 60 replace anti-body with antibody.
Fixed.

25) On page 62 “Data from the FCAP cytometric bead arrays were” OR “Data from the FCAP cytometric bead array were”
Fixed

26) On page 62 the candidate provides information about the statistical analyses performed relevant to the bead arrays, but there is no other information about statistical comparisons for any other type of experiment in this chapter. Was the immunofluorescence quantified and any statistical comparison made?
Immunofluorescence was not quantified, or statistically analysed. The other experiments in this thesis, aside from Cytometric Bead Array, were yes/no outcomes. For example, the immunofluorescence performed was used to confirm the presence of key microglial identity genes. If the antibody was visible in terminally differentiated microglia, it was not visible in hESCs which had not been differentiated. Additional data analysis methods (such as for the motility of microglia) are included in the Appendices, which the reader is directed towards later.

27) The introduction to Chapter 3 on page 63 doesn’t really say anything that resembles introductory material. It indicates that “It has been subdivided into sections to assist with clarity” which should be true of all scientific writing and should not be stated, and includes a declaration about who made a contribution to the project.
This section should instead include a clear articulation of the major unanswered question and aims of this Chapter, to set the scene for the experiments to follow. That should then be followed by an explanation of the promoters selected to drive fluorescence in the reporter stem cell lines i.e. information currently on pages 64 and 65 should be condensed into a few nicely linked paragraphs without subheadings to comprise the introduction. It does not have to be long, but it should certainly bring together the most critical points from the larger introduction to remind the reader of the major research question or goal i.e. why do you want to make these reporters and what is their purpose?
The declarations of contributions have been removed from this and the other experimental chapters. The introduction to Chapter 3 has been edited and condensed to clearly identify the need for novel stem-cell based reporter lines for use in microglia studies.

28) Chapter 3.2 from page 66 also needs to be reconfigured as it does not follow the conventions of scientific writing. For example, please remove the subheadings “CX3CR1”, “CX3CR1 Southern Blot results”, and “CX3CR1 Karyotyping by Monash Pathology” and replace with a single subheading “Generation of a human stem cell line expressing a CX3CR1-driven
flourescent reporter” or the like. Then please write paragraphs describing the generation and evaluation of this stem cell line that include key pieces of information currently missing from the narrative. The results section of a thesis should (broadly speaking) follow a pattern where the paragraph outlines your purpose, the methodology used and the outcome. For example: In order to generate a human stem cell line that expresses XXX under control of the CX3CR1 promoter, such that expression of the XXX protein is turned on as the cells differentiate into microglia, (or whoever did the work) first XXX (briefly state what you did from the beginning so you tell a story). Following XX genome editing, 24 stem cell colonies were selected for PCR screening using primers designed to amplify a ~1800bp region of the XXX gene, demonstrating insertion of XXX into the genomic DNA (if this is what you did). Each PCR was performed in duplicate and 14 of the 24 clones examined were found to express XXX (Figure 8).

Subheadings have been removed, and the section on generating a CX3CR1-reporter line has been edited to follow a more consistent story-telling narrative, as opposed to individual sections.

29) The paragraph that follows should start with something like: In order to confirm that the XXXX gene was correctly expressed within the CX3CR1 gene locus of edited clones (if this is where it was targeted to?), I next performed a Southern blot analysis of DNA extracted from 3 of the edited clones, using a probe against XXX. I found that XXX. At present you state that “To confirm insertion of the vector had been performed correctly, Southern Blotting of the CX3CR1 was performed”, but this does not tell us which vector, what it was supposed to be correctly inserted in to or how Southern blotting told you anything about where the DNA sequence may have been inserted i.e. what constitutes correctly. A person should be able to read your results section without having read your introduction or methods and still understand exactly what you did, why you did it and what you found, as each paragraph should tell them! At present, the lay out does not make it easy to follow.

The entire subsection of CX3CR1 gene targeting has been edited to follow this style of writing.

30) Similarly restructure the TREM2, TMEM119, IRF8 and Pu1 sections.

This has been done.

31) On page 67 “CX3CR1’s” is not an accurate name for the cell line. Please name this cell line and each of the others using standard genetic nomenclature and refer to it accordingly. Please delete your reference to the morphology and behaviour of the cell line on page 67 and move the karyotyping to a single section (after the Pu1 cell line generation) where you deal with all karyotyping together under one subheading e.g. “Karyotyping Human ES reporter cell lines”. It seems as though all of your edited cell lines end up being Trisomy 12. At present it is very repetitive to have that repeated for each line. Indeed, I assume this is actually the result of the starting H9 human ES cell line having the Trisomy 12, as it would be a strange coincidence if all of your gene editing produced this effect. If you combine all karyotyping data together, you can present your findings and draw a simple conclusion for the reader that will be less confusing than it currently is.

This is a fair point. The 5 sections on the individual lines and their screening, southern blotting and karyotyping results have been edited. There is now a single section focusing on karyotyping results for all lines (including the parental WT H9 human ES cell line). The other sections on generation of the H9-CX3CR1-tdTomato, TREM2-E2CRIMSON, TMEM119-GFP, IRF8-mCherry, and PU.1-GFP lines have been edited to follow the examiners suggested narrative.

32) Please simply paragraph 2 on page 76 and combine with paragraph 3: “To determine whether XXX cell lines were capable of differentiating to produce microglia, I utilised two distinct
microglial differentiation protocols. HPCs were not successfully generated using the Abud et al. (2017) protocol, however, were successfully generated using the Stem Diff Haematopoietic Progenitor Kit (Stem Cell Technologies). hESCs developed into large cystic structures and phase bright cells were observed to "bud" from these structures (example in Figure 14 from the XXX cell line). These budding cells were non-adherent and, when analysed by FACS or MACs to quantify CD43-expression, were confirmed to be CD43⁺ microglial progenitor cells.

These two paragraphs have been simplified and joined into one continuous section as suggested.

33) On page 78 and elsewhere in the thesis it looks as though the candidate has inserted a plus in underlined font (+) instead of the symbol ±
This has been fixed.

34) In Figure 16 the candidate says “Zoe images” but this does not tell us what type of microscopy this is.
This has been fixed. The text now reads “Bio-Rad ZOE fluorescent cell imager”.

35) On page 81 the candidate says “Cultures maintained with 1µg/ml of Matrigel developed a characteristic rounding of microglia and clustering which was not observed in cultures maintained in the increased 2.5µg Matrigel” however it is unclear what the rounding tells them and therefore why microglial development required the increase in concentration. The observation is interesting but the explanation is lacking. Please clarify.
This section has been re-written, and has now been expanded to justify why microglia required an increase in Matrigel concentration. The section now reads as follows: “Attempts to rectify this rounding included the addition of cholesterol at 1µg/ml, but this did not improve the clumping observed, so was not included in further experiments performed within this thesis, with the exception of those experiments described in Appendix V. Increasing the concentration of Matrigel from 1µg/ml to 2.5µg/ml following isolation of CD43⁺ HPCs was determined to be the most effective way to prevent rounding and clustering of microglia. It is theorised that the observed clumping and rounding of microglia during culture with 1µg/ml of Matrigel indicated that the microglia were in a reactive, amoeboid state, although this was not confirmed by RNA sequencing. This theory was formed because microglia are known to adopt an amoeboid morphology, involving the retraction of filopodia and processes, in response to a range of cellular and environmental insults (227, 231, 366-369). The observation that an increase in Matrigel concentration reduced clustering and allowed lobed processes to form on cells suggested that this was a more favourable environment for the cells to be grown in, and therefore, 2.5µg/mL of Matrigel was used to support microglial development in all future experiments.”

36) On page 84 What is “Figure 21.12”? Also you need to refer to the specific figure panels including the letters not just the numbers. On this page Figure 20 is entirely skipped. Please also make it very clear which data are from the WT h9 ES cells. If this section is all proof of concept differentiation analysis, make that clear, but if any of it is done with your modified lines, please clearly indicate which images etc are from those altered lines.
“Figure 21.12” has been rectified

37) On page 86 the candidate refers to Figure 2013, please correct this typo.
All figures have been corrected, and their cross-references within the text have been updated to accurately reflect this.

38) On page 90 the candidate is talking about major differences between the medium types and names phenol-red as a major difference. As media often comes with and without a pH
indicator, I am not sure I would have included this as an example of a major difference – usually growth factors and salt concentrations etc are considered major differences in media type.

Phenol red was mentioned within this paragraph because personal communications with Abud and colleagues highlighted that inclusion of Phenol red in differentiation media alters the capacity for differentiation towards microglia in HPCs. This has been included within the paragraph describing differences within media composition. Additionally, brief mention of growth factors included in midbrain differentiation media which are not present in microglia differentiation media has been included. The section now reads as follows:

“….media used for culture of midbrain monocultures contains Phenol-red, whereas microglial media does not, and personal communications from Abud and colleagues described difficulties in differentiating/promoting survival of microglia from CD43+ HPCs in media containing phenol red. Additionally, media used for differentiating neurons contains a number of growth factors (such as TGF-β3, BDNF, GDNF and activin A) which are not present in microglia terminal differentiation media, and may act to alter microglia survival or function…

39) Pages 96-97 appears to recap the findings of the Chapter, but doesn’t really discuss them at all. However the final paragraph jumps the gun a bit, stating that they can secrete multiple cytokines in response to stimulation with various factors – however, that is the whole point of the next thesis Chapter, so it would be more appropriate to indicate that the capacity of these cells to respond to inflammatory cues and participate in the inflammatory response is yet to be established and understanding that will be critical to defining the role of microglial in CNS pathology.

This section has been rearranged and expanded upon to add further discussion to the findings of the chapter. A small discussion on the need for regular karyotyping of hESC lines, and the potential use for the karyotypically abnormal lines has been included. Discussion of the complexities in the protocol for differentiation of hESCs to microglia, and the possible causes for differentiation failures has been expanded upon. Finally, discussion of the functional capacity of microglia

40) On page 100 is the first instance (of many) where referencing errors have resulted in the insertion of (see Error! Reference source not found) into the thesis text. Please correct each of these. There are 3 instances on page 100 but many in the pages that follow.

Fixed. During uploading of the thesis files to the MGRO portal – the file was partially corrupted and resulted in multiple “Error! Reference source not found” messages. The thesis file has been corrected, and references for both papers and figures have been corrected throughout the document to the best of the author’s ability.

41) On page 100, when giving multiple p values in the results text please make it clear which relates to a comparison of changes over-time, which relates to culture type comparisons etc. These results sections denote from the outset whether the comparison is between culture types, or over time within cultures. Where needed, a p-value relationship to either changes over time or between culture types has been clarified.

42) On page 119 the sentence “LPS is an endotoxin …” should be included when you are first explaining what LPS is and why it is used in this study – not in the discussion.

This has been removed from the discussion. The description and explanations of the inflammatory molecules, and the reasons for including them in this thesis have been moved to Chapter Three. This is now part of the narrative set up for the preliminary investigation into microglial cytokine response to inflammatory molecules.
43) Alternating between Results and Discussion sections for each cytokine in Chapter 4 is very unconventional and is certainly not something I have ever seen before. Please have all results sections in sequence so they follow one after the other, then combine the discussion sections. This will also cut down on repetition. Please also avoid using colloquial expressions such as (on page 123) “makes for some interesting discussion”.

Following thorough discussion with Supervisors, it has been decided that the rearrangement of Chapters Four and Five as suggested will not be performed. Multiple attempts to write this chapter were performed, but it was difficult to adequately describe the data and discuss it in the suggested format. While it is acknowledged that the current format of these Chapters is unconventional, there are no “hard” rules for how a thesis must be set out in regard to the arrangement of results and discussions within chapters. MGRO Procedure Document for “Graduate Research Thesis Examination Procedures” Section One, subsection 1.7 “Thesis Content and Academic Integrity Provisions” article 65 states that “The student is responsible for determining the layout of the thesis and selection of the title in consultation with their supervisory team.” The current layout was decided upon following strong suggestion from the thesis supervisors and careful decision on the Author’s part. Discussing all cytokines simultaneously meant that not all facets of the results obtained could be considered.

44) An overall comment is that the candidate should make every effort to reduce the level of repetition in the thesis. That includes removing discussion points covered in the Chapter discussions from the Chapter 6 discussion – which should be reserved for bigger picture “Future Directions” content.

It is fair to say that the thesis itself is repetitive. This is largely because most of the data discussed within the thesis came from a specific assay – the Cytometric Bead Array, and this assay was repeated to measure different culture conditions for Chapters Four and Five. Many papers investigating multiple cytokines simultaneously will take the opportunity to discuss the results of the individual cytokines separately, as has been done here.

To reduce the level of repetition, multiple actions have been taken:
Firstly, as the description and explanation for the statistical analysis performed in this thesis is provided within the Chapter Two as well as in the introductory preambles of Chapters Three, Four and Five, and the specific analysis test is included as part of the figure legends, mention of the specific test (Two Way ANOVA with post-hoc Tukey’s Test) has been removed from the text, except where appropriate.
Secondly, some points covered in the chapter discussions – for example, the discussion of Trisomy 12 in patients with Chronic Lymphocytic Leukaemia and its relevance to CX3CR1-expressing myeloid cells – have been moved from the individual chapter discussions and condensed into the final discussion chapter.
Examiner: Dr Mauricio Castro Cabral-da-Silva

<table>
<thead>
<tr>
<th>Title</th>
<th>Dr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Mauricio Castro Cabral-da-Silva</td>
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<td>Current institution</td>
<td>University of Wollongong</td>
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<td>Recommendation</td>
<td>Pass, with major revisions, certified by Monash Chair of Examiners</td>
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<td>Numerical result</td>
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The thesis makes a significant contribution to knowledge and understanding of the field of research.
Report on Teresa Helena Vandekolk Master’s of research thesis.

The thesis written by Vandekolk describes the generation of five embryonic stem cell-based (H9) reporter lines and their validation by differentiation to microglia, followed by a partial assessment of function of the generated cells.

Evidence was presented that a few clones were properly targeted for all 5 genes targeted, and consequently, cell lines were generated. Clones were validated by submitting them to a high impact, now widely used, validated microglia differentiation protocol first described in Abud et al. (2017), which is highly valuable and understandably strategic.

To this work, Vandekolk attaches two original articles where she figures as co-author. Both papers are in the context of the thesis and reflect some level of technical competency of the candidate.

The task of generating multiple reporter lines (first aim of this thesis) is a repetitive, systematic and labour intensive one. It consists of multiple checkpoints where the experimenter is supposed to assess the quality of the input cells in addition to any of the derived cell lines. If achieved with perfection, the reporter lines generated would be of extreme relevance for the scientific community and would serve as a great aid for the development of alternative differentiation protocols as well as helping further improvement of current protocols. Sadly, all the generated cell lines were found to harbour genomic aberrations, inherited from their parent cell line, and should not be used for further studies.

Negative results are also reportable and have their value but they need to be reported in a way that values the experience of the experimenter. The reader should be made aware of the skills and concepts acquired by the writer along their research period. The presentation of this thesis, in many aspects, falls short of it.

On many occasions the author fails to properly describe the complete conditions set for a given experiment. Incomplete figure legends, bad/wrong referencing or confusing description of the obtained results made it really hard to judge the context and relevance of some findings and if the author has full understanding of what is being described.

While the compiled literature contained in this thesis is pertinent with its object of study, and encompasses all the relevant papers, it is clearly overdone. This thesis lists more than 600 references and, in many instances, the author cites papers from which the title mentions the terms in need of support but are hardly giving support to the author’s statements by its core findings, meaning some references are not appropriate.

There is a clear lack of consistency in this piece of text, making me question at some points if I indeed received the final version of it!
To loosely serve as a guide for the author I have listed most of the problems found in this thesis below. This list is in no ways exhaustive and only aims to illustrate some of the important points which need to be addressed before it is a final version. Further notes and comments can be found in the body of the thesis (uploaded PDF file). As a disclaimer, repetitive mistakes were not all marked down individually. The author should not rely solely on responding to my notes and comments in order to improve this thesis.

Large pieces of text and figures need to be reworked and restructured before it reaches the standards of a master’s thesis.
Minor issues:

- **The abstract is beyond the 500-word limit by quite a few words.**
  
  *This has been fixed. The abstract is now 499 words long.*

- **Some immunocytochemistry images miss the description of the colour used for a given marker in their legend (e.g. p.93).**
  
  *This has been fixed. All immunocytochemistry images within this thesis have the legend updated to accurately reflect the colours of the markers used.*

- **The author doesn’t seem to have any notions of the use of page and line breaks, making this thesis extra long by wasting page space.**
  
  *This has been fixed, as it was also brought up by the other examiner. Where possible, the pages have had page-breaks removed, and figures/figure legends adjusted to remove extra space.*

- **Duplicated aims (p.37), a clear example of lack of attention for details:**

  
  | 3) | Determine which of a selected set of cytokines are secreted by stem cell derived microglia when in media used for midbrain neuron differentiation |
  | 4) | Determine which of a selected set of cytokines are secreted by stem cell derived microglia when in media used for forebrain neuron differentiation |

  *Although Examiner B feels that these are a duplication of aims, they are in fact separate aims, and are investigated in individual chapters. Aim 3) is investigated in Chapter Four, and Aim 4) is investigated in Chapter 5. To make it clearer for future readers, the aims have been re-written and are included below:

  3) Determine which of a selected set of cytokines are secreted by stem cell derived microglia, stem cell derived midbrain neurons, and co-cultures of midbrain neurons with microglia, when cultured in media used for midbrain neuron differentiation

  4) Determine which of a selected set of cytokines are secreted by stem cell derived microglia when cultured in media used for forebrain neuron differentiation

- **n.m. and n/m are used interchangeably. “n.m.” is not listed in the abbreviations list.**
  
  *This has been corrected. n.m. is now used throughout the text, and was included in the abbreviations list.*

- **In some instances, the candidate refer to herself in the third person in a way that resembles the way the contributions of each author is made for a paper but I am not so sure such style is suitable for a thesis.**
  
  *This has been fixed. All references to the author in any capacity have been removed.*

- **CHIR-99021 appears all times as CHIR99026.**
  
  *This has been fixed.*

- **Figure legends miss key descriptive essentials, such as the type of microscopy (phase-contrast/bright field, confocal) or the colour associated with each marker for immunocytochemistry.**
  
  *This has been fixed. All microscopy images now contain the type of microscope used, the objective, colours associated with antibody markers, and the type of microscopy performed.*

- **An elusive “Electronic appendix” is mentioned seven times but is nowhere to be found. Where would the reader be able to access the cited movies? How does the candidate plan to provide access to it? No link to them was provided in this version.**
  
  *During writing, an electronic appendix was made to incorporate the movies and extra data*
generated for the thesis. However, at the time of submission, there was no way to upload the electronic appendix files to the system. The submission process only allowed for uploading of one document in PDF format, and did not allow for inclusion of an electronic appendix. The physical copies of the thesis submitted to the supervisors at the time each contained a disc with the full electronic appendix. As there is currently no other way to upload this data to the Monash Thesis Submissions Portal, the electronic appendices are located on the student’s shared google drive (link provided below), and the link is publicly available to those who request it. To the student’s knowledge, there were no requests made to either supervisor or student for access to the shared drive. Additionally, the link is now included at the end of the table of contents in the main body of the thesis.

https://drive.google.com/drive/folders/1x1Ylt4-TAcuuy7g4-J7bShYdd1uw8vTS?usp=sharing

- **Figure 17:** The effect illustrated by this figure should have been also pictured in the absence of cholesterol. Is the aggregation a result of the added cholesterol? Not discussed in the thesis. Cholesterol is only mentioned again on the attached paper.

The aggregation was not the result of the added cholesterol. The aggregation was the result of insufficient Matrigel included in plating. The “clumping” effect observed in this figure was also observed in the absence of cholesterol. These were exploratory studies with performed with minimal laboratory support, as at the time of culture, the student was the first and only person on the Parkville campus to attempt culturing microglia from stem cells. Therefore, there were many early attempts which were abandoned due to poor differentiation/death/clumping of the cells.

To illustrate that inclusion of cholesterol did not improve clumping and rounding of microglia, an image from a separate Day 17 culture of H9-WT has been included, to show observed clumping beginning a few days following FACs CD43$^+$ isolation. This has also now been discussed in the thesis, in the same section.

- **Figure 18:** The nuclear staining figures conventionally come first to give the reader an idea of how many cells overall are being looked at. The “day 0” of the first column barely shows any cells (DAPI).

The figure referred to here shows very few cells in Day 0 DAPI due to the seeding technique used for experiment set up. Setting up a differentiation required approximately 60 “clumps” of undifferentiated hESCs to be seeded into a single well of a 12 well plate approximately 24 hours prior to beginning of differentiation. These clumps were comprised of between 5-20 cells each, and as such, it was difficult to find a large clump of Day 0 cells in a part of the well that was not affected by the light of the microscope refracting through the plastic. These were the best images able to be obtained for this time point.

For ease, the figure has been rotated 90°, and the scale bars thickened. Additionally, the Merged images have been moved to the right hand side of the figure, and the DAPI images are now first.

- **Figure 19:** A colour scale should be added to this figure. Inconsistent with many other figure legends contained in this work, this one does not present figure letters in brackets, i.e. “A” vs (A). This has been addressed. The colour scale has been added beneath the figure, and the figure legend has been corrected so that figure letters (A), (B) and (C) are bracketed, consistent with other figures.

- **Most of the scale bars are not readable.**

Where possible, this has been fixed.

As some confocal microscopy images within this thesis are used with permission of others, the scale bars on those figures is unalterable as the author of the thesis is not in possession of the original file. For those figures, the author has thickened the scale bar, and has included the size of the scale bar in the legend below.

Additionally, images taken using the Bio-Rad ZOE fluorescence cell imager automatically have a scale bar burned into place in the lower right corner of the image, but as the images taken using this equipment are of lower resolution, these scale bars cannot be altered. Therefore, for images used that were taken on the Bio-Rad ZOE fluorescence cell imager, the scale bar has been thickened,
and the size of the scale bar is included in the figure legend below.

- **Figure 20**: The legend describes a figure that isn’t there (no filter for TREM2-…)
  This does not refer to a figure which is not included. It refers to the fact that the TREM2-E2-CRIMSON fluorophore was not imaged, because the E2-CRIMSON was not easily detected on the A1R microscope, so imaging experiments such as this phagocytosis assay did not attempt to visualize it. For clarity, the note “No filter for TREM2” has been removed.

- Chapter 2 starts numbering subitems from 1.x and progresses to 2.x following to 3.x while chapter 3 starts from 3.x, as expected.
  This has been fixed. All sections are now numbered consistently.

- In reference #23 “International Stem Cell I”, figure as the first author. International Stem Cell Initiative, the "I" should not be abbreviated since this is not an individual's name.
  This has been fixed.

- Reference #133 lacks access date. No clue of what “D.” means. No space between the website address and year.
  This has been fixed. Endnote struggles to correctly export organisation names from Endnote into Word. “D” stands for Dementia. Reference now reads as follows:

- Neither the references section nor the appendices section figure in the table of contents.
  This has been fixed. The References, figures, tables and appendices are now all listed in the table of contents.

- No consistency is observed in the references section. Some journals’ titles figure as full, some as an abbreviation. Some articles are listed with all words of the title in capitals, some are presented in all lower case.
  This has been fixed. All journal names are now present in full. The article listed with the title in all capitals has been manually corrected.

- After page 290 all page numbers are either corrupted or non existent. This thesis + appendices has 432 pages.
  This has been fixed. This was due to a formatting problem with Word that was not recognised prior to submission.

- The karyotyping technique used is frequently referred as “g-Band” instead of “G-banding”
  This has been fixed.

- Appendix IV refers to figure 33 as figure 1 and shows the wrong value in the example equation (25.89 instead of 35.89).
  These issues have both been fixed.

- Appendix V: In “et al.”,”al.” is an abbreviation for “alii”, “aliae” or “alia” and should have a period sign after it.
  Changes to Appendix V, a submitted manuscript, will not be made as this manuscript is currently already under peer review. However, on this advice, all instances of “Et al” in the main body of the thesis and appendices have been fixed to correctly say “et al.”.

- The sequencing data referred to doesn’t have a defined GEO accession number.
  The accession number referred to is GSE89189. The appendix and thesis references have been
The beginning of each chapter isn't in the index. This has been fixed. Unless specifically directed to, Word does not automatically include Chapter Titles as part of a Table of Contents

Too many to be listed:

• Generalised lack of consistency in paragraph format, indentation, capitalisation, bolding and everything else format related. Where possible, this has been fixed. The author has made an effort to make consistent the figure legends, titles, indentation, capitalization and paragraph layout.
• Missing spacing between words. Fixed where possible.
• Missing commas. Fixed where possible.
• Grammatical issues. Fixed where possible, and detected by Microsoft grammar check.
• Capital letters appearing in the middle of words. Fixed where possible using Microsoft spelling and grammar checker.
• Suppression of articles (the). Fixed as per Examiner A’s comments earlier, and in other parts of the methods chapter as found.
• Unnecessary use of capitalisation. Capitals not at the beginning of a title/sentence or part of an acronym/gene name have been removed.
• Unconventional use of well-established acronyms (e.g. FACS/MACS vs FACS/MACs). This has been fixed where possible. FACS/MACS have been replaced with FACS/MACs.
• Non italicsed gene names. Gene names are only italicized when referring to the gene itself, and not the protein. This has been fixed where possible throughout the thesis and appendices.
• Inappropriate use of italics. Italics have been removed when not part of a figure legend, subheading, or gene name.
• Corrupted Greek symbols. Greek symbols have been fixed where possible.
• Missing “:” after many “Figure XX” in figure legends. Fixed in thesis and in Appendices where possible.

The candidate has made a significant effort to go through the thesis and appendices again to find instances of the mistakes indicated by Examiner B, and has made changes where possible to address these issues.

Major issues:

• There is no figures list or figure index. This could have helped to avoid skipping figures or duplicate figure numbers, another major issue with this thesis. There is now a list of figures for the thesis, immediately following the Table of Contents.

• The number of wrong references to figures is the highest I have ever seen on any thesis (most occurrences in chapter 3).
  These references have been corrected and the figure numbers updated to accurately reflect their position within the thesis.

• It is unfortunate, but given the number of mistakes referencing internal chapter figures produced by the author, I feel insecure about the accuracy of the literature references. As I have mentioned previously, the referencing is excessive, which possibly points out to referencing issues.
  Literature references are accurate, and were chosen with care during writing. The chapters were written as separate documents, and in compiling the files together for the thesis, Word appears to have shifted the internal referencing incorrectly. This has been corrected, and to the candidate’s knowledge, all figures are referred to and numbered correctly.
- Two-way ANOVA is found as “two-way ANOVA” but also as “TWO-Way ANOVA”, “2-way ANOVA” and even simply as “Two-Way”. This is a very well-established term and it is unacceptable that such variance is found along the text.

  This has been fixed. All references to a Two-way ANOVA are now in the format of “Two-way ANOVA”.

- There is a lack of consistency in the terminology used to describe the generated cells, e.g: “SCDmicroglia” (abstract), “scdmicroglia” (pp.36-37), “scdMicroglia” (p.150).

  From p.197 and ahead the same terminology as the abstract was used (SCDmicroglia). In the large gaps between the noted pages, including the whole results chapters, other terms are used to refer to the PSC-derived microglia. No “SCD microglia” or SCD-microglia”, or more appropriate forms, were ever used in the examined thesis. Unfortunately, this term is not minor but the main object of study in this thesis, which reflects a lack of care. Also, the author doesn’t seem to follow her own established rule: “All stem cell-derived microglia hereafter will be referred to as “microglia”, for easy...” (p.63).

  This has been fixed. When not referring to a specific line generated within this thesis, the term used is SCDmicroglia throughout the thesis. When referring to a specific line, the full name of the line (eg. H9-CX3CR1-tdTomato microglia) is used.

- Fifteen occurrences of “ERROR! Reference source not found”.

  This has been fixed as noted for Examiner A.

- Sections 3.1a-e are very similar and make the thesis unnecessarily repetitive and could have been consolidated in a single item.

  This has been fixed. Examiner A also pointed this out. This section has been re-written to accomplish this.

- Most figures seem to be just to illustrate observations (lack of quantitative data) and the titles don’t always reflect their contents.

  The figures within Chapter Three are for the validation of SCDmicroglia differentiation, and function to answer yes/no qualitative questions. For example: Did the microglia exhibit membrane-ruffling? Yes, as illustrated by Figure 21 with colourised Wiggle index. Did undifferentiated cells express the same markers as differentiated microglia? No, as shown by Figure 20 immunochemistry images.

  While Examiner B notes that most figures seem to just illustrate observations, and this is somewhat true for Chapter Three, Chapters Four and Five are comprised almost wholly of figures of quantitative data as are Appendix XI and Electronic Appendix VI.

- Missing figures, figures out of order or with duplicate numbering.

  This has been fixed. As noted above, the chapters were written individually, and compilation appears to have been somewhat unsuccessful in translating figure order and numbering. Both the thesis and all appendices contain the correct figures in the correct order.

- The generated cell lines are labelled arbitrarily by the author, but the author fails to stick with a single denomination. For example, the TREM2 targeted PSC line was named in 11 different ways (!). Sometimes multiple denominations would appear in a single page or in a very short range of pages. The following are examples of how the TREM2 targeted cell line was referred to in this thesis: “TREM2-clone#2” (p.70), “Clone #2” (pp.69, 70), “Clone 2” (pp.69, 70), “TREM2.2 clone” (p.71), “TREM2-2 clones” (p.71), “TREM2 2 clone” (p.72), “H9-TREM2” (pp.78, 79, 96, 279), “TREM-2- CRIMSON” (p.86), “TREM2”, “TREM2-reporter cell line”, “TREM-Microglia cells”.

  This has been fixed. All references to each of the reporter lines is now consistent throughout the thesis. The CX3CR1 line is referred to as “H9-CX3CR1-tdTomato”; the TREM2 line is referred to as “H9-TREM2-E2CRIMSON”; the TMEM119 line is referred to as “H9-TMEM119-GFP”; the IRF8 line is referred to as “H9-IRF8-mCherry”, and the PU.1 line is referred to as “H9-PU.1-GFP”.
● Overuse of “demonstrate” and “illustrate” with the wrong meaning.  
   This has been fixed.

● PSC to microglia differentiation table/protocol omitting all small molecules.
   
   Except for small molecule Y27632, used in plating out the cells prior to differentiation, there are no small molecules used in the protocol by Abud et al. (2017). If this comment is in reference to Table 3 in the thesis, inclusion of the small molecules and proteins used for these 12 differentiation protocols is outside the intended scope of the table.

● Some figures borrowed from papers or the internet lack the proper referencing, against the copyright notice.
   
   Only one figure is obtained from the internet (Figure 3), and it is cited correctly in the figure legend. There is no copyright that the candidate is aware of prohibiting use of this figure with citation, as has been done in this thesis. Figure 33 was adapted from knowledge contained in two papers, which were cited with the figure. All other figures were made by the candidate using BioRender.com software and are of her own design, and do not infringe on copyright. The figures made in BioRender.com are all cited as such in the figure legends, as required by BioRender user licensing.

● Chapter 3 discussion: A better discussion about why an extremely high number of cells were dying when sorted by FACS would be desirable here. FACS is a well-established technique which is widely used. One can’t really blame the technique for not being able to make it work. It is expected from a master’s candidate to be able to understand what is “going on” not just “what happened”.
   
   While FACS is a well-established technique, every cytometer is different, and a technique that works in Cytometer A in Laboratory A by Researcher A may not work when using Cytometer B in Laboratory B by Researcher B, even if the protocol, cell lines and reagents are identical. Ultimately, the cause for the cell death during FACS was never confirmed, only suspected. It was believed to be due partially to the delicacy of the progenitor cells, and partially to the shear forces to which cells are subjected to during FACS. This has now been discussed in Chapter Three, and is included below for the examiner’s benefit.
   
   “Isolation of CD43\(^+\) HPCs was determined to be best performed using MACs to avoid the high numbers of cell death that occurred during FACS. As mentioned earlier, multiple alterations to pressure, nozzle size, resuspension volume and flow rate were made to the protocol in attempt to support the CD43\(^+\) progenitor cells through FACS processing, but these were unsuccessful. It is suspected that CD43\(^+\) cells are potentially more sensitive to shear stresses, such as are experienced during FACS, and this might explain why the cells were dying during FACS. Additionally, the length of time for FACS harvesting was approximately four to five hours, most of which was spent on ice. Potentially, this extended time also contributed to the large numbers of cell death during FACS. In contrast, MACs could be performed in as few as three hours, did not subject CD43\(^+\) cells to shear stress, and did not require the use of resin columns. These factors made MACs a gentler option for CD43\(^+\) isolation, and resulted in very little cell death.”

● Chapter 3 discussion: Cytokine secretion does not belong to chapter 3 but it was mentioned in the chapter’s discussion as “proof” that the derived microglia-like cells were functional.
   
   Cytokine secretion does belong in Chapter 3, and in its discussion. Chapter Three, Section 3.34 describes preliminary cytometric bead array data which establishes that the differentiated H9-CX3CR1-tdTomato targeted line can respond to known inflammatory modulators of microglia (being LPS, TNF, IFNγ and Amyloid-β). The preliminary data itself in Appendix VII. While not the focus of Chapter Three, a secretory response to inflammatory stimuli is an important function of microglia, and is the basis for the work performed in future chapters. Therefore, inclusion of cytokine secretion in Chapter Three is necessary before moving on to Chapters Four and Five.
In “appendix V” there is a lack of consistency when listing the cytokines measured. Slashes, commas, spaces or nothing at all are used interchangeably.

This has been fixed.

Appendix VII: The rationale behind the axis range selection for the graphs in this section isn’t clear (see image below) and the same is observed for other measurements.

The graphs in this comment have been taken out of context. The graph for IL-4 comes from Appendix VII, Figure 40, the graph for IL-6 comes from Appendix VII, Figure 41, and the graph for IL-10 comes from Appendix VII Figure 42. The axis ranges for each of these graphs enables comparison of secretion of the cytokine being investigated (for example: IL-4), by incubation with inflammatory stimulators (LPS, TNF, IFNγ and α-Synuclein). The figures are not meant to be compared to each other. For clarity, the introduction to the appendix now includes the following statement: “Scales are specific to the cytokine being interrogated, and figures are not designed to be compared to each other.”.

The manuscript listed in the Appendix does not figure in the listed “publications during enrolment”. The one listed as submitted to Nature Neuroscience in 2018 (Mouse and human microglial phenotypes... through Hif1α), figures in the biorxiv.org as posted in May 2019, a year later (see below). This information is conflicting, please clarify.

At the time of submission, the student had not received notification that the above paper had been published and was under the impression that the paper listed above was still under review. This paper was listed as a “Submitted Manuscript” in the original thesis, but has now been listed as a Publication.

The title for the submitted manuscript in Appendix VI has changed several times. The original title was listed in the thesis as a submitted manuscript but should have been updated to reflect the new title as appears in Appendix VI. This has been corrected. The correct title “A CX3CR1 reporter hESC line facilitates integrative analysis of in vitro derived microglia and reveals improvement of microglia identity upon neuron-astrocyte co-culture” has now been listed in the Submitted publications.

Extensive proofreading, editing and formatting are highly recommended!
In summary, this thesis represents the necessary experimental work to form an acceptable thesis and the candidate seemed to have discussed it to some depth, but major changes to its presentation, the overall formatting and extensive proofreading are required before it will be at the standard of a Master’s thesis.

Please do not hesitate to contact me with further questions in regards to this examination. Faithfully,

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Table of Contents

Table of Contents .................................................................................................................... 1
Figures and Tables ................................................................................................................... 7
Chapter One ............................................................................................................................. 10
Introduction to Literature review ......................................................................................... 10
Stem cells ............................................................................................................................... 10
What are stem cells? ............................................................................................................... 10
Gene Editing in the context of stem cells ............................................................................ 12
Homologous recombination and non-homologous end joining ...................................... 12
Gene Editing Techniques ..................................................................................................... 12
CRISPR-Cas9 .......................................................................................................................... 13
Ethics and concerns regarding gene editing ........................................................................ 14
The Central Nervous System ............................................................................................... 15
CNS development ................................................................................................................ 15
CNS composition .................................................................................................................. 16
CNS immune privilege ......................................................................................................... 18
CNS plasticity ......................................................................................................................... 19
Neurodegenerative Disease ................................................................................................. 19
Parkinson’s Disease .............................................................................................................. 21
Alzheimer’s Disease .............................................................................................................. 22
Microglia ................................................................................................................................ 23
Microglia development ......................................................................................................... 23
Microglia morphology .......................................................................................................... 26
Functional roles and behaviour of microglia ....................................................................... 26
Microglia distribution and regional heterogeneity ............................................................... 28
Key microglial genes ............................................................................................................ 29
Microglial interaction with other cells of the CNS parenchyma ....................................... 31
Microglia for Research ......................................................................................................... 32
Bone marrow derived monocytes ....................................................................................... 32
Immortalised lines ............................................................................................................... 33
Primary cultures .................................................................................................................... 33
Stem cell derived microglia ................................................................................................. 34
Tying it all together ............................................................................................................... 35
HYPOTHESIS: ....................................................................................................................... 36
AIMS: ..................................................................................................................................... 36
Chapter Two

2.1 Targeting and validation of clones

2.1.1 – Plasmid design and CRISPR targeting .................................................. 37
2.1.2 – Manual picking and Expansion of targeted clones .................................. 40
2.1.3 – Validation through PCR Screening ....................................................... 40
2.1.4 – Validation through Southern Blotting ................................................... 42

Chapter Two: Maintenance and differentiation of human embryonic stem cells (hESCs) ........ 45

2.21 – Maintenance of hESCs ........................................................................... 45
2.21a – Preparation of mitotically inactive fibroblasts ........................................ 45
2.21b – hESC culture and maintenance on Mouse Embryonic Fibroblasts .......... 45
2.21c – hESC culture and maintenance on Feeder Free system .......................... 46

2.22 – Differentiation of hESCs towards microglia ......................................... 47
2.23 – Differentiation of hESCs towards midbrain neurons ............................... 48
2.24 – Differentiation of hESCs towards forebrain neurons ............................... 50
2.25 – Co-culture methodology for neurons and microglia ................................. 51
2.26 – Treatment of cultures with inflammatory stimuli ..................................... 51

Chapter Two: Assays, molecular techniques, and data analysis ......................... 52

2.31 Cytometric Bead Array ............................................................................. 52
2.32 – Preparation for immunocytochemistry .................................................. 53
2.32a – Fixation of cells .................................................................................. 53
2.32b – Permeabilisation of microglial monocultures using PBS-Triton-X .......... 54
2.32c – Permeabilisation of neuronal mono- and co-cultures using modified iDISCO protocol .......................................................... 54
2.32d – Primary and secondary antibody staining ............................................ 54
2.32e – Imaging slides and plates .................................................................. 55
2.33 – Phagocytosis assay with pHrodo-Green ester conjugated to E. coli .......... 55
2.34 – Data Analyses ..................................................................................... 56

Chapter Three .................................................................................................. 57

Introduction to Chapter ..................................................................................... 57

Chapter Three: Targeting ................................................................................ 58

3.1 – Generation of a hESC line expressing a CX3CR1-driven fluorescent reporter ... 58
3.1.1 – Generation of a hESC line expressing a TREM2-driven fluorescent reporter ... 60
3.13 – Generation of hESC lines expressing a TMEM119-driven, IRF8-driven, or PU.1-driven fluorescent reporter .......................... 62
3.14 – Karyotyping of CX3CR1-, TREM2-, TMEM119-, IRF8-driven reporter lines and H9-WT parental cells .................................................. 65
Chapter 3.2 – Differentiation towards microglia .......................................................... 66
3.21 – Differentiation using modified protocol published by Abud et al. (2017) .............. 66
3.22 – Differentiation towards haematopoietic progenitor cells ................................... 67
3.23 – Quantification of HPCs using Flow and Magnetic cytometry ............................... 68
3.24 – Maturation of HPCs towards microglia using modified Abud et al. (2017) protocol .... 70
Chapter 3.3 – Validation of differentiated microglia ...................................................... 72
3.31 – Immunocytochemistry of differentiated microglial cells ................................. 73
3.32 – Microglial membrane ruffling ............................................................................. 75
3.33 – Phagocytosis of pHrodo-green labelled E. coli .................................................. 76
3.34 – Preliminary cytometric bead array data .............................................................. 78
3.4 Co-culture of microglia with forebrain or midbrain cultures ................................... 79
3.41 – Co-culture with midbrain cultures ................................................................. 79
3.42 – Co-culture with forebrain ................................................................................. 83
Discussion of Chapter 3 ............................................................................................... 85
Chapter Four ................................................................................................................. 89
Introduction to chapter. ............................................................................................... 89
4.1 - IL-1α secretion .................................................................................................... 90
Results ......................................................................................................................... 90
Comparison between cultures – pro-inflammatory stimuli mediated secretion of IL-1α .... 90
Effects within cultures – time and inflammatory mediators ........................................ 91
Discussion ................................................................................................................... 92
4.2 - IL-1β secretion .................................................................................................. 94
Results ......................................................................................................................... 94
Comparison between cultures – pro-inflammatory stimuli mediated secretion of IL-1β .... 94
Effects within cultures – time and inflammatory mediators ........................................ 94
Discussion of IL-1β .................................................................................................... 96
4.3 - IL-4 secretion .................................................................................................... 98
Results ......................................................................................................................... 98
Comparison between cultures – pro-inflammatory stimuli mediated secretion of IL-4 .... 98
Effects within cultures – time and inflammatory mediators ........................................ 98
Discussion ................................................................................................................... 99
4.4 - IL-6 secretion .................................................................................................... 100
Results ......................................................................................................................... 100
Comparison between cultures – pro-inflammatory stimuli mediated secretion of IL-6 .... 100
Effects within cultures – time and inflammatory mediators ........................................ 101
Discussion ................................................................................................................... 103

3
Chapter Five

5.1: IL

Introduction to chapter

4.12

4.11

4.8

4.5

Results

Comparison between cultures – pro-inflammatory stimuli mediated secretion of IL-8

Effects within cultures – time and inflammatory mediators

Discussion

4.6 - IL-10 secretion

Results

Comparison between cultures – pro-inflammatory stimuli mediated secretion of IL-10

Effects within Cultures – time and inflammatory mediators

Discussion

4.7 - MIP1α secretion

4.8 - TNF secretion

Results

Comparison between cultures – pro-inflammatory stimuli mediated secretion of TNF

Effects within cultures – time and inflammatory mediators

Discussion

Results

Comparison between cultures – pro-inflammatory stimuli mediated secretion of IFNγ

4.10 - VEGF Secretion

Results

Comparison between cultures – pro-inflammatory stimuli mediated secretion of VEGF

Effects within cultures – time and inflammatory mediators

Discussion

4.11 - CX3CL1 secretion

Results

Comparison between cultures – pro-inflammatory stimuli mediated secretion of CX3CL1

Effects within cultures – time and inflammatory mediators

Discussion

4.12 – Chapter Summary

Chapter Five

Introduction to chapter

5.1: IL-1α Secretion

Results

Comparison of inflammatory stimuli within forebrain media monocultures

Comparison of IL-1α secretion between microglial monocultures in forebrain and midbrain media
5.2: IL-1β Secretion .................................................................................................................. 141

Results .................................................................................................................................. 141

Comparison of inflammatory stimuli within forebrain media monocultures ......................... 141
Comparison of IL-1β secretion between microglial monocultures in forebrain and midbrain media .................................................................................................................... 142

Discussion ............................................................................................................................. 143

5.3: IL-6 Secretion ..................................................................................................................... 144

Results .................................................................................................................................. 144

Comparison of inflammatory stimuli within forebrain media monocultures ......................... 144
Comparison of IL-6 secretion between microglial monocultures in forebrain and midbrain media .................................................................................................................... 145

Discussion ............................................................................................................................. 146

5.4: IL-8 Secretion ..................................................................................................................... 148

Results .................................................................................................................................. 148

Comparison of inflammatory stimuli within forebrain media monocultures ......................... 148
Comparison of IL-8 secretion between microglial monocultures in forebrain and midbrain media .................................................................................................................... 149

Discussion ............................................................................................................................. 150

5.5: IL-10 Secretion .................................................................................................................. 152

Results .................................................................................................................................. 152

Comparison of inflammatory stimuli within forebrain media monocultures ......................... 152
Comparison of IL-10 secretion between microglial monocultures in forebrain and midbrain media .................................................................................................................... 153

Discussion ............................................................................................................................. 154

5.6: MIP-1α Secretion .............................................................................................................. 157

Results .................................................................................................................................. 157

Comparison of inflammatory stimuli within forebrain media monocultures ......................... 157
Comparison of MIP-1α secretion between microglial monocultures in forebrain and midbrain media .................................................................................................................... 158

Discussion ............................................................................................................................. 159

5.7: TNF Secretion .................................................................................................................. 160

Results .................................................................................................................................. 160

Comparison of inflammatory stimuli within forebrain media monocultures ......................... 160
Comparison of TNF secretion between microglial monocultures in forebrain and midbrain media .................................................................................................................... 161

Discussion ............................................................................................................................. 162
Appendices

Chapter Six

References

Future investigations

Thesis

Summary and significance of Chapter Four

Summary and significance of Chapter Three

Chapter Summary

5.10: CX3CL1 Secretion .......................................................................................................................... 169
Results .................................................................................................................................................. 169
Comparison of inflammatory stimuli within forebrain media monocultures................................. 169
Comparison of CX3CL1 secretion between microglial monocultures in forebrain and midbrain media ................................................................................................................................. 170
Discussion ............................................................................................................................................ 171
Chapter Summary ............................................................................................................................... 172
Chapter Six .......................................................................................................................................... 176
Summary and significance of Chapter Three ...................................................................................... 176
Impact of methodology choice ........................................................................................................... 176
Strengths and limitations .................................................................................................................... 177
Summary and significance of Chapter Four ......................................................................................... 178
Parkinson’s Disease and stem cell modelling ..................................................................................... 178
Major outcomes of Chapter Four ....................................................................................................... 179
Strengths and limitations .................................................................................................................... 182
Summary and significance of Chapter Five ......................................................................................... 183
Contextual differences in microglia ...................................................................................................... 183
Major outcomes of microglia monocultures modelling forebrain conditions ..................................... 184
Major outcomes of comparison between microglia monocultures .................................................... 185
Strengths and limitations .................................................................................................................... 185
Thesis results in the context of stem cell modelling ........................................................................... 186
Future investigations ........................................................................................................................... 188
References ........................................................................................................................................... 189
Appendices .......................................................................................................................................... 229
Figures and Tables

Figure 1: Summary diagram of the two methods of stem cell generation.......................................................... 11
Figure 2: A simplified illustration of CRISPR-Cas9 gene editing...................................................................... 14
Figure 3: Illustration of neural tube formation.................................................................................................. 16
Figure 4: Graphic comparison of the CNS environment during neurodegeneration and homeostatic conditions......................................................................................................................... 20
Figure 5: Simplified illustration of CD43+ progenitors migrating from yolk sac blood islands, through the embryo to the neural tube, where they continue development into microglia......................... 24
Figure 6: (Previous Page): Maps of the donor vector plasmids for late and early microglia markers. 40
Figure 7: Graphical representation of primer design. .......................................................................................... 41
Figure 8: Graphic comparison of the requirements for differentiation of CD43+ cells using the protocol by Abud et al. (1) and Stem Cell Technologies................................................................. 47
Figure 9: PCR screen results of CX3CR1 targeting............................................................................................ 59
Figure 10: Original and enhanced blots to highlight probed membranes of CX3CR1 targeted cells... 60
Figure 11: PCR screen results of H9-TREM2-E2CRIMSON targeting.............................................................. 61
Figure 12: 3' Southern blot indicates that H9-TREM2-E2CRIMSON-clone#2 is a heterozygote........ 62
Figure 13: PCR screen results of TMEM119 targeting.................................................................................... 63
Figure 14: PCR screen results of IRF8 targeting............................................................................................... 64
Figure 15: PCR screen results of PU.1 targeting ............................................................................................... 65
Figure 16: Progression of differentiation from H9-WT hESCs to HPCs over a 12-day time course..... 68
Figure 17: Comparison of FACs vs MACs for isolation of CD43+ cells from the total % of live cells ..... 69
Figure 18: Fluorescent images of Day 18 H9-CX3CR1-tdTomato microglia..................................................... 71
Figure 19: (A) Day 24 microglia H9-WT cultured with cholesterol on 1µg/mL Matrigel coated plate (B) Day 17 microglia H9-WT cultured in the absence of cholesterol on 1µg/mL Matrigel coated plate ... 72
Figure 20: Demonstration of expression of key microglial cell surface markers by immunocytochemistry. ........................................................................................................................................ 74
Figure 21: Visualisation of microglial membrane ruffling present on Day 17 CD43+ MACs sorted cells, using the Wiggle Index published in (370)............................................................................................ 76
Figure 22: H9-TREM2-E2CRIMSON targeted microglia incubated with pHrodo-green E. coli for 90 minutes. ........................................................................................................................................ 77
Figure 23: Montage of microglial engulfment, morphology changes and migration over a period of 5 minutes. ........................................................................................................................................ 78
Figure 24: Fixed D38 + 24 hours control stem cell derived CX3CR1-tdTomato microglia and midbrain neuron co-culture ................................................................................................................ 81
Figure 25: D29 H9-LMX1A-eGFP cells differentiated towards midbrain dopaminergic neurons............. 82
Figure 26: Immunocytochemistry image of D40 midbrain monoculture .................................................... 83
Figure 27: Day 70 WT-h9 cells differentiated towards forebrain neurons..................................................... 84
Figure 28: Co-culture of forebrain neurons with D38 H9-CX3CR1-tdTomato microglia. .......................... 85
Figure 29: IL-1α secretion by culture type, graphed by incubation condition. .......................................... 91
Figure 30: IL-1α secretion by incubation, graphed by culture type.............................................................. 92
Figure 31: IL-1β by culture type, graphed by incubation condition........................................................... 95
Figure 32: IL-1β secretion by incubation, graphed by culture type............................................................. 95
Figure 33: Simplified illustration of IL-1β positive feedback loop............................................................... 97
Figure 34: Comparison of IL-4 secretion by culture type, and by incubation with inflammatory stimuli. .......................................................................................................................... 98
Figure 35: IL-4 secretion by incubation, within culture type over time........................................ 99
Figure 36: Comparison of IL-6 secretion by culture type, and by incubation with inflammatory stimuli. .................................................................................................................................. 102
Figure 37: IL-6 secretion by incubation, within culture type over time........................................ 102
Figure 38: Graphical representation of pathways involved in IL-6 secretion and positive feedback loop formation .................................................................................................................................. 105
Figure 39: Comparison of IL-8 secretion by culture type, and by incubation with inflammatory stimuli. .................................................................................................................................. 107
Figure 40: IL-8 secretion by incubation, graphed by culture type and over time. .......................... 108
Figure 41: Comparison of IL-10 secretion by culture type, and by incubation with inflammatory stimuli .................................................................................................................................. 112
Figure 42: IL-10 secretion by incubation, within culture type over time ....................................... 112
Figure 43: Comparison of MIP1α secretion by culture type, and by incubation with inflammatory stimuli .................................................................................................................................. 115
Figure 44: MIP1α secretion by incubation, within culture type over time ....................................... 115
Figure 45: Pathways contributing to MIP-1α secretion ..................................................................... 117
Figure 46: Comparison of TNF secretion by culture type, and by incubation with inflammatory stimuli .................................................................................................................................. 118
Figure 47: TNF secretion by incubation, within culture type over time ......................................... 119
Figure 48: Comparison of IFNγ secretion by culture type, and by incubation with inflammatory stimuli .................................................................................................................................. 121
Figure 49: IFNγ secretion by incubation, graphed by culture type ................................................. 122
Figure 50: Comparison of VEGF secretion by culture type, and by incubation with inflammatory stimuli .................................................................................................................................. 124
Figure 51: VEGF secretion by incubation, graphed by culture type ................................................. 125
Figure 52: Simplified illustration of signalling pathways involved in VEGF secretion ..................... 127
Figure 53: Comparison of CX3CL1 secretion by culture type, and by incubation with inflammatory stimuli .................................................................................................................................. 129
Figure 54: CX3CL1 secretion by incubation, graphed by culture type ............................................. 130
Figure 55: A visual summary of the differences in cytokine secretion by stem cell derived cultures of midbrain neurons/astrocytes, microglia, and co-cultures when modelling inflammation of the substantia nigra as in PD.................................................................................................................................. 134
Figure 56: IL-1α secretion by microglial monocultures in FB media ................................................. 139
Figure 57: Comparison of IL-1α secretion by microglial monocultures in either mixed forebrain (Red) or mixed midbrain (Blue) media .................................................................................................................................. 140
Figure 58: IL-1β secretion by microglial monocultures in FB media .................................................. 142
Figure 59: Comparison of IL-1β secretion by microglial monocultures in either mixed forebrain (Red) or mixed midbrain (Blue) media .................................................................................................................................. 143
Figure 60: IL-6 secretion by microglial monocultures in FB media .................................................... 145
Figure 61: Comparison of IL-6 secretion by microglial monocultures in either mixed forebrain (Red) or mixed midbrain (Blue) media .................................................................................................................................. 146
Figure 62: IL-8 secretion by microglial monocultures in FB media .................................................... 149
Figure 63: Comparison of IL-8 secretion by microglial monocultures in either mixed forebrain (Red) or mixed midbrain (Blue) media .................................................................................................................................. 150
Figure 64: IL-10 secretion by microglial monocultures in FB media .................................................... 153
Figure 65: Comparison of IL-10 secretion by microglial monocultures in either mixed forebrain (Red) or mixed midbrain (Blue) media. ................................................................. 154
Figure 66: MIP-1α secretion by microglial monocultures in FB media ....................................................... 158
Figure 67: Comparison of MIP-1α secretion by microglial monocultures in either mixed forebrain (Red) or mixed midbrain (Blue) media. ................................................................. 159
Figure 68: TNF secretion by microglial monocultures in FB media ............................................................. 161
Figure 69: Comparison of TNF secretion by microglial monocultures in either mixed forebrain (Red) or mixed midbrain (Blue) media. ................................................................. 162
Figure 70: IFNγ secretion by microglial monocultures in FB media in FB media ............................................ 164
Figure 71: Comparison of IFNγ secretion by microglial monocultures in either mixed forebrain (Red) or mixed midbrain (Blue) media ................................................................. 164
Figure 72: VEGF secretion by microglial monocultures in FB media ............................................................ 167
Figure 73: Comparison of VEGF secretion by microglial monocultures in either mixed forebrain (Red) or mixed midbrain (Blue) media ................................................................. 167
Figure 74: CX3CL1 secretion by microglial monocultures in FB media ......................................................... 170
Figure 75: Comparison of CX3CL1 secretion by microglial monocultures in either mixed forebrain (Red) or mixed midbrain (Blue) media ................................................................. 171
Figure 76: A visual summary of the differences in cytokine secretion by microglia in FB media.................. 171
Figure 77: Summary of validation work performed in Chapter Three ........................................................... 174
Figure 78: Summary of the significant alterations to cytokine secretion in models of midbrain inflammation.. 178

Table 1: Comparison of thermocycling conditions using Phusion HF or Phusion GC Rich PCR Master Mix Kits................................................................................................................. 42
Table 2: Summary of significant increases to cytokine secretion by microglia monocultures, classified by media type and incubation condition................................................................. 174
Table 3: Current published protocols for derivation of Microglia from stem cells ................................... 176
Chapter One

Introduction to Literature review.

The work performed in this thesis relies on several key concepts. For ease, this review has been divided into the following sections: Stem Cells; Gene editing; the Central Nervous System; Neurodegenerative Disease; and Microglia, which are described in detail below. These concepts will then be linked together to describe the purpose of this thesis and the work that has been performed within it.

Stem cells.

What are stem cells?

Stem cells are cells with the capacity to differentiate towards other cell types, a trait called pluripotency. Pluripotent cells are typically characterised by expression of the markers OCT4, NANOG, and SOX2 (2-4), as well as by their unlimited proliferative potential, undifferentiated morphology, and their ability to differentiate into cells from all three germ layer lineages (5). Stem cells can be derived in two ways. Firstly, they can be obtained from the inner cell mass of human embryos (termed human embryonic stem cells, or hESCs), as demonstrated by Thomson et al. in the late 1990’s (6). Alternatively, as shown by both the Takahashi and Thomson laboratories, fibroblasts from a tissue biopsy can be induced into a pluripotent state (induced Pluripotent Stem Cells, or iPSCs) following viral transfection of OCT4, SOX2 and NANOG (7, 8) (see Figure 1). Stem cells have been widely adopted for modelling development and disease in a number of tissues, with protocols now existing for differentiation towards cardiomyocytes (9), skeletal muscle cells (10), osteoclasts and osteoblasts (11), red and white blood cells (12-14), and multiple types of neurons and astrocytes (15-20).

One of the key difficulties in culturing stem cells is maintaining their pluripotency and their capacity to differentiate. It was initially believed that stem cells were capable of being cultured for many passages without genetic mutations occurring (6-8, 21), however, this is now recognised to be incorrect (22-27). Long term culture can cause hESCs and iPSCs to accrue genetic and epigenetic
characteristics which make them more both adapted to in vitro growth (28) and potentially cancer-like in terms of gene expression and mutations. Common mutations include trisomy 1, 12, 17 and 20 (23, 24), and mutations in the proto-oncogene p53 (26, 29, 30). This slow accumulation of mutations over time in stem cells is known as “drift”.

The primary method used to maintain pluripotency of hESC/iPSC cultures is through addition of a cytokine known as Fibroblast Growth Factor 2 (FGF2) to cultures (31-35). Removal of FGF2 from the in vitro hESC/iPSC environment causes downregulation of genes controlling pluripotency, which in turn results in uncontrolled and spontaneous differentiation (32, 33, 36).

Figure 1: Summary diagram of the two methods of stem cell generation. (i) Individual cells are taken from the Inner Cell Mass of donated embryos, and are clonally expanded (6). (ii) Fibroblasts from a skin or muscle biopsy are isolated, and virally transfected with OCT4, SOX2 and NANOG. This essentially de-differentiates the fibroblasts back to a pluripotent state. (iii) once stem cells have been obtained, they can be differentiated into cardiac, blood, bone, muscle, neural and other cell types for research. For this thesis, blood and brain (in squares) cell differentiations were performed. Figure designed in BioRender.com.
Gene Editing in the context of stem cells.

One of the most exciting prospects for stem cells was that researchers could utilise gene editing for the purposes of studying development via fluorescent or enzymatic reporter lines (37), which would allow greater understanding of disease progression, and therefore better inform therapeutic design. Gene editing has also been used to rectify the effects of deleterious genetic mutations, such as occurs in retinal degeneration (38), Huntington’s Disease (39) and in β-thalassaemia (40, 41).

Homologous recombination and non-homologous end joining.
To edit the genome, normal DNA repair mechanisms such as Non-Homologous End Joining (NHEJ) or Homology Directed Repair (HDR) are artificially manipulated through use of specially designed nucleases or guide transcripts. Originally, it was believed that HDR was a high fidelity process, because HDR uses the original sequence as the template for repair where possible (42), and that NHEJ was error prone, because it does not utilise a template sequence (43-45). However, these concepts have recently been questioned, and it appears as though both HDR and NHEJ accuracy may be dependent on cell type, gene locus, the nuclease used for editing (43, 46, 47) and the age of the cells in question (48). For the purpose of discussion during this thesis, the traditional concepts of HDR and NHEJ will be applied.

Gene Editing Techniques
Gene editing methods include Zinc Finger Nucleases (ZFNs), Trans Activating Like Effector Nucleases (TALENs), Clustered Regularly Interspaced Palindromic Repeats-Cas9 (CRISPR-Cas9), single stranded Oligodeoxyribonucleotides (ssODNs) and recombinant adenoviral vectors (rAAV). Most of these methods are outside the scope of this review, but they are discussed in detail by Maggio and Gonçalves (49), Collin and Lako (50), and by Malankhanova et al. (39).

The earliest genomic edits performed in hESCs used ZFNs (51). ZFNs remain a popular technique for hESC/iPSC editing, due to the versatility of ZFN design (40, 50, 52-54). The advent of the CRISPR-Cas9 system has recently become extremely prevalent within stem cell research (41, 52, 55-
CRISPR-Cas9

CRISPR-Cas9 is an enzymatic system used by bacteria as part of their immunity against phage invasion (62). During phage invasion, viral genomic material is incorporated into the host genome, into CRISPR regions, flanked by Protein Adjacent Motif (PAM) recognition sites, and this is passed on to future generations. Future phage invasions cause the Cas9 nuclease complex to bind to exogenous DNA which matches the bacterial CRISPR region, and matching phage-bacterial sequences are translated into CRISPR-RNA (crRNA) and trans-activating RNA (tracrRNA) (63), forming a heterodimer that binds to Cas9 (63-66). The Cas9 complex is then able to degrade invading genetic material.

The ability of the CRISPR-CAS9 endonuclease system to specifically recognise and target foreign DNA (67) meant that it was quickly adapted for use in mammalian systems by introduction of short guide RNA sequences (sgRNA) (68). CRISPR, due to its specificity, can be used to introduce new sequences, to remove genes from the genome (see Figure 2), or to repair faulty gene sequences, and has been used for many human (41, 55-59, 61, 69) and murine (70-72) studies since its inception over the last seven years. CRISPR has rapidly been adopted and is being continually evolved to improve specificity, efficiency, and the number of recognition sequences that can be used. This rapid evolution of CRISPR technology has also resulted in calls for more stringent ethics requirements for application of CRISPR-Cas9 in stem cell research (73-79).
Figure 2: A simplified illustration of CRISPR-Cas9 gene editing. The Protein Adjacent Motif (PAM) site (in green) guides the Cas9 complex to the correct region of DNA (blue), which is unwound. The short guide RNA (sgRNA) hybridises to the DNA, and signals CAS9 to nick or cleave the DNA at that place. The nick/cleaved DNA then undergoes homologous recombination to generate either a deletion, or to insert a new sequence. Figure designed in BioRender.com

Ethics and concerns regarding gene editing

One of the primary concerns regarding gene editing is transferral of a genetic alteration to future generations (germ line editing) and direct editing of embryos. To combat this concern, Australia, and many other countries, have strict regulations and laws which restrict the types of research that can be performed using genetic modification of somatic cells and embryos (73, 77). Gene editing studies utilising hESC/iPSCs in vitro to investigate developmental pathways and gene function have fewer restrictions but are still required to satisfy both institutional and government regulations and ethics committees. This involves transparency regarding funding sources and conflicts of interest, detailed record keeping of experiments and outcomes, freely given and fully informed consent, and all work should be given the opportunity for peer review.

Phase I and II clinical trials using genetically modified human cells for a variety of conditions have already begun around the world (80-83), although not all parties believe genome editing is ready
to progress from the laboratory into clinical trials. It was strongly recommended by the Hinxton Group and others, that before human trials take place, clarification of possible off-target effects, optimisation of editing tools and their delivery, further use of appropriate animal models and extensive genomic sequencing should be performed (74, 75, 84).

It would be remiss to discuss CRISPR and ethics without mention of the very recent and egregious misstep of Dr Jiankui He. In 2018 He announced that he had used CRISPR on viable embryos, which were carried to term, to insert a homozygous mutation in the CCR5 gene to prevent contraction of HIV from their father (85). Dr He was widely condemned by both the scientific and broader community, for what has been described as an inappropriate and unnecessary alteration to human embryos (76, 78, 86). Furthermore, the supposed genetic alteration that He introduced using CRISPR has since been indicated to potentially reduce the survival rate by approximately 20% in humans possessing this genotype (79).

While gene editing techniques are incredibly useful and have immeasurably improved scientific knowledge of key developmental pathways, there is clearly a need for a more thorough grasp of genome editing consequences. This can be accomplished through further research using both hESCs and patient derived iPSCs. Using well characterised and commercially available hESCs lines would serve as useful positive controls, which could then be compared to patient derived iPSCs, to identify key differences in outcomes.

The Central Nervous System

The Central Nervous System (CNS), comprised of the brain and spinal cord, integrates all incoming sensory information from the Peripheral Nervous System (PNS), and coordinates appropriate responses automatically (87, 88). The CNS, but not the PNS, will now be briefly described.

CNS development

During gastrulation of the embryo, bone morphogenetic protein (BMP) and activin/nodal signalling pathways are activated to induce neuroepithelial cell formation from the ectodermal layer
(89, 90) (illustrated in Figure 3). This ectodermal layer eventually closes to form the neural tube, in a process known as neurulation (88, 90-93). From the neural tube, all regions of the adult central and peripheral nervous systems will continue to develop into three distinct regions of the embryonic brain – the prosencephalon, the mesencephalon and the rhombencephalon, also known as the forebrain, midbrain and hindbrain, respectively. With the exception of microglia, all neurons and glia are derived from the ectodermal germ layer (88). As the CNS continues to develop, the brain and spinal cord tissue organise themselves into gray- and white-matter. Gray-matter tissue is outermost, and contains the cell bodies, synapses and dendrites, while white matter tissue is observed within the deeper regions of the brain, and is comprised of myelin sheaths and axons (87).

Figure 3: Illustration of neural tube formation by Encyclopedia Britannica (94). (A) the ectodermal layer of the embryo begins to form neuroepithelial cells, which thicken to form a neural plate. The mesodermal layer gives rise to the notochord, and the endoderm is derived from the innermost layer of the gastrula and forms epithelial tissues. (B) The neural plate deepens into a neural groove, with neural folds, and neural crest cells. (C). The neural folds fuse together (neurulation). (D) The neural tube has completely formed, and neural crest cells delaminate from the neural tube, migrating along the length of the embryo to form peripheral cells and tissues.

CNS composition

The cells which compose the CNS fall into two subtypes: neurons, and glia. The primary function of neurons is to communicate to other cells across synapses, using electrochemical signals (87), thereby coordinating all physical systems. There have been many attempts to determine the true
numbers of neurons in the brain; it is estimated that there are between 67-85 billion neurons contained within the human brain, though the density of neurons varies considerably by region, and each neuron has thousands of connections to other neurons and glia, (88, 95). Neurons are classified into subtypes based on a variety of factors, including their size, electrophysiology, the types and shape of spines present on their dendrites, types of receptors, neurotransmitter release, and location (87, 88). Finally, neurons are delicate – once they reach maturity, there is limited capacity for repair, replication, or regeneration in the event of damage or death (88).

Glial cells in the CNS comprise oligodendrocytes, astrocytes, and microglia. Oligodendrocytes are critical for normal neuron function, as they form the myelin sheaths which encase axons to insulate neurochemical signalling, and also provide trophic support (96). Oligodendrocyte precursors arise in three distinct waves, and are first observed in humans at approximately 10 gestational weeks within the forebrain, whereupon they migrate to colonise the ventricular and subventricular zones (97, 98). Mature oligodendrocytes, defined by the expression of Myelin Basic Protein, do not appear until at least 20 gestational weeks in the subcortical layers (97). Classification of oligodendrocyte lineage cells by single cell RNA-sequencing in mice has revealed 12-subpopulations, separated temporally and spatially (98), including six distinct types of mature oligodendrocytes, indicating a high degree of heterogeneity in oligodendrocyte populations. To allow oligodendrocyte extension of filopodia for ensheathing of axons, continuous cytoskeletal rearrangement occurs at the level of microtubules and microfilaments (97). Lastly, to determine which axons are myelinated, and to communicate with neurons, oligodendrocytes express ion channels and neurotransmitter receptors (96-98).

Astrocytes are derived from both NG2-glia and neural stem cells (99, 100), and are the most abundant cell within the brain. It is estimated that astrocytes occupy between 20-40% of brain tissue volume across regions (95, 101), and exist in a tile formation which minimally overlap with other astrocytes. Once believed to be a purely homogenous population which existed solely to physically support neurons, astrocytes are now understood to be a heterogenous population with numerous
context dependent regional, morphological and transcriptomic differences (96, 99, 101, 102). Astrocytes are more complex in the human CNS compared to rodents (101). Multiple types of Ca\textsuperscript{2+} signalling events have been observed within astrocytes, and are believed to influence release of synaptogenic and trophic factors, as well as uptake and secretion of neurotransmitters and cytokines (101). By releasing and responding to these factors, astrocytes provide critical context cues for synapse formation between neurons and astrocytes and are generally thought of as being neuroprotective. However, astrocytes also have the capacity adopt a morphologically, functionally and transcriptionally altered profile when the CNS is diseased or damaged, an inflammatory state known as astrogliosis which can be neurotoxic (101, 102). This is partially due to the presence of another cell type, microglia, which will be discussed later in detail. Astrogliosis can result in astrocytes which physically segregate distinct regions of damaged CNS tissue from healthy tissue, resulting in “scar-formation”. Scar-forming astrocytes are characterised by extensive proliferation and migration towards the site of damage (101). Astrogliosis can also be characterised by astrocytes which do not proliferate or migrate towards the site of pathology, but still alter their molecular profile at a transcriptomic and proteomic level, though these astrocytes are not as well understood as their scar forming counterparts (101).

As it is now becoming clear that astrocytes are extremely complex, it is more important than ever to attempt to understand their ability to communicate with neurons and other cells within the CNS, as well as to understand their role in CNS pathology.

**CNS immune privilege**

Immune privilege refers to the capacity of a tissue to tolerate introduction of foreign tissues without eliciting an immune response (103). In the case of the CNS, it used to be thought that the brain was completely separated from the peripheral immune system, and thus, was immunologically privileged (103). However, this view has been revised – it is now clear that the CNS not only interacts with the peripheral immune system during periods of disease, but also relies on the peripheral immune system, and on its own immune cells, microglia, for maintaining homeostasis (103, 104). Microglia, and peripheral immune cells, provide trophic support to neurons and other glial cells, and actively monitor
the CNS for infection and cellular pathologies (96, 103, 104). The interaction between the CNS and peripheral immune system is possible due to the presence of a previously unrecognised lymphatic system within the dural sinuses (105, 106). This lymphatic system allows drainage of CSF and interstitial fluids from the subarachnoid spaces, and also for trafficking of immune cells into and out of the brain through the lymphatic endothelium (103, 105, 106). The field of CNS immune privilege is discussed in detail by Louveau et al. (2015), and by Negi and Das (2018)(103, 104).

CNS plasticity
In the context of the CNS, plasticity refers to the capacity of the brain and spinal cord tissue to adapt to persistent and/or variable demands (107), specifically regarding remodelling or alteration to neuronal synapses and circuitry (108). This concept is applicable to the CNS across the lifespan of an organism, and is essential for memory formation and learning (108, 109). In the CNS, plasticity occurs at a cellular, synaptic and molecular level and is observed in neurons, astrocytes and microglia (110). Physical alteration of neural structures, such as occurs in neurogenesis during pre- and post-natal development, and functional changes to increase efficiency of neuron firing, are both examples of CNS plasticity under homeostatic conditions (111).

In the context of Alzheimer’s Disease (AD) pathology, plasticity refers to the CNS attempting to adjust to an insult (111), for example, the accumulation of amyloid-β oligomers, and accompanying inflammation, during the disease progression. Unfortunately, there is limited capacity for neurons within the CNS to adjust to continued insults and inflammation (111). During sustained insults to the CNS, neurons are susceptible to degradation, malfunction or death, a process termed neurodegeneration (112).

Neurodegenerative Disease
As described earlier, neurodegeneration describes the malfunction, degradation, and death of neurons within the CNS. Diseases which are neurodegenerative are progressive, incurable, and are strongly associated with increased age (113, 114).
In the healthy CNS, neurons, astrocytes and microglia are in constant communication, and homeostatic conditions are maintained (see Figure 4). However, during neurodegeneration, communication between neurons, astrocytes and microglia is altered, and there is a significant increase of inflammatory cytokines released by both microglia and astrocytes (115). Accumulation of insoluble proteins can also occur, contributing to inflammation (116). Additionally, the process of neuronal degradation and death can trigger an inflammatory cascade in nearby neurons and astrocytes, which further contributes to degradation and inflammation, forming a positive feedback loop of inflammation and neuron death.

In this thesis, neurodegenerative disease will be briefly discussed in the contexts of Parkinson’s Disease and Alzheimer’s Disease.

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**Figure 4:** Graphic comparison of the CNS environment during neurodegeneration and homeostatic conditions. In the Homeostatic environment, microglia, astrocytes, and neurons communicate through trophic factors and chemokines (blue and green circles), and brain tissue is intact. During neurodegeneration, microglia adopt an amoeboid morphology, astrocytes...
Parkinson’s Disease

Parkinson’s disease (PD) is a progressive, multifactorial, incurable disease, where neurons within a region of the midbrain known as the substantia nigra are selectively destroyed. This selective destruction leads to loss of movement control, difficulty with swallowing and speech, as well as gastrointestinal problems (114). In Australia, PD affects approximately 69,000 people, and costs the Australian healthcare system an estimated $567.7 million annually (114). Persons with PD have an average lifespan of 12.5 years post diagnosis, and after dementia, PD is the second highest cause of death from a neurological condition (114).

It is clear that new avenues of research are needed to further our understanding of this complex and debilitating disease. Microglia and astrocytes are heavily involved in PD pathology (117-124) in both murine models and in human patients. Previous studies of Parkinson’s disease have been heavily focused on either understanding destruction of dopaminergic neurons (125-127), or on microglia involvement, utilising primary mouse/human, or immortalised cell lines (119, 128, 129).

One of the main factors known to contribute to PD development is accumulation of a protein known as α-synuclein. Multiple system atrophy (MSA), Lewy Body Dementia (LBD) and PD are collectively known as synucleinopathies, because they are all characterised by aberrant α-synuclein accumulation (130). Mutations in the gene for α-synuclein (SCNA) have been linked to familial cases of PD, leading to speculation that α-synuclein is potentially pathogenic (130, 131). When incorrectly folded, α-synuclein can form insoluble aggregates, particularly within the substantia nigra, leading to release of pro-inflammatory mediators by microglia and astrocytes, and also impairing normal neuronal function (114, 116). These misfolded aggregates are believed to trigger misfolding and accumulation of α-synuclein throughout the brain, a hypothesis known as prion-propagation (114, 130-132). This prion-propagation of misfolded α-synuclein has been shown in mice expressing wild-type human α-synuclein, supporting this theory (131). Interestingly, α-synuclein knock out mice also show increased neuroinflammation, indicating α-synuclein expression is required for normal CNS
function in mice, though whether this translates to humans is unclear (116).

Alzheimer’s Disease

Alzheimer’s Disease (AD) is a form of dementia, first described in 1906 by Alois Alzheimer, believed to affect more than 12 million people worldwide (133). In Australia, AD is estimated to account for approximately 70% of all dementia cases, and is more commonly experienced by women than men (134). AD progression is irreversible, and incurable. As in PD, astrocytes and microglia are known to be strongly involved in the pathogenesis and progression of AD (135-139). Progression is strongly influenced by age, sex, the presence of the apolipoprotein ε4 allele, and other genetic and environmental factors (139).

There is a prolonged prodromal phase which occurs over multiple decades, during which patients are asymptomatic, after which memory lapses, aphasia, emotional instability and deterioration in social skills occur (134). Patients with Alzheimer’s can live for between 3-20 years post-diagnosis, with an average of 7-10 years (130, 134, 140). Physiologically, AD is identified by the progressive death of neurons within the cortices, which results in reduced brain volume (140), and systemic increased inflammation with increased circulating cytokines (141-143). At a molecular level, AD is characterised by the presence of insoluble amyloid-β plaques in the intercellular spaces, and by neurofibrillary tau aggregates (140, 144, 145). Fibrillar tau deposition is discussed in detail by (144, 145), but amyloid-β will be briefly described below.

Amyloid-β is the naturally occurring, cleaved product of Amyloid Precursor Protein (APP), and exists in several isoforms in the normally functioning CNS, each with distinct physical properties (145). The normal function of amyloid-β is uncertain, but in vitro studies have indicated that amyloid-β is capable of exerting significant anti-microbial effects on S. pneumoniae and Candida albicans (146). Amyloid-β has also been shown to bind to 5’ regions of the AD-associated genes APOE, APP and BACE, to trigger transcription of these genes in a concentration dependent manner (147), and may also be involved in cholesterol trafficking in mice (148). Additionally, inhibition of amyloid-β
production in murine cortical neurons using γ-secretase inhibitors in vitro has been demonstrated to be neurotoxic, which suggests that amyloid-β may be important for normal neuron function (149). However, in the context of AD, overproduction and plaque formation of the Amyloid-β_{1-42} isoform occurs in both familial and sporadic AD development (140, 145), creating a neurotoxic environment. Accordingly, it is thought that an inability to clear toxic amyloid-β_{1-42} aggregates is a significant driving force behind the development of AD (130, 133, 135, 140, 145, 150, 151).

Microglia

Microglia are small, mesodermal glia cells of the brain and spinal cord, first described in 1919 by Pio Rio Del-Hortega (152). Since that time, arguments regarding microglia lineage pointed to microglia having either a bone marrow derived haematopoietic stem cell origin, or a neuroectodermal origin (153-155). The mesodermal, primitive haematopoiesis origin of microglia was not conclusively proven until fate-mapping studies were performed approximately 100 years after their initial discovery (154, 156, 157). As the only resident haematopoietic cells of the CNS, microglia communicate with astrocytes, neurons and oligodendrocytes, prune synapses and axons during early neural development, as well as modulate inflammatory responses in the CNS (112, 158-161).

Microglia development

Within the developing embryo, an early wave of haematopoietic development, known as primitive haematopoiesis, occurs in the Yolk-sac, forming “blood islands” (162). Primitive haematopoiesis occurs between weeks 3-6 of gestation in humans, generating tissue resident macrophages, such as microglia, as well as early megakaryocyte and erythrocyte cells (162-164). Definitive haematopoiesis, which occurs later in gestational development, occurs in the aorto- gonadal- mesonephros region of the embryo, and has a broader range of potential cell types, including lymphoid and myeloid cells, but does not contribute to microglial development (165-168). Microglia are one of the earliest cells to develop in the embryo. In mice, primitive cKit⁺ haematopoietic precursors are detected at Embryonic day 8 in yolk sac blood islands, which migrate to and colonise the developing neural tube prior to closure, where they simultaneously lose cKit
expression and upregulate CD43 and CX3CR1 expression (160). This is also observed in humans (154, 156, 157, 169). Primitive haematopoietic progenitors expressing CD43, emerge from the yolk sac, and utilise early blood vessels to migrate to the neuroepithelial and cephalic mesenchymal tissues (154, 157, 169-177). The opportunity to migrate is limited, as murine parabiont studies have conclusively proven microglia do not migrate into the brain from blood vessels in adult physiological systems (178, 179), and neither do bone marrow derived monocytes, except during periods of dyshomeostasis where blood brain barrier integrity is compromised (180, 181).

In terms of human physical distribution and development, amoeboid microglia have been observed entering the cerebral wall from ventral luminal tissues at 4.5 gestational weeks (GW) and the spinal cord from 9 GW (178, 179, 182, 183). Once the neural tube has been colonised, microglia migrate in an outward trajectory from the inner most regions of the early telencephalon, cerebellum and midbrain, in parallel with the expansion of radial glia (171, 173-175, 178, 179, 182-193). The
presence of microglia at this early stage of development is believed to directly influence numbers of Neural Progenitor Cells (NPCs) and Neural Stem Cells (NSCs) throughout the CNS. In particular, in utero microglial ablation in mice correlates with an increase in NPCs and NSCs, and a decrease in differentiated neurons (194-198). It is believed that the presence of microglia helps to encourage NPCs and NSCs to differentiate, rather than to proliferate (199, 200). Apoptotic NPCs and NSCs also encourage phagocytosis by microglia, to contribute to maintaining a constant level of progenitor and stem cells (199). Microglial distribution also coincides with the appearance of astrocytes within the CNS and retinal tissues, indicating communications and interaction between astrocytes and microglia to modulate embryonic neuronal development (201-206). Microglia have been observed within all parts of the CNS but are not observed in the peripheral nervous system.

For maturation, microglia rely on the transcription factors spi-1 proto oncogene (PU.1) (156, 207-209) and interferon regulatory factor 8 (IRF8) (156, 210-212). The essential nature of PU.1 signalling is evident as research has revealed complete disruption of PU.1 signalling in mice leads to embryonic and neonatal lethality, delayed B-cell development, and a total lack of macrophage lineage cells, including microglia (208, 213, 214). Human in vitro studies using small interfering RNA (siRNA) knockdown of PU.1 also highlight that primary microglia culture viability and function is critically dependent on PU.1 expression (215). PU.1 is now recognised to be a master regulator of important downstream haematopoietic factors such as runt-related transcription factor 1 (RUNX1), IRF8 and colony stimulating factor 1 receptor (CSF1R) (160, 208, 213, 216), which all act to support haematopoietic cell survival and function. In contrast to PU.1, IRF8 disruption does not appear to cause embryonic or neonatal lethal defects in mice but does interfere with correct microglia development. Knockout studies using IRF8⁻/⁻ mice consistently show microglial defects in allograft inflammatory factor 1 (AIF1) expression, motility, migration and downregulation of genes involved in purinergic and metabotropic signalling (211, 212, 215, 217).
Microglia morphology

Microglia physiology ranges from ramified – where the soma is small, but with extensive processes, through to completely amoeboid, with a very large soma and almost no processes (218). Microglial physiology is dependent on the needs of the local environment; ramified microglia are associated with maintaining homeostasis through contact with the surrounding cells (209, 219), whereas amoeboid microglia are associated with pro-inflammatory functions, including phagocytosis and autophagy (220-222).

Functional roles and behaviour of microglia

It was initially believed that unless directly stimulated due to infection or damage to the CNS, microglia were largely quiescent within the CNS (223, 224). In actual fact, “quiescent” microglia are highly active in maintaining communication with surrounding neurons and glial cells, continually monitoring the CNS, and assisting with maintaining homeostasis (225). This active monitoring is accomplished in two ways. Firstly, the branching processes of microglia are extremely motile, making constant contact directly with neuron synapses (225, 226). Secondly, microglia possess many receptors for neurotransmitters (227, 228), cytokines (229, 230), purines such as ATP/ADP (231-233) and ion channels (234-236), which allow for rapid response to fluctuations in these signalling molecules. For a detailed discussion on microglia surveillance, please see the discussions by Ransohoff and Cardona (2010), Kettenham et al. (2011, 2013), and by Kierdorf and Prinz (2017) (160, 190, 196, 197, 219).

Translating microglial phenotype and function from peripheral macrophage nomenclature, microglia have been described as adopting an “M1” or “M2”-like state, describing their pro- and anti-inflammatory profiles and functions (237). An M1 state indicates pro-inflammatory polarisation of macrophages produced by Interferon-γ (IFN-γ) or tumour necrosis factor α (TNF-α) signalling (238, 239). Commonly, this state was characterised by production of reactive oxygen and nitrogen species via inducible nitrogen oxygen synthase (iNOS), major histocompatibility complex class II (MHCII), as well as CD86 presentation to stimulate T-helper cells (237). However, the idea of applying M1/M2 nomenclature to macrophages, including microglia, is controversial. Original papers describing M1 and
M2 phenotypes were performed using isolated bone marrow macrophages incubated with stimulants in vitro (240). Hence, these terms are not considered to be representative of microglia either as highly adapted tissue resident macrophages or the in vivo environment (237, 241-243). However, conventional nomenclature is still commonly used. For a full description of M2 sub-states, please see Orihuela et al. 2016, or Ransohoff 2016 (237, 243).

Within the CNS, dead and dying neurons release adenosine triphosphate (ATP), which facilitates chemotaxis of microglia by binding to $P_2Y_{12}$ receptors (231, 244, 245). Microglia will also migrate in response to chemokines such MIP-1α, as well as to nitric oxide species, and astrocyte signalling (232, 244-249). The wide variety of stimuli which can initiate a migratory response, allows microglia to effectively monitor and respond to changes in homeostasis throughout the CNS.

One of the earliest recognised functions of microglia is directing neuronal growth during development, first described in 1968 by Blinzinger and Kreutzberg, who identified microglia removing synaptic boutons following cranio-facial axotomy injury, and termed the process “synaptic stripping” (250). This function has since been studied in detail in both humans and rodents. Colonisation of the CNS by microglia coincides with synapse formation between neurons, axon-target directed growth, and neuronal differentiation (172, 178, 179). This timing of colonisation allows microglia to be actively involved in synaptogenesis, the pruning of excess synapses on neurons, and to maintain a set number of neural precursor cells during embryogenesis and in post-natal development (184, 186, 197). Inhibition of microglial phagocytosis (and thus, inhibition of synaptic pruning) during embryogenesis results in increased numbers of neural precursors and the adoption of a reactive phenotype by microglia (184). Additionally, a mouse model of Rhett Syndrome, which has impaired microglial function, used the introduction of WT microglia to rescue the physical symptoms, including apnoea and low body weight (251). It was shown that the introduction of WT microglia allowed for phagocytosis of neuronal debris within the CNS, a process which is reduced in microglia of patients and mice with Rhett Syndrome (251). These examples indicate that the presence of excessive numbers of neurons is just as detrimental to CNS function as too few, and that the role of microglia
in synaptic pruning is essential for maintaining homeostasis and proper function within the CNS.

**Microglia distribution and regional heterogeneity**

Originally thought to be a homogeneous population in terms of gene expression and density, it is now understood that microglia are extremely heterogeneous. Throughout the human CNS, microglia are unevenly distributed and are regionally distinct at a transcriptomic level (252-257). A very recent study has used single cell mass cytometry to identify at least four transcriptionally distinct microglial subtypes within the adult human CNS (252), distinguished by expression of **CD206, CD11c, CCR5, CD45** and other markers. There is also a subset of microglia within the subventral zone (a known neurogenic niche) which has a much higher rate of proliferation and expression of cell cycling genes compared to microglia elsewhere within the brain (253, 258-260). From a distribution perspective, microglia have been documented to be least concentrated in the cerebellum, frontal, parietal and occipital grey matter, when compared to the hippocampus, basal ganglia, substantia nigra pars compacta and thalamus white matter (257). While this study was restricted by only counting ramified microglia, it has served as an important demonstration of the diverse distribution of microglia within the human CNS, and inspired further research into microglial heterogeneity (261-263).

Microglia also demonstrate significant differences in functionality depending on their location. For example, murine microglia located within the cerebellum more actively phagocytose debris compared to microglia from the striatum or the cortices, due to the higher level of neuronal apoptosis occurring within the cerebellum (264). This study also showed that microglia isolated from the striatum or cortex adopted a phagocytic transcriptomic profile when exposed to apoptotic neurons, highlighting the inherent flexibility of microglia response to stimuli (264). Responses are not only limited to phagocytosis, as murine hippocampal microglia have also been reported to express **TNF, CD4** and Fcy receptor II more strongly compared to the cerebellum, cortex, diencephalon or tegmental regions of the CNS (265). Similarly, a murine model of amyotrophic lateral sclerosis showed spinal dorsal horn microglia, but not cortical microglia, upregulated **VEGF** over the course of disease progression (266).
Thus, heterogeneity of microglia in several key areas – distribution, gene expression and functionality, is important for CNS function, and is now beginning to be recognised and studied in detail.

**Key microglial genes**

Microglia express a number of proteins and receptors such as *PU. 1* and *IRF8* (described above), as well as *AIF1/IBA1, TREM2, CX3CR1, TMEM119*, and *CSF1R* (described below). Recently, microglia cultures *in vitro* have also been described as having a unique transcriptional signature, involving the following genes: *P2RY12, C1QA, PROS1, MERTK1, GAS6* and *GP34*, which are dependent on the presence of exogenous TGF-β1 (267). However, further studies are required to fully determine the functions and responsibilities of these TGF-β signature genes in humans.

Fractalkine receptor, also known as CX3C Chemokine Receptor 1 (*CX3CR1*), is expressed on both microglia (268-270), and astrocytes (269, 271) within the CNS. The endogenous ligand, fractalkine (or CX3CL1), is expressed in both soluble and membrane bound forms by neurons and astrocytes (271-278). *CX3CR1-CX3CL1* communication is integral for maintaining CNS homeostasis and nervous system development (272). Knock-out of the *CX3CR1* receptor in mice results in increased production of inflammatory cytokines such as IL-1β from microglia (159). This is due to an inability of CX3CR1 knock-out microglia to respond to CX3CL1 secreted by neurons and astrocytes, which causes microglia to secrete IL-1β and other inflammatory molecules (279). Fractalkine binding to CX3CR1 on microglia elevates intracellular calcium and rearrangement of cytoskeletal actin filaments to allowing migration and motility of processes (271, 278).

*TMEM119* is a transmembrane protein whose ligand and function is currently unknown in the CNS, however, has been shown to be exclusively expressed on microglia (280, 281). As *TMEM119* is not expressed by neurons, astrocytes or oligodendrocytes, it is a useful marker of microglial identity (267, 280). To that end, there have already been strides towards generation of TMEM119-fluorescent reporter mice (282), and generation of antibodies (280). However, the use of antibodies to study *TMEM119* in the context of the CNS is problematic. Isolation of microglia from human and mouse tissue results in rapid and massive downregulation in *TMEM119* expression, which indicates current
culture methods do not meet the intrinsic requirements for maintaining microglial identity (280), as least, to maintain expression of \textit{TMEM119}. Determining the factor(s) involved in \textit{TMEM119} expression by microglia would undoubtedly improve scientific knowledge of microglia function. Possibly, \textit{TMEM119} expression relies on physical or neurochemical contact with the other cells present in the CNS, and the absence of these cells post-isolation results in downregulation.

Colony Stimulating Factor 1 Receptor (\textit{CSF1R}) in microglia is a receptor for both macrophage colony stimulating factor 1 (M-CSF1) and for interleukin 34 (\textit{IL-34}), and is critical for normal cerebral development (283-286). \textit{IL-34} and \textit{CSF1} have a coordinated spatio-temporal overlap during embryogenesis in mice, resulting in development of tissue-specific macrophages, including microglia (199, 283, 284). The \textit{CSF1R} promotor region contains recognition sequences for important haematopoietic transcription factors, such as \textit{PU.1} and \textit{RUNX1} (287), implying that \textit{CSF1R} expression is reliant on these transcription factors. \textit{CSF1R} knockout mice do not survive to adulthood, with neonates displaying a total lack of microglia within the CNS and impaired olfactory bulb development (284). Disruption of \textit{CSF1R} in adult mice using small molecules results in microglial apoptosis, whereas overexpression of \textit{CSF1R} causes increased microglial proliferation and altered response to LPS (287).

Allograft inflammatory factor 1 (\textit{AIF1}), also known as \textit{IBA1}, is unique to microglia, and is an actin-cross-poly-linker (288-290). AIF1 proteins bind to actin, allowing direct modulation of the actin filaments, creating an identifiable ruffle-like motion at the ends of microglial processes, as well as allowing formation of lamellipodia (291, 292). This binding of \textit{AIF1} to actin is believed to occur through the Phospholipase-C-\gamma pathway, and allows migration to sites of inflammation or injury (291). Due to its ubiquitous expression in microglia, AIF is often used as a marker for microglia in microscopy.

\textit{TREM2}, or triggering receptor expressed on myeloid cells 2, is a type 1 transmembrane receptor which acts to regulate microglial number, phagocytosis and inflammatory cytokine output (293-297). Defects in \textit{TREM2} expression in microglia result in reduced phagocytic capacity, and increased production of inflammatory molecules TNF-\alpha, IL-1\beta and NOS2 (294, 298). Additionally, mutations in \textit{TREM2} sequence, or protein overexpression, can increase risk of development and
progression of Alzheimer’s Disease (299-302). Treml2 is present within the CNS as both a membrane bound and soluble protein. High levels of soluble Treml2 are associated with poorer outcomes for patients with inflammatory CNS diseases (295). Treml2 signals intracellularly through phosphorylation of a molecule called Dap12 – allowing activation of the ERK pathway to initiate phagocytosis and cytoskeletal rearrangement of F-actin fibres (294). Mutations in either Dap12 or Treml2 in humans causes a progressive, fatal illness known as Nasu-Hakola disease, characterised by bone cysts, dementia and loss of motor control (294, 298, 303). Not all microglia within the CNS express Treml2; murine studies show Treml2 is almost entirely absent from the hypothalamus, but is strongly upregulated in the Cingulate Cortex, a region immediately superior to the corpus callosum (293). This heterogenous pattern of expression is also reflected in humans, where Treml2 is significantly increased in the hippocampus compared to the brain stem and cerebellum in patients with late stage AD (304).

Microglial interaction with other cells of the CNS parenchyma.

Microglia are not restricted to interactions with neurons within the CNS. Increasing evidence suggests that interactions between microglia, astrocytes and oligodendrocytes are far more complex than previously believed. Following gamma radiation exposure in mice, microglia release TNF1α and IL-1β, which causes astrogliosis and inflammatory cytokine production (305). Stimulation with lipopolysaccharide (LPS) causes microglial secretion of C1q, TNF1α and IL-1α, which in turn cause astrocytes to adopt a neurotoxic profile in mice (102). This results in fewer synapses forming between neurons, as well as death of healthy neurons and mature oligodendrocytes through release of soluble toxins (102, 201). Microglia have also been demonstrated to communicate with oligodendrocytes in murine cortical slice cultures during LPS stimulation by production of Golli proteins (306), and to influence proliferation of oligodendrocytes and their precursors (307), potentially indicating microglia act in both a protective and detrimental manner towards oligodendrocytes depending on the context. As a consequence of these and numerous other studies, it is now well recognised that cross communication between microglia and -neurons, -astrocytes and -oligodendrocytes occurs in the CNS.
Microglia for Research.

As described in the above paragraphs, there are many genetic, phenotypic, and functional differences between human and animal microglia. Additionally, the recent study by Masuda et al. (2019) confirmed that there were clear spatial and temporal differences between human and murine gene profiles. Although there were also similarities between some gene profiles within this study, it is important to accept that the observed differences in genetic profiles mean that work conducted in mice may not be relevant to human microglia. This cumulative knowledge indicates that murine microglia and monocyte-derived macrophages do not appropriately replicate all facets of microglia function across human disease, which can be read about in papers by Gomez-Nicola et al. (2015), Martin et al. (2017), in Stansley et al. (2012) and others. Therefore, new models of microglia should be sourced, as contradictory results between animal studies can cause difficulty for translation into therapeutic avenues. In spite of their limitations, well characterised animal models are very useful as initial starting points for investigation of microglial roles and function. As an alternative to animal studies, there are a number of in vitro systems that are readily used to study microglial function.

Bone marrow derived monocytes

Until microglia were definitively shown to be primitive transcriptional-activator-MYB-independent cells, it was thought that peripheral blood monocytes and microglia shared a common progenitor, and thus may have both contributed to microglial populations. While peripheral monocytes can enter the brain and contribute to inflammation under specific circumstances, peripheral monocytes generally contribute only minimally to the CNS haematopoietic parenchyma, and are therefore not a useful model of microglia. This is underscored by the recent paper by the Barres group, which showed bone marrow derived monocytes inserted into the CNS of microglia depleted mice were unable to develop the transcriptional identity of microglia, despite apparent successful integration into the parenchyma. In this study, only yolk sac derived microglial precursors were able to successfully engraft into the CNS and adopt a microglia
transcriptome (267, 321, 322).

**Immortalised lines**

Murine and human studies have shown adult microglia have a low turnover rate unless activated, and are generally long lived (169, 323, 324). A recent study using C\textsuperscript{14} dating in cancer patients, established only 28% of human microglia were able to divide over 12 months, and that microglia can be decades old (324). By comparison, SV40 immortalised human microglia have a doubling time of only 24 to 48 hours (325). This difference in turnover rate between immortalised and *in vivo* human microglia indicates distinct genetic and phenotypical changes. Additionally, Butovsky’s 2014 paper also notes that immortalised microglial lines, regardless of species origin, do not share the same signature gene expression by human foetal and adult primary microglia (267). This is further supported by functional studies which have shown immortalised microglia display differential release of cytokines, chemokines and migratory responses to LPS when compared to primary human microglia (326, 327). These differences in turnover rates, transcriptomic, and functional data indicate immortalised microglia lines might not generate data which translates to realistic phenotypes in the human CNS, and so should be avoided.

**Primary cultures**

Primary culture of human microglia was until recently, arguably the most appropriate source for studying microglia. However, the options for primary human culture are limited to either autopsy tissue, or removal of epileptic/glioma tissue (328), which results in isolated microglia adopting a pro-inflammatory state.

Microglia isolated from autopsies have often been exposed to hypoxia for as long as 48 hours (311-313). Hypoxia triggers microglia into an inflammatory and reactive state in a dose dependent manner, altering the profile of cytokine secretion and autophagy capacity (329). Therefore, the reactive nature of microglia isolated from autopsy patients may act as a confounding variable in experiments. Similarly, microglia are attracted to gliomas by secreted factors such as glial derived neurotrophic factor (GDNF) and macrophage colony stimulating factor 1, and CX3CL1 (330-333). However, these factors can also prime microglia into a pro-tumorigenic state (330, 331). For this
reason, unless studies are actively investigating tumour associated microglia, this is not an optimal method for isolation of primary microglia.

Primary microglia have also been shown to behave in distinct ways when cultured under different conditions, such as an organ slice or in monoculture, (311-313, 328, 334, 335). These changes in behaviour can likely be attributed to the presence (or lack thereof) of other CNS cells to communicate with and extracellular scaffolding. Additionally, almost all culture methods for primary human microglia use FBS in culture media for “trophic support” (311, 313, 328, 335, 336). FBS, as described earlier, is subject to batch variations in composition, is not chemically defined, and risks exposing isolated microglia to pathogens (337-340). For these reasons, although primary human microglia cultures are probably most appropriate for glioma studies, they are difficult to obtain, are subject to exposure to conditions which render them reactive and pro-inflammatory.

*Stem cell derived microglia*

In the last three years, multiple protocols describing stem cell derived microglia have been published (341-352). As this is area is still new, most of these protocols have focused on demonstrating the similarity of stem cell derived microglia to primary human foetal and adult microglia, as well as their functional capacity. The primary advantage of utilising stem cells to derive microglia (SCDmicroglia) is that they allow precise control of development, from primitive blood through to maturation, and are most likely to represent human cells accurately. These protocols have used both embryonic stem cell lines, as well as patient derived iPSCs, demonstrating robust generation of microglia from all backgrounds thus far. As SCDmicroglia are derived from stem cells, they can be genetically modified to express either fluorescent or enzymatic reporter tags, which allows for efficient tracking during differentiation and engraftment studies. Additionally, some of these protocols can be performed using chemically defined media, allowing for manipulation of key signalling pathways.

The most considerable downside to these protocols is that, like most stem cell protocols, they are laborious, time-consuming, reagent intensive, and expensive. However, the successfully
differentiated SCD microglia can be used in a wide variety of settings, including engraftment studies and strictly in vitro work, making this an enticing option for those who already have stem cell culture facilities available.

**Tying it all together.**

In this thesis, hESCs will be genetically modified using CRISPR to express fluorescent and enzymatic reporters for key microglial genes *CX3CR1, TREM2, TMEM119, IRF8* and *PU.1*. At least one of these hESC-CRISPR lines will be genotypically validated, and then differentiated into microglia using a recent published protocol (341), for phenotypic and functional characterisation. This characterisation will include analysis of phagocytic capacity, expression of key markers such as P2RY12 and AIF1/IBA1 by immunocytochemistry, and secretion of cytokines upon stimuli with known inflammatory mediators.

This thesis will then explore the heterogeneity of SCD microglia in terms of cytokine responses to known mediators of inflammation, including amyloid-β monomers, and α-synuclein aggregates (as present in AD and PD respectively). This will be accomplished by utilising SCD microglia monocultures, as well as stem cell derived forebrain and midbrain neural monocultures, and finally SCD microglia and midbrain co-cultures. Stem cell derived neuronal cultures naturally contain astrocytes as both neurons and astrocytes differentiate from neural stem cells (353, 354), and this will serve as a model of microglia-neuron-astrocyte communication. By using forebrain and midbrain neuronal cultures, and interrogating the conditions separately, this will allow for determination of differences in cytokine secretion due to cell/culture type.

Previously, most work describing microglia function has been performed using either primary murine, immortalised lines, or primary human cultures. While these have provided a strong foundation, there is now the opportunity to interrogate microglia function using stem cells, an arguably more relevant approach. As there are relatively few explorations of microglial functional changes in the context of stem cell studies, this thesis aims to fill this gap in the literature by using the CRISPR targeted lines to interrogate microglial cytokine secretion in vitro.
HYPOTHESIS:

Stem cell derived microglia will secrete cytokines in response to known mediators of inflammation, and the level of cytokine secretion will be altered by co-culture with neurons.

AIMS:

1) Generation of targeted hESCs for differentiation towards microglia

2) Demonstrate how microglia respond when stimulated by known inflammatory mediators (such as LPS, Amyloid-β, α-synuclein, TNF-α, or IFNγ) compared to vehicle

3) Determine which of a selected set of cytokines are secreted by stem cell derived microglia, stem cell derived midbrain neurons, and co-cultures of midbrain neurons with microglia, when cultured in media used for midbrain neuron differentiation

4) Determine which of a selected set of cytokines are secreted by stem cell derived microglia when cultured in media used for forebrain neuron differentiation

5) Demonstrate that media type influences cytokine secretion by stem cell derived microglia during in vitro experiments
Chapter Two: Methodology and Reagents

Please refer to Appendix I – Reagents and Consumables for a full list of reagents required for these methods.

Chapter 2.1: Targeting and validation of clones.

2.11– Plasmid design and CRISPR targeting

The donor vector plasmids were designed to generate dual enzymatic and fluorescent reporter lines for specific markers of microglia lineage. As microglia are identified in vivo by the presence of IBA1, SPI1/PU.1, TREM2, TMEM119 or CX3CR1, separate plasmids were designed for each gene (see Figure 6). To ensure plasmids were integrated into the genome correctly, both positive (neomycin/kanamycin) and negative (Diphtheria Toxoid A [DTA]) selection strategies were used. Positive selection was conferred on the transfected cells by addition of geneticin (G418), killing all cells that have not taken up the plasmid. Negative selection further refines the population by removing cells in which the plasmid has randomly integrated into the genome. Because the DTA gene is located outside the region of homology, this gene will theoretically be excised only in correctly targeted plasmids, while maintained in randomly integrated plasmids. Maintenance of the DTA gene results in expression of the toxin and subsequent cell death.

The donor vector plasmids were designed based on the S. pyogenes CRISPR-Cas9 system described by Ran et al. and Mali et al. (65, 66). This CRISPR-Cas9 system relies on dual nickases to cut single strands of DNA, to create 5’ overhangs instead of double stranded breaks. This acts to increase both the specificity of targeting and likelihood of correct insertion into the genome of the donor vector. H9 Human Embryonic Stem cells (hESCs) were initially grown on Mouse Embryonic Fibroblasts (MEFs) (see section 2.1 for details on culture) during targeting and selection. Two days prior to targeting, a 6cm dish was seeded with mitotically inactive MEFs (Section 2.1) at a density of 12K/cm². On the day of targeting, hESCs were dissociated into single cells by gentle treatment with Accutase™ for 15 minutes at 37°C and manually counted. One million cells were prepared for nucleofection using
the LONZA P3 Primary cell Human 4D kit as per the manufacturer’s instructions, using the LONZA 4D Nucleofector unit pre-loaded CX-156 settings. The cells were immediately plated onto a 6cm dish pre-seeded with MEFs, in hESC media supplemented with 10 μM Y27632 (see section 2.1 for hESC media composition). Targeted cells were left to recover for approximately 72 hours at 37°C, 5% CO₂, with media changes performed daily. On the third day, media was aspirated, the plate gently rinsed with warmed Phosphate Buffered Saline (PBS), and fresh hESC media containing 50ng/mL of G418 was added. Selection was continued for ten days, with fresh media being replaced every day.
2.12 – Manual picking and Expansion of targeted clones

Following selection, G418 was removed from hESC media to allow surviving cells to proliferate into small colonies. Two days before picking colonies to expand as clones, 15 wells each of 24-well and 12-well plates were each seeded with MEFs at a density of 12K/cm$^2$. On the day of colony dissection, 1mL of hESC media supplemented with 10uM Y27632 and 50ng/mL FGF2 was added to 15mL Falcon tubes. Using a dissection microscope, the MEFs immediately surrounding the colony of interest were removed using a sterile 1mL pipette tip. Next, an 18-gauge needle was used to score a grid into the colony of interest, and the innermost squares of the colony were transferred into a 15mL Falcon tube. Each colony was gently resuspended to dissociate into smaller clumps by pipetting. 250µL of this cell suspension was plated into a single well of the 24-well plate, and the remaining 750µL was plated into a single well of a 12-well plate. Media was changed every day with fresh hESC media containing 20ng/mL FGF2, and the cells were expanded by passaging. Once the colonies from the 24-well plate had been expanded to a 12-well plate, cells were frozen down at a ratio of 1:2 in hESC freezing medium (Section 2.2.1). Once the colonies from the 12-well plate had been expanded to 6cm dishes, the cells were harvested using Accutase™, and frozen down to use for PCR screening.

2.13 – Validation through PCR Screening

For each gene, two sets of PCR primers were designed using SnapGene® software in conjunction with web-based Basic Local Alignment Search Tool (BLAST) to align with the genomic DNA and with the insert of the donor plasmid (see Figure 7).
Figure 7: Graphical representation of primer design. A) primers (1) and (2) were designed to amplify within the reporter insert region of the plasmid, which is not present in gDNA. B) gDNA primers were designed to bind either side of the insert size. Primers 1 and 2 confirm plasmid DNA, Primers 3 and 4 confirm genomic wildtype DNA, and Primer pairs (1 and 4) or (2 and 3) can be used to confirm insertion of reporter into genomic DNA.

To determine correct annealing temperatures, both sets of primers were tested with either plasmid or genomic DNA, using Phusion High Fidelity or Phusion GC-Rich Master mixes (Life Technologies), with and without 5% DMSO, on the Applied Biosystems Veriti 96 well Thermal Cycler, using a temperature gradient. PCR amplicons were run on a 0.8% agarose gel, in 1x TAE, at 90V, for 1 hour, visualised using the DNR Bio-Imaging Systems apparatus with Live-Off Gel Capture software. Optimal temperature was determined based on several factors: 1) The PCR amplicon, visualised as a band on the agarose gel, needed to be the appropriate size as predicted by either SnapGene® or BLAST software; 2) the band should be bright and clear; and 3) there should be minimal non-specific bands present. Following optimisation, gDNA isolated from clones was screened by PCR performed following the manufacturer’s instructions as illustrated in Table 1. Following PCR, samples were visualised as above. The clones were identified as correctly targeted if they possessed a band of the appropriate size. Incorrectly targeted clones were not used further, except as negative controls for Southern blotting.
Table 1: Comparison of thermocycling conditions using Phusion HF or Phusion GC Rich PCR Master Mix Kits.

<table>
<thead>
<tr>
<th></th>
<th>Two step thermocycling</th>
<th>Three step thermocycling</th>
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</thead>
<tbody>
<tr>
<td><strong>Initial Denaturation x1</strong></td>
<td>98°C, 2 minutes</td>
<td>98°C, 2 minutes</td>
</tr>
<tr>
<td><strong>Amplification stage x 35</strong></td>
<td>98°C, 10 seconds</td>
<td>98°C, 10 seconds</td>
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<tr>
<td></td>
<td>72°C, 30 seconds/kb</td>
<td>55-68°C, 30 seconds</td>
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<td></td>
<td>72°C, 10 minutes</td>
<td>72°C, 30 seconds/kb</td>
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2.14—Validation through Southern Blotting

Although PCR is used to screen colonies, Southern blotting is used as a backup validation to ensure the clones are correctly targeted. This is done by electrophoresing restriction endonuclease digested gDNA, followed by transferring the DNA onto a filter membrane and subsequently probing the membrane with a radioactive labelled probe. Visualisation is accomplished using a phosphor screen. Depending on the probe, the observed band sizes will indicate whether the clone is homozygous or heterozygous for the transgene, as well as whether random integrations are present. Southern blotting was originally described in 1975 by Edwin Southern (355) but the protocol has since been updated (355). This protocol can be substantially modified between labs depending on available buffers, membrane types and probe design, therefore the methodology used in this thesis is described below.

gDNA was extracted using sarkosyl lysis buffer and the adapted protocol by Ramirez-Soliz et al. (356). Cells were first harvested from 6cm² dishes by removing the media, then incubating with 1mL Accutase™ for 15 minutes at 37°C. The cells were pelleted in 15mL Falcon tubes at 160xg for 5 minutes at room temperature, and the pellet frozen at -80°C for at least two hours. On the day of lysis, pellets were thawed at room temperature for 5 minutes, before 1.4mL of Sarkosyl Lysis buffer
(0.5% Sarkosyl, 10mM EDTA pH 8.0, 10mM NaCl, 10mM TrisCl pH 7.5, 1mg/mL proteinase K, made to volume in nuclease free water) was added to each tube. The tubes were placed in a container with wet paper towels, which was then sealed and incubated at 60°C overnight. The next day, a saturated salt-ethanol solution was prepared by addition of 150μL 5M NaCl for every 10mL of pre-chilled 100% ethanol. 2.8mL of this saturated salt-ethanol solution was added to each Falcon tube, which were incubated at room temperature for 30 minutes. Following incubation, 4.2mL of 70% ethanol was added to each tube, which was then centrifuged for 1 minute at 10,000g at room temperature, forming a gDNA pellet. The supernatant was discarded, and the pellet washed twice more using the same volume of 70% ethanol centrifuging between washes each time. The pellet was allowed to partially air dry at room temperature for half an hour, before being resuspended in T0.1E solution (10mM TrisCl pH:8.0, 0.1mM EDTA pH:8.0, made to volume in nuclease free water). gDNA was quantified using the ND-1000 Nanodrop Spectrophotometer, using the DNA settings.

To determine the most appropriate restriction endonuclease (RE) to use, the donor vector was analysed in SnapGene® to find an enzyme that would give a minimum of 3-4 cuts. Once the appropriate RE had been chosen, up to 25μg of gDNA from both WT and targeted clones was digested overnight at 37°C to ensure complete digestion. Following overnight digestion, 10μg digested DNA was loaded into wells of a 0.8% gel, in 1x TAE, and run for a minimum of 16 hours at 25V. Gels were imaged as described in above. The gel was then rinsed in distilled H₂O and placed on a rocker for 30 minutes at 15rpm, in 0.25M HCl. After 30 minutes, the HCl was poured off, the gel was again rinsed three times in distilled H₂O, and then washed twice in Denaturation Solution (1.5M NaCl, 0.5M NaOH), and rocked for 20 minutes at the same speed each time. Following washing with Denaturation Solution, the gel was rinsed three times in distilled H₂O, then washed twice in Neutralisation Solution (1.5M NaCl, 0.5M TrisCl, pH = 7.0), on the rocker at the same speed as before. To transfer the DNA from the gel to the Amersham Hybond N+ membrane, a reservoir containing approximately four to six centimetres of 20x Saline Sodium Citrate buffer (3M NaCl, 3.3M NaCitrate, pH=7.0) (SSC) was prepared. A Perspex sheet was placed over the reservoir at a 90° angle, with space
on either side for three layers of Whatman 3MM paper to hang over the edges into the reservoir for capillary transfer. The gel was sandwiched between the Whatman 3MM paper, and the Amersham Hybond N+ membrane, which had been equilibrated for 20 minutes in 20x SSC buffer. Finally, the Whatman covered membrane had at least 10cm of paper towels placed on top and was weighted down with a Perspex sheet and a 400g weight. The capillary transfer system was left at room temperature for 16 to 24 hours. To confirm transfer of the DNA from the gel to the membrane, the gel was imaged as in described earlier. The transfer was deemed complete if no trace of bands from the day before were present during visualisation using DNR Bio-Imaging Systems apparatus with Live-Off Gel Capture software. The membrane was then rinsed in 2x SSC, dried between sheets of 3MM, wrapped in foil and baked at 80°C for 2 hours to fix DNA to the membrane.

To isolate probe sequences from the plasmid, up to 5ug of guide plasmid was digested overnight with the appropriate RE at 37°C. The digested plasmid was then run on a 1.5% gel at 90V for 45-60 minutes to fully separate the fragments, after which was the fragment of interest was excised from the gel using a scalpel. The digested fragment was extracted from the gel using the Bioline Isolate II PCR and Gel Kit as per manufacturer’s instructions. The concentration of the isolated fragment was determined using a NanoDrop ND-1000 Spectrophotometer. The DecaLabel DNA Labelling Kit was used to radioactively labelling isolated fragments, as per the manufacturer’s instructions, which were then used immediately for hybridisation to the membrane. During preparation of the radioactively labelled probes, the southern membrane was blocked by incubation at 42°C in 20mL Ambion UltraHyb buffer for one hour. The probes were boiled for 5 minutes, added directly to the membrane and UltraHyb buffer, and incubated overnight at 42°C. Following overnight hybridisation, the buffer/radioactive probe was aspirated, and the membrane washed twice at 42°C in 2x SSC buffer containing 0.1% sodium dodecyl sulphate (SDS) for 5 minutes. The membrane was washed a further two times in 0.1x SSC buffer containing 0.1% SDS, at 42°C for 5 minutes. A Geiger-Muller β-emissions detector was used to confirm that residual emissions were below 5μSv after washes. Once the membrane was determined to be below 5μSv, the membrane was wrapped in cling
film, and loaded into a cassette containing a Phosphor screen in a darkroom and stored at -80°C for seven days. Following this, the cassette was removed from the -80°C freezer, and allowed to warm to room temperature, and the phosphor screen was visualised using the Amersham Typhoon 5 Biomolecular imaging apparatus. The image of the membrane was saved as a .tiff file format.

Chapter 2.2: Maintenance and differentiation of human embryonic stem cells (hESCs)

2.21 – Maintenance of hESCs.
As hESCs can be maintained either in the presence of absence of MEFs, with both systems having been used at various stages throughout this thesis, the two methods are described below.

2.21a – Preparation of mitotically inactive fibroblasts
MEFs were thawed into a 0.1% gelatine coated T75 in MEF Media (1% Penicillin/Streptomycin and 10% Foetal Bovine Serum in DMEM) (3). Cells were passaged at a ratio of 1:4 into 4x T150 flasks twice when at 80% confluency, for a total of 16x T150 flasks at time of mitotic inactivation. MEFs were treated with mitomycin C at a concentration of 10µg/mL as per (4), and incubated for 3 hours at 37°C. Following incubation with mitomycin C, MEFs were washed three times with warmed PBS (-/- Ca²⁺/Mg²⁺), lifted from the flasks using 5mL TryPLE/T150 flask, incubated at 37°C for 5 minutes and collected into a 50mL Falcon tube. The tube was centrifuged at 300xg for 5 minutes at room temperature, and the supernatant aspirated. The pellet was resuspended in 10mL MEF media, cells counted on a haemocytometer, and frozen down in MEF media supplemented with 10% DMSO at -80°C. Long term storage was in a vapour phase liquid nitrogen tank.

2.21b - hESC culture and maintenance on Mouse Embryonic Fibroblasts
Two days before passaging or thawing of hESCs, mitomycin C treated MEFs were thawed and plated onto a 0.1% gelatine coated 6cm dishes at a concentration of 12K/cm² in MEF media. hESCs thawed onto MEFs were cultured in hESC media (20% Knock-Out Serum Replacement, 1% Penicillin/Streptomycin, 1% Non-Essential Amino Acids, 0.5% Glutamax, 0.01% β-mercaptoethanol, 100µg/mL FGF2) supplemented with 10µM Y27632 (ROCK-inhibitor). Media was changed daily.
hESCs were allowed to grow for 5-6 days, or until 80% confluent. To passage hESCs for freezing, media was aspirated from the 6cm dish and the cells washed twice with warm PBS. The cells were then incubated with 1mL of pre-warmed TrypLE for 5 minutes at 37°C. Cells were collected in a 15mL Falcon tube, which was centrifuged at 160xg for 5 minutes. The supernatant was discarded, and the cell pellet resuspended in 4mL Freeze Mix (50% hESC media, 40% FBS, 10% DMSO). Once frozen, these vials were transferred to a vapour phase nitrogen tank for long term storage. To passage hESCs for expansion or targeting, media was aspirated and the 6cm dish was washed twice with warm PBS. Cells were incubated with 1mL of pre-warmed Accutase™ for 5 minutes at 37°C. hESCs were collected into a 15mL Falcon tube and were centrifuged at 160xg for 5 minutes at room temperature, and the supernatant removed. hESCs were counted on a haemocytometer, and seeded at a density of 10K/6cm dish, supplemented with 10µM Y27632.

2.21c – hESC culture and maintenance on Feeder Free system
hESCs maintained in the absence of MEFs were termed “Feeder Free”. They were maintained in Essential 8 media on tissue culture plates coated with 1µg/cm² recombinant human Laminin-521. Media was replaced daily. hESCs were converted from MEFs onto a feeder free system by passing hESCs cultured in MEFs, media was aspirated, and the dish washed twice with warmed PBS. 1mL ReLeSR per 6cm² dish was added, then removed after 30 seconds. The dish was then incubated for 7 minutes at 37°C. Following incubation, 2mL PBS was added to the dish and hESCs were collected into a 15mL Falcon tube. Cells were pelleted at 160xg for five minutes at room temperature, and the supernatant discarded. Cells were resuspended in 1mL Essential 8 supplemented with 10µM Y27632 and passaged at a 1:4 ratio. All further passages of hESCs maintained in the absence of Feeders was performed using ReLeSR as described, generally once every four to five days, when plates appeared to be approximately 80% confluent. To freeze feeder free hESCs, cells were harvested using ReLeSR as described for passaging, and resuspended in 4mL Essential 8 medium supplemented with 10% DMSO. Cells were frozen at -80°C and subsequently transferred to a vapour phase nitrogen tank for long term storage.
Differentiation towards microglia is a stepwise process where first, hESCs are differentiated towards haematopoietic progenitors, which are isolated and then differentiated towards microglia. Differentiation of hESCs to CD43+ haematopoietic progenitors following the protocol by Abud et al. (1), was determined to be too difficult after multiple failed attempts to generate CD43+ cells (Figure 8A). Instead, the Stem Diff Haematopoietic Progenitor Kit by Stem Cell Technologies was employed and used as per the manufacturer’s instructions (Figure 8B); cytokine specifics and concentrations are confidential and are unavailable to end users. On Day 12 of differentiation, floating CD43+ cells were harvested by MACs.

Attempts to harvest CD43+ cells by FACs resulted in large numbers of dead cells. Therefore, a column-less MACs method by Stem Cell Technologies, the Easy Sep Human APC Positive Selection
Kit, was used to isolate CD43+ progenitors as per the manufacturer’s instructions. Following isolation, CD43+ cells were resuspended in 1mL Microglia Media (comprised of 2% ITS-G, 1% B27, 1% Glutamax, 1% Non-Essential Amino Acids, 0.5% N2 Supplement, 200µM monothioglycerol, supplemented with 100ng/mL IL-34, 50ng/mL TGFβ1, 25ng/mL MCSF and 4µg/mL extra insulin). Cells were counted on a haemocytometer, then plated onto 12-well plates at a density of 100K/cm² for further differentiation towards microglia in 1mL/well microglia media, or frozen down as pellets for D12 controls, and stored at -80°C for up to three months. Once CD43+ cells were plated, 500µL fresh microglia media was added to the wells every two days to differentiate CD43+ progenitors towards microglia. On Day 24, all media in the well except for 1mL was collected, including non-adherent microglia, into a 15mL Falcon tube, and centrifuged at 300xg for five minutes at room temperature. The supernatant was discarded, the pellet resuspended in fresh microglia media, and then seeded back into wells containing old media. Every two days, 500µL fresh microglia media was added to the wells up to Day 34. On Day 34, cells were either collected for terminal differentiation and co-culture with neurons or were prepared for terminal differentiation in monoculture. For set up of terminal microglial differentiation in monoculture, media and non-adherent microglia were collected as above, and seeded back into the wells containing old media. On Day 35, all media and cells were collected, as for a Day 24 or Day 34 wash. Instead of keeping 1mL old media, cells were resuspended in 1mL fresh terminal microglia media (comprised of microglia media supplemented with both CD200 and CX3CL1 at 100ng/mL each, as well as the cytokines described earlier, seeded back into their original wells, and returned to incubation at 37°C at 5% CO₂. On Day 37, media was refreshed with the addition of an extra 500µL terminal microglia media. On Day 38, microglia were treated with inflammatory stimuli as described below.

2.23 – Differentiation of hESCs towards midbrain neurons

Differentiation of hESCs to midbrain floorplate neurons was performed based on the protocol by Kriks et al. (357) with some minor modifications. On Day 0, hESCs grown in feeder free conditions were seeded onto Matrigel coated plates at a density of 120,000 cells/cm², in 100%
Midbrain Differentiation Media 1 (Knock-out Serum Replacement, 15%, 1% Glutamax, 1% Non-Essential Amino Acids, 1% Penicillin/Streptomycin, 0.1% β-Mercaptoethanol, to volume in Knock-Out DMEM) (MDM1) supplemented with 100nM LDN-193189 and 10μM SB431542. On Day 1, Day 0 media was removed and supplemented with 100% MDM1 containing 100nM LDN-193189, 10μM SB431542, 100ng/mL Sonic Hedgehog and 2μM Purmorphamine. On Day 3, Day 1 media was replaced with 100% MDM containing 100nM LDN-193189, 10μM SB431542, 100ng/mL Sonic Hedgehog, 2μM Purmorphamine and 3μM ChiR-99021. On Day 5, Day 3 media was replaced with a 75:25 mixture of MDM1 and Midbrain Differentiation Medium 2 (1% glucose, 2.5% sodium bicarbonate in DMEM/F12 powder made to 1L, 1% Penicillin/Streptomycin, 1% N2 supplement, 1% human Apo-transferrin and 0.05% insulin) (MDM2) containing 100nM LDN-193189, 100ng/mL Sonic Hedgehog, 2μM Purmorphamine and 3μM ChiR-99021. At Day 7, a 50:50 mix of MDM1:MDM2 replaced the Day 5 media, containing only 100nM LDN-193189 and 3μM ChiR-99021. On Day 9, 25:75 MDM1:MDM2 media containing 100nM LDN-193189 and 3μM ChiR-99021 replaced the Day 7 media. This marked the end of the initial patterning towards neuronal progenitors, and on Day 11, maturation media (comprised of 1% Penicillin/Streptomycin, 1% Glutamax, 2% B27 Supplement without Retinoic Acid in Neurobasal Medium) was introduced, supplemented with 20ng/mL each of BDNF and GDNF, 200μM Ascorbic Acid, 2.5μM DAPT, 1ng/mL TGFβ-3, 0.5mM dibutyryl cyclic AMP and 3μM ChiR-99021. Every 2 days, the maturation media was completely replaced, without the addition of ChiR-99021 from Day 13 onwards.

On Day 25, midbrain cells were harvested for replating by removing old media and incubation with Accutase for 15 minutes at 37°C. Cells were washed twice with PBS and collected in a 15mL Falcon tube, centrifuged at 300xg for 5 minutes at room temperature to pellet, and resuspended in 1mL midbrain maturation media containing 10μM Y27632. Resuspended cells were counted on a haemocytometer, and seeded into wells that had been coated first with Poly-L-Ornithine (250μL/well 24-well plate, 24 hours at 37°C), and then with natural mouse laminin (2μg/cm² in PBS, 24 hours at 37°C) over the previous two days, containing maturation media supplemented with 10μM Y27632 to
assist survival of neurons post re-plating. Media was changed every two days until approximately Day 35. At this point, cells were incubated in a 50:50 Midbrain maturation media: microglia terminal differentiation media for 3 days, regardless of whether these neurons were destined for mono- or co-culture. At approximately Day 38 of differentiation, midbrain neurons were incubated with inflammatory stimuli as described in Section 2.6.

2.24 – Differentiation of hESCs towards forebrain neurons
To differentiate hESCs towards forebrain neurons rapidly, the protocol published by Qi et al. (358) was utilised with some minor modifications. hESCs grown in feeder free conditions were harvested using ReLeSR for 15 minutes as described above. hESCs were seeded at a density of 100,000 cells/cm² onto 6-well plates coated with VTN-n at 0.5µg/cm². Days 0-5 were performed in 100% Essential 6 medium (with 1% Penicillin/Streptomycin and 10ng/mL FGF2) supplemented with LDN193189 100nM, SB431542 10µM, XAV939 2µM on Days 0-2, and with LDN193189 50nM, SB431542 5µM, XAV939 1µM, PD0325901 0.4µM, SU5402 2µM, and DAPT 5 µM on Days 3-5, with a full media change every day. Day 6 was performed in 66% Essential 6 medium : 33% N2/B27 medium (comprised of 0.5% N2 Supplement, 1% Penicillin/Streptomycin, 1% Glutamax, 1% B27 supplement without Retinoic Acid, to volume in 50% Knock-Out DMEM-F12 and 50% Neurobasal Medium), supplemented with LDN193189 50nM, SB431542 5µM, XAV939 1µM, PD0325901 8µM, SU5402 10µM, and DAPT 10µM. Day 7 was performed in 33% Essential 6 medium : 66% N2/B27 medium, supplemented with PD0325901 8µM, SU5402 10µM, and DAPT 10µM. Day 8 was 100% N2/B27 medium, supplemented with PD0325901 8µM, SU5402 10µM, and DAPT 10µM. From Day 9 onwards, cells were incubated in Forebrain Maturation Media, comprised of Neurobasal Media supplemented with 2% B27 without retinoic acid, 1% Glutamax and 1% Penicillin/Streptomycin (FBM). On Day 9, cells were dissociated using 1mL Accutase, collected in a 15mL Falcon tube, centrifuged at 160xg for 5 minutes at room temperature, and seeded into 24-well plates (coated first with 15µg/mL Poly-L-Ornithine for 24 hours, then with natural mouse laminin at 10µg/mL and 20µg/mL fibronectin for 24 hours) in FBM (supplemented with 20ng/mL BDNF, 0.5mM dibutyryl cyclic AMP, 0.2mM Ascorbic
Acid, 50μM D-Serine and 10μM Y27632) at 100,000 cells/cm². A half media change was performed on Days 10 and 12, in FBM media identical to Day 9 (without the presence of 10μM Y27632). On Day 13, cells were gently dissociated and replated as on Day 9.

From Day 14 onwards, media was refreshed every two to three days. Cells were not replated or dissociated again during differentiation. To maintain secure attachment between the developing neuronal cells and the tissue culture plastic, 1μg natural mouse laminin was included in media changes once every 7 days. Cells were able to be maintained in this manner for as long as two months. On day 35 of differentiation, FBM was totally removed, and replaced with 50:50 Forebrain maturation media: microglia terminal differentiation media for 3 days, regardless of whether these neurons were destined for mono- or co-culture. On day 38 of differentiation, forebrain neurons were incubated with inflammatory stimuli as described in Section 2.7.

2.25 – Co-culture methodology for neurons and microglia
On Day 34 of microglial differentiation, microglia destined for co-culture were seeded into neuron containing wells at a ratio of 1 microglia:10 neurons after being harvested as described in Section 2.3. For a single well of a 24-well plate with 300,000 neurons, this equated to 30,000 microglia per well. Microglia were co-cultured in media that was 50% terminal microglia media, and 50% neuronal maturation media (either forebrain maturation or midbrain maturation media depending on whether microglia were co-cultured with forebrain or midbrain neurons). Microglia were co-cultured with neurons for three days prior to the addition of inflammation promoting stimuli (see below), to avoid prejudicing cells towards an inflammatory phenotype from simultaneous media composition changes and harvesting.

2.26 – Treatment of cultures with inflammatory stimuli
Cells were treated with five different proinflammatory stimuli. Interferon-γ (IFNγ), Tumour Necrosis Factor (TNF), lipopolysaccharide (LPS), α-synuclein aggregates (α-syn), amyloid-β monomers (Aβ), or with a control. IFN (20ng/mL), TNF (100ng/mL), LPS (100ng/mL) and α-syn (2.5μM) were all solubilised in PBS, while Aβ (60μM) was solubilised in DMSO. The control wells contained PBS and
the 0.01% DMSO. Wells were treated for a maximum of 48 hours, with media samples taken initially at 5 minutes and then at 2, 4, 8, 24 and 48 hours, and cells pelleted at 2, 4, 8, 24, and 48 hours respectively. All media and cell pellet samples were immediately frozen at -80°C and stored for up to three months before use. All microglial and neuronal monoculture samples were treated with inflammatory stimuli on day 38 and 40 respectively. Due to the difficulties associated with long-term cultures, co-cultures were treated on microglial day 38, with neurons between day 38 and 43, regardless of neuronal type.

Chapter 2.3: Assays, molecular techniques, and data analysis

2.3.1 Cytometric Bead Array

Cytometric Bead Array (CBA) utilises antibody-labelled microbeads to adhere to proteins in media, which are in turn labelled by fluorescent beads, and are quantified using flow cytometry. The BD Biosciences Human Soluble Proteins Flex-Kit was used for this thesis, in conjunction with the BD FACs Canto FACs apparatus and FCAP Array software. As this is a proprietary kit, the composition of reagents is listed as confidential, however, the volumes of reagents within the protocol have been halved to allow double the number of experiments to be performed. Aside from this difference, the protocol has been performed exactly as per manufacturer’s instructions. While preparing for analysis, except for thawing reagents on ice, all steps were performed at room temperature. Following set up of voltage settings, thresholds and compensations, samples and standards were loaded sequentially into the FACSCanto™ and run on the “medium” pressure settings. 300 events per bead were determined to be optimal for sufficient data to be collected. Here, 11 proteins were investigated, therefore a minimum of 3300 events were collected for analysis. Data from the FCAP cytometric bead array experiment was exported as .fcs files and analysed by FCAP Array software (BD Biosciences) for quantification of cytokine levels. Samples were imported into the program and arranged as either standards or tests. Next, the FCAP Array database of cytokine beads was consulted for the appropriate CBA-beads, and these were selected for samples. Samples were analysed by the software to determine whether all cytokines could be detected for all samples. If all cytokines could be
automatically detected, then the next step was to input the concentrations of the standards. If some cytokines were unable to be automatically detected, manual selection was required within the program, and was then applied to all samples to ensure equal detection, before standard concentrations were input. Standard curves using a 5-point logarithmic calculation were automatically generated by the FCAP array software. Should calculation of a curve fail, the concentration of that cytokine could not be determined. Standard curves typically had an $R^2$ value of over 0.998 for correlation of calculated concentration vs. actual concentration as determined by FACs. Once standard curves were calculated, FCAP array software was able to automatically calculate concentration of the cytokines of interest in the samples in pg./mL. These calculated concentrations were then exported to Excel for data rearrangement and later analysis in GraphPad Prism.

2.32 – Preparation for immunocytochemistry
Monoculture microglia do not form dense clumps, and so can easily be prepared for immunocytochemistry using PBS with 0.1% Triton-X100. Monoculture neurons or neurons co-cultured with microglia tend to form large, dense ganglionic structures, which need stronger detergents to enable permeabilisation of antibodies.

2.32a – Fixation of cells
To fix monoculture microglia, 200µL of 37°C 8% PFA was added directly to each well and incubated at room temperature for two minutes. Wells were rinsed twice with PBS, and replaced with 4% PFA, which was incubated at room temperature for 10 minutes. The 4% PFA was aspirated, and the wells rinsed twice with PBS. If the cells were not to be immediately prepared for immunolabelling, 1mL of PBS with 0.01% sodium azide was added to each well, the plate was wrapped in parafilm, and stored at 4°C for up to 6 weeks. To fix neurons in monoculture, or in co-culture with microglia, media was aspirated carefully from the plate, and wells were rinsed twice with PBS. 4% PFA was added to wells and allowed to sit at room temperature. The 4% PFA was aspirated, and the wells rinsed twice with PBS. Cells not to be immediately prepared for immunolabelling were treated as above.
2.32b – Permeabilisation of microglial monocultures using PBS-Triton-X
To permeabilise microglial monoculture, the PBS was first aspirated from wells, before being replaced with PBS containing 0.1% Triton-X. The plate was placed on a rocker at 15rpm, at room temperature, for a minimum of 1 hour. Following permeabilisation, the wells were blocked with PBS containing 3% Donkey Serum at room temperature for a minimum of 1 hour. Following blocking, the wells were rinsed twice with PBS before being prepared for primary staining.

2.32c – Permeabilisation of neuronal mono- and co-cultures using modified iDISCO protocol.
iDISCO is a protocol published by Renier et al. (359) used primarily for whole mount staining of embryos or tissues. Here, it was found that a modified version of this protocol greatly enhances the ability of antibodies to penetrate into the large clusters of cells formed by neurons in mono- and co-cultures. Permeabilisation Solution 1, (1x PBS, 20% DMSO, 0.2% TritonX-100, and 0.2% TWEEN-20) was added to wells of neuron mono- or co-cultures at 0.5mL/well of a 24-well plate, and incubated overnight at room temperature, on a plate rocker at 15rpm. Permeabilisation Solution 1 was then aspirated from wells, which were washed once with PBS. Permeabilisation Solution 2 (1x PBS, 20% DMSO, 0.1% Tween20, 0.1% TritonX, 0.1% Deoxycholate 0.1% tergitol/NP40) was added to wells at 0.5mL/well per well of a 24-well plate and incubated overnight on a plate rocker at 15rpm. Permeabilisation Solution 2 was then aspirated from wells, and wells were washed gently with 1x PBS. Non-specific anti-body binding was blocked using 3% Donkey Serum in Permeabilisation Solution 2 for 1 hour at room temperature, on a rocker at 15rpm.

2.32d – Primary and secondary antibody staining
For microglial monoculture wells, primary and secondary antibody staining was performed in PBS containing 3% donkey serum. For neuronal monocultures and co-cultures with microglia, primary and secondary staining was performed in Permeabilisation Solution 2 with 3% Donkey serum. In either case, the primary antibodies were prepared at the desired concentration, and added to the wells. For secondary control wells, no primary antibodies were added, and the wells were covered in either PBS containing 3% donkey serum or Permeabilisation Solution 2 with 3% donkey.
serum. The plate was wrapped in foil and incubated overnight at 4°C on a plate rocker at 15rpm. The wells were then washed gently three times with PBS. Secondary antibodies were prepared at their appropriate concentration in either PBS containing 3% donkey serum or Permeabilisation Solution 2 with 3% Donkey serum and were added to wells. The plate was wrapped in foil and incubated at room temperature for a minimum of 1 hour on a plate rocker at 15rpm. Following incubation of secondary antibodies, wells were washed three times with PBS. After washing, cells that were grown on coverslips were mounted onto slides using Prolong Gold with DAPI and allowed to cure for 24 hours at room temperature in the dark. Cells grown directly on the bottom of the wells were covered in PBS containing 0.02% sodium azide, with 5µg/mL Hoechst 33258 added. Plates and slides were wrapped in foil and stored in the dark at 4°C before imaging. Slides were stored at 4°C in the dark.

2.32e – Imaging slides and plates.
Plates with plastic bottoms were imaged using the Nikon A1R microscope, on objectives up to 20x. Plates with glass bottoms or glass slides were imaged using the Leica SP8. All images were analysed using FIJI software for Windows.

2.33 – Phagocytosis assay with pHrodo-Green ester conjugated to E. coli
This assay was performed with modifications to the manufacturer’s instructions. One vial of α-select E. coli from Bioline was thawed on ice and resuspended in Hank’s buffered salt solution containing the pHrodo-green ester. The pHrodo-green ester was resuspended in 150µL of DMSO for a stock solution of 8.9mM, which was diluted in HBSS for a final concentration of 1mM. The E. coli and resuspended pHrodo-green were incubated at room temperature in the dark, for 40 minutes. The labelled E. coli were rinsed twice with 1mL HBSS, centrifuging at 14,000RPM in a benchtop centrifuge to remove unincorporated dye. 50µL of labelled E. coli were incubated at 37°C per well of 96-well plate of microglia for up to 90 minutes in the dark, prior to visualisation on the Nikon A1R microscope.
2.34 — Data Analyses.

Data was imported from the BD Systems FACSCanto™ as .fcs files, analysed in FCAP Array software and converted into excel format. This data was rearranged for ease of analysis in GraphPad Prism V.7.0. Data from FCAP Cytometric Bead Array were analysed as pg./mL changes over time and by treatment condition. A Two-way ANOVA with post-hoc Dunnett’s test to allow for multiple comparisons between treatment groups and time was performed, with a minimum of n=3, and significance being p=<0.05.
Chapter Three.
Targeting and differentiation validation

Introduction to Chapter

This chapter aims to validate the reporter lines generated and used for this thesis. Reporter lines are often used to highlight a specific cell type when performing complex culture conditions. To date, most microglial reporter lines have not been of a stem cell lineage. A robust stem cell derived microglial reporter, which fluoresces under the control of a key microglial gene, would be a valuable addition to the research world.

For this thesis, CX3CR1, TREM2, TMEM119, PU.1/SPI1, and IRF8 were chosen for this purpose. As discussed in Chapter One, microglia constitutively express CX3CR1 upon maturation, regardless of stimulation status or location within the CNS. Therefore, CX3CR1 is an ideal prospect for a reporter line, as reporter expression would serve as confirmation of microglial identity when paired with functional validation methods such as phagocytosis and immunocytochemistry. As microglia mature, TREM2 is expressed on the plasma membrane (295). TREM2 mutations and expression deficits have been strongly linked to late onset dementia and Alzheimer’s disease (301, 303, 345), and a functional microglia reporter for TREM2 would be a useful asset for future in vitro studies. TMEM119, as described in Chapter One, is exclusively expressed on microglial cells within the central nervous system (222, 280, 281), yet its role has not yet fully elucidated. Likely, this is partially due to the rapid and massive drop in expression of TMEM119 in isolated primary microglia (102, 322). It appears that an as-yet-unidentified signalling factor required for TMEM119 expression is absent during culture of primary microglia, and while detection of TMEM119 by immunocytochemistry is possible (as shown later in this chapter), it is variable across studies. Therefore, a fluorescent and enzymatic reporter line was designed, to facilitate use by other researchers in testing and optimisation of in vitro microglial culture conditions. PU.1 is a key pioneer and master regulatory transcription factor and controls the initial pathway fates for myeloid lineage development (360-362). It is commonly used and
acknowledged in primary, cell line and stem cell derived microglia studies as a key identifying marker of microglia (208, 363). Within the CNS, microglia are the only cells able to express *PU.1*, therefore, use of a *PU.1* fluorescent reporter in co-culture conditions will allow for clear identification and tracking of microglial lineage development. Microglia originate from a *PU.1/IRF8* dependent pathway, as described in *Chapter One*, and *IRF8* regulation of myeloid genes contributes to age-dependent alterations to the CNS transcriptome (364). Aberrant signalling of *IRF8* is also known to contribute to neuroinflammation by mediating TGF-β signalling (365). Understanding these considerations, design of a fluorescent reporter which can be used to identify microglial cells as they develop, age, and deteriorate under disease conditions would be useful for further investigations.

To facilitate development of a novel reporter line for investigation into microglia derived from stem cells, a dual enzymatic-fluorescence reporter plasmid was designed, and inserted into wild-type H9 hESCs. *CX3CR1*- and *TREM2*- targeted hESCs were validated by dual-selection, southern blot, and PCR. The functional capacity of the targeted cells to develop into microglia was then tested by utilising a known protocol for microglial development, and microglial phagocytosis, motility, gene expression and cytokine release were all measured to validate the reporter.

**Chapter 3.1 – Targeting**

**3.11 – Generation of a hESC line expressing a *CX3CR1*-driven fluorescent reporter.**

In order to generate a H9-CX3CR1 reporter line, several steps were required. Firstly, as described in *Chapter Two*, hESCs were nucleofected with a donor vector containing the *CX3CR1*-tdTomato sequence, and then selected using Diphtheria Toxoid A and Geneticin. Following selection, individual colonies were expanded as clones, which were screened by PCR in duplicates for an amplicon of approximately 1800bp. PCR primers were validated prior to screening clones. Please see *Appendix II – Primer Validation, Section 1 – CX3CR1 targeting* for details. In total, 24 clones were picked, expanded, and isolated for gDNA screening. 14 clones were indicated to be correctly targeted
by PCR screening, as illustrated by Figure 9. However, only clones #6.8, #7.2 and #7.12 survived the freeze-thaw process when revived for further expansion for southern blotting.

To confirm correct insertion of the donor vector containing the CX3CR1-tdTomato sequence into the CX3CR1 locus of the targeted clones, Southern blot analysis of the H9-CX3CR1-tdTomato clones was performed. Southern blot analysis also enabled determination of whether targeting had generated heterozygous or homozygous clones. This technique was performed by extracting gDNA from expanded colonies, digesting them with specific restriction endonucleases, and visualisation of the digested gDNA bound to a membrane with P32 hybridised probes, as outlined in Chapter Two. Only correctly inserted CX3CR1-tdTomato vectors would be able to be detected using Southern blot, as the process of digestion with restriction endonucleases generates gDNA fragments of known sizes. 5’ membrane gDNA fragments are 4.9kb (Knock in) and 6.5kb (WT), while the expected band sizes for the 3’ membrane are 6.8kb (Knock in) and 10.8kb (WT). Additionally, the P32 labelled probe will only hybridise to the specific sequences of the WT and CX3CR1-tdTomato digested fragments, allowing

![Figure 9: PCR screen results of CX3CR1 targeting, run in duplicate on 1% Agarose gel. Correctly targeted clones have a band of approximately 1800bp, and are indicated by white boxes. Ladder on each side is Generuler 10kB ladder. Correctly targeted clones are #6.1, #6.4, #6.6, #6.8, #6.9, #6.10, #6.13, as well as #7.2, #7.4, #7.6, #7.8, #7.10, #7.12 and #7.14.](image-url)
diagnosis of genotype. Figure 10 shows that the 5’ insertion of CX3CR1 vector into clone #6.8 compared to WT is a heterozygote, and this is also observed in the 3’ insertion blot.

![Figure 10: Original and enhanced blots to highlight probed membranes of CX3CR1 targeted cells. Lane order from L-R is as follows: Clone #6.2 (negative control), Clone #6.8, H9-WT, and Non-template control. A=original membrane, B = enhanced image of membrane. Enhanced image was generated in FIJI by using the mplmagma LUT, despeckle, 1.25gamma, 2.00 Gaussian blur, and auto brightness contrast settings. White arrows indicate bands of interest in Figure 10b. Top membrane is probe with 5’ sgRNA, bottom membrane is probed with 3’ sgRNA. Expected band sizes for 5’ membrane are 4.9kb (Knock in) and 6.5kb (WT). Expected band sizes for 3’ membrane are 6.8kb (Knock in) and 10.8kb (WT).](image)

3.12 – Generation of a hESC line expressing a TREM2-driven fluorescent reporter.

In order to generate a H9-TREM2 reporter line, the same processes were employed as for generation of the H9-CX3CR1 line. As described in Chapter Two, hESCs were nucleofected with a donor vector containing the TREM2-E2CRIMSON sequence, and then selected using Diphtheria Toxoid A and Geneticin. Following selection, individual colonies were expanded as clones, which were screened by PCR in duplicate for an amplicon of approximately 1500bp. PCR primers were validated prior to screening clones. Please see Appendix II – Primer Validation, Section 2 – TREM2 targeting for details.

In total, 20 clones were picked, expanded, and isolated for gDNA screening following targeting and selection as described in Chapter Two. Clones were determined to be “correctly targeted” if on PCR
amplification, they were able to generate an amplicon of approximately 1500bp. 8 clones were indicated to be correctly targeted by PCR screening, however, only one clone (Clone #2) survived the freeze-thaw process. Although disappointing, this was not unexpected as many of the other clones grew slowly, suggesting that targeting had possibly disrupted key cell cycling genes. A 1500bp amplicon is visible for Clone #2, but not by Clone #9, WT or NTC reactions in Figure 11.

Once shown by PCR to be correctly targeted, the H9-TREM2-E2CRIMSON clone was expanded further for Southern Blot, as for H9-CX3CR1-tdTomato. This would enable confirmation that the TREM2-E2CRIMSON vector had been correctly inserted into the TREM2 gene sequence, and whether Clone #2 was a homozygous or heterozygous targeted clone. The 5’ Southern membrane failed to hybridise correctly, however, the 3’ Southern membrane (Figure 12) clearly shows that Clone #2 is heterozygous, with both 8.7kb (WT) and 5.9kb (targeted) DNA fragments visible, while the WT-H9 lane only shows a single, darker fragment DNA at 8.7kb.
3.13 – Generation of hESC lines expressing a TMEM119-driven, IRF8-driven, or PU.1-driven fluorescent reporter

TMEM119 is a key marker of microglial identity, however, its function in microglia is currently not known. Therefore, a fluorescent reporter would be highly advantageous. As with the H9-CX3CR1-tdTomato and H9-TREM2-E2CRIMSON reporter lines, H9-WT cells were nucleofected with a donor vector containing a TMEM119-GFP reporter. Cells were selected using Diphtheria Toxoid A and Geneticin, then picked, expanded, and screened for possible correctly targeted clones by PCR. Prior to screening, primers were validated as described in Appendix II – Section 3: TMEM119. A clone was
determined to be “correctly targeted” if an amplicon of approximately 1800bp was present following PCR. In total 14 clones were picked following targeting, but only one appeared to be correctly targeted (Clone #2), as illustrated in Figure 13.

![PCR Screen Results](image)

*Figure 13: PCR screen results of TMEM119 targeting, run in singlicate on 1% Agarose gel. Correctly targeted clones have a band of 2156bp. Ladder on each side is Generuler 10kB ladder. Correctly targeted clone 2 appears next to clones 9 and 12, which are not correctly targeted, wild type (WT) and non-template controls (NTC).*

To generate a H9-IRF8 line expressing a fluorescent reporter, as for other targeting experiments, H9-WT cells were nucleofected with an IRF8-mCherry vector, selected under Diphtheria Toxoid A and Geneticin, and then picked and expanded as clonal colonies. IRF8 targeting was easily the most successful nucleofection performed for this thesis, with eight out 14 clones indicated as correctly targeted (see Figure 14), possessing an amplicon of approximately 4500bp. Unfortunately, screening of H9-IRF8-mCherry clones was complicated by problems with initial primers. Despite best efforts in primer design, the first set of primers were not specific and could not be improved on by alterations to primer concentration, annealing temperature, inclusion of 5% DMSO or by using other polymerase mixes. This meant primers for IRF8 needed to be redesigned and validated a second time.
Although this second set of primers was successfully able to detect the band of interest, the initial difficulties and need for primer redesign delayed the use of this line, and ultimately contributed to not using the IRF8 clones for experiments this thesis.

![Image of PCR screen results of IRF8 targeting]

*Figure 14: PCR screen results of IRF8 targeting, run in singlicate on 0.8 % Agarose gel. Correctly targeted clones #3, #4, #8, #9, #10, #11, #12 and #13 all appear to have a band of approximately 4500bp. Ladder on each side is Generuler 10kB ladder. Clones #1 and #4, along with Wild type (WT) and non-template controls (NTC), do not present with band of required size to indicate they have been correctly targeted.*

The final gene selected for targeting, PU.1, was extremely difficult to target. Two attempts at nucleofection with a PU.1-GFP vector were required for successful clones to be identified. In total, out of 24 clones, only one was indicated as being correctly targeted by PCR screening (as seen in ). A clone was determined to be correctly targeted if an amplicon of approximately 1600bp was observed on an agarose gel following PCR. However, the one H9-PU.1-GFP clone indicated to be correctly targeted was slow to expand, and the expected green of the GFP insertion was not visible during differentiation towards microglia. For these reasons, this line was not used in future studies. Although the use of multiple reporter lines would have been valuable, time constraints limited the capacity of to use and
screen them all in this thesis. Therefore, southern blotting was not performed on the TMEM119-GFP, IRF8-mCherry, or PU.1-GFP clones.

![Figure 15: PCR screen results of PU.1 targeting, run in singlicate on 1% Agarose gel. Correctly targeted clone 6 has a band of approximately 1600bp (indicated by arrow). Ladder on each side is Generuler 10kB ladder. Wild type (WT) and non-template controls (NTC), do not present with band of required size.]

3.14– Karyotyping of CX3CR1-, TREM2-, TMEM119-, IRF8-driven reporter lines and H9-WT parental cells

As H9-CX3CR1-tdTomato cells appeared morphologically identical to untargeted cells, proliferated normally, and differentiated towards microglia (discussed later within this chapter), they were believed to be suitable for use in submitted manuscripts and for the experiments within this thesis. A manuscript (Appendix V) was written espousing use of H9-CX3CR1-tdTomato as a valid \textit{in vitro} model. Reviewer comments for this manuscript requested karyotyping to confirm normal karyotype. Unfortunately, it transpired that the H9-CX3CR1-tdTomato line possesses Trisomy 12 (see Appendix III – Karyotyping Results). However, RNA-seq data performed by manuscript collaborators has conclusively shown that there are no statistically significant differences in Chromosome 12 gene expression between the H9-CX3CR1 cells differentiated towards microglia, and published data on
stem-cell derived microglia (see Appendix III, accession: GSE89189). As with the H9-CX3CR1-tdTomato targeted line, the H9-TREM2-E2CRIMSON, H9-TMEM119-GFP and H9-IRF8-mCherry clones in an undifferentiated state appeared morphologically normal. They proliferated as expected, growing colonies indistinguishable from WT cells, and could successfully differentiate towards microglia. However, when the H9-CX3CR1-tdTomato karyotyping indicated a Chromosome 12 trisomy, it was deemed necessary to karyotype these lines as well. Samples were sent to Monash Pathology, and karyotyping results (Appendix III) indicated the same Trisomy 12 abnormality observed within the H9-CX3CR1-tdTomato line. Further investigations regrettably revealed that the parental H9 WT line used for targeting was in possession of Trisomy 12, and that this was passed on to all subsequent generations and targeted lines. Although PU.1-GFP #6 was not karyotyped, given that it too was generated from the same H9-WT parental line as the CX3CR1-tdTomato, TREM2-E2CRIMSON, TMEM119-GFP and IRF8-mCherry lines, it is extremely likely that this line also possesses Trisomy 12. Appendix III details the karyotype reports. The TREM2-E2CRIMSON, TMEM119-GFP, IRF8-mCherry and PU.1-GFP plasmids have since been sent to MCRI for re-targeting in karyotypically normal H9-WT cells, but the re-targeted lines were not generated in time for use within this thesis.

Chapter 3.2 – Differentiation towards microglia

3.21 – Differentiation using modified protocol published by Abud et al. (2017)

The differentiation protocol used in this thesis, outlined in Chapter Two, was based on the protocol by Abud et al. 2017 (341). This protocol is comprised of two steps: First, differentiation of hESCs to Haematopoietic Progenitor Cells, then secondly, maturation towards microglia-like cells. Since publication, modifications to Abud et al. have been described (347), using the same differentiation kit. The published modifications have provided evidence that microglia generated using the differentiation kit are functionally and transcriptionally similar to those derived using the original protocol.
3.22 – Differentiation towards haematopoietic progenitor cells

To determine whether H9-WT, H9-CX3CR1-tdTomato and H9-TREM2-E2CRIMSON lines were capable of differentiation towards microglia, two distinct microglial differentiation protocols. The first method used the original protocol for derivation of Haematopoietic Progenitor Cells (HPCs) outlined in Abud et al. 2017 (341). Unfortunately, derivation of HPCs using this method were unsuccessful for unknown reasons, and this was observed across the H9-WT, H9-CX3CR1-tdTomato and H9-TREM2-E2CRIMSON lines. However, adopting the Stem Diff Haematopoietic Progenitor Kit by Stem Cell Technologies successfully generated HPCs from the outset. The hESCs developed into large, cystic structures, from which small, phase bright cells budded (see Figure 16 for example of H9-WT cell differentiation). These budding cells were non-adherent, and analysis by FACs or MACs to quantify CD43+ expression confirmed these cells to be the CD43+ progenitor cells required for microglial differentiation. Utilisation of the Stem Diff Haematopoietic Progenitor Kit has also recently been published by the same laboratory as the original Abud et al. paper (347), describing minimal differences to the transcriptome between the two protocols, and highlighting that the terminally differentiated microglia were functionally identical to those in the original paper. Therefore, using the Stem Diff Haematopoietic Progenitor Kit for generation of HPCs was performed for all differentiations and experiments within this thesis.
Figure 16: Progression of differentiation from H9-WT hESCs to HPCs over a 12-day time course. A = Day 0, B = Day 2, C = Day 3, D = Day 5, E = Day 10, F = Day 12. Scale bar in all figures = 100μm. (A) An early colony is indicated by the black arrow. (B) The colony expands and begins to form a dense inner region. (C) The dense inner region begins to aggregate (black arrow) and the rest of the colony flattens out into an epithelial layer. (D) The dense inner region typically forms a large mound (black arrow), while the rest of the colony forms cystic structures and flattened regions simultaneously. (E) At Day 10, the mound has tightly compacted, and many round, small, floating cells can be observed. (F) On Day 12, the floating cells are harvested for MACs analysis. Brightfield microscopy images taken on the Bio-Rad ZOE fluorescence cell imager.

3.23– Quantification of HPCs using Flow and Magnetic cytometry

Upon successfully differentiating hESCs towards CD43+ HPCs, it was important to both isolate and quantify the HPCs from the pool of differentiated cells. As HPCs are typically non-adherent, collection of the supernatant was usually sufficient to obtain most of the CD43+ population. However,
to enrich the CD43\(^+\) population, it was necessary to sort the harvested cells. During this time, it is important to note that the H9-CX3CR1-tdTomato line had not yet been screened by southern blot, so all data in this section is based on analysis with H9-Wildtype and H9-TREM2-E2CRIMSON targeted cells.

Initial attempts to sort CD43\(^+\) cells were performed by FACs, where an average %total live cells CD43\(^+\) was only 11.89± 2.16\% (H9-TREM2-E2CRIMSON cells, n=3), and 6.25±3.10 \% (H9-wildtype cells, n=3). Cells were only taken for FACs if, after harvesting, Trypan Blue cell counts indicated less than 5\% cells were dead. The discrepancy between live cells during harvesting, and FACs counts, indicated that cells did not survive FACs. To rectify this extremely low survival rate, adjustments to flow rate, pressure, nozzle size and the volume of resuspension were made, but were ultimately unsuccessful. It was decided to abandon FACs and investigate sorting using MACs. CD43\(^+\) positive selection using columnless MACs sorting as described in Chapter Two – Section 2.3. This method was more effective for isolation of CD43\(^+\) cells, with an average % of total live cells CD43\(^+\) of 94.12±0.65\% (H9-TREM2-E2CRIMSON cells, n=4) and 86.39±1.76 \% (H9-WT cells, n=5). When compared using an unpaired t-test with Welch’s correction (to account for differences in standard deviation) between FACs and MACs, MACs was determined to significantly improve the yield of CD43\(^+\) cells, which is illustrated in Figure 17.

\[ \begin{align*}
\text{FACs} & \quad \text{MACs} \\
\begin{array}{c}
\begin{array}{c}
\text{H9 WT} \\
\text{TREM2}
\end{array}
\end{array}
\end{align*}\]

**Figure 17:** Comparison of FACs vs MACs for isolation of CD43\(^+\) cells from the total % of live cells, across H9-WT and H9-TREM2-E2CRIMSON cells. ****p<0.0001, ***p<0.0005
3.24– Maturation of HPCs towards microglia using modified Abud et al. (2017) protocol

On successful isolation of CD43⁺ progenitors, cells were plated and matured as described in Chapter Two – Section 2.4. Using the CX3CR1-H9’s to trace development, the CX3CR1 td Tomato reporter was typically observed to be clearly fluorescing at approximately Day 18 of differentiation, six days post MACs isolation (see Figure 18). This is in line with expected expression of CX3CR1 as reported in Abud et al. (2017). Although Abud et al. describes CX3CR1 being present from Day 16(341), their CD43⁺ population is isolated on Day 10, not Day 12 as performed here. Given the two-day lag between sorting, it is not unreasonable that CX3CR1 reporter expression in these experiments is also two days behind. The main difficulty with imaging early microglia is their constantly moving processes interfere with taking clear still images. Therefore, early microglia were typically imaged in the form of time lapses, to observe both motility and fluorescence (for more details, see Electronic Appendix I Movie: Day 18 CX3CR1 fluorescence). Using the modified Abud method, CD43⁺ cells began to show lobed somas and branching processes from as early as Day 16, which indicated maturation and adoption of microglial morphology (see Figure 18 for example of branching and lobed soma morphology). Microglia were not defined as “fully mature” until they had received the final maturation media containing CD200 and CX3CL1 (100ng/mL) for a final 3 days, in line with the protocol described by Abud et al. (2017)(341).
During maturation of CD43\(^+\) cells towards microglia, it was evident that microglial development was being prohibited by an external factor. Cultures of CD43\(^+\) cells would clump together within days of seeding, and the cells would adopt a rounded, large morphology. Additionally, these rounded cells would clump together, a change typically associated with death of the cells (see Figure 19). Attempts to rectify this rounding included the addition of cholesterol at 1\(\mu\)g/mL, but this did not
improve the clumping observed, so was not included in further experiments performed within this thesis, with the exception of those experiments described in Appendix V. Increasing the concentration of Matrigel from 1µg/mL to 2.5µg/mL following isolation of CD43+ HPCs was determined to be the most effective way to prevent rounding and clustering of microglia. It is theorised that the observed clumping and rounding of microglia during culture with 1µg/mL of Matrigel indicated that the microglia were in a reactive, amoeboid state, although this was not confirmed by RNA sequencing. This theory was formed because microglia are known to adopt an amoeboid morphology, involving the retraction of filopodia and processes, in response to a range of cellular and environmental insults (227, 231, 366-369). The observation that an increase in Matrigel concentration reduced clustering and allowed lobed processes to form on cells suggested that this was a more favourable environment for the cells to be grown in, and therefore, 2.5µg/mL of Matrigel was used to support microglial development in all future experiments.

Figure 19: (A) Day 24 microglia H9-WT cultured with cholesterol on 1µg/mL Matrigel coated plate – cells have both rounded and clustered together (B) Day 17 microglia H9-WT cultured in the absence of cholesterol on 1µg/mL Matrigel coated plate – cells are partially rounded and are clustered together. Scale bar = 50µm. Brightfield image taken on the Nikon A1R confocal microscope with Hoffman contrast, 10x objective

Chapter 3.3 – Validation of differentiated microglia

Once microglia were observed to be maturing through morphological changes and CX3CR1 fluorescence, it was important to confirm that these cells were truly microglia-like. First, expression of key genes through immunocytochemistry was determined and compared against Day 0 hESCs and
Day 12 CD43+ cells. Next, the functional capacity in the form of phagocytosis, migration, and secreted cytokine response to known inflammatory mediators was tested. Finally, a submitted manuscript (see Appendix V for full manuscript and figures) was drafted in collaboration with researchers at Monash University (Clayton), where H9-CX3CR1-tdTomato microglia were validated by co-culture with ReN neurons, the enzymatic luciferase reporter was tested, and RNA-seq was performed to compare the H9-CX3CR1-tdTomato microglia against Abud et al. (2017)’s published data (using accession GSE89189 for reference).

3.31- Immunocytochemistry of differentiated microglial cells.

As microglia are known to express several key surface markers, it was felt prudent to demonstrate the presence of these markers in the Day 38 H9-CX3CR1-tdTomato microglia. Here, it is shown that the H9-CX3CR1-tdTomato fluorescent reporter is functional in Day 38 H9-CX3CR1-tdTomato differentiated microglia, but not in Day 0 undifferentiated H9-CX3CR1-tdTomato cells. P2RY12, TMEM119 (both visible in green in Figure 20), IBA1 and TREM2 (both visible in white in Figure 20) are all clearly present in Day 38 differentiated H9-CX3CR1-tdTomato microglia, but are not present in undifferentiated cells. Monocultured microglia were visible as lobed, branching cells with multiple processes.
Figure 20: Demonstration of expression of key microglial cell surface markers by immunocytochemistry. Fluorescence microscopy images taken on the Leica SP8 microscope, 20x objective. Scale bar all images = 50 µm. DAPI = nuclear staining (blue), CX3CR1 = CX3CR1 tdTomato reporter line fluorescence (red), TREM2 and IBA in white, P2RY12 and TMEM119 in green.
3.32 – Microglial membrane ruffling

One of the defining morphological features of cultured microglia was the description of “ruffling” (291) caused by IBA1 binding to f-actin filaments within the cytoskeleton. This ruffling is key to facilitating phagocytosis of cellular debris. It was therefore vital to determine whether this feature was present on the differentiated microglia, and whether phagocytosis was possible.

In Figure 21, an example microglia has been focused upon at 20x magnification, and ruffling movement of the microglial membrane is illustrated by the rainbow regions (blue = less movement, yellow-red = more ruffling) using the Wiggle Index (first described in (370)). For further demonstration of this ruffling movement, please see Electronic Appendix II – Movie: Microglia Ruffling I and II.

Ruffling and branching processes were evident early as early as Day 16-Day 17 in microglial differentiation.

In Figure 23, a montage of 5 minutes 40x footage, a single WT stem-cell derived microglia has is shown as an example of microglial motility and morphological changes. In Figure 23, Frames B through G, the long, thin processes which extend off the main body of the microglia can be observed thickening and retracting towards the soma. The microglial soma changes from being centrally located with multiple branching processes (Frames A to E), to lobular with shorter processes (Frames F to H), and finally to amoeboid with only a couple of short, thickened processes (Frames I to L). Additionally, observed in Figure 23, the microglial body moves from roughly centre of Frame A towards the top left of Frame L. Using Fiji calculations and Pythagorean theorem (see Appendix IV) to determine the distance moved, it was determined that over the course of 5 minutes, the microglial cell moved 28.38µm. For the full footage, please see Electronic Appendix II – movie: Phagocytosis and Migration.
3.33 – Phagocytosis of pHrodo-green labelled *E. coli*

As described in Chapter One, microglia are known to phagocytose synapses as part of their normal function to support directional neuron growth (371-373). Microglia are also known to migrate to facilitate phagocytosis of cellular debris or pathogens that manage to cross the blood brain barrier (371, 373, 374). Here, the stem cell derived microglia are motile during inflammatory conditions, and they are able to phagocytose pHrodo-green labelled *E. coli*.

In Figure 22, H9-TREM2-E2CRIMSON microglia are observed having internalised pHrodo-green labelled *E. coli* after a 90-minute incubation. The H9-TREM2-E2CRIMSON fluorescent reporter was not clearly visible, despite clear expression of TREM2 by immunocytochemistry as observed in Figure 21. For this reason, the fluorescent reporter has not been shown here. Microglia, stained with Hoechst (in blue), can clearly be observed co-localising with green “specks”. pHrodo-green labelled *E. coli* only fluoresce once internalised into lysosomes with low pH (375).
Figure 22: H9-TREM2-E2CRIMSON targeted microglia incubated with pHrodo-green E. coli for 90 minutes, imaged on Nikon A1R., 20x Phagocytosis test (n=3, representative image). (A) combined image of (B), (C) and (D). B= Phalloidin (blue), C= pHrodo-green E. coli, D = brightfield image. Scalebar = 100µm all images.
3.34– Preliminary cytometric bead array data

A key feature of microglial identity is to secrete cytokines in response to inflammatory stimuli, such as, LPS, TNF, IFNγ, Amyloid-β and α-synuclein. LPS is an endotoxin found on the cell membranes of gram-negative bacteria, and has been described as the “gold standard” for stimulation of microglia (239, 327). TNF is an inflammatory and pleiotropic cytokine, produced by in the CNS by microglia and astrocytes (376), as well as by stem cell derived microglia (341, 343, 349, 351, 352) in response to LPS incubation. IFNγ, like TNF, is a pleiotropic and proinflammatory cytokine, capable of stimulating microglial secretion of other pro-inflammatory factors such as TNF, IL-1α and IL-6 (239, 377). As mentioned in Chapter One, amyloid-β is neuroinflammatory and contributes to neuronal death in Alzheimer’s Disease, and previous studies have suggested amyloid-β is capable of directly influencing microglia behaviour (377). Finally, studies suggest that α-synuclein aggregates are pathogenic under
specific circumstances (130, 131), and that mutations in the SNCA gene contributes to familial PD. For these reasons, LPS, TNF, IFNγ, Amyloid-β and α-synuclein were used to stimulate microglia and to elicit a secretory response in this thesis. As a proof of concept, H9-CX3CR1-tdTomato microglia monocultures were incubated with either Vehicle (PBS + 0.01% DMSO), LPS (100ng/mL), TNF (100ng/mL), IFNγ (20ng/mL) or α-synuclein (2.5µM) as described in Chapter Two. Conditioned media was collected after 2-, 4-, 8-, 24- or 48-hours as described in Chapter Two, and the cytokines IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, MIP-1α, TNF, IFNγ, VEGF and CX3CL1 were investigated using CBA (see Chapter Two).

The preliminary data show (see Appendix VII) the H9-CX3CR1-tdTomato microglia were capable of secreting low levels (<50pg/mL) of IL-1α, IL-1β, and IL-4, IFNγ and VEGF when incubated with Vehicle, LPS, TNF and α-synuclein. High levels of IL-6, IL-8, IL-10, MIP-1α, TNF, CX3CL1 were also observed during incubation. As all experiments for CBA in this chapter are n=1, no statistical comparisons or judgements can be made with any confidence, however, these data indicate the functional capacity of H9-CX3CR1-tdTomato microglia to respond appropriately to known inflammatory stimuli. This ability was pursued in Chapters Four and Five, where H9-CX3CR1-tdTomato microglia were cultured either alone, or in co-culture with neurons/astrocytes, to determine whether culture conditions influenced cytokine production.

3.4 Co-culture of microglia with forebrain or midbrain cultures.
One of the key features of microglia in vivo is that they are integrated within the CNS, and constantly interact with neurons and astrocytes. Therefore, it was important to confirm that the microglia generated here were able to survive in co-cultures with either forebrain or midbrain neurons.

3.41 – Co-culture with midbrain cultures.
Stem cell derived neuronal cultures are often heterogeneous populations, formed primarily of neurons, with an increasing population of astrocytes over time (378). This is because neurons and astrocytes are both from the ectodermal lineage, and the ratio of neurons: astrocytes varies between
stem cell lines (379). Here, H9-WT cells were used, and astrocytes were observed in low numbers in all differentiations towards midbrain floorplate neurons. For this reason, stem cell derived midbrain neuron cultures will be referred to as midbrain monocultures throughout this thesis.

Demonstration of co-cultures between stem-cell derived midbrain cultures and microglia has not previously been performed. It was important to determine whether microglia were able to survive in co-culture with midbrain cultures, especially, given the differences in the composition of the media used to culture these cell types. For example, media used for culture of midbrain monocultures contains Phenol-red, whereas microglial media does not, and personal communications from Abud and colleagues described difficulties in differentiating/promoting survival of microglia from CD43+ HPCs in media containing phenol red. Additionally, media used for differentiating neurons contains a number of growth factors (such as TGF-β3, BDNF, GDNF and activin A) which are not present in microglia terminal differentiation media, and may act to alter microglia survival or function. H9-CX3CR1-tdTomato-microglia differentiated from hESCs (as per Chapter Two) were plated into stem cell derived midbrain neurons at a ratio of 1:10, as described in Abud et al. (341) on Day 34 of differentiation. Cells were imaged on D38 after 4 days of co-culture with midbrain neurons (see Figure 24). Surviving H9-CX3CR1-tdTomato microglia were visible under a microscope, and tended to be rounded, with larger cell bodies and shorter processes, with fluorescent tdTomato expressed throughout their soma and processes. After fixation and immunolabelling, neurons were visible as β-III-tubulin positive cells, and astrocytes were present as S100β positive cells. DAPI was used to stain the nuclei, while microglia were visualised using the CX3CR1-td-Tomato fluorescent reporter. As the protocol for differentiation towards midbrain neurons is well established, confirmation of midbrain neuron identity was not performed outside of parallel cultures using the H9-LMX1A-eGFP reporter line (see Figure 25 and Figure 26) and identical protocols. LMX1A is an early transcription factor which contributes to dopaminergic neuron fate, and the karyotypically normal H9-LMX1A-GFP reporter line was generated within the same laboratory as the other cell lines used in this chapter (380).
Figure 24: Fixed D38 + 24 hours control stem cell derived CX3CR1-tdTomato microglia and midbrain neuron co-culture, taken on Nikon A1R, 20x objective, 1024x1024 resolution. (A): βIII-tubulin = neuronal marker, in white, (B): S100β = early astrocytes in green, (C) DAPI = cell nucleus, in blue (D): CX3CR1-tdTomato = microglia, in red (E): merged channel maximum intensity projection image of (A-D).
Figure 25: D29 H9-LMX1A-eGFP cells differentiated towards midbrain dopaminergic neurons. Image taken using Nikon A1R, 10x magnification, scale bar = 200µm. Image used with kind permission of A. Quaran.
3.42 – Co-culture with forebrain

Most co-culture studies performed with stem cell derived microglia have utilised forebrain neurons. Again, there were some heterogeneity to forebrain culture differentiation, as astrocytes were observed in all forebrain cultures (see Figure 27). For this reason, all forebrain cultures of neurons will be referred to as Forebrain monocultures. As Forebrain co-cultures were not the main focus of this thesis, the co-culture images presented are from live cells, imaged on the Bio-Rad ZOE Fluorescence cell imager (See Figure 28). As in the midbrain co-cultures, microglia co-cultured with forebrain neurons tended to be more rounded, with shorter and thicker processes than their monocultured counterparts. This may have been due to cellular debris within both cultures but was not investigated further due to time restraints.

Figure 26: Immunocytochemistry image of D40 midbrain moniculture, stained for β-III-tubulin in white (A), S100β (B) in green, and with DAPI in blue (C). (D) is a merged channel image of A-C. Image taken on Nikon A1R, 20x objective, at 1024x1024 resolution.
Figure 27: Day 70 WT-h9 cells differentiated towards forebrain neurons. Scale Bar = 100µm. (A) β-III-tubulin = neurons in green, (B) S100β = astrocytes in red (indicated by white arrows), (C) DAPI = nuclei in blue, (D) merged image. Nikon A1R, 20x objective, 1024x1024 resolution. Image used with kind permission of E. Kwak.
**Discussion of Chapter 3.**

The first aim of Chapter Three was to generate a fluorescent reporter line, where fluorescence was driven by a key microglial gene such as *CX3CR1*, to allow for co-cultures with neurons, and to traverse development of microglia from hESCs and HPCs. To that end, H9-WT cells were nucleofected with a
variety of plasmids to generate H9-CX3CR1-tdTomato, H9-TREM2-E2CRIMSON, H9-TMEM119-GFP, H9-IRF8-mCherry and H9-PU.1-GFP lines. All of these lines were confirmed by PCR to be correctly targeted, while the H9-CX3CR1-tdTomato and H9-TREM2-E2CRIMSON were also confirmed by Southern blot analysis to be correctly targeted. It was felt focusing primarily on one line (H9-CX3CR1-tdTomato) would allow for deeper and more thorough investigation, than a surface level investigation of five targeted lines. Unfortunately, the H9-CX3CR1-tdTomato, H9-TMEM119-GFP and H9-TREM2-E2CRIMSON lines possess Trisomy 12, and H9-IRF8-mCherry possesses an isochromosome 12. This is believed to have been caused by the parental H9-WT hESCs already being in possession of either isochromosome 12 or Trisomy 12 at time of targeting. The confirmation that the parental H9-WT hESC stock used for targeting IRF8 shares the same isochromosome 12 (Appendix III), supports this notion. It is crucial that future studies aiming to generate stem cell reporter lines should regularly screen their working stocks of hESCs for karyotypic abnormalities, especially prior to reporter line generation. Although the H9-CX3CR1-tdTomato, H9-TMEM119-GFP and H9-TREM2-E2CRIMSON lines possess Trisomy 12, they are potentially useful for studies outside the scope of this thesis. 

The next aim of this chapter was to replicate the methodology for generation of microglia using the H9-CX3CR1-tdTomato, H9-TREM2-E2CRIMSON and H9-WT hESCs. The protocol by Abud et al. (341) was optimised and modified, as it was found that the differentiation towards CD43⁺ HPCs was most easily accomplished using the StemDiff Haematopoietic Progenitor kit, and not by using the growth factors described by Abud et al. (2017) (341). Isolation of CD43⁺ HPCs was determined to be best performed using MACs to avoid the high numbers of cell death that occurred during FACs. As mentioned earlier, multiple alterations to pressure, nozzle size, resuspension volume and flow rate were made to the protocol in attempt to support the CD43⁺ progenitor cells through FACs processing, but these were unsuccessful. It is suspected that CD43⁺ cells are potentially more sensitive to shear stresses, such as are experienced during FACs, and this might explain why the cells were dying during FACs. Additionally, the length of time for FACs harvesting was approximately four to five hours, most of which was spent on ice. Potentially, this extended time period also contributed to the large numbers
of cell death during FACs. In contrast, MACs could be performed in as few as three hours, did not subject CD43+ cells to shear stress, and did not require the use of resin columns. These factors made MACs a gentler option for CD43+ isolation, and resulted in very little cell death.

Early microglial cells were observed from Day 16 of differentiation, but required a higher concentration of Matrigel than published (341) to cope with during maturation, otherwise they tended to form clusters and adopted a rounded morphology. It is not unusual to “tweak” a published protocol to enable a more efficient or easier method, however, as is done here – changes must be noted so that others can reproduce your findings (381). Additionally, adopting a commercial differentiation kit for generation of CD43+ HPCs will help to reduce user error, as it requires only a base media, and two supplements. In contrast, the protocol described by Abud et al. (341) requires multiple base media, multiple growth factors at various concentrations, and multiple CO2:O2 ratios. Each of these requirements in the original Abud protocol introduces the risk of error, and therefore risk of altered or unsuccessful differentiations.

Finally, this chapter aimed to validate the functional capacity of the H9-WT, H9-TREM2-E2CRIMSON and H9-CX3CR-tdToma lines. When differentiated, SCDmicroglia expressed key microglial markers of identity as shown by immunocytochemistry; demonstrated the capacity to phagocytose e. coli particles, and were morphologically similar to microglia described elsewhere (341, 349). Stem cell derived microglia were also able to survive co-culture with forebrain or midbrain cells in media comprised of 50% microglia terminal differentiation media, and 50% neuronal differentiation media. Additionally, and most importantly, preliminary data showed that microglia were able to respond to a variety of stimulatory molecules such as LPS, TNF and α-synuclein. However, the capacity of stem cell derived microglia to secrete cytokines needs further exploration, as most studies have described secretion from stem cell derived microglia only in monoculture (as performed here for proof of concept), and without the interaction or communication from neurons/astrocytes. This means that the understanding of stem cell derived microglial response when part of a co-culture vs as a
monoculture is virtually unknown, and critically hampers the understanding the role of microglia in a CNS environment. For this reason, the next two chapters will look at cytokine secretion by H9-CX3CR1-tdTomato microglia in monoculture, and compared to co-culture with neurons, and neuronal monoculture.
Chapter Four
Effect of inflammatory mediators on cytokine and chemokine signalling in midbrain modelling systems

Introduction to chapter.

The aim of this Chapter is to clarify the secretory profile of stem cell derived microglia/midbrain cultures when incubated with inflammatory mediators present in Parkinson’s Disease. In the last three years, nearly a dozen protocols have been published detailing methods for generation of microglia-like cells from iPSC/hESCs (for details, see Chapter One). None of these new protocols, however, have investigated how these cells perform under conditions which mimic the midbrain environment during Parkinson’s Disease (PD). Here, this was achieved by investigating protein levels of pro- and anti-inflammatory cytokines known to be involved in neuroinflammation. These cytokines were IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, MIP-1α, TNF, IFNγ, VEGF and CX3CL1. Although the methods used here are obviously simplified, they provide an excellent starting point for future studies into microglial behaviour in stem cell models of PD.

To assist with clarity, this chapter has been divided into individual cytokines. In addition, each set of cytokine/chemokine results are discussed separately throughout the chapter, and an overall discussion of the results will occur in the Discussion Chapter.

The methodology used in this chapter is summarised as follows: All experiments described in this chapter are n=3. H9-CX3CR1-tdTomato cultures were differentiated towards microglia, and H9-WT cultures were differentiated towards midbrain floorplate neurons as per Chapter Two. All cultures were incubated in the presence of Vehicle (PBS + 0.01% DMSO), LPS (100ng/mL), TNF (100ng/mL) or α-Synuclein (2.5μM). Midbrain monocultures and Co-cultures of microglia and midbrain neurons were also incubated with IFNγ (20ng/mL), but microglial monocultures were not incubated with IFNγ due to cell number restrictions. All analysis performed was Two-way ANOVA with post-hoc Tukey’s test. Data for specific timepoints (2-, 4-, 8-, 24- and 48-hours) was pulled from ANOVA output using VSIG-Lookup tables in EXCEL (for Two-way ANOVA with Post-hoc Tukey’s test data, please see electronic
Appendix XI). The same data was used for comparison between culture types as well as within culture types. For comparisons between culture types the following symbols were used to indicate significant differences: # difference between microglia and neuron, *difference between microglia and co-culture, ¥ Difference between co-culture and neurons. Differences within culture types as a function of time or due to incubation condition are noted separately on graphs. Detailed methodology can be found in Chapter Two.

The results for each cytokine (IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, MIP-1α, TNF, IFNγ, VEGF and CX3CL1) will now be described and discussed in turn, with a small overall summary at the end of the chapter.

4.1 - IL-1α secretion

Results

Comparison between cultures – pro-inflammatory stimuli mediated secretion of IL-1α

All three culture types (co-cultures, microglial monocultures, and midbrain monocultures) secreted minimal levels of IL-1α in response to vehicle (PBS + 0.01% DMSO) or pro-inflammatory stimuli (LPS 100ng/mL, TNF 100ng/mL or α-synuclein 2.5µM)

Comparing IL-1α secretion between culture types (see Figure 29) revealed significant elevations of IL-1α secretion were evident at 48-hours in microglial monocultures compared to midbrain monoculture and to co-cultures in Vehicle (2.43 ± 0.95 pg./mL vs 0.36 ±0.27pg/mL and 0.13±0.013pg/mL, p<0.0001 and p=0.0019 respectively) and LPS (3.03±0.70pg/mL vs 0.29 ±0.26pg/mL and 0.08 ± 0.08pg/mL, p<0.0001 both) incubations respectively. Incubation with α-synuclein also resulted in a small, but significant increase to secretion of IL-1α in microglial monocultures when compared to co-cultures (2.57 ± 0.44pg/mL vs 0.08 ± 0.07pg/mL, p=0.0162). but this did not reach significance with midbrain monocultures. Incubation with TNF did not significantly alter secretion of IL-1α between culture types.
Effects within cultures – time and inflammatory mediators

Once investigation between culture types was performed, it was pertinent to investigate whether there were differences in IL-1α secretion within culture types due to time or incubation conditions. Using the data from Figure 29, arranged for incubation type, or by time, it was revealed that there were no significant differences within midbrain monocultures or co-cultures (see Figure 30). However, microglial monocultures show a small but significant increase in IL-1α secretion over time in vehicle (0.47 ± 0.31pg/mL at 2-hours vs 2.43 ± 0.95 pg/mL at 48-hours, \(p<0.0001\) both) and in LPS incubation conditions (0.41 ±0.21pg/mL at 2-hours vs 3.03 ± 0.71pg/mL, \(p<0.0001\) both). There were no differences between incubation conditions within microglial monocultures.

Figure 29: IL-1α secretion by culture type, graphed by incubation condition. Vehicle (PBS+ 0.01% DMSO), LPS (100ng/mL) TNF (100ng/mL) or α-synuclein (2.5µM). Two-way ANOVA using post-hoc Tukey’s test for significance was performed. \(N=3\). * = significant difference between microglial monoculture and co-culture. # = significant difference between microglial monoculture and midbrain monoculture ****/#### \(p<0.0001\), ### \(p<0.005\), *\(p<0.05\)
Figure 30: IL-1α secretion by incubation (PBS+DMSO, LPS (100ng/mL) TNF (100ng/mL) or α-synuclein (2.5µM)) graphed by culture type. Graphs show each culture type, and secretion of IL-1α on incubation with Vehicle (*Black), LPS (+Red), TNF (#Green), IFNγ (¤Purple) or α-Synuclein (¥Blue). Two-way ANOVA using post-hoc Tukey’s test for significance was performed. N=3 ****p <0.0001 vehicle, ++++p <0.0001 LPS

Discussion

IL-1α is a major proinflammatory cytokine secreted by both astrocytes (382) and microglia (383), known to mediate innate immune responses to bacterial and viral proteins (384). IL-1α is typically membrane bound, and is only released from cells undergoing necroptosis or by Ca²⁺ influx (385, 386), such as occurs when stimulation with LPS. Based on this knowledge, it was speculated that incubation with inflammatory stimuli (specifically, with LPS) would lead to significant production of IL-1α in all cultures. The general lack of IL-1α secretion across all culture type and incubation conditions was therefore unexpected. Previous studies using human primary microglial and mixed glial cultures have shown extremely variable IL-1α secretion levels (383, 387) after incubation with LPS for 24-hours, which may be attributed to differences in analysis techniques. ELISA studies showing high levels of IL-1α may have been performed using lysed cells (387), whilst low levels of IL-1α seem to only observed in studies investigating supernatant (383, 386). The results presented here come from supernatant only, and do not contain cells. It is logical, therefore, that the IL-1α protein levels detected in this study were extremely low across all culture types and indicate that a) microglial and astrocyte cells have not undergone necroptosis and b) that incubation with inflammatory mediators was not sufficient to raise intracellular calcium. These results are supported by other protocols deriving microglia from stem cells, which show low (341) to negligible (349, 388) IL-1α secretion when analysing conditioned media, as was performed here. It is also further supported by rodent studies, which show intraperitoneal
injection of LPS does not significantly elevate IL-1α secretion in vivo (102), and that IL-1α protein levels are typically lower than the limit of detection by ELISA when analysing the supernatant of in vitro primary rodent glia (389). As a consequence, it is likely that the minimal secretion of IL-1α across culture types and incubation conditions observed here is reflective of a consistent in vitro phenotype: minimal IL-1α secretion occurs in the absence of substantial cell death or calcium flux.

While microglia monocultures appear to show a significant increase at 48-hours during incubation with Vehicle, LPS and α-synuclein, it is possible that this is due to several factors. Firstly, 48-hours is the longest time period without a media refreshment. This means that the microglia may not be receiving adequate levels of exogenous cytokines to maintain a resting state and may be becoming more reactive as a result. Alternatively, microglia may be in the process of dying at this time point and may be releasing IL-1α as a result. Finally, it is important to bear in mind that when two conditions express very low (<1pg/mL) concentrations of cytokines, small increases to secretion will often end up looking significant by comparison as an artefact. Here, it is believed that this last explanation is most likely to accurately describe IL-1α secretion during Vehicle, LPS and α-synuclein incubations by microglia monocultures.

No inflammatory condition measured within the culture types was able to exert any significant effect on IL-1α secretion. This is probably because the incubation concentrations of the inflammatory proteins used were well below the threshold for initiating either Ca²⁺ flux or necroptosis. Without reaching this threshold, the cells will not release IL-1α.

In summary, IL-1α is secreted at a very low level across all culture types, and under all incubation conditions. This is most likely due to IL-1α being membrane bound and only released by cells due to necroptosis or sufficient Ca²⁺ flux. IL-1α was secreted by microglial monocultures at a significantly higher concentration compared to midbrain monoculture and co-cultures following incubation in basal, LPS for 48-hours, and following incubation in α-synuclein for 24- or 48-hours. Within each culture, TNF significantly increased IL-1α secretion following 48-hours only within co-
culture conditions, and no other culture type or incubation condition was able to elicit significant alterations to IL-1α secretion.

4.2 - IL-1β secretion

Results

Comparison between cultures – pro-inflammatory stimuli mediated secretion of IL-1β

IL-1β secretion was significantly elevated in microglial monocultures compared to co-cultures at 48-hours when incubated with either vehicle (PBS + 0.01% DMSO) (13.30 ± 9.47 pg/mL vs 0.02 ± 0.02 pg/mL, \( p=0.0007 \)) or with LPS (100 ng/mL) (12.05 ± 7.87 pg/mL vs 0.00 pg/mL, \( p=0.0240 \)). IL-1β was also elevated in microglial monocultures compared to midbrain monocultures at 48-hours when incubated with vehicle (13.30 ± 9.47 pg/mL vs 0.00 ± 0.00 pg/mL, \( p<0.01 \)). Incubation with TNF (100 ng/mL) or α-synuclein (2.5 µM) (Figure 31) did not significantly alter IL-1β secretion between culture types.

Effects within cultures – time and inflammatory mediators

Once investigation between culture types was performed, it was pertinent to investigate whether there were differences in IL-1β secretion within culture types due to time or incubation conditions. Using the data from Figure 31, arranged by culture type and time, revealed IL-1β secretion was significantly increased over time in vehicle conditions (PBS + 0.01% DMSO) within microglial cultures (0.38 ± 0.22 pg/mL at 2-hours vs 13.30 ± 9.47 pg/mL at 48-hours, \( p=0.0002 \)), but not within midbrain monocultures or co-culture conditions (see Figure 32). IL-1β secretion was not significantly altered due to incubation with pro-inflammatory mediators within microglial monoculture, midbrain neuron-astrocyte monoculture, or co-culture conditions.
**Figure 31**: IL-1β by culture type, graphed by incubation condition. Vehicle (PBS + 0.01% DMSO) and LPS (100ng/mL) conditions, but not in TNF (100ng/mL) or α-synuclein (2.5µM) conditions. Two-way ANOVA using post-hoc Tukey’s test for significance was performed. N=3, *=significant difference between microglial monoculture and co-culture. # = significant difference between microglial monoculture and midbrain monoculture ***/### p<0.0001, ### p<0.005, *p<0.05

*p<0.05, **p<0.005

**Figure 32**: IL-1β secretion by incubation (PBS + 0.01% DMSO, LPS (100ng/mL) TNF (100ng/mL) and α-synuclein (2.5µM), graphed by culture type. Vehicle (Black), LPS (Red), TNF (Green), IFN (Purple) or α-Synuclein (Blue). Two-way ANOVA using post-hoc Tukey’s test for significance was performed. N=3, ***p<0.0005
Discussion of IL-1β. 

IL-1β is a major proinflammatory cytokine, and is elevated in Parkinson’s Disease patient CSF, indicating IL-1β involved in the progression and inflammation known to occur in PD in vivo (390). As IL-1β is known to be mediated by multiple pathways, including caspase-1 (384, 391) THIK-1 (392) P2X7R (393, 394) and TLR signalling (393), it was expected that secretion of IL-1β would occur more readily than IL-1α, as it is not membrane bound, and does not rely on Ca²⁺ flux or cell death for release into the extracellular space.

The generally low secretion of IL-1β, similar to IL-1α, was somewhat unexpected. Other protocols describing microglia derivation from stem cells, show large differences in measured IL-1β secretion in response to LPS challenge – from less than 10pg/mL (346), to 150pg/mL (343) to more than 20,000pg/mL (345). These variations create difficulties in predicting IL-1β secretion in stem cell derived cultures. The data presented here may be reflective of some aspects of microglial function described in primary human microglia (383). This idea and data are supported by studies analysing human primary microglia supernatant, which secreted extremely low levels of IL-1β in response to LPS incubation (383), analysis of PD patient CSF has been proven to contain low levels of IL-1β in the absence of contaminating leukocytes (390). Additionally, the protocol used in this thesis (341), has not previously shown IL-1β secretion at all.

IL-1β is also involved in a positive feedback loop (384, 385) (see Figure 33) – which might explain the high concentrations of IL-1β described elsewhere, but which were not observed here. A positive feedback loop may also explain how IL-1β secretion was observed to be significantly altered in vehicle conditions over time within microglial monocultures, but not in midbrain monocultures or co-cultures. As astrocytes do not have the same TLRs as microglia, they are unable to respond to the same stimuli to secrete IL-1β (393), and could not have contributed to this positive feedback loop. One way to determine whether positive feedback loop is involved, could be to perform studies in microglial monocultures using siRNA targeted to IL-1β, or to use chemical inhibition of pathways known to
initiate IL-1β secretion already demonstrated in other cell and model types (such as using A740003 to inhibit P2X7 receptors (395) or Z-WEHD-FMK to inhibit Caspase1/ICE (396)).

Figure 33: Simplified illustration of IL-1β positive feedback loop. 1) IL-1β binds to the IL-1 receptor complex, which activates 2) one of the NLRP3, Caspase1 or TRAF/IRAK signalling cascades. These signalling cascades initiate transcription within the nucleus (3), which increases IL-1β mRNA within the cell. This mRNA is translated and packaged into vesicles (5) and is then released into the extracellular space to start the cycle again (6). Image adapted from (384, 385), using BioRender.com.

In summary, IL-1β secretion was low across all culture types and in all incubations. Microglial monocultures secreted significantly more IL-1β compared to midbrain monocultures and co-cultures when incubated in vehicle or LPS conditions, but not when incubated with TNF or α-synuclein. Inflammatory stimuli were unable to influence secretion of IL-1β within culture types. Further investigation of IL-1β in microglial monocultures should focus on determining whether secretion is mediated by a positive feedback loop using inhibitors. Finally, secretion of IL-1β in microglial monocultures is possibly reflective of primary human microglial cultures, and may be appropriate for use in further studies investigating PD.
4.3 - IL-4 secretion

Results

Comparison between cultures – pro-inflammatory stimuli mediated secretion of IL-4

IL-4 was secreted in low levels by all culture types. Co-cultures incubated in vehicle (PBS +0.01% DMSO) showed significantly increased IL-4 secretion (5.67 ±3.60pg/mL) at 48-hours compared to both microglial (0.32 ± 0.18pg/mL, \( p<0.0001 \)) and neuronal monocultures (not detected, \( p<0.0001 \)) (see Error! Reference source not found.). Incubation with LPS (100ng/mL), TNF (100ng/mL) or \( \alpha \)-synuclein (2.5\( \mu \)M) did not alter IL-4 secretion between cultures.

Effects within cultures – time and inflammatory mediators

Once investigation between culture types was performed, it was pertinent to investigate whether there were differences in IL-4 secretion within culture types due to time or incubation
conditions. Using the data from Figure 34, arranged by culture type and time, indicated time significantly influenced IL-4 secretion within co-cultures (not detected at 2-hours vs 5.68 ± 3.61pg/mL, p<0.0001) but only during incubation with vehicle (see Figure 35). Secretion of IL-4 in microglial and neuronal monocultures was not significantly altered due to time or incubation conditions.

![Figure 35: IL-4 secretion by incubation (vehicle (PBS + 0.01% DMSO), LPS (100ng/mL), TNF (100ng/mL), IFNγ (20ng/mL) and α-synuclein (2.5µM)), within culture type over time. Graphs show each culture type, and secretion of IL-4 on incubation with Vehicle (*Black), LPS (+Red), TNF (#Green), IFNγ (×Purple) or α-Synuclein (¥Blue). N=3 Two-way ANOVA with post-hoc Tukey’s Test for multiple comparisons was performed. ****p<0.0001](image)

**Discussion**

IL-4 is a powerful anti-inflammatory cytokine secreted by T-cells in response to local injuries (397), as well to LPS (398). The majority of human studies investigating neuroinflammatory diseases, such as Parkinson’s or MS, have focused on looking at measurable changes within the CSF fluid and blood serum (122, 398-400). Attempts to investigate IL-4 in brain tissue (123, 401) have found that IL-4 is not expressed in the substantia nigra (123), and is only minimally expressed by astrocytes (401), while *in vitro* human microglia have been revealed to secrete IL-4 when cultured with T-cells (402). IL-4 has also been indicated as a potential differentiating marker between atypical parkinsonism and early onset Parkinson’s disease (403). Most published literature does not typically focus on IL-4 secretion from specific cell types in human CNS tissue, but instead focuses on IL-4 as a peripheral cytokine with effects on the CNS.

Knowing the importance of IL-4 in modulating inflammation, it was surprising to observe such low levels of IL-4 secretion across culture types and incubation conditions. It was expected that culture with inflammatory stimuli would encourage secretion from either astrocytes or microglia, since both are supposed to produce IL-4 in response to disease states(401, 402). However, the only attempt to
determine IL-4 secretion in stem-cell derived microglia protocols was unsuccessful (349); similarly, investigations of the effects of Dengue and Zika virus in stem cell derived microglia also failed to detect IL-4 \textit{in vitro} (404). This might mean stem cell derived models of microglia are missing a key component which would enable IL-4 secretion. If, like in rodents, the major source for IL-4 in the human CNS is T-cells (399, 405, 406), then this could be tested in future studies, by using parallel T-cell/microglia/neuron mixed cultures on microfluidics chambers, and performing ELISA with the conditioned media.

Another possible explanation for the low levels of IL-4 could be that the all cultures used here are not functionally mature enough to produce IL-4 to a significant level. Using RT-PCR or single-cell RNA-sequencing to confirm whether IL-4 is present at a transcriptional level would be an ideal starting point. As the transcriptional profiles of microglia (407-409), astrocytes (410) and neurons (256, 411-413) in humans are known to be altered by age, region and disease state, RNA-sequencing of these stem cell derived cultures would assist in determining where these cultures fit into known data, and whether IL-4 transcript is upregulated in midbrain culture models of microglia, neurons and co-cultures relative to published data.

In summary, IL-4 is minimally secreted in microglial and neuronal monocultures, and secretion in co-culture is significantly upregulated at 48-hours under vehicle conditions. IL-4 secretion is not significantly altered by incubation with inflammatory stimuli. The low levels of IL-4 secretion across culture types could be due to lack of maturity, or due to absence of a key co-factor required for signalling within culture. Future investigations into the lack of IL-4 should focus on determining whether T-cells are required for IL-4 production \textit{in vitro} and RNA-sequencing studies.

4.4 - IL-6 secretion

\textbf{Results}

\textit{Comparison between cultures} – pro-inflammatory stimuli mediated secretion of IL-6

IL-6 was secreted at widely varying levels between culture types and incubation conditions. Secretion of IL-6 was significantly increased in co-cultures compared to microglial monoculture at 24-
and 48-hours (4128.4 ± 1902.0 pg/mL vs 1.47 ± 0.533 pg/mL, p<0.0001, and 5816.90 ± 2886.0 pg/mL vs 0.97 ± 0.97 pg/mL, p<0.0001 respectively) when incubated with TNF. The same trend, though to a lesser degree, was observed with midbrain monocultures, which secreted significantly more IL-6 than microglial monocultures at 24- and 48-hours (807.38 ± 195.81 vs 1.47 ± 0.533 pg/mL p<0.0001, and 1814.12 ± 91.78 pg/mL vs 0.97 ± 0.97 pg/mL, p<0.0001 respectively). There was no significant difference in IL-6 secretion between co-cultures and midbrain monocultures. Additionally, secretion of IL-6 was not observed in microglial monocultures when incubated with LPS (100 ng/mL) and was only minimally observed at 48-hours when incubated with α-synuclein.

**Effects within cultures – time and inflammatory mediators**

After comparison of IL-6 secretion between culture groups, it was important to investigate secretion within individual culture types. Using the data in Figure 36, incubation conditions and time were analysed within each culture type. Co-culture secretion of IL-6 was significantly increased at 24- and 48-hours (27.29 ± 14.29 pg/mL vs 4128.38 ± 1901.61 pg/mL, p<0.0001 and 65.39 ± 31.89 pg/mL vs 5816.90 ± 2886.0 pg/mL p<0.0001, respectively) when incubated with TNF compared to vehicle secretion at the same time point (see Error! Reference source not found.). Additionally, secretion of IL-6 significantly increased over time, specifically at 24- and 48-hours compared to 2-hours (74.94 ± 36.13 pg/mL vs 4128.38 ± 1901.61 pg/mL p<0.0001, and 5816.90 ± 2886.0 pg/mL p<0.0001, respectively), when incubated with TNF, indicating time affects IL-6 secretion under these conditions. Although TNF also appears to substantially increase IL-6 secretion within neuronal monocultures at 24- and 48-hours compared to vehicle, statistical analysis indicated this was not significant. Finally, secretion of IL-6 in microglial monocultures was not significantly altered due to time or incubation conditions at any point.
Figure 36: Comparison of IL-6 secretion by culture type, and by incubation with inflammatory stimuli. Incubations are vehicle (PBS + 0.01% DMSO, LPS (100ng/mL), TNF (100ng/mL) and α-synuclein (2.5µM)). Two-way ANOVA with post-hoc Tukey’s test for significance was performed. Scale is logarithmic. N=3. ****/####p<0.0001 *significant difference between co-culture and microglial monoculture. # significant difference between midbrain monoculture and microglia monoculture.

Figure 37: IL-6 secretion by incubation (vehicle (PBS + 0.01% DMSO, LPS (100ng/mL), TNF (100ng/mL), IFNγ (20ng/mL) and α-synuclein (2.5µM)), within culture type over time Graphs show each culture type, and secretion of IL-6 on incubation with Vehicle (*Black), LPS (+Red), TNF (#Green), IFNγ (#Purple) or α-Synuclein (XBlue). Two-way ANOVA with post-hoc Tukey’s test for significance was performed. Scale bars are set for each culture type and are not equal between groups. N=3 ****p<0.0001 between TNF and Vehicle. #### p<0.0001 between 2-hours and later timepoint.
Discussion

The role of IL-6 within the CNS is extremely complex. IL-6 absence inhibits the generation of neurons and glial cells (414), whereas IL-6 is initially neuroprotective in the early stages of cerebral infarction (415), and has been established in mice to be protective against loss of neural progenitor cells during infection with herpes simplex virus-1 (416). On the other hand, IL-6 has also been shown to be significantly upregulated in both the brain tissue (417) and CSF of patients (122, 390) with PD, suggesting an inflammatory role in PD pathology.

Stem cell derived cultures of microglia (341, 346, 348, 349) have previously exhibited increased IL-6 protein levels when treated with a variety of inflammatory stimuli, whilst astrocytes (418, 419) and neurons (420) are also able to respond to and produce IL-6. Based on this, it was anticipated that microglial monocultures would produce substantial levels of IL-6, However, IL-6 secretion over time in microglial monocultures across all incubation types was minimal. This suggests IL-6 secretion by stem cell derived microglia may be inhibited by one of the factors included in the culture media for these experiments, though identification of the specific factor was not attempted here.

Unexpectedly, co-cultures secreted the greatest amount of IL-6 when compared to both microglial and neuronal monocultures. Additionally, within co-cultures, secretion of IL-6 was strongest during incubation with TNF. TNF has previously been shown to stimulate secretion of IL-6 and IL-8 in human astrocytes (421). TNF is known to initiate transcription and secretion of molecules associated with inflammation (including IL-6) via the JAK-STAT pathway in astrocytes (418, 419, 421, 422), which activates the transcription factor Nuclear Factor kappa B (NF-κB), as illustrated in Error! Reference source not found.. Here, midbrain monocultures and co-culture secretion of IL-6 in response to TNF stimulation is likely to due to activation of the JAK-STAT pathway, in combination with IL-6 autocrine and paracrine signalling to form a positive feedback loop (419, 422-424) (see Figure 38). Recent advances in the field of neuroscience have confirmed the importance of cross-talk between microglia, neurons and astrocytes (201). Potentially, these experiments with co-cultures of midbrain neurons
(which also contain astrocytes, see Chapter Three) and microglia allow for reinforcement of TNF signalling, thereby amplifying secretion of IL-6. This is supported by the data which show neither midbrain or microglial monocultures are able to secrete more IL-6 than co-cultures (see Figure 36).

IL-6 secretion by microglial monocultures was not produced at all in response to LPS, contrary to previous papers (341, 343, 346, 347, 351, 404). LPS, like TNF, is able to activate JAK-STAT, but instead signals through TLR’s (see Figure 38). However, LPS activation of the STAT pathway also stimulates Suppressor of Cytokine Signalling (SOCS) to inhibit further JAK-STAT activation, which, in turn, limits the transcription and secretion of proinflammatory molecules such as TNF and IL-6(422, 423, 425-427). This difference in JAK-STAT pathway signal modulation between LPS and TNF stimulation may explain why incubation with TNF, not LPS, was more effective at stimulation of IL-6 secretion in the stem cell derived co-cultures here. JAK-STAT signalling could be verified in future experiments by inclusion of JAK or STAT inhibitors during incubation with TNF. If the production of IL-6 is significantly altered, then this would indicate JAK-STAT pathway involvement. This could be further supported by performing an ELISA for phosphorylated vs. non-phosphorylated JAK/STAT proteins to conclusively demonstrate JAK-STAT activity.

In summary, secretion of IL-6 is occurs most strongly in co-cultures when stimulated with TNF, not in microglial monocultures with LPS as expected. This is most likely due to TNF stimulation of JAK-STAT signalling, which promotes a positive feedback loop of IL-6 secretion/stimulation. Future experiments seeking to understand that mechanism should investigate cultures using JAK/STAT inhibitors.
Figure 38: Graphical representation of pathways involved in IL-6 secretion and positive feedback loop formation. Pathway (a) 1- TNF binds to TNF receptor, which triggers JAK-STAT signalling (2). JAK-STAT signalling initiates (3) NF-κB promotion of IL-6 and TNF, which are transcribed (4). IL-6 and TNF are packaged into vesicles and bud out from the cell to be released into the extracellular space (5), where IL-6 and TNF are able to bind to their respective receptors (6). Pathway (b) 1- LPS binds to Toll Like Receptor 4, which initiates JAK-STAT signalling. Some promotion of NF-κB occurs, but SOCS signalling is also concurrently promoted. SOCS blocks further initiation of JAK-STAT signalling, preventing positive feedback loop from occurring. Image created with Bio-render.com.

4.5 - IL-8 secretion

Results

Comparison between cultures – pro-inflammatory stimuli mediated secretion of IL-8

Analysis of secretion of IL-8 between microglial monoculture, neuronal monoculture and co-culture conditions revealed co-cultures incubated with TNF (100ng/mL see Figure 39) consistently secreted more IL-8 than microglial monocultures at 4-hours (7851.44 ± 1822.0pg/mL vs 1232.88 ± 1116.55 pg/mL, p=0.0021), 8-hours (14013.0 ± 2183.0pg/mL vs 4286.38 ± 4103.76pg/mL, p<0.0001), 24-hours (13996.0 ± 482.4pg/mL vs 2957.42 ± 2310.52pg/mL, p<0.0001) and 48-hours (11340.0 ± 2350.0 pg/mL vs 330.84 ± 330.80pg/mL, p<0.0001). Co-cultures also secreted more IL-8 than midbrain neuronal monocultures at 4-hours (7851.44 ± 1822.0pg/mL vs 1513 ± 431.92pg/mL,
p<0.0001) and 8-hours (14013.0 ± 2183.0pg/mL vs 2104.15 ± 671.23pg/mL p<0.0001) when incubated with TNF but secreted significantly less IL-8 at 48-hours (11340.0 ±2350pg/mL vs 18489.65 ± 445.28pg/mL p<0.0001) compared to neuronal monocultures at the same timepoint. At 24- and 48-hours, neuronal monocultures incubated with TNF secreted significantly more IL-8 compared to microglial monocultures (13476.87 ± 3096.57 vs 2957.42 ± 2310.52pg/mL, p<0.0001, and 18489.65 ± 445.28pg/mL vs 330.84 ± 330.80pg/mL, p<0.0001 respectively).

No significant differences in IL-8 secretion were observed between culture types during incubation with Vehicle (PBS + 0.01% DMSO), LPS (100ng/mL), or α-synuclein (2.5 µM). These incubations resulted in secretion of less than 500pg/mL IL-8 over a 48-hour period.

In addition, IL-8 secretion kinetics appear to be altered by the presence or absence of microglia. The presence of microglia in culture (microglial monoculture and co-culture conditions) results in peak IL-8 secretion after 8-hours of incubation. Contrastingly, the absence of microglia (neuronal monocultures) results in peak secretion of IL-8 at 48-hours. The significantly increased IL-8 production in co-culture conditions compared to microglial monoculture from 4-hours onward indicates communication between microglia and neurons/early astrocytes is occurring, potentially amplifying initial IL-8 secretion.
Figure 39: Comparison of IL-8 secretion by culture type, and by incubation with inflammatory stimuli. Vehicle (PBS + 0.01% DMSO, LPS (100ng/mL), TNF (100ng/mL) and α-synuclein (2.5µM). Two-way ANOVA with post-hoc Tukey’s test for significance was performed. N=3. # difference between microglia and neuron, *difference between microglia and co-culture, ¥ Difference between co-culture and neurons **p<0.005, ****p<0.0001

Effects within cultures – time and inflammatory mediators

After comparison of IL-8 secretion between culture groups, it was important to investigate secretion within individual culture types. Using the data in Figure 38, incubation conditions and time were analysed within each culture type. Analysis indicated incubation with TNF significantly altered IL-8 secretion within neuronal monoculture and co-cultures, but not within microglial monocultures (see Figure 40). Midbrain monocultures incubated with TNF secreted significantly more IL-8 at 24-hours (13477 ± 3096pg/mL vs 72 ±18pg/mL, p<0.0001) and 48-hours (18489 ± 445pg/mL vs 162 ± 39pg/mL, p<0.0001) compared to vehicle. Co-cultures incubated with TNF secreted significantly more IL-8 at 4-hours (7851 ± 1811pg/mL vs 72 ± 54pg/mL, p<0.0001), 8-hours (14013 ± 2182pg/mL vs 89 ± 107
41pg/mL, \( p<0.0001 \), 24-hours (13996 ± 482pg/mL, vs 255 ± 118pg/mL, \( p<0.0001 \)) and 48-hours (11340 ± 2350pg/mL vs 278 ± 112pg/mL, \( p<0.0001 \)) compared to vehicle.

Additionally, time significantly contributed to secretion of IL-8 in midbrain monocultures and co-cultures, but only when incubated with TNF. Midbrain monoculture secretion of IL-8 was significantly increased at 24-hours (13477 ± 3096pg/mL, \( p<0.0001 \)) and 48-hours (18489 ± 445pg/mL, \( p<0.0001 \)) compared to 2-hours (261 ± 54pg/mL). Co-culture secretion of IL-8 was significantly increased at 4-hours (7851 ± 1811pg/mL \( p<0.0001 \)), 8-hours (14013 ± 2182pg/mL, \( p<0.0001 \)), 24-hours (13996 ± 482pg/mL, \( p<0.0001 \)) and 48-hours (11340 ± 2350pg/mL, \( p<0.0001 \)) compared to 2-hours (1718 ± 480pg/mL). Time did not significantly alter IL-8 secretion in microglial monoculture under any incubation conditions.

Discussion

IL-8 is a pro-inflammatory cytokine secreted by astrocytes (428) and microglia (429-431) in vitro (327, 432, 433) in response to LPS. In addition, IL-8 concentration in patient CSF has been positively correlated with severity of PD (434, 435). Unexpectedly, LPS was not the most potent stimulus of IL-8 secretion. Previous experiments suggest LPS stimulation of macrophages and microglia may require “priming” with IFNγ to potentiate LPS signalling (436, 437). Supporting this, multiple protocols (341, 346, 349) deriving microglia from stem cells have shown secretion in response to combined LPS/IFNγ incubation. LPS incubation was performed alone in the experiments presented.
here, and the absence of IFNγ may have contributed to the lack of substantial IL-8 secretion. This could be investigated in future studies by using parallel cultures which contain LPS and/or IFNγ combinations. However, as LPS is unlikely to be present in human Parkinsonian brain without substantial disruption to the blood-brain-barrier, these future studies would serve only to prove the requirement for “priming” microglia with IFNγ prior to incubation with LPS. If so, then current in vitro models of inflammation using incubation with LPS may not be the most appropriate for modelling PD.

Here, IL-8 secretion was most strongly promoted in midbrain monocultures and co-culture conditions during incubation with TNF, but this was not observed in microglial monocultures. TNF is known to modulate production of IL-8 through NF-κB (438), and expression of TNF is also known to be significantly increased in the striatal tissue and lumbar CSF of PD patients (390, 439-441). Potentially, TNF incubation and subsequent IL-8 secretion observed in the monoculture midbrain and co-culture systems is reflective of the conditions observed in human floorplate.

Another interesting aspect of the data in these experiments is that cultures with microglia produce less total IL-8 than cultures without microglia (see Figure 39). TNF has been demonstrated to stimulate IL-8 production by human astrocytes (421, 442) and neurons (443), which explains why IL-8 was observed in midbrain monoculture and co-culture conditions. As microglial monocultures produced the least IL-8 in all incubation conditions, and because production of IL-8 was significantly upregulated when microglia were co-cultured with midbrain neurons/astrocytes, this strongly indicates that communication between microglia and neurons/astrocytes is occurring in vitro. Microglia are known to interact with almost all cell types present in the CNS (432, 444) (160, 268, 407) through a variety of cytokines and chemokines, under both inflammatory and homeostatic conditions (201). It is likely that communication within co-cultures between midbrain neurons/astrocytes and microglia has positively reinforced the production of IL-8, while the absence of neurons/astrocytes in microglial monocultures inhibited IL-8 secretion. This idea is supported by the presence of TNFR1 receptors on both microglia and astrocytes (239, 421, 445-447), allowing IL-8 secretion in response to
both exogenous and endogenous TNF-α, and by the data in Figure 39, which shows co-cultures generally secrete the greatest amount of IL-8 in all incubation conditions.

The kinetics of IL-8 secretion also appear to be influenced by the presence of microglia: cultures containing microglia exhibit a curved of IL-8 secretion, peaking at 8-hours, whereas cultures without microglia appear to increase secretion of IL-8 over time, and peak at 48-hours. A possible explanation for these differences in IL-8 secretion across culture types is involvement of prostaglandin E2 (PGE2) signalling, previously shown in macrophages to regulate proinflammatory signalling by TNF (448). Studies using primary human microglia have also confirmed IL-8 regulation by PGE2 (431, 442), indicating PGE2 regulation of IL-8 secretion may be occuring here. Alternatively, TGFβ1, a regulatory molecule known to inhibit IL-8 secretion and secreted by microglia may be involved (430). All three culture conditions contained exogenous TGFβ1 to maintain microglial identity (267, 449) and to remove differences in media as a confounding factor. It is possible, though, that the absence of microglia in midbrain monocultures resulted in reduced TGFβ1 concentration, compared to cultures containing microglia. If so, this may have contributed to increased IL-8 production over time as observed in Figure 39. Equally possible is that both scenarios are true to an extent, and IL-8 secretion here is modulated by a combination of PGE2 and TGFβ1 signalling. Unfortunately, neither PGE2 nor TGFβ1 presence were measured during these experiments, but this could be investigated in future studies using ELISA to determine whether IL-8 secretion coincides with alterations to PGE2/TGFβ1. This could also be investigated by using TGFβ1/PGE2 inhibitors (such as pirfenidone (450) or indomethacin/ibuprofen (451)) to determine whether IL-8 expression increases in the presence of TNF.

In summary, IL-8 secretion was most strongly induced by incubation with TNF, not LPS as expected. Neither LPS nor α-synuclein were able to significantly alter secretion of IL-8 within culture types compared to vehicle. Microglial presence in culture alters the kinetics of IL-8 secretion, and microglial monocultures produce the least amount of IL-8 when compared to co-culture or midbrain.
monoculture. TNF-regulated production of IL-8 is possibly mediated in these cultures by TGFβ1 and/or PGE2. Future studies seeking to understand the mechanism behind altered IL-8 production in TNF incubated cultures could utilise TGFβ1/PGE2 inhibitors.

4.6 - IL-10 secretion

Results

Comparison between cultures – pro-inflammatory stimuli mediated secretion of IL-10

In contrast to expectations, IL-10 secretion was not significantly altered due to culture type. A Two-way ANOVA with post-hoc Tukey’s test to compare culture types against each other, determined microglial monocultures, midbrain monocultures and co-cultures of midbrain neurons and microglia were not statistically different at any given timepoint measured (see Figure 41), and that incubation with LPS (100ng/mL), TNF (100ng/mL), α-synuclein (2.5 µM) or Vehicle (PBS + 0.01% DMSO) did not significantly alter IL-10 secretion.

Effects within Cultures – time and inflammatory mediators

Although analysis indicated there were no significant differences in IL-10 secretion between culture types, it was important to determine whether incubation with inflammatory mediators would significantly alter IL-10 secretion within culture types. Using the data from Figure 41, comparison of incubation conditions and changes over time within culture types was performed (see Figure 42). No significant differences due to incubation condition were observed within microglial monocultures, midbrain monocultures or co-cultures, at any time point.
IL-10 is typically considered an anti-inflammatory cytokine which known to be integral for modulation of IL-8 (430, 431), while increased CSF levels in patients with Parkinson’s disease is associated with later onset of symptoms (390). IL-10 is secreted by both microglia (452) and astrocytes...
and has been previously shown to be produced in stem cell derived microglial monocultures treated with LPS and/or IFNγ (341, 346).

The lack of significant alterations to IL-10 secretion is intriguing. As stem-cell derived microglia have previously been demonstrated to significantly alter IL-10 secretion in response to LPS (341) (albeit from a negligible concentration to approximately 40pg/mL), does this mean that the culture methodology utilised here is impacting on microglial secretion? Investigations on primary human microglia found IL-10 secretion was absent when T-cells were not present (454), indicating IL-10 secretion by microglia relies on communication with the T-cells normally located within the subarachnoid space. The absence of significant alterations to IL-10 secretions in midbrain neuronal cultures, which were confirmed in Chapter Three to contain astrocytes, was also puzzling, as astrocytes have also been specifically shown to secrete IL-10 (452, 453, 455).

If microglial secretion of IL-10 requires communication between microglia and T-cells, this could be investigated in future studies by using microfluidics chambers and ELISA. Another explanation for the minimal secretion of IL-10 involves TGFβ and Wnt signalling. Canonical Wnt-signalling via GSK-3β, is known to inhibit IL-10 secretion in murine cortical glia (456, 457). Wnt signalling is closely integrated with TGFβ to modulate key transcription factors such as NF-κB (458), which act to promote transcription of IL-10 during inflammation (459). It is possible that the exogenous TGFβ1/TGFβ3 included in the culture media for maintenance of microglial identity and continued support of neurons, interfered with secretion of IL-10 in these experiments. Investigation of Wnt-TGFβ signalling and influence on IL-10 secretion cultures would be a good starting point for future studies.

In summary, IL-10 secretion was not significantly altered between culture types, or within culture types due to incubation with inflammatory mediators. This may be due to either an absence of T-cells, or aberrant WNT signalling. Future studies should look to include T-Cell co-cultures with
microglia, and to consider inhibition/overexpression of WNT signalling prior to inflammatory mediator challenge, to examine the effect on IL-10 secretion.

4.7 - MIP1α secretion

Results

Comparison between cultures – pro-inflammatory stimuli mediated secretion of MIP1α

Analysis of MIP1α secretion showed that secretion of MIP1α was significantly increased in co-culture compared to midbrain monocultures at 2-hours (17.18 ± 3.62pg/mL vs 0.35 ± 0.17pg/mL, p=0.0002), 4-hours (19.14 ± 3.63pg/mL vs 0.35 ± 0.20 pg./mL, p<0.0001), 8-hours (38.03 ± 2.66pg/mL vs 0.37 ± 0.18pg/mL, p<0.0001) and 24-hours (26.18 ± 2.99pg/mL vs 1.65 ± 0.34pg/mL, p<0.0001) when incubated with TNF (100ng/mL). MIP1α was also significantly increased in co-cultures compared to microglial monocultures at 8-hours (38.03 ± 2.66 vs 2.35 ± 1.23pg/mL, p<0.0001) and 24-hours (26.18 ± 2.99pg/mL vs 1.60 ± 0.54pg/mL, p<0.0001). MIP1α secretion was not significantly altered between cultures when incubated with Vehicle (PBS + 0.01% DMSO), LPS (100ng/mL) or α-synuclein (2.5µM). (Figure 44).

Effects within cultures – time and inflammatory mediators

After comparison of MIP1α secretion between culture groups, it was important to investigate secretion within individual culture types. Using the data from Figure 43, arranged by culture type and time, it was revealed that TNF incubation within co-cultures significantly increased MIP1α secretion compared to vehicle at 2-hours (17.18 ± 3.62pg/mL vs 0.19 ± 0.14pg/mL, p=0.0002), 4-hours (19.14 ± 3.63pg/mL vs 3.08 ± 1.89pg/mL, p=0.0058), 8-hours (38.03 ± 2.66pg/mL vs 0.00, p<0.0001) and 24-hours (26.18 ± 2.99pg/mL vs 0.27 ± 0.20pg/mL, p<0.0001). These changes were not observed in midbrain or microglial monocultures (see Figure 44). MIP1α secretion was also significantly influenced by time within co-cultures when incubated with TNF, peaking at 8-hours compared to 2-hours (38.03 ± 2.66pg/mL vs 17.18 ± 3.62pg/mL, p<0.0001).
Figure 43: Comparison of MIP1α secretion by culture type, and by incubation with inflammatory stimuli. Incubations are vehicle (PBS + 0.01% DMSO, LPS (100ng/mL), TNF (100ng/mL) and α-synuclein (2.5µM)). Two-way ANOVA with post-hoc Tukey’s test for significance was performed. N=3. ***p<0.0001, **p<0.0005 # difference between microglia and neuron, *difference between microglia and co-culture, ¥ Difference between co-culture and neurons.

Figure 44: MIP1α secretion by incubation (vehicle (PBS + 0.01% DMSO, LPS (100ng/mL), TNF (100ng/mL), IFNγ (20ng/mL) and α-synuclein (2.5µM)), within culture type over time. Vehicle (Black), LPS (Red), TNF (Green), IFN (Purple) or α-Synuclein (Blue). Two-way ANOVA with post-hoc Tukey’s test for significance was performed. Scale bars are set for each culture type and are not equal between groups. N=3. **** p<0.0001 between 2 and 8-hours during incubation with TNF. ####p<0.0001 between TNF and Vehicle at indicated timepoint.
**Discussion**

MIP-1α is a proinflammatory chemokine produced in human microglia (460-462) and astrocytes (463-465). It is a neutrophil attractant in humans (466), and a variety of models show secretion in response to LPS (467) TNF, IFNγ and other inflammatory mediators (468, 469). Stem cell derived microglia have previously demonstrated MIP-1α secretion in response to IFNγ/LPS challenge (341, 349), but have not investigated whether the MIP-1α secretion follows the classic bi-phasic “wave” described in human macrophage and foetal microglia studies (467, 469). Based on these studies, it was expected that stem-cell derived cultures of microglia would produce MIP-1α in a wave-like manner.

Surprisingly, MIP-1α was minimally expressed by microglial monocultures, peaking at 4 hours in Vehicle and TNF incubations. Again, TNF induced the strongest secretion of MIP-1α, and by co-cultures. Most probably, the combination of microglia and astrocytes in co-cultures allows for paracrine and autocrine signalling loops to form, leading to increased secretion of MIP-1α. As a consequence, these data are potentially reflective of an in vivo environment. This is supported by the data in Figure 44, which demonstrates that secretion of MIP-1α is lower in microglial monocultures, and almost absent from midbrain neural monocultures. MIP-1α secretion is known to be differentially induced between microglia and astrocytes (470), and it is possible that the absence of either microglia or astrocytes from culture inhibits MIP-1α production in stem cell derived cultures. To determine the influence of communication between microglial and astrocyte signalling, future experiments could use a combination of MIP-1α antibodies and small molecule inhibitors to investigate why the response of co-cultures incubated with TNF is both delayed and exaggerated compared to incubation with LPS.

Probably the most interesting facet of the data presented is MIP-1α secretion in co-cultures is both delayed and exacerbated when incubated with TNF when compared to incubation LPS, or α-synuclein. This might be explained by the multiple regulatory pathways known to be involved in MIP-1α production. The receptor for MIP-1α, CCLR5, is a g-protein coupled receptor which allows binding of MIP-1α and other proteins, and is a critical point of the HIV infection pathway (471, 472). Although
substantial research has been performed to understand the role of this receptor in HIV, comparatively little has been undertaken to investigate the role of MIP-1α and its receptor specifically in PD. Therefore, the mechanisms governing secretion of MIP-1α in a midbrain model are somewhat uncertain. A simplified summary of some of the known pathways stimulating MIP-1α secretion in microglia has been illustrated in Figure 45 below.

In summary, MIP-1α secretion was significantly increased in co-cultures incubated with TNF compared to microglial monocultures and midbrain neuronal monocultures. No significant alterations to secretion were observed within culture types, with the exception of co-culture incubation with TNF. It is likely that the increased MIP-1α secretion response to TNF in co-cultures is due to communication between microglia and astrocytes. Future experiments should look to investigate the mechanism behind TNF incubation altering the secretion of MIP-1α compared to vehicle and LPS.

![Figure 45: Pathways contributing to MIP-1α secretion. Stimulation of TLR, TNFR and the CCLR5 receptors work through multiple signalling mechanisms to initiate transcription within the nucleus, by way of transcription factors NF-κB, CDP and C/EBP-β.](image)
4.8 - TNF secretion

Results

Comparison between cultures – pro-inflammatory stimuli mediated secretion of TNF

TNF secretion was universally low between culture types and was only significantly elevated in midbrain neuronal cultures incubated with LPS (100ng/mL). TNF secretion in neuronal cultures at 2-hours was significantly increased compared to microglial monoculture or co-culture conditions (14.35 ± 13.94pg/mL vs 0.26 ± 0.16pg/mL and 0.25 ± 0.25pg/mL respectively). TNF secretion was not altered between cultures incubated with vehicle (PBS + 0.01% DMSO) or α-synuclein (2.5 µM) at any point.

![Figure 46: Comparison of TNF secretion by culture type, and by incubation with inflammatory stimuli. Incubations are vehicle (PBS + 0.01% DMSO), LPS (100ng/mL) and α-synuclein (2.5µM). Two-way ANOVA with post-hoc Tukey’s test for significance was performed. # difference between microglia and neuron, ¥ Difference between co-culture and neurons N=3. *p<0.05](image)

Effects within cultures – time and inflammatory mediators

Rearranging the data used to plot Figure 46, arranged by culture type and time allowed investigation into these factors as possible influences on TNF secretion. With the exception of LPS incubation in neuronal monocultures, there were no significant alterations to TNF secretion as a consequence of incubation condition within any culture condition.
Discussion.

TNF levels in CSF can be correlated with Parkinson’s Disease progression (390), and was one of the earliest inflammatory factors demonstrated to be present in the brain tissue of patients with PD (122, 417, 440, 473). TNF levels in CSF, serum and cerebral tissues can be used to discriminate between healthy and PD patients (434, 474-476), and is also associated with increased anxiety and depressive symptoms in PD patients (477).

Different protocols for stem-cell derived microglia report widely varying levels of TNF secretion in response to LPS/IFN stimulation (341, 343, 346, 349, 351), which made predictions regarding the magnitude of TNF secretory response difficult. However, it was expected based previous protocols (341, 343, 346, 351), that secretion would be somewhere in the low hundreds of pg./mL.

Surprisingly, secretion of TNF was almost entirely absent across all conditions measured, with the exception of the 2-hour time point in midbrain monocultures incubated with LPS. Although statistically significant, the size of the error bars, and the universally low secretion in all other conditions, timepoints and culture systems, makes one doubt the validity of this significance. An exhaustive search through the literature has suggested two potential reasons for the lack of TNF secretion. Firstly, microglia require CX3CL1 for maintaining identity and a homeostatic profile (341, 368, 478-480), as such, as described in Chapter Two, all cultures include exogenous CX3CL1 at 100ng/mL. Exogenous CX3CL1 has been shown in murine microglia to suppress TNF signalling in a dose
dependent manner both in vitro and in vivo (481, 482). Potentially, the exogenous CX3CL1 included in culture media is inhibiting the microglial and astrocyte production of TNF as a response to LPS or α-synuclein here. Secondly, TNF secretion relies on Ca$^{2+}$ flux out of the cell (483, 484). Multiple studies have highlighted that TNF secretion is severely compromised when cells are incubated with dibutyryl cAMP, and is believed to be due to the accumulation of intracellular Ca$^{2+}$ as a consequence of adenylate cyclase signalling (483-486). As described in Chapter Two, cultures used for experiments used a 1:1 mixture of Midbrain to Microglial maintenance media. Midbrain media contains dibutyryl cyclic AMP, which could be increasing intracellular Ca$^{2+}$, and restricting TNF secretion. It is strongly suspected that the combined presence of both CX3CL1 and dibutyryl cyclic AMP is preventing microglia and astrocytes, from secreting TNF in response to pro-inflammatory mediators. This is supported by the preliminary secretory data discussed in Chapter Three, where microglial media only conditions saw concentrations of TNF of between 150-300pg/mL (see Appendix II). Future studies could confirm this by repeating these experiments in the presence of cAMP and CX3CL1 antagonists, such as cAMPs-Rp (487), and anti-CX3CL1 antibodies respectively.

In summary, secretion of TNF in response to inflammatory mediators was much lower than expected. This is believed to be a result of culture media containing both CX3CL1 and dibutyryl cyclic AMP, both of which are known to inhibit TNF production in a variety of models. Future studies could confirm this by using inhibitors of CX3CL1 and adenylate cyclase signalling to demonstrate improved TNF response.

4.9 - IFNγ secretion
Results
Comparison between cultures – pro-inflammatory stimuli mediated secretion of IFNγ
IFNγ was minimally expressed across all culture types and incubation conditions. Comparisons of culture types (microglial monoculture, midbrain neuronal monoculture, and co-culture of midbrain neurons with microglia), revealed no significant differences in IFNγ secretion under any conditions (Vehicle PBS +0.01% DMSO, LPS (100ng/mL), TNF (100ng/mL) or α-synuclein (2.5µM)).
After confirming culture type did not influence IFNγ secretion, the next step was to confirm whether incubation with inflammatory mediators or time altered IFNγ secretion within culture types. Using the data from Figure 46, arranged by culture type and time, revealed no significant alterations to secretion of IFNγ within culture types as a result of incubation with Vehicle (PBS +0.01% DMSO), LPS (100ng/mL), TNF (100ng/mL) or α-synuclein (2.5µM) (see Figure 49). IFNγ secretion was not affected by time.

Figure 48: Comparison of IFNγ secretion by culture type, and by incubation with inflammatory stimuli. Incubations are vehicle (PBS + 0.01% DMSO, LPS (100ng/mL), TNF (100ng/mL) and α-synuclein (2.5µM). Two-way ANOVA with post-hoc Tukey’s test for significance was performed. N=3

Effects within cultures – time and inflammatory mediators
**Discussion.**

IFNγ is typically believed to be secreted only by CD4⁺ T-cells and natural killer cells within the perivascular spaces (488-490) of the CNS. There is some evidence indicating IFNγ may also produced by neurons (491, 492) and/or microglia (194, 493) under specific circumstances, but this is still questioned, as other studies show IFNγ is not secreted by either neurons or microglia in murine slice cultures (494). To date, only two protocols for deriving microglia from stem cells (349, 352) have demonstrated secretion of IFNγ. Additionally, the role of IFNγ in the CNS is contested; some studies demonstrate IFNγ involvement in neuroprotection and neurogenesis (238, 495), while others show aberrant IFNγ signalling can contribute to the destruction of the nigrostriatal pathway and calcification of basal ganglia (496, 497). The many inconsistencies for the role of IFNγ during neuroinflammatory disease progression made IFNγ an intriguing target for investigation. It was expected, based on data from primary murine neurons (491, 492) and microglia (194) that IFN would be significantly secreted under inflammatory conditions.

Contrary to expectations, IFNγ secretion was not significantly altered between culture types, or by incubation conditions, and was minimal at all time points. The data presented here does not support the previous data for stem cell derived microglia (349, 352). However, the data does support previous work which describes very low concentrations of IFNγ in human brain tissue in both healthy control and PD patients (<3pg/mL (403) and approximately 14pg/mL respectively (475)). Possibly, as IFNγ secretion by microglia and macrophages has previously been demonstrated to occur only in response to combined IL-12 and IL-18 signalling (493, 498), it is possible that these cytokines are
absent from the cultures, and therefore, IFNγ secretion is minimal. To confirm this theory would require analysis of conditioned media by ELISA or cytometric bead array to determine whether IL-12 or IL-18 are present in supernatant. This could be further supported by experiments which include exogenous IL-12 and IL-18 to attempt to stimulate IFNγ production. Alternatively, if CD4⁺ lymphocytes in the perivascular spaces are the actual source for IFNγ within the CNS (497), as mentioned above, then the absence of CD4⁺ cells from these cultures will naturally mean IFNγ secretion is also absent. This could be investigated in future experiments by using conditioned media from stimulated CD4⁺ lymphocytes to stimulate cultures, or by performing a co-culture with these lymphocytes.

In summary, IFNγ secretion was not different between culture types or due to incubation conditions and was minimal overall. This supports previous human and murine studies and could be due to either a lack of IL-12 or IL-18 signalling or caused by the absence of CD4⁺ lymphocytes. Future studies should investigate these avenues using exogenous IL-12 and IL-18 incubation, or by co-culture with CD4⁺ Lymphocytes.

4.10 - VEGF Secretion

Results
Comparison between cultures – pro-inflammatory stimuli mediated secretion of VEGF
Initial investigations into VEGF secretion focused on determining whether there were differences between culture types. Co-cultures incubated with TNF (100ng/mL) secreted significantly more VEGF than microglial or midbrain monocultures at 48-hours (724.31 ± 313.71pg/mL vs 7.93 ± 7.93pg/mL and 78.72 ± 47.08pg/mL respectively, p<0.0001), see Figure 50. Differences in VEGF secretion between cultures when incubated with vehicle (PBS+ 0.01%), LPS (100ng/mL) and α-synuclein were not observed. Additionally, VEGF secretion was also significantly increased by co-cultures incubated with IFNγ (20ng/mL) at 48-hours when compared to midbrain monocultures (724.31 ± 313.71pg/mL vs 56.79 ± 35.45pg/mL) (see Appendix VII for details).
Effects within cultures – time and inflammatory mediators

Once differences in VEGF secretion between culture types were determined, the next step was to determine whether VEGF secretion was significantly altered within culture types due to incubation conditions or time. Using data from Figure 50 arranged by culture type and time, determined VEGF secretion within midbrain and microglial monocultures was unaffected by incubation conditions. However, incubation with TNF in co-cultures resulted in significantly increased VEGF secretion at 48-hours when compared to vehicle (see Figure 51) (724.31 ± 313.71 pg/mL vs. 290.15 ± 92.98 pg/mL). Additionally, VEGF secretion was also significantly altered over time in TNF, IFNγ and α-synuclein incubations when comparing 2-hours to 48-hours for each incubation.

Figure 50: Comparison of VEGF secretion by culture type, and by incubation with inflammatory stimuli. Incubations are vehicle (PBS + 0.01% DMSO), LPS (100ng/mL), TNF (100ng/mL) and α-synuclein (2.5μM). Two-way ANOVA with post-hoc Tukey’s test for significance was performed. N=3 ****p<0.0001 *difference between co-culture and microglia, ¥ Difference between co-culture and neurons
Figure 51: VEGF secretion by incubation (PBS+DMSO, LPS (100ng/mL) TNF (100ng/mL), IFNγ (20ng/mL) or α-synuclein (2.5µM)) graphed by culture type. Graphs show culture type, and secretion of VEGF during incubation with Vehicle (*Black), LPS (+Red), TNF (#Green), IFNγ (iPurple) or α-Synuclein (VBlue). Two-way ANOVA with post-hoc Tukey’s test for significance was performed. N=3 **p<0.005

Discussion.

Vascular Endothelial Growth Factor (VEGF) is a neuroprotective (499), angiogenic factor critical for vasculature development (500) and neurogenesis (501) within the CNS. During homeostatic conditions, VEGF is secreted by astrocytes (502) and neurons (503) to promote neurogenesis in the subventricular zone and the dentate gyrus (501, 504). However, patients with PD have significantly upregulated VEGF in the CSF (505) and the substantia nigra tissue (506), indicating a potential inflammatory role in the pathology of PD. Only one study using stem cell derived microglia to date has shown VEGF secretion in microglia, and secretion only occurred when microglia were co-cultured with iPSC derived cortical neurons in the presence of LPS/IFNγ (346). Based on previous work (346) it was expected that co-cultures would produce the greatest amount of VEGF.

Co-cultures generally secreted the most VEGF across all conditions, in line with expectations, but only to a significant degree when incubated with TNF, and not when incubated with LPS or α-synuclein. Additionally, TNF was the only inflammatory mediator to significantly increase VEGF secretion within co-culture conditions compared to vehicle incubation. These findings beg the questions: (a) Why is TNF the only cytokine to significantly increase VEGF secretion? (b) why is co-culture able to secrete significantly more VEGF compared to microglial and midbrain monocultures? (c) Why didn’t LPS initiate a significant response?
The most probable reason for TNF to significantly increase VEGF secretion is stimulation of the NF-κB transcription factor via Inhibitor of κB Kinase (IKK) (507, 508). TNF signalling through IKK or c-Jun N-terminal Kinase (JNK) pathways determines if cells survive or die in response to stimulation with inflammatory mediators (509). As NF-κB signalling is required for VEGF secretion by macrophages and mesenchymal stem cells (507, 508), it is reasonable to think TNF-induced-NF-κB signalling may be involved in modulation of VEGF secretion here. This theory is supported by previous work demonstrating dependence on TNF induced NF-κB signalling for VEGF signalling in murine TNF-knockout models (510); and by human studies demonstrating VEGF secretory regulation by NF-κB in glioblastoma (511). Also, TNF is also able to stimulate production of VEGF-promoting factors, such as IL-6 (512, 513), IL-8 (514, 515) and TNF itself (516, 517), allowing for the formation of autocrine and paracrine feedback loops in cultures (see Figure 52 for illustration).

Co-cultures were probably able to secrete the greatest amount of VEGF as due to cross-talk between microglia, astrocytes, and neurons (201, 494). These cell types are known to be in constant communication (201, 271, 309, 494, 518), and it is clearly observed here that co-cultures secrete significantly more VEGF compared to either midbrain or microglial monocultures. It is possible that as all three cell types have receptors for TNF (37, 376), paracrine signalling is amplified. Future studies could investigate whether direct contact between microglia and neurons/astrocytes is required for amplified VEGF signalling, by using TNF-conditioned astrocyte or microglial media to stimulate VEGF secretion.

Finally, LPS incubations may have been insufficient to initiate VEGF secretion in the absence of IFNγ. VEGF secretion in stem cell derived microglia has previously occurred only during incubation with a combination of LPS and IFNγ (346). This is further supported by research showing IFNγ acts to potentiate LPS mediated signalling through multiple pathways and in multiple cell types, as mentioned previously in this chapter (436, 519-521). Future studies could confirm the requirement for IFNγ during
LPS stimulation to significantly increase the levels of VEGF over time by including an IFNγ/LPS condition.

In summary, VEGF secretion was significantly increased in co-culture conditions compared to midbrain neurons and microglial monocultures when incubated with TNF. This is most likely due to NF-κB signalling and potentiation of signalling by the formation of paracrine and autocrine feedback loops. Future studies should investigate both the requirement for cell-cell contact for initiation of secretion, and the ability of IFNγ to potentiate the ability of LPS to stimulate VEGF secretion.

Figure 52: Simplified illustration of signalling pathways involved in VEGF secretion. (1) TNF binds to TNFR, and initiates signalling of IKK pathway (2). IKK stimulates NF-κB transcription of key genes (3). (4) Transcription of VEGF (508), IL-6 (522), IL-8 (430, 431) and TNF occurs in response to NF-κB, while VEGFR is transcribed due to SP1 (which is also initiated by TNF) (523). (5) Proteins are packaged for release into the extracellular space (6). Once in the extracellular space, (7) VEGF is able to bind to its receptor and trigger JAK-STAT (524), PI3K (525), ERK1/2 (526, 527) or other key molecules. Figure designed in BioRender.com
4.11 - CX3CL1 secretion

Results

Comparison between cultures – pro-inflammatory stimuli mediated secretion of CX3CL1

The final cytokine analysed in this exploratory panel was CX3CL1. Analysis revealed that incubation with either LPS (100ng/mL), TNF (100ng/mL) or α-synuclein (2.5μM), but not incubation with vehicle (PBS + 0.01% DMSO), significantly altered secretion of CX3CL1.

The concentration of CX3CL1 in microglial monocultures incubated with LPS was significantly reduced in microglial monocultures compared to neuronal monocultures at 8-hours (28608 ± 9074pg/mL vs 60636 ± 5718pg/mL, p=0.0282), 24-hours (649 ± 205pg/mL vs 46537 ± 4147pg/mL, p<0.0001) and 48-hours (202 ± 64pg/mL vs 50000 ± 3367pg/mL, p<0.0001), and compared to co-culture at 24-hours (649 ± 205pg/mL vs 44259 ± 2045pg/mL, p<0.0001) and 48-hours (202 ± 64pg/mL vs 40363 ± 2436pg/mL, p<0.0001) (see Figure 53).

The concentration of CX3CL1 in microglial monocultures when incubated with TNF was also significantly reduced compared to both neuronal monoculture and co-cultures at 48-hours (3207 ± 3207pg/mL vs 47146 ± 4610pg/mL and 41459 ± 2742pg/mL, p= 0.0015 and 0.0059 respectively).

Finally, concentration of CX3CL1 in microglial monocultures when incubated with α-Synuclein was also reduced when compared to neuronal monocultures and co-cultures at 24-hours (5794 ± 5418pg/mL vs 45377 ± 2881pg/mL and 42639 ± 1999pg/mL, p=0.0029 and 0.0125, respectively) and 48-hours (328 ± 3 pg./mL vs 44974 ± 2260pg/mL and 41759 ± 2088pg/mL, p=0.0147 and p=0.0264 respectively). There were no significant differences between midbrain monoculture or co-culture secretion of CX3CL1 at any point, under any incubation conditions.
Effects within cultures – time and inflammatory mediators

Once differences in CX3CL1 secretion between culture types were determined, the next step was to determine whether CX3CL1 secretion was significantly altered within culture types due to incubation conditions or time. Using data from Figure 53 but arranged by culture type and time, the concentration of CX3CL1 dropped significantly in microglial monocultures between 2- and 24-hours when incubated with Vehicle (PBS + 0.01% DMSO) (60587 ± 3286pg/ml vs 28279 ± 6767pg/mL, \( p<0.0001 \)) and LPS (100ng/mL) (32401 ± 10247pg/mL vs 649 ± 205pg/mL, \( p=0.0042 \)). The concentration of CX3CL1 also dropped significantly in microglial monocultures between 2- and 48-hours in Vehicle (60587 ± 3286pg/ml vs 18039 ± 7266pg/mL, \( p<0.0001 \)), LPS (32401 ± 10247pg/mL vs 202 ± 64pg/mL, \( p=0.0031 \)) and α-synuclein (2.5µM) (44295 ± 973pg/ml vs 328 ± 3pg/mL, \( p=0.0448 \)).
Figure 54 demonstrates differences in secretion due to time were not observed within midbrain monocultures or co-culture systems – and indicates CX3CL1 concentration was being sustained by neuronal production in these populations.

Additionally, Figure 54 shows LPS incubation in microglial monocultures significantly reduced CX3CL1 compared to Vehicle incubation at 24-hours (649 ± 205pg/mL vs 28279 ± 6764 pg./mL, p=0.0168), but was not significantly altered by incubation with other mediators of inflammation. Differences in CX3CL1 concentrations due to incubation conditions were not observed in midbrain monocultures or co-cultures.

Discussion

CX3CL1 is the only ligand for CX3CR1. Within the human CNS, CX3CL1 is primarily expressed by neurons (308) and to a lesser degree, by astrocytes (269). It is generally accepted that while microglia are incapable of producing CX3CL1 (159, 444, 528), the receptor for CX3CL1 is exclusively expressed by microglia in a healthy environment (269), but can be upregulated on astrocytes during inflammatory conditions (269). Increased CX3CL1 concentrations in the CSF of Parkinson’s Disease patients is positively correlated with symptom severity and disease progression (529). Additionally, both CX3CL1 and its receptor are upregulated in glioblastomas, allowing recruitment and infiltration of tumour associated microglia (332, 530). CX3CL1 signalling can also be protective; high levels of CX3CL1 in human plasma immediately following ischemic stroke has been associated with reduced
stroke severity and improved patient recovery (S31). Also, multiple rodent studies have demonstrated CX3CL1-CX3CR1 signalling is protective against loss of neurons in α-synuclein toxicity and MPTP models of PD through downregulation of inflammatory proteins such as IL-1β, IL-6 and TNF (275, 532-534). This dual protective and degenerative role for CX3CL1 is highly context dependent, and the subtle nuances are still not completely understood.

Here, CX3CL1 secretion was consistently highest in midbrain monocultures during incubation with inflammatory stimuli (see Figure 53), while CX3CL1 levels in microglial monocultures dropped significantly over time, which correlates with publications demonstrating CX3CL1 production by neurons but not by microglia (269, 535). CX3CL1 was present initially in microglial monocultures due to addition of exogenous CX3CL1 to ensure terminal differentiation of microglia, as per Abud et al. (341) and as described in Chapter Two. Knowing that microglia are unable to produce CX3CL1, the drop in CX3CL1 concentration in microglial monocultures over time is logical and is likely the result of either receptor-ligand binding between the exogenous CX3CL1 and microglial CX3CR1, or degradation of CX3CL1 by microglial metalloproteases. This could be confirmed in future studies through radioligand binding, as described previously in rodents (536), or by using metalloprotease inhibitors.

To date, most studies investigating stem cell derived microglia have investigated CX3CR1, but not CX3CL1, presence at an mRNA and protein level. However, one protocol for iPSC derived microglia has shown low levels of CX3CL1 produced when stimulated with LPS for 24 hours (348). This might be attributed to the slight differences in identity that are known to occur in derivation of microglia-like cells from iPSCs/hESCs when compared to human foetal/adult microglia using principal components analysis and gene ontology (267, 322, 341, 343, 345, 347, 349). The data presented here for stem cell derived microglial monocultures supports primary human data (269, 308), and is believed to be the first direct investigation into potential CX3CL1 secretion over time by stem cell derived microglia.

As seen in Figure 54, incubation with pro-inflammatory mediators (LPS 100ng/mL, TNF 100ng/mL IFNγ 20ng/mL or α-synuclein 2.5µM), does not significantly alter CX3CL1 concentration over
time in either midbrain monoculture or co-culture systems when compared to vehicle (PBS +0.01% DMSO). This is extremely interesting, as the CX3CL1-CX3CR1 signalling axis between microglia and neurons is critical for maintaining homeostasis within the CNS (for full reviews, see (159, 271, 444)), and dysregulation of this axis is known to affect neurogenesis (537) and to activate microglia in diabetic retinopathy, PD models and glioblastoma models (206, 330, 532). Here, the absence of CX3CL1 secretory changes may indicate that the inflammatory incubation conditions used were not strong enough to disrupt CX3CL1-CX3CR1 signalling in vitro. Alternatively, TGF-β1 signalling may be involved. Exogenous addition of TGF-β1 to rodent microglia induces CX3CR1 mRNA and protein in a time and concentration dependent manner (538); and as the cultures here contain TGF-β1 to maintain microglial identity (267, 322), it is possible that the exogenous TGF-β1 is somehow modulating the CX3CL1-CX3CR1 axis. This could be investigated in future studies by either temporarily inhibiting TGF-β1, or by removing it from culture, though this would run the risk of loss of microglial identity and function, as described by Butovsky et al. in 2014 (267).

Finally, LPS was the only inflammatory molecule to significantly alter the concentration of CX3CL1 in microglial monocultures compared to vehicle (see Figure 53). Microglia have previously demonstrated downregulation of CX3CR1 in response to LPS in mice models (539), while lung epithelial cells decrease CX3CL1 secretion in response to LPS (540). However, there have been no studies to determine whether microglia can actively degrade CX3CL1, and therefore, it is uncertain as to what is happening to the exogenous CX3CL1 in these microglial monocultures. It is possible that matrix metalloproteases are degrading the CX3CL1 (541), or it may be actively sequestered by endocytosis (274). If CX3CL1 were bound to CX3CR1 on microglia, this would not be detected in a cytometric bead array which measures only the supernatant. This unusual finding is a point future studies should focus on.

In summary, stem cell derived microglial monocultures do not produce CX3CL1, and this is observed here in the significant drop of CX3CL1 over time. Midbrain monocultures and co-cultures are
able to maintain CX3CL1 concentrations, and are not affected by incubation with TNF, LPS, IFNγ or α-Synuclein. Midbrain monocultures and co-cultures secrete significantly more CX3CL1 than microglial monocultures when incubated with LPS, TNF and α-synuclein incubation conditions. Microglial monocultures are unable to sustain CX3CL1 levels over time, and incubation with LPS significantly reduces the amount of exogenous CX3CL1 in microglial monocultures compared to vehicle.

4.12 – Chapter Summary

As stem cell derived microglia have not previously been co-cultured with midbrain neurons/astrocytes, it was difficult anticipate experimental outcomes. Additionally, the duration and magnitude of stem cell derived midbrain neuron/astrocyte secretions in response to inflammatory factors have not been well characterised previously. To overcome this, each cell type (microglia or midbrain neurons/astrocytes) was cultured individually, and co-cultured. In an effort to accurately represent the in vivo environment, where microglia occupy between 8-20% of CNS tissue by volume in humans (95), and replicating previous stem cell studies (341), microglia were seeded at a 10% density when co-cultured with midbrain neuron/astrocytes. Finally, to remove media composition as a confounding variable, all cultures were incubated in media composed of 1:1 microglia media to midbrain media for 3 days prior to incubation with inflammatory factors. It was predicted that the inflammatory proteins IL-1α, IL-1β, MIP-1α, TNF and IFNγ would be secreted in high concentrations, however, this was not observed. Instead, these cytokines were all minimally secreted, across all culture types. It is possible that the inflammatory incubation conditions used here were insufficient to stimulate IL-1α secretion, as this typically only occurs during Ca²⁺ flux or necroptosis. Similarly, although the levels of IL-1β and IFNγ were low, this potentially reflects the human in vivo tissue and CSF concentrations in patients (383, 390, 475). The unexpectedly low levels of MIP-1α production by microglial monocultures compared to co-cultures may have been due to a requirement for crosstalk between microglia/neurons/astrocytes for substantial secretion. Finally, the uniformly minimal expression of TNF across culture types appears to be likely due to suppression of signalling by the
inclusion of both dibutyrylcyclic AMP and CX3CL1 in culture media, both of which are known to inhibit TNF signalling (481-484).

Although microglia monocultures also expected to secrete high concentrations of IL-6, IL-8 and VEGF, the concentrations measured were moderate at best, when compared to the co-cultures of microglia and midbrain neurons/astrocytes, which secreted significantly greater amounts. This is believed to be due to the critical role of crosstalk between microglia/neurons/astrocytes, and clearly highlights the need to investigate CNS inflammation using a multi-cell-type system to more accurately model in vivo conditions. The anti-inflammatory proteins, IL-4 and IL-10, were expected to be secreted strongly by microglial monocultures, but this was not observed. It is possible that IL-4 signalling in the CNS is due to T-cell production in the subarachnoid space, in which case the data in this thesis supports previous work. The cause of the low concentration of IL-10 in response to inflammatory mediators is uncertain but could be due to impaired Wnt signalling from the inclusion of TGF-β1 and TGF-β3 in
culture media. Finally, CX3CL1 secretion was on par with expectations. As microglia are known to be incapable of secretion, it was expected that the level of CX3CL1 in spiked media would drop over time without recovery, which was observed. Instead, the midbrain cultures, and co-cultures of midbrain neurons/astrocytes with microglia were able to sustain CX3CL1 levels over the duration of the experiment.

When investigating the impact of incubation, LPS, TNF, IFNγ and α-synuclein were chosen. TNF, IFNγ and α-synuclein are known to be dysregulated during PD, while LPS is often used for stimulation of microglial secretion (327, 432, 433, 542). However, the data in this chapter indicate that for investigating PD using a stem cell derived model of both microglia and midbrain neurons/astrocytes, LPS may not be the best choice. LPS did not universally induce the strongest secretion or modulation of cytokines in this model system. Instead, TNF produced the most robust responses, especially when investigating IL-6, IL-8, MIP-1α, and VEGF. Future studies investigating PD inflammation on stem cell derived models should consider using TNF, instead of LPS.

In conclusion, this chapter represents the first detailed investigation into the secretory behaviour of stem-cell derived microglia in an *in vitro* model of the midbrain environment. Nine pro-inflammatory, and two anti-inflammatory proteins were investigated and compared in conditions known to stimulate microglial responses. Furthermore, it has clearly been shown that the secretory profiles of stem cell derived microglia and neurons/astrocytes are distinct. Finally, an amplified and exaggerated secretory profile occurs during co-culture of these microglia with stem-cell derived midbrain neurons/astrocytes, particularly when incubated with TNF.
Chapter Five

Introduction to chapter

The primary aims of this chapter were to (a) show microglia monoculture cytokine responses to inflammatory stimuli during culture in media containing 50% forebrain media; and (b) to compare microglia secretory responses between cultures in microglia media containing either 50% forebrain or 50% midbrain media. Evidence is emerging (as described in Chapter One) which indicates that microglia are a heterogeneous population within the CNS. Differences in microglial functional responses, density, transcription profile and morphology are not due to aging alone, but are altered by regional location and by inflammation associated with disease (252, 253, 263, 543-546). When translated into in vitro experiments, these differences could mean the functional and transcriptional differences observed between published protocols for microglial derivation are partially due to media composition. Each protocol for stem cell derived microglia utilises a unique media composition (547), which might contribute to the unique profiles of the microglia generated. Here, microglial monocultures were cultured in microglial media which contained either 50% forebrain or 50% midbrain media. Media was classified as either “forebrain” or “midbrain”, depending on whether it was used for stem cell derivation of floorplate (548) or forebrain (358) neurons.

The secondary aim of this chapter was to demonstrate microglial secretion in the context of a model of forebrain differentiation. As described in Chapter Four and in Appendix IV, stem cell derived microglia (SCDmicroglia) are often co-cultured together with stem cell derived forebrain (FB) neurons to assess microglial integration and behaviour in an environment which more accurately recapitulates the CNS. Derivation of FB cultures from iPSCs/hESCs is now well established in many laboratories around the world, due to their comparative ease of differentiation from iPSCs/hESCs, using dual-SMAD
inhibition protocols. More information on forebrain differentiation can be found in the excellent reviews by (19) and (549).

As in Chapter Four, the cytokines chosen for investigation were IL-1α, IL-1β, IL-6, IL-8, IL-10, MIP-1α, TNF, IFNγ, VEGF and CX3CL1 allowing for direct comparison between microglia monocultures containing either 50% forebrain or 50% midbrain media. In addition, these cytokines are dysregulated in both PD and AD in vivo (434, 441, 550, 551). IL-4 secretion was omitted from investigation due to shipping delays.

The results for this chapter have been divided into individual cytokines, with each set of results discussed briefly throughout the chapter. This chapter focused on alterations to secretion by microglial monoculture in forebrain media. The microglial monocultures in forebrain media were then compared against microglial monoculture in midbrain media to look for differences in secretion due to media composition. Due to restrictions in the number of forebrain neurons able to be generated, this chapter does not include forebrain neuron monocultures, or co-cultures of forebrain neurons with microglia. However, preliminary investigations into these conditions were performed, and this data can be found in Appendix VIII.

The methodology in this chapter is summarised as follows:

All forebrain differentiations described in Appendix VIII were performed using H9-WT cells until Day 40, where they were either used in monoculture, or in co-culture with 10% microglia. All microglial differentiations were performed using H9-CX3CR1. All cultures were incubated in the presence of Vehicle (PBS +0.01% DMSO), LPS (100ng/mL), TNF (100ng/mL), IFNγ (20ng/mL) or Amyloid-β (60nM) as described in Chapter Two. All analysis performed within this chapter was Two-way ANOVA with post-hoc Tukey’s test, to ameliorate the risk of false positives. Data for specific timepoints (2-, 4-, 8-, 24- and 48-hours) was pulled from ANOVA output using VSIG-Lookup tables in Excel (for Two-way ANOVA with Post-hoc Tukey’s test data, please see Electronic Appendix IV).
same data was used for comparison between microglial monocultures (media composition) as well as within microglial monocultures (Time and/or inflammatory mediator incubation). For the sake of simplicity, although microglia media composition was comprised of either 1:1 forebrain media to microglial media or 1:1 midbrain media to microglial media, these conditions will hereafter be referred to as forebrain (FB) or midbrain (MB) media. Detailed methodology can be found in Chapter Two. When comparing cytokine secretion between FB and MB media, only Vehicle, LPS and TNF incubation conditions were used for analysis. IFNγ was not included because microglia in MB media were not incubated with IFNγ due to limited cell numbers. Amyloid-β was also excluded from this comparative analysis, because microglia in MB media were incubated with α-synuclein instead of amyloid-β, in attempt to mimic the inflammatory circumstances of Parkinson’s Disease.

The results for each cytokine will now be described individually, with a small discussion at the end of each section. A chapter summary of the results will follow at the end of the chapter.

5.1: IL-1α Secretion

Results

Comparison of inflammatory stimuli within forebrain media monocultures.

The first stage of investigation was to determine whether IL-1α secretion in microglial monocultures containing forebrain media was significantly altered by incubation with Vehicle (PBS + 0.01%DMSO), LPS (100ng/mL), TNF (100ng/mL), IFNγ (20ng/mL) or Amyloid-β (60nM). Secretion of IL-1α was very low across all incubation types (see Figure 56), and analysis demonstrated incubation with pro-inflammatory mediators did not significantly alter IL-1α secretion compared to Vehicle at any point. Additionally, IL-1α secretion was not significantly altered over time during incubation with inflammatory mediators.
Comparison of IL-1α secretion between microglial monocultures in forebrain and midbrain media

Once incubation with inflammatory mediators had been analysed, the next step was to determine whether microglia cultured in FB or MB media responded differently to the same stimuli. Using the same data from Figure 56, arranged by culture type and time, revealed that while secretion was low in both conditions, microglial monocultures containing midbrain media (MB) secreted significantly more IL-1α compared to cultures containing forebrain (FB) media at 48-hours when incubated with either vehicle (PBS + 0.01% DMSO) (2.44 ± 0.95 pg./mL vs 0.18 ± 0.09 pg/mL, p=0.0185) or in LPS (100ng/mL) (3.03 ± 0.70 vs 0.33 ± 0.15 pg/mL, p=0.002) (see Figure 57). There was no difference in IL-1α secretion between microglia cultured in MB or FB media incubated with TNF.
Discussion

As discussed in Chapter Four, IL-1α is a proinflammatory cytokine known to be produced by microglia, and typically released by cells during calcium flux or cell death (385, 386) The results from Chapter Four, where IL-1α secretion was low due to insufficient cell death/lack of Ca\(^{2+}\), helped to form the expectation that IL-1α secretion would be low in these microglial monocultures too. IL-1α mRNA has been demonstrated in murine microglia to be upregulated in response to amyloid-β (552), and has also been shown in human hippocampus to be increased in patients with Alzheimer’s disease (553).

When investigating secretion of IL-1α by microglial monocultures in FB media, in line with expectations, there were no significant alterations due to time or incubation conditions. It is likely that incubation with inflammatory mediators (LPS (100ng/mL), TNF (100ng/mL), IFNγ (20ng/mL), and Amyloid-β (60nM)) was again insufficient to instigate necroptosis or Ca\(^{2+}\) flux with microglia cultured in FB media, as in MB media. This is also probably likely to be affected by the choice of material for analysis. Here, supernatant of cultures was used, as in Chapter Four. Recent studies have shown that supernatant from cultures typically yields much lower IL-1α (383, 386) concentrations compared to lysed cells (387), which is supported by the data in Chapter Four. The lack of IL-1α secretion by microglia monocultures in FB media supports the previous work in this thesis and is in line with previous publications.
When comparing microglia monocultures in FB and MB media, it was interesting to note IL-1α release was modestly, but significantly, increased at 48-hours in MB media cultures. This occurred during incubation with either vehicle or LPS, but not during incubation with TNF, when compared to FB media cultures. A couple of theories may explain these results. One, FB media might contain a component which inhibits IL-1α secretion, or MB media might contain a component which promotes IL-1α secretion. As started in Appendix IX, FB media contains D-serine, but does not contain GDNF, DAPT or TGF-β3, which are included in MB media. Though none of these molecules have been indicated to promote secretion of IL-1α, it is possible that one (or all) of these molecules are influencing IL-1α secretion but demonstrating this would require further experiments. Two, it is possible that the inherent variability of stem cell cultures may influence significance, especially since n=3. Although an n of three is generally considered in stem cell biology to be the threshold at which data can be determined to be significant, this is still a small sample size, meaning the experiment may be underpowered and may allow false positives to occur (554).

In summary, microglia in FB media did not secrete IL-1α and was not significantly altered over time or by incubation with inflammatory mediators. Comparison between microglia incubated with MB and FB media indicated a small but significant increase in secretion of IL-1α between microglial monocultures during culture with Vehicle and LPS, but not during culture with TNF. This difference in secretion of IL-1α is probably more attributable to the small scale of secretion, and less to the FB/MB media composition.

5.2: IL-1β Secretion

Results
Comparison of inflammatory stimuli within forebrain media monocultures.
The first stage of investigation was to determine whether IL-1β secretion in microglial monocultures in FB media was significantly altered by incubation with Vehicle (PBS + 0.01%DMSO), LPS (100ng/mL), TNF (100ng/mL), IFNγ (20ng/mL) or Amyloid-β (60nM). IL-1β secretion was low
(<10pg/mL) across all conditions, and analysis revealed that secretion was not significantly altered over time or between incubation conditions.

![Graph showing IL-1β secretion over time](image)

**Figure 58:** IL-1β secretion by microglial monocultures, comparisons between Vehicle (PBS + 0.01% DMSO), LPS (100ng/mL), TNF (100ng/mL), IFNγ (20ng/mL) and Amyloid-β monomers (60nM). Two-way ANOVA with post-hoc Tukey’s Test performed. N=5.

**Comparison of IL-1β secretion between microglial monocultures in forebrain and midbrain media**

Once incubation with inflammatory mediators had been analysed, the next step was to determine whether secretion of IL-1β was different between microglia cultured in FB or MB media. Using the same data from Figure 58, arranged by culture type and time, revealed that while secretion was generally low for the first 24-hours in both conditions, secretion of IL-1β was significantly increased during incubation with Vehicle (PBS + 0.01% DMSO) by MB microglial monocultures compared to FB monocultures at 48-hours (13.3 ± 9.47pg/mL vs 0.15 ± 0.07pg/mL, p=0.0011) (see Figure 59). Secretion of IL-1β was not significantly altered between cultures incubated with LPS (100ng/mL) or TNF (100ng/ml).
**Discussion**

IL-1β is a proinflammatory cytokine (described in *Chapter Four*), which is elevated in the serum (142, 555) and CSF (556) of patients with Alzheimer’s Disease (AD). It was expected, based on the work in *Chapter Four*, that secretion would be generally low over time, and in all incubation conditions.

It was also expected, based on the results from *Chapter 5.1* (IL-1α secretion), that there would be only minimal differences to IL-1β secretion between microglial monocultures in either FB or MB media.

As expected, IL-1β production by microglial monocultures in FB media was low at all time points and was not significantly altered by incubation with inflammatory mediators. As in *Chapter Four*, this low level IL-1β secretion might be reflective of the functions described in primary human microglia (383).

When comparing IL-1β secretion between microglial monocultures in MB and FB media, IL-1β secretion in MB microglia was significantly increased during incubation with vehicle (PBS + 0.01% DMSO) at 48-hours. Significant differences were not observed between microglial monocultures during incubation with LPS or TNF. It is possible that this difference is an artefact – MB media monocultures appear to have much larger error bars at 48-hours, which are not present in the FB microglial cultures, indicating a large degree of variability in IL-1β secretion between n’s. Increasing the number of n’s for microglia cultured in MB media could assist in determining whether IL-1β secretion is truly this variable during culture with MB media.
Finally, it is important to highlight the low levels of IL-1β secretion microglial monocultures in FB and MB media. When comparing FB and MB media, several reagents are common to both formulations, one of which is dibutyrylcyclic AMP (dbcAMP). DBCAMP is known to significantly reduce microglial branching in rats (557), and may explain why microglia co-cultured with midbrain and forebrain neurons were rounded with fewer processes (described in Chapter Three). More importantly, dbcAMP is known to inhibit secretion of IL-1β (558) in rat microglia, in a concentration dependent manner. It is possible that the inclusion of dbcAMP in FB and MB media in Chapters Four and Five, may inhibit microglial function, and results in reduced IL-1β secretion. The preliminary CBA data in Appendix VI, indicates that microglia cultured in 100% microglia media (i.e. do not contain dbcAMP) appears to result in more pronounced IL-1β secretion. This needs further investigation, preferably comparing current results with more microglial monocultures incubated in the absence of dbcAMP.

In conclusion, IL-1β was minimally secreted across all timepoints and in all incubation conditions within FB microglial monocultures. Vehicle incubation resulted in increased secretion of IL-1β by MB microglia but was not significantly altered by LPS or TNF. The inclusion of dbcAMP in FB and MB media is likely to be contributing to the low levels of IL-1β and should be investigated further.

5.3: IL-6 Secretion

Results

Comparison of inflammatory stimuli within forebrain media monocultures. The first stage of investigation was to determine whether IL-6 secretion in microglial monocultures in FB media was significantly altered by incubation with Vehicle (PBS + 0.01%DMSO), LPS (100ng/mL), TNF (100ng/mL), IFNγ (20ng/mL) or Amyloid-β (60nM). IL-6 production was high (>100pg/mL), and secretion significantly increased over time in LPS incubated cultures (see Figure 60) at 24-hours (164.5 ± 36.5 pg./mL, p<0.0001) and 48-hours (163.0 ± 29.0pg/mL, p<0.0001) compared to 2-hours (3.8 ± 0.7pg/mL). Additionally, analysis indicates that incubation with LPS compared to vehicle significantly increased the amount of IL-6 secretion at 24-hours (164.5 ± 36.5 pg./mL vs 0.4 ± 0.2pg/mL, p<0.0001).
and 48-hours (163.0 ± 29.0pg/mL vs 0.40pg/mL, p<0.0001). Secretion of IL-6 was otherwise not significantly altered in FB microglial monocultures over time or by incubation conditions.

**Figure 60:** IL-6 secretion by microglial monocultures, comparisons between Vehicle (PBS + 0.01% DMSO), LPS (100ng/mL), TNF (100ng/mL), IFNγ (20ng/mL) and Amyloid-β monomers (60nM). Two-way ANOVA with post-hoc Tukey’s Test performed. N=5. **** p<0.0001 between 2-hours and indicated timepoint. #### p<0.0001 between LPS and Vehicle at indicated timepoint.

**Comparison of IL-6 secretion between microglial monocultures in forebrain and midbrain media**

Once incubation with inflammatory mediators had been analysed, the next step was to determine whether secretion of IL-6 was different between microglia cultured in FB or MB media. Secretion of IL-6 by microglia in FB and MB media was minimal during incubation with either Vehicle or TNF. However, using the same data from Figure 60 arranged by culture type and time showed incubation with LPS (100ng/mL) caused microglial monocultures in FB media to secrete significantly more of IL-6 compared to microglia in MB media at 24-hours (164.5 ± 36.5 pg./mL vs 0.00pg/mL, p<0.0001) and 48-hours (163.0 ± 29.0pg/mL vs 0.00pg/mL, p<0.0001) (see Figure 61).
**Discussion**

As described in Chapter Four, IL-6 is a pleiotropic cytokine with a complex role in the CNS. IL-6 plasma concentration in patients with AD is significantly upregulated compared to control patients (559, 560) and is associated with increased neuroinflammation. However, the level of expression of IL-6 at both the protein and mRNA levels within the CNS is more complex. Morimoto and colleagues demonstrated in a study of 100 patients that IL-6 was not differentially expressed between control and AD patients within the temporal cortex at an mRNA level (141). Similarly, microarray data from human primary microglia treated with Amyloid-β also show no difference in IL-6 gene expression between control and AD patients (561). On the other hand, multiple studies have demonstrated IL-6 to be significantly increased at a protein level in the CSF and plasma of patients with AD and patients experiencing delirium (561-563). It appears as though determining the significance of IL-6 in relation to AD might be influenced by the compartment used for analysis (serum vs plasma vs CSF vs CNS tissues), and that there is a discrepancy between results for mRNA and protein analysis. These discrepancies in the literature made it difficult to anticipate the microglial secretory response in monocultures containing FB media.

IL-6 secretion has been demonstrated by stem cell derived microglia (341, 346, 349), in response to either LPS, LPS/IFNγ, or LPS/IL-1β. Reassuringly, FB media microglia did secrete substantial levels of IL-6, specifically in response to LPS (see Figure 60). This LPS response supports previous work, although secretion of IL-6 is not as pronounced as observed elsewhere (341, 346). After 24 hours...
exposure to 100ng/mL LPS, Abud et al. demonstrated significant IL-6 secretion by microglial monocultures. However, Abud et al. used only microglia media, whereas these experiments have used microglia media in conjunction with either FB or MB media. Possibly, a reagent contained in either FB or MB media is blunting the effect of LPS incubation on IL-6 secretion in these experiments.

Nevertheless, the lack of IL-6 secretion in response to IFNγ, TNF and Amyloid-β was somewhat unexpected. Both primary and immortalised human microglia have been shown to upregulate IL-6 in response to Amyloid-β (561, 564). Conversely, studies have also demonstrated that murine microglial IL-6 secretion in response to Amyloid-β incubation is dependent on the structural form of Amyloid-β (565). Possibly, the structure of Amyloid-β used in these experiments was not appropriate for stimulation of IL-6 secretion by stem cell derived microglia in FB media. Primary and immortalised human microglia have also shown increased IL-6 secretion in response to incubation with IFNγ (566). However, another study indicated that IFNγ incubation alone was unable to stimulate secretion of IL-6 (564). Possibly, the absence of a co-stimulatory factor (such as TNF or LPS) during IFNγ incubation in the experiments presented here may have prevented microglia from secreting large amounts of IL-6 (349, 566, 567). Finally, TNF has also previously been shown to stimulate IL-6 secretion in astrocytes (418, 419, 421), but has not been demonstrated previously to stimulate IL-6 secretion by microglia (420). Likely, the absence of astrocytes in these experiments likely contributed to the lack of IL-6 secretion during incubation with TNF.

Finally, the difference in IL-6 secretion between microglial monocultures must also be discussed. Microglial cultures incubated with LPS secreted significantly greater amounts of IL-6 in FB media cultures compared to cultures with MB media. The observation that IL-6 secretion is universally low across all incubation types by microglia cultured with MB media, while microglial incubation with LPS in FB media results in significant IL-6 production, strongly indicates that media composition is affecting cytokine release. When comparing the media composition (See Appendix IX), FB media does not contain GDNF or DAPT, both of which are present in MB media. GDNF is a protective neurotrophic
factor, which has been demonstrated to potently reduce IL-6 secretion at a protein level, and to reduce IL-6 mRNA expression in murine microglia (567-570). Additionally, DAPT, a direct inhibitor of γ-secretase signalling in the NOTCH pathway, has also been shown to decrease the amount of IL-6 secreted by murine macrophages (571), primary microglia (572, 573) and BV-2 microglia (573), specifically by modulating NF-κB and p38/MAPK. It is likely that the combined presence of GDNF and DAPT within microglial MB media cultures is able to effectively inhibit secretion of IL-6 during incubation with inflammatory mediators. Microglial FB cultures, in the absence of GDNF and DAPT, do not have to overcome these inhibitory signals, and are therefore able to produce IL-6 in response to LPS.

In summary, microglial FB media cultures were able to secrete IL-6 in response to incubation with LPS. This response was significantly different to that observed in microglial monocultures with MB media and is likely a result of media composition. Most probably, the presence of GDNF and DAPT in MB media cultures is actively suppressing IL-6 production, while the absence of these two molecules in FB media microglia cultures allows for IL-6 secretion.

5.4: IL-8 Secretion

Results

Comparison of inflammatory stimuli within forebrain media monocultures.
The first stage of investigation was to determine whether IL-8 secretion in microglial monocultures in FB media was significantly altered by incubation with Vehicle (PBS + 0.01%DMSO), LPS (100ng/mL), TNF (100ng/mL), IFNγ (20ng/mL) or Amyloid-β (60nM). IL-8 secretion was extremely pronounced (>1000pg/ml), and analysis revealed both LPS (100ng/mL) and TNF (100ng/mL) significantly increased IL-8 secretion over time and when compared to Vehicle (see Figure 62).

IL-8 secretion during incubation with LPS was increased compared to 2-hours (218.1 ± 38.5 pg./mL) at 24-hours (8110 ± 1806 pg./mL, p<0.0001) and 48-hours (9163 ± 2069 pg./mL, p<0.0001). TNF incubation stimulated IL-8 in a similar manner, and compared to 2-hours (273.6 ± 60.9pg/mL), secretion was significantly increased at 24-hours (9199 ± 1886pg/mL, p<0.0001) and 48-hours (9095
± 2106 pg./mL, p<0.0001). Incubation with Vehicle, IFNy or Amyloid-β did not significantly alter IL-8 secretion over time. Finally, LPS incubation increased IL-8 secretion at 24-hours (8110 ± 1806 pg./mL vs 181 ± 73 pg/mL, p<0.0001) and 48-hours (9163 ± 2069 pg./mL vs 159 ± 32 pg/mL, p<0.0001) hours compared to vehicle (PBS + 0.01% DMSO). TNF incubation also increased IL-8 secretion at 24-hours (9199 ± 1886 pg/mL vs 181 ± 73 pg/mL, p<0.0001) and 48-hours (9095 ± 2106 pg./mL vs 159 ± 32 pg/mL). Incubation with IFNy (20ng/mL) or Amyloid-β (60µM) did not significantly alter IL-8 secretion compared to Vehicle at any point.

Comparison of IL-8 secretion between microglial monocultures in forebrain and midbrain media

Once incubation with inflammatory mediators had been analysed, the next step was to determine whether secretion of IL-8 was different between microglia cultured in FB or MB media. Using the same data from Figure 62, arranged by culture type and time, showed that IL-8 secretion was significantly altered by microglial monocultures in FB media during incubation with both LPS (100ng/mL) and TNF (100ng/mL) (see Figure 63).
LPS incubation caused FB microglia to secrete significantly more IL-8 than MB microglia at 24-hours (8110 ± 1806 pg./mL vs 63 pg./mL, p<0.0001) and 48-hours (9163 ± 2069 pg./mL vs 89 ± 29 pg./mL, p<0.0001). FB microglia incubated with TNF also secreted significantly more IL-8 than MB cultures at 24-hours (9199 ± 1886 pg./mL vs 2957 ± 2310 pg./mL, p=0.0228) and 48-hours (9095 ± 2106 pg./mL vs 330 ± 330 pg./mL, p=0.0002). IL-8 secretion by microglial monocultures in FB or MB media was not significantly altered during incubation with vehicle (PBS + 0.01% DMSO).

![Graph showing IL-8 secretion by microglial monocultures in either mixed forebrain (Red) or mixed midbrain (Blue) media.](image)

**Discussion**

IL-8, as described in Chapter Four, is a potent and proinflammatory cytokine produced by both astrocytes and microglia (421, 428, 430, 431). Primary microglia isolated from AD patients have shown significantly increased IL-8 expression during incubation with Amyloid-β at both a protein and mRNA level (561). Additionally, systematic meta-analysis has indicated peripheral IL-8 concentrations can be used for discrimination between people who are healthy, and people who have AD (574). Additionally, high concentrations of IL-8 in patient CSF has been associated with increased phosphorylated-tau, indicating more severe disease progression (575).

Stem cell derived microglial monocultures are capable of producing IL-8 in response to LPS and/or IFNγ (341, 345, 346). In addition, Chapter Four demonstrated microglial monocultures in MB media secreted significant levels of IL-8 in response TNF. It was therefore expected that microglial monocultures in FB cultures would be capable of secreting IL-8 in response to both TNF and LPS. As expected, IL-8 secretion was significantly increased by incubation with either LPS or TNF (see Figure
compared to vehicle. This is exciting, as it fits with published protocols detailing IL-8 production in response to LPS, (341, 345, 346) while simultaneously supporting early primary human data (431) and confirming the capacity of stem cell derived microglia to secrete IL-8 in response to TNF under multiple media conditions. To date, stem cell derived microglia have not previously demonstrated IL-8 secretion in response to TNF, making this a novel finding. It is interesting that the magnitude of IL-8 secretion in response to TNF is on par with the response to LPS, and potentially indicates that these are equally potent stimulators of microglia during in vitro cultures with FB media.

It was also interesting to observe that during incubation with LPS or TNF, there were clear differences in IL-8 secretion between microglia cultured in either MB or FB media. Microglia cultured with FB media clearly secrete more IL-8 than MB media during incubation with both LPS and TNF, again, hinting that media composition is affecting microglial secretion. As discussed previously in this chapter, MB media contains GDNF, DAPT, and TGF-β3, whereas FB media does not. There is minimal data available for TGF-β3 and its effect on microglial (or macrophage) production of inflammatory cytokines – most studies investigating microglia and TGF have focused on TGF-β1 or -β2. However, inclusion of GDNF in murine primary cultures is protective for midbrain dopaminergic neurons by regulating microglial activation (576). This is supported by other studies (567, 577) which demonstrated GDNF actively suppressed the P38/MAPK and NF-κB pathways, resulting in reduced inflammatory cytokine production. Similarly, NF-κB is also promoted in BV-2 microglia by NOTCH signalling (573), and inhibition of NOTCH using DAPT has been shown to inhibit NF-κB activation. P38/MAPK and NF-κB transcription factors all contribute to controlling IL-8 production (430, 438, 578). Therefore, it is not unreasonable to believe that the presence of GDNF and DAPT in MB media are minimising the IL-8 secretory response. This could easily be tested in future experiments by simply removing GDNF and/or DAPT from MB media microglial cultures and observing whether IL-8 secretion is increased.
In summary, IL-8 secretion was significantly upregulated in FB cultures in response to both LPS and TNF, to a startlingly similar degree. The use of TNF to successfully stimulate IL-8 production is a novel finding and supports early primary human data. Additionally, there are substantial differences in IL-8 secretion between microglia cultured in MB or FB media – where MB media cultured microglia produce much less IL-8 over time. This is suggestive that media composition is affecting the ability of microglial monocultures to respond to typical stimuli that have previously demonstrated IL-8 production. It is believed that the most likely components of the MB media which might be contributing to this reduced IL-8 response are GDNF and DAPT, which signal through known IL-8 transcriptional pathways. Investigation of the role of GDNF and DAPT in regulation of stem-cell derived microglia should be performed in future studies.

5.5: IL-10 Secretion

Results
Comparison of inflammatory stimuli within forebrain media monocultures.
The first stage of investigation was to determine whether IL-10 secretion in microglial monocultures in FB media was significantly altered by incubation with Vehicle (PBS + 0.01%DMSO), LPS (100ng/mL), TNF (100ng/mL), IFNγ (20ng/mL) or Amyloid-β (60nM). IL-10 secretion was high (>100pg/mL, and similar to IL-6, LPS significantly altered secretion of IL-10 over time (see Figure 64). During incubation with LPS, IL-10 secretion was significantly increased compared to 2-hours (15.49 ± 3.06pg/mL) at 24-hours (378.5 ± 90.4pg/mL, p<0.0001) and 48-hours (302.2 ± 119.1pg/mL, p<0.0001). Secretion was not significantly altered over time during incubation with Vehicle, TNF, IFNγ or Amyloid-β. Additionally, analysis also revealed that incubation with LPS significantly increased IL-10 secretion compared to incubation with Vehicle at 24-hours (378.5 ± 90.4pg/mL vs 13.37 ± 5.22pg/mL, p<0.0001) and at 48-hours (302.2 ± 119.1pg/mL vs 9.42pg/mL, p<0.0001). Incubation with TNF, IFNγ or Amyloid-β did not significantly alter IL-10 secretion compared to Vehicle at any point.
Comparison of IL-10 secretion between microglial monocultures in forebrain and midbrain media

Once incubation with inflammatory mediators had been analysed, the next step was to determine whether secretion of IL-10 was different between microglia cultured in FB or MB media. Using the same data from Figure 64 arranged by culture type and time, revealed microglial monocultures in FB media secreted significantly more IL-10 than MB cultures during incubation with LPS (100ng/mL), but not during incubation with vehicle (PBS + 0.01% DMSO) or TNF (100ng/mL) (see Figure 65). LPS incubated FB cultures secreted more IL-10 than MB cultures at 24-hours (378.5 ± 90.4pg/mL vs 0.00pg/mL, p<0.0001) and 48-hours (302.2 ± 119.1pg/mL vs 2.30 ± 0.76pg/mL, p<0.0001).
Discussion

IL-10 is a usually considered to be a neuroprotective and anti-inflammatory cytokine (455), and as described in Chapter Four, it is secreted by both microglia and astrocytes (452, 453). However, whether IL-10 can be considered “beneficial” or “neuroprotective” in the context of AD appears increasingly doubtful, as studies have demonstrated detrimental associations between IL-10 and AD in humans (141, 579, 580) and mice (581). Adenoviral-mediated IL-10 expression in murine systems is associated with increased Amyloid-β plaque burden (581), whereas deficiency of IL-10 can allow increased phagocytosis of Amyloid-β (579). In humans, single base polymorphisms in the promotor region of IL-10 have been identified as a significant genetic risk factor for development of AD (580), and overexpression of IL-10 within the CNS of patients with AD seems to indicate a damaging role. IL-10 expression within the cortex is upregulated (141) and increased signalling seems to occur in the hippocampus (579) of patients with AD. The peripheral expression of IL-10 in patients with AD is more contentious. IL-10 serum concentrations are reduced by up to four-fold compared to healthy controls in one study (142), while another suggests IL-10 serum concentrations correlate with both cognitive decline and ventricle volume deficits (582). The overall picture appears to indicate that IL-10 production is dysregulated within the CNS of patients with AD compared to healthy controls.

IL-10 secretion can be induced in murine microglia through incubation with LPS, Adenosine and TNF (455), and stem cell derived microglia have also been shown to produce IL-10 in response to...
LPS incubation (341, 346). However, data from Chapter Four showed minimal IL-10 secretion across all culture types and incubation conditions. It was hoped that microglial cultures in FB media would display a different IL-10 secretion profile compared to microglial monocultures in MB media, as the media composition is different between MB and FB media. It was expected that IL-10 secretion would likely be strongest in LPS stimulated cultures, as this has been used in published stem cell protocols (352).

IL-10 was substantially elevated in microglial monocultures grown in FB media, particularly in response to LPS, and to a much greater degree than previously published monoculture data ((341, 346). The analysis of IL-10 secretion performed by Abud et al. is specifically relevant to these experiments, as the protocol detailed by them was used in this thesis for differentiation of microglia. Abud et al. observed IL-10 secretion using microglial monocultures incubated in 100% microglial media (341), whereas in this chapter, microglial monocultures were grown in 50% forebrain neuron media (FB) to 50% microglial media. This strongly indicates that secretion of IL-10 by stem cell derived microglia is context dependent – that is, the media in which microglia are grown and incubated, affects their ability to respond to exogenous stimuli. This is supported by the statistical comparison between microglia grown using either FB or MB media, which demonstrated microglia in FB media secreted significantly more IL-10 in response to LPS compared to microglia in MB media. In Chapter Four, it was postulated that the minimal secretion of IL-10 by microglia in MB media could be due to not including T-cells in culture, as earlier human primary microglia studies have showed negligible IL-10 secretion in the absence of T-cells (454). However, as clearly demonstrated, stem cell derived microglia are capable of secreting IL-10 when cultured in either 100% microglial media (see Appendix VI) or in 50% FB media (see Figure 64 and Figure 65). This indicates in vitro secretion of IL-10 by stem cell derived microglia is more likely to be affected by media composition, than by the absence of T-cells. If IL-10 secretion relied on the presence of T-cells, then IL-10 secretion would not be observed during culture in media containing 50% FB media as is presented here, nor would secretion have been observed in other stem cell microglia publications (341, 346, 352). This data supports previous works (341, 346, 352), while
also highlighting that microglia responses are context-dependent. The advent of multiple stem cell derivation of microglia methods, and a lack of uniformity between them, means it is imperative for researchers to be aware of the inherent limitations within their method of choice. It is important that researchers consider these limitations during interpretation of experiment results.

It is becoming increasingly obvious that microglia within the brain are a heterogeneous population, capable of a wide variety of responses (220, 322, 407, 547). In this thesis, microglia also appear to be affected by media composition. As discussed earlier within this chapter, FB media and MB media are remarkably similar, save for four key reagents. It is likely that the inclusion of one or more of these reagents is contributing to the lack of IL-10 response in MB-media microglial cultures, and each will be briefly considered here. The first reagent, D-serine, is contained only in FB-media. Although dysregulated D-serine has been linked to increased pathophysiology in AD patients (583), D-serine itself has not been linked to modulation of IL-10 expression. The next three reagents, TGF-β3, DAPT, and GDNF are contained only in MB-media. There is minimal information regarding TGF-β3 specific modulation of IL-10, so no real hypotheses as to TGF-β3 regulation of IL-10 can be made. GDNF has been directly linked to prevention of LPS neurotoxicity in murine midbrain dopaminergic neurons, potentially through modulation of microglial activation (576), although whether this resulted in reduced IL-10 production was not investigated. The final reagent in MB media is DAPT – a γ-secretase and NOTCH inhibitor. Inhibition of NOTCH signalling has shown to reduce IL-10 production in macrophages (584, 585), BV2 (586) and N9 (587) immortalised microglia, as well as in primary murine (377, 588) microglia. This suggests NOTCH-mediated production of IL-10 is conserved across microglial model types. This is further supported by reviews which describe dysregulation of NOTCH signalling in AD (589). Based on this information, it seems likely that the reason for the discrepancy in IL-10 secretion between microglial monocultures in FB and MB media is due to the inclusion of DAPT, although the involvement of GDNF, TGF-β3 and D-serine cannot be ruled out without further investigation. MB media microglia show significantly reduced IL-10 secretion, mirroring the observations in previous primary murine and immortalised microglial cell line studies. As FB media
does not contain DAPT, IL-10 secretion by microglia is allowed to occur in response to incubation with
LPS, because NOTCH signalling is not blocked by inhibition of γ-secretase. This could be confirmed in
future studies, simply by including DAPT in FB-media during microglia culture. If secretion of IL-10 in
drops significantly in FB media containing DAPT, compared to FB media without DAPT, then this would
indicate NOTCH inhibition prevents IL-10 signalling in stem cell derived microglia. Another option
could be to remove DAPT from MB media, and to compare IL-10 secretion to MB media containing
DAPT. If IL-10 secretion by microglia in MB media in the absence of DAPT, this would further support
the theory that NOTCH inhibition prevents IL-10 secretion.

In summary, incubation with LPS caused significant secretion of IL-10 by microglia in FB media,
and FB media microglia incubated in LPS produced more IL-10 compared to microglia cultured in MB
media. This adds to the argument that microglial responses are context dependent, and that media
composition is able to alter microglial production of cytokines. It is most likely that in these
experiments, IL-10 production by microglia in FB media occurs due to the absence of DAPT, a γ-
secretase inhibitor which is present in MB media.

5.6: MIP-1α Secretion

Results
Comparison of inflammatory stimuli within forebrain media monocultures.
The first stage of investigation was to determine whether MIP-1α secretion in microglial
monocultures in FB media was significantly altered by incubation with Vehicle (PBS + 0.01%DMSO),
LPS (100ng/mL), TNF (100ng/mL), IFNγ (20ng/mL) or Amyloid-β (60nM). MIP-1α secretion was
significantly altered over time and by incubation with LPS (see Figure 66). Maximal MIP-1α secretion
occurred at 4-hours (160.2 ± 39.86pg/mL, *p<0.0001*) and 8-hours (125.1 ± 45.44pg/mL, *p<0.0001*)
compared to 2-hours (51.09 ± 10.49pg/mL), before falling (Figure 66). Additionally, at 4-hours,
secretion of MIP-1α was significantly greater in LPS incubated microglia (160.2 ± 39.86pg/mL,
*p<0.0001*) compared to vehicle (14.05 ± 5.01pg/mL). Incubation with TNF, IFNγ or Amyloid-β did not
significantly alter MIP-1α secretion over time or compared to vehicle at any point.
Comparison of MIP-1α secretion between microglial monocultures in forebrain and midbrain media

Once incubation with inflammatory mediators had been analysed, the next step was to determine whether secretion of MIP-1α was different between microglia cultured in FB or MB media. Using the same data from Figure 66, arranged by culture type and time revealed microglia in FB media incubated with LPS (100ng/mL) produced significantly more MIP-1α compared to microglia in MB media at 4-hours (160.2 ± 39.86pg/mL vs 0.00pg/mL, p<0.0001) and at 8-hours (125.1 ± 45.44pg/mL vs 0.00pg/mL, p<0.0001) (see Figure 67). MIP-1α secretion was not different between microglial monocultures incubated with vehicle (PBS + 0.01% DMSO) or TNF (100ng/mL).
Figure 67: Comparison of MIP-1α secretion by microglial monocultures in either mixed forebrain (Red) or mixed midbrain (Blue) media. Two-way ANOVA with post-hoc Tukey’s test was performed. Forebrain media n=5, in midbrain media n=3 ****p<0.0001 between midbrain and forebrain media at indicated timepoint.

Discussion

MIP-1α, as described in Chapter Four, is a proinflammatory chemokine produced by human microglia in response to LPS and Amyloid-β (460-462, 590), and by stem cell derived microglia in response to LPS/IFNγ incubation (341, 349). The consensus on MIP-1α production and involvement in the pathology of AD in patients is uncertain. MIP-1α in plasma has been used to discriminate between AD patients with high and low burdens of amyloid-β plaque (591) and is increased in serum of AD patients (592, 593). Other studies have failed to show an association between MIP-1α concentration and AD (594-596). Additionally, in Chapter Four, minimal MIP-1α was observed across all cultures, and under all incubatory conditions.

As it turned out, MIP-1α was secreted in microglial monocultures in FB media, but only during incubation with LPS. MIP-1α production by microglia in FB media was also much greater than by microglia in MB media. This suggests that the conditions used for microglial maintenance and growth may affect MIP-1α secretory responses. It is likely that the altered MIP-1α production between microglia in either FB or MB media is due to a reagent included in the media (see earlier).

Early studies have demonstrated that foetal human microglia production of MIP-1α was inhibited by pre-treatment with TGF-β (469) and that TGF-β3 was a potent inhibitor of MIP-1α in murine macrophages and bone marrow derived monocytes (467, 597). It is possible that the inclusion of TGF-β1 in microglial media, in conjunction with the TGF-β3 in MB media, contributed to the reduced
MIP-1α secretion by microglia monocultures in MB media. This would require substantial further investigation, ideally using parallel culture studies for clarification.

Induction of MIP-1α has been more thoroughly investigated in microglia. In murine systems and immortalised lines, MIP-1α is upregulated at both a protein and mRNA level, by MAPK and by NF-κB (598, 599). This is echoed in a study which also demonstrated dependence on MAPK in human microglia (460). MB media contains two indirect inhibitors of the MAPK and NF-κB pathways (GDNF and DAPT, see Appendix IX). GDNF and DAPT both interfere with NF-κB and MAPK signalling (567, 573). It is conceivable that either one or both GDNF and DAPT are modulating the MAPK and NF-κB pathways in the stem cell derived microglia, thereby reducing MIP-1α secretion. A definitive answer as to whether GDNF or DAPT are influencing MIP-1α secretion in these circumstances can only be obtained through further investigations. Potentially, this could be done through a combination of RT-PCR for MAPK and NF-κB, western blotting for analysis of the ratio of phosphorylated vs total protein levels, and the use of parallel culture studies with and without GDNF/DAPT.

In summary, microglia in FB media secreted significantly more MIP-1α during incubation with LPS when compared to vehicle, and when compared to microglia in MB media, simply by virtue of the fact that MB microglia failed to secrete any detectable MIP-1α. The difference in secretion between FB and MB media microglia potentially indicates media composition can affect MIP-1α secretion. MB media contains GDNF and DAPT, which can indirectly inhibit MIP-1α transcriptional regulators MAPK and NF-κB, and TGF-β3, a potent MIP-1α inhibitor. To enable better understanding of MIP-1α secretion mechanisms, requires deeper investigation in future experiments.

5.7: TNF Secretion

Results

Comparison of inflammatory stimuli within forebrain media monocultures.

The first stage of investigation was to determine whether TNF secretion in microglial monocultures in FB media was significantly altered by incubation with Vehicle (PBS + 0.01%DMSO), LPS (100ng/mL), IFNγ (20ng/mL) or Amyloid-β (60nM). Incubation with LPS resulted in significant
differences in TNF secretion over time, and when compared to vehicle (see Figure 68). Secretion of TNF was minimal (<1pg/mL) to low (<20pg/mL), depending on the incubation condition. TNF secretion by microglia was significantly increased at 2-hours (9.77 ± 3.04pg/mL) compared to 24-hours (0.06 ± 0.05pg/mL, p<0.0001) and 48-hours (0.00pg/mL, p<0.0001) during incubation with LPS. Incubation with Vehicle, IFNy or Amyloid-β did not significantly alter TNF secretion.

**Figure 68:** TNF secretion by microglial monocultures, comparisons between Vehicle (PBS + 0.01% DMSO), LPS (100ng/mL), IFNy (20ng/mL) and Amyloid-β monomers (60nM). Two-way ANOVA with post-hoc Tukey's Test performed. N=5. ****p<0.0001 between 2-hours and indicated timepoint.

Comparison of TNF secretion between microglial monocultures in forebrain and midbrain media

Once incubation with inflammatory mediators had been analysed, the next step was to determine whether secretion of TNF was different between microglia cultured in FB or MB media. Using the same data from Figure 68 arranged by culture type and time, revealed microglial monoculture in FB media significantly increased secretion of TNF during incubation with LPS (100ng/mL) compared to microglia in MB media (see Figure 69). FB cultured microglia secreted significantly more TNF at 2-hours (9.77 ± 3.04pg/mL vs 0.72 ± 0.39pg/mL, p=0.001) and at 4-hours (12.13 ± 3.81pg/mL vs 0.55 ± 0.28pg/mL, p<0.0001). There was no significant difference in TNF secretion during incubation with vehicle (PBS + 0.01% DMSO).
Figure 69: Comparison of TNF secretion by microglial monocultures in either mixed forebrain (Red) or mixed midbrain (Blue) media. Two-way ANOVA with post-hoc Tukey’s test was performed. Forebrain media n=5, in midbrain media n=3 **p<005, ****p<0.0001 between forebrain and midbrain cultures at indicated time point.

Discussion

The production of TNF by stem cell derived microglia has varied widely between papers, from less than 200pg/mL in 24 hours (341), to as much as 5000pg/mL in 18 hours (346). In addition, the work performed in Chapter Four demonstrated minimal TNF produced across all conditions measured. These factors made it difficult to predict the magnitude of TNF secretion by microglial monocultures in FB media. Unfortunately, TNF secretion was again very low in microglial monocultures, especially when considered in the context of previous stem cell microglial publications (341, 343, 349, 351, 352). Although a small but significant increase was observed during initial LPS incubation, TNF concentrations did not exceed 20pg/mL. This indicates that media composition is again potentially influencing microglia. As discussed in Chapter Four, microglial media contains CX3CL1 to assist in maintaining microglial identity and homeostasis (206, 341). Intriguingly, CX3CL1 has also been shown to inhibit TNF production in a dose dependent manner in murine modelling systems (481, 600). It is possible that, as hypothesised in Chapter Four, CX3CL1 presence in microglial media contributes to suppression of TNF secretion.

FB media, like MB media, contains dbcAMP to maintain neurons. However, dbcAMP has been directly shown to inhibit TNF secretion in multiple studies, by inhibiting the Ca\(^{2+}\) flux required for TNF release (483, 484, 486). Likely, the inclusion of dbcAMP in FB media also contributes to the low
concentration of TNF secreted by microglia. This could be investigated in future studies by repeating the current experiments but excluding dbcAMP from media.

Interestingly, there was a significant difference in TNF secretion between microglial cultures in FB and MB medias during incubation with LPS. *In vitro* models of hypoxia with BV2-line microglia have shown that TNF production is partially ameliorated by pre-treatment with DAPT (573). MB, but not FB media, contains DAPT, and microglia in MB media show reduced TNF secretion compared to microglia in FB media. Conceivably, inclusion of DAPT in MB media suppresses TNF production by microglia. Future studies could investigate this by removing DAPT from MB microglia monoculture experiments, to see whether TNF secretion is altered.

In summary, TNF secretion by microglial monoculture in FB media was very low (less than 20pg/mL) in all in conditions measured, though it was significantly increased compared to vehicle when incubated with LPS. Secretion of TNF was also increased by microglial monocultures in FB media compared to MB media during LPS incubation. Most likely, media composition is affecting TNF secretion. Both FB and MB media contain exogenous CX3CL1 and dbcAMP, which are known to directly inhibit TNF production, while MB media also contains DAPT, which can reduce TNF signalling as well. Future studies should investigate whether CX3CL1, dbcAMP and/or DAPT definitively inhibit TNF secretion in stem cell derived microglia, as they do in BV-2 and murine primary cultures.

5.8: IFNγ Secretion

Results
Comparison of inflammatory stimuli within forebrain media monocultures.

The first stage of investigation was to determine whether IFNγ secretion in microglial monocultures in FB media was significantly altered by incubation with Vehicle (PBS + 0.01%DMSO), LPS (100ng/mL), IFNγ (20ng/mL) or Amyloid-β (60nM). Secretion of IFNγ was minimal (<1.0pg/mL) at all time points, analysis confirmed there were no significant alterations to IFNγ secretion over time or as a result of incubation condition (see Figure 70).
Comparison of IFNγ secretion between microglial monocultures in forebrain and midbrain media

Once incubation with inflammatory mediators had been analysed, the next step was to determine whether secretion of IFNγ was different between microglia cultured in FB or MB media. Using the same data from Figure 70 arranged by culture type and time again confirmed there was no difference in IFNγ production between microglia cultured with FB or MB media, under any incubation conditions (see Figure 71).

Figure 70: IFNγ secretion by microglial monocultures, comparisons between Vehicle (PBS + 0.01% DMSO), LPS (100ng/mL), TNF (100ng/mL), IFNγ (20ng/mL) and Amyloid-β monomers (60nM). Two-way ANOVA with post-hoc Tukey’s Test performed. N=5.

Figure 71: Comparison of IFNγ secretion by microglial monocultures in either mixed forebrain (Red) or mixed midbrain (Blue) media. Two-way ANOVA with post-hoc Tukey’s test was performed. Forebrain media n=5, in midbrain media n=3.
Discussion

Three protocols for stem cell derivation of microglia to date have shown IFNγ secretion (346, 349, 352), although two of these protocols demonstrate only minimal secretion in microglial monocultures incubated with LPS (346, 349). The data from Chapter Four also showed minimal IFNγ secretion across all culture and incubation conditions measured. Based on these previous works, it was expected there would be minimal secretion of IFNγ by microglia monocultures in FB media.

The majority of studies show that patients with AD typically have very low concentrations of IFNγ in the blood and CSF (601-603), and previous stem cell microglia work has also demonstrated minimal levels of IFN-γ after 18-24 hours of stimulation with LPS (346, 349). In contrast, other studies have indicated significant upregulation of IFNγ in AD patient plasma (604), and during incubation of peripheral blood monocytes with amyloid-β oligomers (143). Because of this, the general consensus for the role of IFNγ in AD is not clearly defined (for detailed reviews, see (551, 605, 606)), adding difficulty to interpretation of results. However, it was expected that IFNγ secretion by microglial monocultures in FB media would be minimal, based on the previous demonstrations in stem cell cultures (346, 349).

Since IFNγ secretion was minimal (<1pg/mL), and not significantly altered between microglial cultures in FB and MB media, this may indicate either that IFNγ secretion is somehow inhibited by a component of the media, or, that the microglia in this thesis are incapable of substantial IFNγ production as a response to incubation with inflammatory stimuli. If media composition is the cause, it is possible that TGF-β1 (a component of microglial maturation media, see Chapter Two) may be involved. In microglia, TGF-β1 has been demonstrated to inhibit IFNγ induction of MHC II antigens (333, 606, 607), indicating that TGF-β1 might act as an IFNγ antagonist in these circumstances. As proof of principle, TGF-β1 directly inhibits IFNγ signalling by preventing expression of key the IFNγ signalling molecules STAT4 and T-bet in CD4+ T-cells (608). The role of TGF-β1 in modulation microglial cytokine production was not investigated in these experiments, but it is conceivable that TGF-β1 may
be acting as an inhibitor of IFNγ secretion. However, this requires extensive further studies for clarification.

In summary, microglia in FB media produced minimal levels of IFNγ, and was not significantly increased in response to incubation with inflammatory stimuli. IFNγ secretion was also not significantly altered between microglial cultures in FB or MB media. The low level of IFNγ secreted by microglia in these experiments supports previous stem cell work which also showed minimal secretion (346, 349). It is possible that the lack of IFNγ production in response to inflammatory stimuli is due to either a lack of microglial functional capacity, or that a common component in the media is inhibiting microglial secretion. Given that other stem cell microglia protocols also report low levels of IFNγ, it may be that reagent required for microglial maintenance is inhibiting IFNγ secretion. One of the more likely candidates for this is TGF-β1, a cytokine responsible for maintaining microglial identity and function (267), and also common to the experiments described here and in other stem cell works (346, 349).

5.9: VEGF Secretion

Results

Comparison of inflammatory stimuli within forebrain media monocultures.

The first stage of investigation was to determine whether VEGF secretion in microglial monocultures in FB media was significantly altered by incubation with Vehicle (PBS + 0.01%DMSO), LPS (100ng/mL), IFNγ (20ng/mL) or Amyloid-β (60nM). Secretion of VEGF was moderate (>20pg/mL, less than 50pg/mL), and was significantly increased at 48-hours (37.94 ± 9.68pg/mL) compared to 2-hours (1.39 ± 0.39pg/mL, p=0.0089) during incubation with LPS (see Figure 72). VEGF production during incubation with LPS, TNF, IFNγ or Amyloid-β was not significantly different to vehicle secretion at any point and was also not significantly altered by incubation with vehicle, TNF, IFNγ or Amyloid-β over time.
Figure 72: VEGF secretion by microglial monocultures, comparisons between Vehicle (PBS + 0.01% DMSO), LPS (100ng/mL), TNF (100ng/mL), IFN\(\gamma\) (20ng/mL) and Amyloid-\(\beta\) monomers (60nM). Two-way ANOVA with post-hoc Tukey’s Test performed. N=5. **p<0.005 between 2-hours and indicated timepoint.

Comparison of VEGF secretion between microglial monocultures in forebrain and midbrain media

Once incubation with inflammatory mediators had been analysed, the next step was to determine whether secretion of VEGF was different between microglia cultured in FB or MB media. Using the same data from Figure 72 arranged by culture type and time indicated no difference in VEGF secretion between microglia cultured with FB or MB media, under any incubation conditions (see Figure 73).

Figure 73: Comparison of VEGF secretion by microglial monocultures in either mixed forebrain (Red) or mixed midbrain (Blue) media. Two-way ANOVA with post-hoc Tukey’s test was performed. Forebrain media n=5, in midbrain media n=3.
Discussion

VEGF, as described in Chapter Four, is a neuroprotective and angiogenic factor, typically produced in the CNS by neurons and by astrocytes (376, 502). Detailed investigations into microglial production of VEGF are few (609, 610), and focus mainly on murine systems. While production of VEGF by human microglia has been investigated (611), it appears microglial secretion of VEGF in patients with AD has not been comprehensively studied. VEGF secretion has been demonstrated in co-cultures of neurons with stem-cell derived microglia (346), and Chapter Four demonstrated low-level secretion by microglia monocultures in MB media. Bearing in mind the data obtained in Chapter Four, it was expected that there would be low-level secretion of VEGF by microglia in FB media, and it was thought that there may be differences in VEGF production between microglia in FB and MB media.

As expected, VEGF secretion was low, across all conditions investigated, except during LPS incubation, where VEGF secretion significantly increased over time. The low level of VEGF secretion here is supported by a large, recent study which demonstrated that VEGF levels are not significantly altered in the CSF of healthy controls, patients with mild cognitive impairment or patients with AD (612). However, other studies investigating VEGF concentration in serum strongly disagree with the data here. Some studies have demonstrated patients with AD have significantly greater serum VEGF concentrations compared to healthy controls (613, 614), while other studies have demonstrated the complete opposite (615, 616). Finally, a large scale systematic meta-analysis of published works statistically determined that VEGF concentrations in blood and CSF were not associated with AD, but did not determine whether this was true for CNS samples (150). The discrepancies in results between studies make it difficult to determine where the data from this chapter sits in the broader context of the literature. As a stand-alone study, the data for VEGF secretion within this thesis present a novel contribution for stem-cell microglia models. No one has previously attempted to investigate whether the production of VEGF by stem-cell derived microglia is influenced by incubation with specific inflammatory stimuli.
In addition, it was clearly demonstrated that there were no significant differences to VEGF secretion between microglia in FB or MB media. This could be due to a number of reasons. Potentially, a media component common to both FB and MB media, or contained within microglia media, is inhibiting VEGF secretion. Alternatively, it could be that in the absence of neurons/astrocytes, there is minimal paracrine between microglia to potentiate VEGF secretion, resulting in the low levels observed here. Future studies could investigate either of these proposed mechanisms to determine why VEGF secretion is low in stem cell derived microglia.

In summary, during LPS incubation, VEGF secretion was modestly increased over time in microglial cultures in FB media. There were no differences to VEGF secretion between microglia in MB or FB media monocultures. As the broader context for VEGF in AD is uncertain, this work should be considered an early and investigation into production of VEGF by stem cell derived microglia in an in vitro model of forebrain conditions.

5.10: CX3CL1 Secretion

Results

Comparison of inflammatory stimuli within forebrain media monocultures.

The first stage of investigation was to determine whether CX3CL1 concentration within microglial monocultures in FB media was significantly altered by incubation with Vehicle (PBS + 0.01%DMSO), LPS (100ng/mL), IFNγ (20ng/mL) or Amyloid-β (60nM). Analysis confirmed no significant differences between incubation conditions and vehicle concentrations at any point. Although the initial concentration of CX3CL1 was extremely high (>1000 pg./mL) and appeared to drop away, the same analysis revealed there were no significant differences in CX3CL1 over time (see Figure 74.
Comparison of CX3CL1 secretion between microglial monocultures in forebrain and midbrain media

Once incubation with inflammatory mediators had been analysed, the next step was to determine whether secretion of CX3CL1 was different between microglia cultured in FB or MB media. Using the same data from Figure 74, arranged by culture type and time, microglia cultured in MB media had a significantly higher concentration of CX3CL1 compared to microglia cultured in FB media (see Figure 75) at 2-hours (60587 ± 3286pg/mL vs 26647 ± 3189pg/mL, p<0.0001), 4-hours (60545 ± 2555pg/mL vs 25565 ± 2917pg/mL, p=0.0001), and 8-hours (53732 ± 3763 pg./mL vs 23536 ± 2528pg/mL, p=0.0003). There were no significant differences in CX3CL1 concentration between microglial monocultures in FB or MB media incubated in either LPS (100ng/mL) or TNF (100ng/mL).
Discussion

CX3CL1, as discussed in Chapters One and Four, is the only ligand known for CX3CR1 in humans. It is not considered to be produced by microglia (159, 444, 528), however its receptor, CX3CR1 is exclusively expressed by microglia during homeostasis (269). The CX3CL1-CX3CR1 axis is critical for maintaining homeostasis in the CNS, and disruption of this axis contributes to neuroinflammation and disease progression. CX3CL1 is not ubiquitously distributed through the CNS either – a recent study demonstrated that, in humans, CX3CL1 appears to be most strongly expressed in the hippocampus, and least in the cerebellum by astrocytes and neurons (304). However, in the context of AD, the dysregulation of CX3CL1 is still uncertain. CX3CL1 has been shown to be significantly decreased across the CNS (304), and in CSF (617) of patients with AD compared to healthy controls. CX3CL1 has also been shown to be not significantly different (618), and to be increased in the plasma of patients with AD (619). This obviously makes it difficult to make conclusive statements about the role of CX3CL1 dysregulation in AD. However, as microglia are known not to express CX3CL1, it was expected that in these experiments, the concentration of exogenous CX3CL1 in culture would drop over time.

As expected, CX3CL1 concentrations fell over time in microglial monocultures in FB media, under any incubation conditions (see Figure 75). This is unsurprising, as there are no astrocytes or neurons to produce CX3CL1 in these cultures, and the only other source is the exogenous addition of CX3CL1 (100ng/mL) during experiment set-up. What is most intriguing is how CX3CL1 concentration is significantly lower in microglia maintained in FB media, compared to MB media, during Vehicle
incubation (see Figure 75), though it is unclear why this is the case. The most likely explanations are that either FB media is somehow less supportive for maintaining CX3CL1 levels, or that MB media is more supportive. It could be that the composition of MB media contains a reagent that directly or indirectly stabilises soluble CX3CL1 in vitro. Alternatively, microglia in FB media might be able to rapidly sequester CX3CL1 internally through endocytosis, previously demonstrated in umbilical vein cells (274), or potentially produce greater concentrations of metalloproteases which can degrade soluble CX3CL1 (274, 620, 621). Unfortunately, none of these avenues were investigated during the course of these experiments, so it is not known whether they are contributing to the reduced concentration of CX3CL1. Future studies wishing to understand these mechanisms should begin by comparing media composition, and work from there.

In summary, microglia in FB media did not significantly alter CX3CL1 concentrations over time, or due to incubation with known inflammatory factors. Additionally, CX3CL1 concentration is significantly decreased in FB media compared to MB media, most probably due to differences in media composition, which are affecting microglial responsiveness. Future studies investigating microglia and CX3CL1 could investigate the potential mechanisms behind these differences.

**Chapter Summary**

Cytokine production by stem cell derived microglia in co-culture with neurons has been studied only minimally (346), and there have been no investigations into alternative inducers of inflammation in stem cell microglia. Typically, LPS is used either alone (343, 348, 351, 352), or in conjunction with IFNγ (341, 346, 349). To date there have been no direct comparisons performed between individual mediators of inflammation and microglia secretion of cytokines. This chapter has gone some way to filling this gap in the literature, by comparing LPS (100ng/mL), TNF (100ng/mL), IFNγ (20ng/mL), Amyloid-β (60nM) against Vehicle (0.1% DMSO in PBS) using a published protocol (341, 347). A shortage off forebrain neuron cultures prevented detailed investigations into co-cultures
of microglia with FB neurons, and FB neuron monocultures, but these preliminary data are presented in Appendix VII.

As in Chapter Four, it was predicted that inflammatory cytokines IL-1α, IL-1β, TNF and IFNγ would all be secreted in high concentrations, but this was not observed (see Figure 76 for summary). IL-1α, IL-1β, TNF and IFNγ were all minimally secreted by microglial monocultures in FB media, regardless of incubation condition. IL-1α release is dependent on Ca²⁺ flux, while IL-1β and TNF have been demonstrated to be downregulated in a dose dependent manner by dbcAMP (385, 386, 483, 484, 486, 558). Both FB and MB media contain dbcAMP, which will elevate intracellular Ca²⁺ (557, 622), thereby preventing release of IL-1α, and potentially also contributing to the minimal IL-1β and TNF secretion in these cultures. The exact reason for the minimal IFNγ secretion is uncertain, but it might be due to inclusion of TGF-β1 in microglial media – a growth factor previously demonstrated to interfere with key parts of the IFNγ signalling pathway in T-cells (608). It was predicted that MIP-1α, IL-6, IL-8 and VEGF would be secreted in response to incubation with inflammatory cytokines, and that IL-10 would be minimally secreted. In line with expectations, MIP-1α, IL-6, and IL-10 were all secreted in response to LPS, while IL-8 was secreted in equally high levels in response to both LPS and TNF. This is extremely interesting, as it contradicts the work performed in Chapter Four, and supports the use of LPS for microglial stimulation (327, 432, 433, 623). VEGF secretion did occur but was not significantly altered by incubation condition over time, while CX3CL1 concentration (although initially high) was also not significantly altered by incubation condition.

Although this was a model of FB conditions, incubation with amyloid-β did not significantly alter secretion by microglia at any point, for any cytokine measured. This could be due to a number of reasons. Here, Amyloid-β stocks were prepared and used as monomers. Previously, Amyloid-β effect on microglia has been indicated to be potentially structure dependent, although there is no clear consensus as to which structure is most able to elicit an immunological response (377, 383, 624).
Potentially, the monomeric stocks used in these experiments were not the most immunogenic, and therefore were unable to stimulate a response. Future studies could further investigate this if desired.

Due to differences in media composition between microglia cultured in FB and MB media (see Appendix IX), it was prudent to investigate whether this affected cytokine secretion. These differences are summarised in Table 1 below.

Table 2: Summary of significant increases to cytokine secretion by microglia monocultures, classified by media type and incubation condition.

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Forebrain Media</th>
<th>Midbrain media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>nil</td>
<td>CX3CL1, IL-1α</td>
</tr>
<tr>
<td>LPS</td>
<td>IL-6, IL-8, IL-10, MIP-1α, TNF</td>
<td>IL-1α</td>
</tr>
<tr>
<td>TNF</td>
<td>IL-8</td>
<td>nil</td>
</tr>
</tbody>
</table>

Intriguingly, it appears as though the secretory responses of microglia monocultures are significantly altered by both the media in which they are maintained, and by the incubation condition used. For example, LPS stimulates the greatest secretion of IL-6, IL-8, IL-10, MIP-1α and TNF by microglia maintained in FB media, but this is not demonstrated by microglia maintained in MB media. Regrettably, cell number restrictions for FB cultures prevented comparisons being performed for FB co-cultures with microglia, and FB neuron monocultures, but these could be investigated in future studies. It also appears as though the inclusion of DAPT and GDNF in MB media may be inhibiting...
microglial monoculture responses. These two reagents contribute to modulation of the NF-κB, and MAPK pathways, which are integral for initiation of inflammatory responses in microglia. Disruptions to these pathways have been demonstrated to affect IL-6, IL-8, IL-10, MIP-1α, VEGF and TNF in microglia (416, 421, 430, 458, 507, 588, 598, 625, 626). Consistent with this data, MB microglial monocultures exhibited low levels of IL-6, IL-8, IL-10, MIP-1α, VEGF and TNF compared to FB microglia. It is possible that this is due to the effects of DAPT and/or GDNF, which could be confirmed through further study.

In conclusion, this chapter has clearly demonstrated that stem cell microglia have vastly different secretory responses depending on the media in which they are maintained, and that it is likely that media composition affects cytokine secretion. This is the first direct comparison of inflammatory stimuli in stem cell derived microglia, and it supports previous studies which suggest in vivo environment dependent identity and cytokine responses (267, 407, 410, 627). This highlights the importance of choosing the most appropriate methodology for modelling disease. To be able to accurately determine the role of microglia, it is critical that the media does not influence the behaviour of your cells of interest. Future work should focus on determining whether inclusion of DAPT and/or GDNF is suppressing activation of the NF-κB, and MAPK pathways in microglia cultured in MB media, as well as whether other forms of Amyloid-β are able to induce an inflammatory response by microglia cultured in FB media.
Chapter Six
Thesis Discussion

Summary and significance of Chapter Three
Impact of methodology choice

Reproducibility and consistency are the foundation for development of accurate models of disease and normal function, and it is critical that future studies of neuroinflammation using SCDmicroglia can demonstrate these qualities. Here, it is argued that adopting a single methodology across laboratories is the most effective way to generate consistent data on microglia functional capacity and gene expression, which can then be applied to different disease modelling systems. This data could take the form of transcriptomics from RNA-sequencing, or proteomics from whole cell lysates or cytometric bead array. Each protocol in Table 3 generates microglia-like cells, but the inherent methodology differences result in similar but distinct populations of microglia-like cells when transcriptomic data is analysed by principle components analysis (see Appendix V for further details).

Table 3: Current published protocols for derivation of Microglia from stem cells

<table>
<thead>
<tr>
<th>Author</th>
<th>iPSC or hESC</th>
<th>2D or 3D</th>
<th>Includes Serum?</th>
<th>Length of Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abud et al. 2017</td>
<td>iPSC</td>
<td>2D</td>
<td>No</td>
<td>38 days</td>
</tr>
<tr>
<td>Amos et al. 2017</td>
<td>iPSC/H1</td>
<td>3D EB's</td>
<td>FBS</td>
<td>40 days</td>
</tr>
<tr>
<td>Brown jhon et al.</td>
<td>iPSC</td>
<td>3D EB's</td>
<td>FBS</td>
<td>25-35 days</td>
</tr>
<tr>
<td>Duovirus et al. 2018</td>
<td>iPSC, RUES, H9</td>
<td>2D</td>
<td>No</td>
<td>60 days</td>
</tr>
<tr>
<td>Muffat et al. 2016</td>
<td>iPSCs</td>
<td>3D EB's</td>
<td>No</td>
<td>74 days</td>
</tr>
<tr>
<td>Pandya et al. 2017</td>
<td>iPSC</td>
<td>2D</td>
<td>FBS</td>
<td>25 days</td>
</tr>
<tr>
<td>Takata et al. 2018</td>
<td>iPSC</td>
<td>2D</td>
<td>no</td>
<td>26 days</td>
</tr>
<tr>
<td>McQuade et al. 2018</td>
<td>iPSC</td>
<td>2D</td>
<td>No</td>
<td>36-38 days</td>
</tr>
<tr>
<td>Mesci et al. 2018</td>
<td>iPSC</td>
<td>2D</td>
<td>No</td>
<td>60 -70 days</td>
</tr>
<tr>
<td>Haensler et al. 2018</td>
<td>iPSC</td>
<td>3D EB's</td>
<td>No</td>
<td>60-75 days</td>
</tr>
<tr>
<td>Xu et al. 2019</td>
<td>iPSC</td>
<td>2D</td>
<td>No</td>
<td>37 days</td>
</tr>
</tbody>
</table>
Strengths and limitations

In this thesis, fluorescent reporters for CX3CR1, TREM2, TMEM119, PU.1 and IRF8 were generated for use in in vitro studies of neuroinflammation and microglia development, and H9-CX3CR1-tdTomato and H9-TREM2-E2CRIMSON lines were functionally validated (see Figure 77 for summary). The H9-CX3CR1-tdTomato and H9-TREM2-E2CRIMSON lines behave like microglia, in that they can perform phagocytosis, produce cytokines, are motile, possess key microglial identity markers, and actively integrate when co-cultured with neurons. The major limitation for using these lines is that they are karyotypically abnormal due to possession of Trisomy 12. Due to time restrictions, repeating these experiments with a karyotypically normal line was not an option. Other limitations (which apply to all stem cell studies), include the time and reagent intensive requirements for maintenance and differentiation, and the cost of reagents.

While the H9-CX3CR1-tdTomato line is karyotypically abnormal, it was still possible to differentiate towards a microglia lineage, highlighting the robustness of the differentiation protocol (347). In addition, when differentiated to microglia, the CX3CR1 reporter line is functional. This functionality is defined by visibility of the fluorescent CX3CR1-tdTomato reporter from approximately day 16 of differentiation, the capacity of H9-CX3CR1-tdTomato differentiated microglia to phagocytose labelled e. coli particles, morphology, production of cytokines on stimulation with LPS, and the ability of microglia to be successfully co-cultured with neurons/astrocytes, as well as transcriptomic data. The specific expression of the CX3CR1-tdTomato reporter during differentiation indicates that the CX3CR1 line could be used for disease modelling outside the context of neuroinflammation and microglia. For example, Chronic Lymphocytic Leukaemia (CLL) and Burkitt’s lymphoma are diseases affecting B-cells, and are both commonly affected by trisomy 12 (628). A recent study demonstrated that the Nuclear factor of activated T-cells (NFAT) pathway is dysregulated in patients with CLL (629). The H9-CX3CR1-tdTomato line could theoretically be used in future studies of CLL or Burkitt’s lymphoma to better understand how this dysregulation of NFAT signalling affects B-cells, and to improve therapeutic design.
Summary and significance of Chapter Four
Parkinson’s Disease and stem cell modelling.
To date, none of the protocols for SCDmicroglia (see Table 3) have investigated microglia function in a midbrain model setting, either in vivo or in vitro. Although most protocols for SCDmicroglia claim that the differentiated cells can be used for multiple models of neurodegeneration, in actuality, these studies typically only utilise LPS (342-344, 348, 351, 352) to stimulate microglial cytokine production, which is unlikely to mimic the inflammatory environment of a PD brain. In Chapter Four of this thesis, microglia were generated using the modified Abud et al. paper (347), and incubated with a variety of known inflammatory stimuli, including α-synuclein aggregates (known to contribute to PD pathology (534, 630-632)). Additionally, the work performed in this chapter indicates that microglia are not the sole producers of inflammatory cytokines. When using a model of midbrain
microglia-neuron-astrocyte connectivity, microglia monocultures typically produced weaker cytokine responses than neuron-astrocyte monocultures, or co-cultures of microglia, neurons, and astrocytes.

**Major outcomes of Chapter Four**

Several aspects of this chapter are important for future research. Firstly, it has clearly shown that co-culture of midbrain neurons/astrocytes with microglia resulted in an amplified secretion of inflammatory cytokines, compared to microglial monocultures (see Error! Reference source not found.). This supports previous research which illustrates the importance of neuron-glial cross talk when attempting to model disease (271, 309, 444, 633, 634). These experiments also indicate microglial monocultures will likely be insufficient to accurately portray the inflammatory milieu of PD. Secondly, these data show that when modelling a midbrain environment, TNF, not LPS, induces cytokine secretion most strongly (see Figure 78 below). This directly contradicts the use of LPS as the “Gold standard” for activation of microglia or astrocytes (429, 432, 542). While the secretory and pathophysiological responses of microglia to LPS are well established (239, 327, 369), LPS is not part of the PD sequelae in vivo. Therefore, LPS is a poor choice when used to “activate” microglia in the context of modelling PD. TNF is, however, upregulated in the CNS, CSF and serum of patients with PD (440, 635, 636), and seems a pathophysiologically more appropriate choice for initiating inflammatory responses when modelling a midbrain environment. Additionally, previous work in this laboratory has demonstrated TNF contributes to neurite retraction and death of PITX3<sup>eGFP</sup> neurons (37). This thesis proposes that TNF should be adopted for eliciting inflammatory microglial responses in future works. Thirdly, neither α-synuclein aggregates or IFNγ were able to significantly alter cytokine secretion in a MB model. To enable thorough dissection of these outcomes, this discussion will first focus on α-synuclein, then on IFNγ.
The lack of response to α-synuclein aggregates in all examined cultures, was extremely unusual, as PD in humans is characterised by the presence of α-synuclein aggregates and oligomers in both astrocytes and microglia (637). Although the recombinant α-synuclein aggregates sourced for this research were described as “biologically active fibrils”, the lack of inflammatory response may have a couple of causes. For instance, a recent study using iPSC derived macrophages demonstrated that monomeric and fibrillar α-synuclein aggregates were incapable of inducing cytokine release, and this was thought to be caused by a lack of pre-exposure to ‘priming cytokines’ (346). Possibly, lack of prior exposure to ‘priming cytokines’ such as LPS or TNF in the experiments presented here prevented H9-CX3CR1-tdTomato microglia from responding to exogenous α-synuclein. It has also been suggested that because recombinant proteins sourced from bacteria are not subjected to post-translational modifications (PTMs), they are potentially less pathogenic than endogenous proteins (638). The recombinant α-synuclein used in these experiments may therefore be unable to induce the same responses in microglia as α-synuclein sourced from a mammalian host. This is supported by reviews
by Zhang et al. (639) and by Oueslati et al. (640), which conclude that PTMs can and do significantly influence the structure and toxicity of α-synuclein. Possibly, using α-synuclein aggregates sourced from donated human tissues or from murine systems (isolated by immunoprecipitation), would have been more effective at eliciting a cytokine response in these experiments. Lastly, a final possible cause for the lack of response to α-synuclein is the aggregation state. The aggregation state of α-synuclein has been indicated to influence cytokine release by immortalised microglia (631) and gene expression \textit{in vivo} (632). Although fibril aggregates were used in this study, it could be that monomers or oligomers are more effective at inducing cytokine secretion in SCDmicroglia. It is suggested that future studies should investigate the role of α-synuclein structure in SCDmicroglia responses.

**IFNγ** incubation for microglia monocultures was not performed due to cell number restrictions, however, no such restrictions applied with MB monocultures and co-cultures. There is clear evidence that cells within the CNS are capable of responding to exogenous IFNγ (641-643), because astrocytes (644) (645), neurons (491, 646) and microglia (647) all possess receptors for IFNγ. When contemplating why IFNγ did not elicit a strong cytokine response in a MB model by and large, a few possible causes arise. For one, incubation of IFNγ alone is not typically performed. Prior studies using IFNγ in microglia have used IFNγ in conjunction with LPS (341, 344, 346, 349) while early human astrocyte studies used combinations of IFNγ/IL-1β or IFNγ/LPS (648). It is possible that the use of IFNγ alone was insufficient to stimulate secretion of most cytokines measured int his thesis. In a similar vein, the concentration of IFNγ used in these experiments was 20ng/mL, based on work by Abud et al. (341), which used combined IFNγ/LPS to stimulate SCDmicroglia. In the experiments presented in this thesis, LPS and IFNγ were used individually to activate cultures, to determine whether IFNγ was capable of eliciting a response when used alone. The concept was supported by murine studies which utilised IFNγ concentrations as low as 20ng/mL (649) and as high as 100ng/mL (650). It is conceivable that for stem cell derived cultures of microglia, IFNγ at 20ng/mL is too low to affect a response when used as a single stimulus for neuroinflammation modelling.
Strengths and limitations

The main strength of this thesis chapter is in the novelty of the work performed. To date, no other studies have been performed which directly compare cytokine output between SCDmicroglia monocultures, midbrain neuron/astrocyte monocultures, or co-cultures of microglia and midbrain neurons/astrocytes. There have been no studies of SCDmicroglia which attempt to emulate MB conditions as seen in PD. Furthermore, there have been no studies which use a stem cell culture system to determine which inflammatory stimuli are most effective for inducing a cytokine response in the context of the midbrain. This novelty means that the work described here can act as a steppingstone for future researchers interested in investigating PD inflammation using stem cell cultures. The data in this chapter also indicate that there are clearly different responses to inflammatory stimuli between culture types, and this should be considered by other researchers performing future experiments. Additionally, this chapter used published protocols, known mediators of inflammation, chemically defined reagents, and conservative data analysis with commonly available software. These factors together make for a robust series of experiments which can be replicated by researchers in other laboratories. However, as outlined earlier, the largest limitation to this thesis is the use of a cell line with trisomy 12 for generation of microglia. While RNA-sequencing data (see Appendix III) demonstrated that differentiated microglia are not significantly different to those already published, it must be cautioned that the results presented here might not be able to be replicated in midbrain models when using karyotypically normal lines. The other major limitation was that all experiments completed used media which contained GDNF, DAPT, and dbcAMP. This was done to remove the possibility of differences in media composition as a confounding variable, as these three reagents are key components in MB maintenance media but are not in microglia media. However, GDNF, DAPT, and dbcAMP are known inhibitors of key inflammatory pathways, and may therefore have contributed to the low levels of IL-1β, IL-4, IL-10, TNF and IFNγ secreted across all analysed culture types.
Summary and significance of Chapter Five
Contextual differences in microglia

Microglia have long been recognised in murine systems to be a heterogenous population. Studies from the early 90’s detail differences in microglia density across brain regions (651), as well as selective expression of neurotrophic factors (652). Later work also highlighted that murine microglia express TNF and other mediators of inflammation in varying degrees, depending on where in the CNS the microglia were isolated from (265). These works have been expanded upon by more recent murine transcriptomic studies, which indicate there may be as many as 10 different microglia subtypes in healthy rodent CNS tissues (260). One study comparing microglia in the cerebellum and striatum, showed cerebellar microglia are more likely to be “primed” towards phagocytic clearance of cell debris (264). Another has demonstrated using RNAseq that microglia in cortex and striatum appear similar; that there are greater transcriptional differences in microglia found in the cerebellum or hippocampus; and that the impact of aging on microglia is both influenced by and dependent on region (254). However, the inherent difficulty in obtaining sufficient numbers of high-quality microglia for human studies has contributed to the dearth of data on regional heterogeneity in the human CNS. Only very recently have there been studies performed using human microglia to investigate regional heterogeneity, at a transcriptomic, functional, or morphological level (252, 260, 263, 653, 654). Early indications are that there are at least four regional subpopulations of microglia that can be classified by transcriptomics (252, 260, 263), and that some of the genes in these profiles may overlap with murine subpopulations. Microglia have also been identified using CD68 and IBA1 antibodies to be most concentrated in the white matter, hippocampus, thalamus, basal ganglia and substantia nigra pars compacta, compared to the cerebellum, frontal, parietal and occipital gray matter (257, 323).

In light of this, it is not surprising that there is no published work describing how SCDmicroglia behave under altered culture conditions. The field of SCDmicroglia is still in its infancy, being only 3 years since the first protocol was published (349). These publications have gone to great lengths to show the similarity of the differentiated microglia to known human data and to demonstrate their
functional capacity (341, 343, 344, 346, 347, 349-351), but there has been minimal work to determine whether this similarity is altered by local cues from their environment *in vitro*. The work performed in Chapter Five can therefore be said to be a first step to demonstrating heterogeneity in SCDmicroglia.

**Major outcomes of microglia monocultures modelling forebrain conditions**

Due to cell number limitations, this chapter focused on microglia monocultures, and attempted to determine which inflammatory mediator was most able to elicit a response over time. This was done by comparing LPS (100ng/mL), TNF (100ng/mL), IFNγ (20ng/mL) and Amyloid-β (60nM) against Vehicle (0.1% DMSO in PBS) using a modified, known protocol (341, 347).

In contrast to Chapter Four, LPS was the most consistent inducer of cytokine secretion by microglia monocultures. This supports the notion of LPS as being the “gold standard” for microglial activation (327, 432, 542), but only in the context of stem cell microglia monocultures in FB media. Intriguingly, accumulation of LPS aggregates has very recently been identified around the nucleus of neurons, microglia, and amyloid-β plaques in the cortex and hippocampus of patients with sporadic AD (655-659). The LPS aggregates are suggested to originate from the gastrointestinal tract microbiome (655, 660), indicating both the intestinal barrier and blood brain barrier (BBB) may be weakened in AD patients, allowing migration of the microbiome to previously uncolonised sites. This is further supported by a recent study in the UK, which used deep ribosomal sequencing to prove the brains of AD patients exhibited increased bacterial RNA concentration compared to healthy controls (661).

Possibly, LPS and SCDmicroglia can be used to interrogate the role of LPS in AD. Although cytokine secretion was not measured in these recent studies (655-659), LPS significantly increased secretion of IL-6, IL-8, IL-10, MIP-1α and TNF in the microglial monocultures described in this thesis. It is possible that these inflammatory cytokines are also released in the tissues of patients with AD during LPS accumulation. It would be useful to perform further studies using microglia in co-culture with forebrain neurons, to determine whether LPS still consistently upregulates these cytokines.
It was expected that Amyloid-β in particular would elicit a secretory response, however this was not observed. Amyloid-β capacity to induce cytokine secretion from SCDmicroglia may be reliant on amyloid-β structure (136, 377, 662-665). Amyloid-β exists in a number of structural forms, and this thesis used monomers prepared in house. It is possible that monomeric amyloid-β is insufficient to induce cytokine secretion.

**Major outcomes of comparison between microglia monocultures**

It is becoming apparent that regional heterogeneity, in the form of gene expression and distribution, is present human microglia, as in murine microglia. However, there has been no work to date which attempts to identify whether SCDmicroglia also exhibit this capacity for heterogeneity. The work presented in *Chapter Five* represents the first direct comparison of SCDmicroglia monoculture cytokine release (specifically for H9-CX3CR1-tdTomato microglia). Microglia monocultures in forebrain and midbrain media demonstrated clearly different cytokine responses to incubation with Vehicle, LPS or TNF. It was observed that the cytokine secretion by microglia in FB media was often stronger than what was observed by microglia in MB media. This might indicate that a component of midbrain media is blunting the stimulatory effect of incubation with LPS or TNF. It was also observed that when comparing microglia monoculture responses, LPS was a more potent stimulator of cytokine secretion than Vehicle or TNF, particularly for microglia cultured in forebrain media.

**Strengths and limitations**

The main strength of *Chapter Five*, as in *Chapter Four*, is in its novelty. As yet, there have not been any publications which demonstrate that microglia derived using the same protocol can respond differently to inflammatory stimuli depending on their environment. Here, microglia in forebrain and midbrain media were shown to have vastly different responses to LPS and TNF, depending on the media in which they were maintained. This indicates for the first time that SCDmicroglia responses are context dependent. Additionally, the work performed in this thesis used commercially available reagents and consumables, meaning that it is more likely to be replicated in other laboratories.
While it would have been preferable to also compare forebrain neuronal monocultures/forebrain-microglia co-cultures against midbrain neuronal monocultures/midbrain-microglia co-cultures respectively, this was not possible due to time and cell number restrictions. Although preliminary forebrain neuronal monoculture and co-culture data is available (see Appendix X), no statistical analysis could be performed due to insufficient n’s. Had analysis been possible, this would have assisted other researchers to determine which method of CNS modelling is most appropriate for their investigations for AD and PD. Additionally, as in Chapter Four, the caveat that this work was performed using the H9-CX3CR1 trisomy line must be noted. However, trisomy 12 has been described as a result of transient Presinilin-1 expression in mice used to model Alzheimer’s Disease (666). Possibly, the C3XCR1 line used in this thesis could be used to explore the effects of trisomy 12 in the context of AD in the future.

Finally, an anticipated criticism of this thesis is that the data analysis is very conservative. However, given the novelty of this research, it was decided that conservative analysis would ensure only the largest changes would be identified as significant. Using the less conservative One-Way ANOVA, particularly in reference to forebrain microglia monocultures, results in data which indicates that Amyloid-β, IFNγ and even Vehicle incubations significantly alter cytokine secretion over time (see Appendix XI). This is because the One-Way ANOVA analyses only a small subsection of data and must be repeated for each condition. Therefore, while effects over time may be significant, this method of analysis is not suitable for comparing effects between ligand additions. By comparison, the conservative Two-way ANOVA with post-hoc Tukey’s test measures all data simultaneously and can be used to detect significant effects over time and between ligand additions. For this reason, the conservative Two-way ANOVA with post-hoc Tukey’s test was used throughout this thesis.

Thesis results in the context of stem cell modelling
Microglia are critical for maintaining homeostasis within the CNS, but their dysregulation during Alzheimer’s and Parkinson’s Disease contributes to neuronal dysfunction and degradation
New models for studying microglia in the context of neurodegeneration have been rapidly evolving over the last 15-20 years. At the commencement of this thesis, there were only two protocols for stem cell derivation of microglia (341, 349) – now there are more than a dozen. However, to date, the work performed using SCDmicroglia has been limited to demonstrations of functionality and similarity to primary human microglia. One study has demonstrated missense *TREM2* mutations in the context of dementia and Nasu-Hakola disease (343), but there have been no investigations as to whether SCDmicroglia are influenced by their *in vitro* environment.

In the context of midbrain models for PD, microglia were demonstrated in this thesis to have minimal cytokine responses during incubation with mediators of inflammation when in monocultures. This may be partially due to the inclusion of fractalkine and TGF-β in culture media, both of which are important for maintaining microglial identity, but are also known to inhibit production of cytokines (206, 333, 467, 671). When cultured with stem cell derived midbrain neurons/astrocytes, cytokine secretion was much greater. This increased secretion is believed to be due to communication between microglia, neurons, and astrocytes, another requirement for proper microglial function and identity (444, 672). Additionally, microglia monocultures showed clearly identifiable differences in cytokine secretion, depending on whether media composition included Midbrain or Forebrain media. Preliminary data for microglial-forebrain co-cultures also indicates here may be an amplified cytokine response during incubation with mediators of inflammation, similar to observations for microglia in co-culture with midbrain neurons.

The main outcome of this thesis is that there is evidence of heterogeneity in SCDmicroglia responses, which are dependent on both the media composition, and whether microglia are in a monoculture or co-culture setting. This is an important step forward for neurodegenerative research, as this supports work showing microglia heterogeneity in models of AD and PD (254, 259, 673) (255, 674).
Future investigations

It would have been interesting to also investigate transcriptional networks of the SCDmicroglia in this thesis, as this would have allowed classification of SCDmicroglia cluster with one of the known human microglia subpopulations during basal and inflammatory conditions. This could be an area for future researchers to investigate. As primary microglia are known to differ across brain regions and with age, knowing exactly where SCDmicroglia sit in this context would be an important step forward. SCDmicroglia are known to be similar to human primary microglia at a transcriptomic and functional level, however, SCDmicroglia have not yet been classified as to which subpopulation they most closely represent.

To facilitate comparison of forebrain and midbrain cultures more thoroughly, it is suggested that further replicates of the forebrain neuronal monocultures, and forebrain-microglia co-cultures are performed. This will help to further prove that microglia cytokine secretion is both dependent on the media (by comparing midbrain and forebrain conditions), and on *in vitro* context (by comparing microglia in monoculture against FB neuron monoculture and FB-microglia co-cultures).

As amyloid-β and α-synuclein were unable to significantly alter cytokine secretion, it is also suggested that investigations should be performed to determine which structures are most able to invoke a response. While these proteins are observed in a variety of forms *in vivo*, it would be helpful to achieve a consensus on the most appropriate structure to use *in vitro*.

Finally, it is suggested that the influence of D-serine (contained in FB media), GDNF, dbcAMP and DAPT (contained in MB media) on cytokine secretion by microglia be investigated. It is strongly suspected that the inclusion of GDNF, dbcAMP, and DAPT are inhibiting microglia during culture in MB media. However, their removal from MB cultures may result in de-differentiation of dopaminergic neurons. Future work should potentially begin with microglia monocultures, to determine whether cytokine secretion is increased in the absence of one or all of these components.
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207


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216


219


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# Appendices

## Index

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appendix I</td>
<td>Reagents and consumables.</td>
<td>231</td>
</tr>
<tr>
<td>Appendix II</td>
<td>Primer validation for screening targeted lines</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>CX3CR1 primer validation</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>TREM2 primer validation</td>
<td>244</td>
</tr>
<tr>
<td></td>
<td>TMEM119 Primer Validation</td>
<td>248</td>
</tr>
<tr>
<td></td>
<td>PU.1 Targeting</td>
<td>252</td>
</tr>
<tr>
<td></td>
<td>IRF8 Primer Validation</td>
<td>256</td>
</tr>
<tr>
<td>Appendix III</td>
<td>Karyotyping results</td>
<td>260</td>
</tr>
<tr>
<td>Appendix IV</td>
<td>Calculations for movement of microglia during phagocytosis.</td>
<td>271</td>
</tr>
<tr>
<td>Appendix V</td>
<td>Comparison of published stem cell derived microglia protocols</td>
<td>273</td>
</tr>
<tr>
<td>Appendix VI</td>
<td>Manuscript submitted to Stem Cell Reports.</td>
<td>276</td>
</tr>
<tr>
<td></td>
<td>A CX3CR1 reporter hESC line facilitates integrative analysis of in vitro derived microglia and reveals improvement of microglia identity upon neuron-astrocyte co-culture</td>
<td>276</td>
</tr>
<tr>
<td></td>
<td>Highlights</td>
<td>276</td>
</tr>
<tr>
<td></td>
<td>Summary</td>
<td>277</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
<td>277</td>
</tr>
<tr>
<td></td>
<td>Results and Discussion</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Experimental Procedures</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Author Contributions</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Acknowledgments</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Figure legends</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>References</td>
<td>18</td>
</tr>
</tbody>
</table>

## Appendix VI

### Preliminary Cytometric Bead Array Data

- Basal microglial secretion in the presence of CX3CL1 and TGFβ1
Basal Microglial secretion in the absence of CX3CL1 and TGFβ1 ..................................................31
Stimulated microglial secretion in the presence of CX3CL1 and TGFβ1 ........................................34
Stimulated microglial secretion in the absence of CX3CL1 and TGFβ1 ........................................40
Appendix VIII ........................................................................................................................................46
Comparison of Midbrain Co-culture and Midbrain monoculture VEGF secretion .......................46
Appendix IX ............................................................................................................................................47
Supplementary forebrain neuron monoculture and forebrain neuron-microglia co-culture data ......47
  Microglial-forebrain co-culture .............................................................................................................47
  Forebrain neural monoculture ..........................................................................................................54
  Summary of forebrain monoculture secretions. .....................................................................................63
Appendix X ............................................................................................................................................64
  Media composition for Forebrain and Midbrain neurons ...............................................................64
Appendix XI ............................................................................................................................................65
  Alternative data analysis of cytometric bead array results, using One-Way ANOVA ..................65
    Microglia Monocultures in midbrain media .....................................................................................65
    Midbrain monocultures in midbrain media ....................................................................................78
    Co-cultures of midbrain neurons with microglia in midbrain media ..........................................90
    Microglia monoculture in forebrain media ....................................................................................102
## Appendix I

### Reagents and consumables.

**Table 1: Reagents required for hESC maintenance**

<table>
<thead>
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<td>Essential 8 Medium + Supplement</td>
<td>A1517001</td>
<td>ThermoFisher Scientific</td>
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<tr>
<td>Penicillin/Streptomycin, 10,000U/mL</td>
<td>15140-122</td>
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<tr>
<td>recombinant human Laminin-521</td>
<td>A29248</td>
<td></td>
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<tr>
<td>DPBS, no calcium, no magnesium</td>
<td>14190359</td>
<td></td>
</tr>
<tr>
<td>ReLeSR™</td>
<td>#05873</td>
<td>Stem Cell Technologies</td>
</tr>
<tr>
<td>Selleck Chemicals Y-27632 dihydrochloride</td>
<td>s1049-50mg</td>
<td>Jomar Life Research</td>
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**Table 2: Reagents required for differentiation of hESCs to microglia**

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<td>STEMdiff™ Hematopoietic Kit</td>
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<td>EasySep™ Human APC Positive Selection Kit II</td>
<td>17661</td>
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<td>MEM Non-Essential Amino Acids Solution (100X)</td>
<td>11140050</td>
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<td>Glutamax</td>
<td>35050-061</td>
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<tr>
<td>N2 Supplement 100x</td>
<td>17502-048</td>
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<td>B-27® Supplement (50X), serum free</td>
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<tr>
<td>StemPro® Accutase® Cell Dissociation Reagent</td>
<td>A1110501</td>
<td>ThermoFisher Scientific</td>
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<tr>
<td>DMEM/F-12, no phenol red</td>
<td>21041025</td>
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<tr>
<td>Insulin-Transferrin-Selenium (ITS -G) (100X)</td>
<td>41400045</td>
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<tr>
<td>Penicillin/Streptomycin, 10,000U/mL</td>
<td>15140-122</td>
<td></td>
</tr>
<tr>
<td>recombinant human M-CSF1</td>
<td>300-25-10</td>
<td>LONZA</td>
</tr>
<tr>
<td>recombinant human TGF-b1</td>
<td>100-21-50</td>
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<tr>
<td>Recombinant human IL-34</td>
<td>200-34</td>
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<tr>
<td>Recombinant human CX3CL1</td>
<td>300-31-50</td>
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<tr>
<td>Recombinant human CD200</td>
<td>C311-50ug</td>
<td>Jomar Life Research</td>
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<tr>
<td>Biolegend CD43-APC Clone 10-G7</td>
<td>343206</td>
<td>Australian Biosearch</td>
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## Table 3: Reagents required for differentiation of hESCs towards midbrain dopaminergic neurons

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<tr>
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<tr>
<td>MEM Non-Essential Amino Acids Solution (100X)</td>
<td>11140050</td>
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<td>B-27® Supplement (50X), minus Retinoic Acid A</td>
<td>12587010</td>
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<td>Knock-Out Serum Replacement</td>
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<td>Glutamax II</td>
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<tr>
<td>N2 Supplement 100x</td>
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<tr>
<td>Natural Mouse Laminin</td>
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<tr>
<td>KnockOut™ DMEM 1x</td>
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<tr>
<td>Neurobasal Media</td>
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<tr>
<td>StemPro® Accutase® Cell Dissociation Reagent</td>
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<tr>
<td>Beta-mercaptoethanol</td>
<td>21985-023</td>
<td>Ajax FineChem</td>
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<tr>
<td>DMEM/F12 powder</td>
<td>12660012</td>
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<td>Glucose</td>
<td>AJA783</td>
<td>In Vitro Technologies</td>
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<tr>
<td>Sodium Bicarbonate</td>
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<td>recombinant human TGFB3</td>
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<td>recombinant human GDNF</td>
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<tr>
<td>recombinant human Activin A</td>
<td>120-14E</td>
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<td>Selleck Chemicals DAPT (GSI-IX)</td>
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<td>Jomar Life Research</td>
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<tr>
<td>Selleck Chemicals LDN-193189</td>
<td>S7507-10mg</td>
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<tr>
<td>Selleck Chemicals SB431542</td>
<td>S1067-50mg</td>
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<tr>
<td>Selleck Chemicals Y-27632 dihydrochloride</td>
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<tr>
<td>Selleck Chemicals ChiR-99026</td>
<td>S2924-25mg</td>
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<tr>
<td>Selleck Chemicals Purmorphamine</td>
<td>S3042-25mg</td>
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<tr>
<td>dibutyryl cyclicAMP</td>
<td>D0627-1G</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Ascorbic Acid</td>
<td>A4544</td>
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<tr>
<td>Human Apo-Transferrin</td>
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<tr>
<td>Poly-L-Ornithine</td>
<td>P4957-50mL</td>
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</tr>
<tr>
<td>Human Insulin (solution) 10mg/mL</td>
<td>I9278</td>
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<tr>
<td>Reagent</td>
<td>Category number</td>
<td>Supplier</td>
</tr>
<tr>
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<td>Tocris PD 0325901</td>
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<tr>
<td>Tocris XAV 939</td>
<td>RDS374850</td>
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<tr>
<td>Tocris SU 5402</td>
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<tr>
<td>Ascorbic Acid</td>
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<tr>
<td>D-Serine</td>
<td>S4250-5G</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>dibutyrylcyclicAMP</td>
<td>D0627-1G</td>
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</tr>
<tr>
<td>poly-L-ornithine</td>
<td>P4957-50mL</td>
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<td>Fibronectin, Liquid, 0.1% solution, BioReagent, suitable for cell culture, 1mg</td>
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<tr>
<td>Recombinant human BDNF</td>
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<td>LONZA</td>
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<td>Selleck Chemicals DAPT (GSI-IX)</td>
<td>S2215-50mg</td>
<td>Jomar Life Research</td>
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<tr>
<td>Selleck Chemicals LDN-193189</td>
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<td>Selleck Chemicals SB431542</td>
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<td>Selleck Chemicals Y-27632 dihydrochloride</td>
<td>s1049-50mg</td>
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<td>Vitronectin-N</td>
<td>A14700</td>
<td>ThermoFisher Scientific</td>
</tr>
<tr>
<td>Essential 6 media, 500mL</td>
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<td>B27 with Retinoic Acid, serum free, 10 mL</td>
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<tr>
<td>Penicillin/Streptomycin, 10,000U/mL</td>
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<td></td>
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<tr>
<td>N2 supplement</td>
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<tr>
<td>Knockout DMEM/F12</td>
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<tr>
<td>Neurobasal media</td>
<td>10829018</td>
<td></td>
</tr>
<tr>
<td>Glutamax</td>
<td>35050-061</td>
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</tr>
<tr>
<td>mouse-laminin</td>
<td>23017015</td>
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### Table 5: Reagents required for Cytometric Bead Array

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<tr>
<th>Reagent</th>
<th>Category number</th>
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<tbody>
<tr>
<td>Human IL-1alpha CBA Flex Set (D6)</td>
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<tr>
<td>Human IL-1beta CBA Flex Set (B4)</td>
<td>558279</td>
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<tr>
<td>Human VEGF CBA Flex Set (B8)</td>
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<tr>
<td>Human TNF CBA Flex Set (C4)</td>
<td>560112</td>
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<tr>
<td>Human IL-4 CBA Flex Set (A5)</td>
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<tr>
<td>Human IL-6 CBA Flex Set (A7)</td>
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<td>Human IL-8 CBA Flex Set (A9)</td>
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<tr>
<td>Human IL-10 CBA Flex Set (B7)</td>
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<td>Human IFN-α CBA Flex Set (E7)</td>
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<tr>
<td>Human MIP-18 CBA Flex Set (B9)</td>
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<td>Human Fractalkine CBA Flex Set (C6)</td>
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<tr>
<td>Human Soluble Protein Master Buffer Kit, 500 tests, (Assay Diluent, Capture Bead Diluent, Capture Bead Diluent for Serum/Plasma Samples, Detection Reagent Diluent, Wash Buffer, Instrument Setup Beads)</td>
<td>558265</td>
<td>BD Biosciences</td>
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Table 6: Reagents required for RNA, DNA and Protein Extractions

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<th>Reagent</th>
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<tr>
<td>ISOLATE II Genomic DNA Kit - 50 reactions</td>
<td>BIO-76001</td>
<td>Bioline</td>
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<td>SensiFAST™ Probe No-ROX One-Step Kit. 100 rxn</td>
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<td>ISOLATE II RNA/DNA/PROTEIN ISOLATION KIT</td>
<td>BIO-52085</td>
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<td>Trisure reagent</td>
<td>BIO-38032</td>
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<td>Agarose</td>
<td>BIO-41025</td>
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<tr>
<td>Proteinase K</td>
<td>BIO-37039</td>
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<tr>
<td>Phusion High-Fidelity PCR Master Mix with HF Buffer (500 tests)</td>
<td>F531L</td>
<td>ThermoFisher Scientific</td>
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<tr>
<td>Phusion High-Fidelity PCR Master Mix with GC Buffer (500 tests)</td>
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<td>Ncol-High Fidelity Restriction Endonuclease</td>
<td>R3193S</td>
<td>NEB</td>
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<tr>
<td>Scal Restriction Endonuclease</td>
<td>R3122S</td>
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</tr>
<tr>
<td>Sacl Restriction Endonuclease</td>
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<tr>
<td>EcoRI-High Fidelity Restriction Endonuclease</td>
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<td>Generuler 1KB ladder</td>
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<tr>
<td>Purple Loading Dye</td>
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### Table 7: Reagents required for CRISPR Targeting and Southern Blotting

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<th>Reagent</th>
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<td>P3 Primary Cell 4D-NucleofectorTM X Kit L</td>
<td>V4XP-3024</td>
<td>LONZA</td>
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<td>2',DeoxyadenosideTriphosphate[Alpha-32P], 10mCi/mL, 250uCi</td>
<td>BLU012H250uC</td>
<td>Perkin Elmer</td>
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<td>Geneticin™ Selective Antibiotic (G418 Sulfate) (50 mg/mL)</td>
<td>10131035</td>
<td>Life Technologies</td>
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<td>ULTRAhyb® Ultrasensitive (125 mL) Hybridization Buffer</td>
<td>AM8670</td>
<td>Life Technologies</td>
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<tr>
<td>DECAprime™ II DNA Labeling Kit (30 reactions)</td>
<td>AM1455</td>
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<td>NucAway Spin Columns</td>
<td>AM10070</td>
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<tr>
<td>Amersham Hybond-N+ membrane</td>
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<td>GE Life Sciences</td>
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### Table 8: Reagents required for culture treatments

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<th>Reagent</th>
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<td>eBioscience™ Lipopolysaccharide (LPS) Solution (500X)</td>
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<td>Life Technologies</td>
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<td>IFN-γ Recombinant Human Protein, 1mg</td>
<td>PHC4033</td>
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<tr>
<td>Recombinant human Alpha-synuclein protein aggregate, 100ug</td>
<td>ab218819</td>
<td>Abcam</td>
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<tr>
<td>Biolegend TNFalpha, 100ug</td>
<td>570106</td>
<td>Australian Biosearch</td>
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<tr>
<td>1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), 25g</td>
<td>105228-25G</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Reagent</td>
<td>Catalogue number</td>
<td>Supplier</td>
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<tr>
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<td>------------------</td>
<td>------------------------------</td>
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<tr>
<td>rabbit Anti-Pu.1 unconjugated antibody, 100uL</td>
<td>ab76543</td>
<td>Abcam</td>
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<tr>
<td>goat anti-Iba1 unconjugated Antibody, 100ug</td>
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<tr>
<td>rabbit anti-IRF8 unconjugated antibody, 100uL</td>
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<td>100uL Anti-human-TMEM119</td>
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<td>Anti-IL1 alpha antibody</td>
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<tr>
<td>Anti-TNF alpha antibody</td>
<td>ab9635</td>
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<tr>
<td>Anti-C1QA antibody [EPR2980Y]</td>
<td>ab76425</td>
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<tr>
<td>Anti-iNOS antibody [EPR16635]</td>
<td>ab178945</td>
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<td>Anti-Arginase antibody [ARG1/1125] - BSA and Azide free</td>
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<tr>
<td>APC Conjugation Kit (30ug)</td>
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<td>Anti-EAAT1 antibody</td>
<td>ab416</td>
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<td>Anti-Beta3 Tubulin, Chicken Polyclonal, 100ug</td>
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<td>donkey anti-mouse IgG H&amp;L secondary antibody, 500ug</td>
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<td>Donkey Anti-Rabbit IgG H&amp;L (Alexa Fluor® 488), 500ug</td>
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<td>anti-human-TREM2 antibody, 25 ug</td>
<td>AF1828-SP</td>
<td>In Vitro Technologies</td>
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<td>50ug anti-human IBA1 antibody</td>
<td>1919741</td>
<td>Novachem [Australia]</td>
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<td>IL-10 Monoclonal Antibody (JES3-9D7), 500ug</td>
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<td>ThermoFisher Scientific</td>
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<td>IL-4 Monoclonal Antibody (8D4-8), eBioscience™, 50ug</td>
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<tr>
<td>IL-1 beta Monoclonal Antibody (ILB1-H67), 500ug</td>
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<td>Dulbecco’s Phosphate Buffered Saline, 1x, no calcium, no magnesium, 10x 500mL</td>
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<tr>
<td>Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, 500uL</td>
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<tr>
<td>CellMask™ Deep Red Plasma membrane Stain</td>
<td>C10046</td>
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<td>8mm Round Cover Glass, #1.5(T), 100/pk</td>
<td>72296-08 PD25,</td>
<td>Emgrid Australia</td>
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rabbit anti-P2RY12 unconjugated antibody, 100ug

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<td>pHrodo Green Ester</td>
<td>P35369</td>
<td>ThermoFisher Scientific</td>
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<td>Hank’s Buffered Salt Solution</td>
<td>14025</td>
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<tr>
<td>DimethylSulphoxide</td>
<td>D8418-250ML</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>α-select chemically competent e.coli</td>
<td>BIO-85027</td>
<td>Bioline</td>
</tr>
<tr>
<td>Slides, microscope, frosted one end, size 25 mm × 75 mm, Box</td>
<td>S8400-1PAK</td>
<td>Sigma Aldrich</td>
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</tbody>
</table>
Appendix II
Primer validation for screening targeted lines

**CX3CR1 primer validation**

Primers were designed using a combination of Snapgene software and Basic Local Alignment Search Tool (BLAST). To confirm primers could successfully bind within and detect the plasmid, primers were designed that would bind to the short homology arm and the PGK-promotor region (see Figure 1). Similarly, primers that were able to detect WT genomic DNA were designed which bound within the short genomic arm (see Figure 2). Table 11 lists the primers used for CX3CR1 targeting, the expected band sizes, and PCR cycling conditions used.

**Table 11: CX3CR1 screening primers.**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5’- 3’</th>
<th>Primer Pairs</th>
<th>PCR conditions</th>
</tr>
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<tbody>
<tr>
<td>CX3CR1 Vec-Gen_FWD</td>
<td>CATGCTCCAGACTGCCTTG</td>
<td>Confirmation Primers</td>
<td>Phusion High Fidelity (HF) with 6% DMSO, 3-step protocol, annealing temp = 66°C, 30sec/kb extension</td>
</tr>
<tr>
<td>CX3CR1 Genomic_FWD</td>
<td>AAATGATGGACCCAATGCA</td>
<td>Validate confirmation primers</td>
<td></td>
</tr>
<tr>
<td>CX3CR1 Gen-Vec_Rev</td>
<td>GTCTGGACGGGTGAATACAG</td>
<td>CX3CR1Vec-GenFWD &amp; CX3CR1 Genomic _REV = 1704 bp</td>
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</tr>
<tr>
<td>CX3CR1 Genomic_Rev</td>
<td>AGCGAGCATAATTGTGGTC</td>
<td>CX3CR1Genomic FWD &amp; CX3CR1Genomic_REV = 1596 bp</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1: A vector map of CX3CR1 targeting donor plasmid. The CX3CR1-Gen-Vec_REV and CX3CR1-VECGen_FWD primers in purple text indicate binding within the PGK promotor and the CX3CR1 Short Homology arm.

Figure 2: Visualisation of the genomic primers and their binding sites within the CX3CR1 gene. Both the Genomic FWD and Genomic REV primers bind to the short homology arm.
Primers for vector DNA were validated using plasmid DNA and by checking the annealing temperature using Phusion HF, with and without DMSO, as seen in Figure 3a-b.

Figure 3: 1% agarose gels, run at 90Volts for 45 minutes. Ladder is GeneRuler 1KB ladder, with 3000, 1500bp, 1000bp and 500bp bands highlighted by arrows. Vector DNA primers are: **CX3CR1 Vec-Gen_FWD**: CATGCTCC AGACTGCCTTG, and **CX3CR1 Gen-Vec_Rev**: GTCTGGACGGGTGAATACAG, expected band size – 1704bp. Gel A is Phusion High Fidelity Buffer without DMSO, and Gel B is Phusion High Fidelity Buffer with 6% DMSO. Both conditions appear to strongly amplify the band of interest across all temperatures tested, but also have a strong secondary band of approximately 200-300bp at the bottom.
Analysis of the vector primers indicated a second binding site for the CX3CR1-GEN-VEC-FWD within the short homology arm, which would give rise to a second band of 238bp. To resolve this, a 10-fold dilution curve for PCR (Figure 4) was performed, and all further work based on the results.

Figure 4: Primer concentration curve for Vector primers using: **CX3CR1 Vec-Gen_FWD**: CATGCTCC AGACTGCCTTG, and **CX3CR1Gen-Vec_Rev**: GTCTGGACGGGTGAATACAG, expected band size – 1704bp and 238bp on a 1% agarose gel, run at 90Volts for 45 minutes. Ladder is GeneRuler 1KB ladder, with 3000, 1500bp, 1000bp and 500bp bands highlighted by arrows. Phusion High Fidelity Buffer with 6% DMSO Enzyme mix used. A single 10-fold dilution (5μM to 0.5μM) was sufficient to remove the amplification of the second band, while retaining the band of interest (1704bp)
Once vector primers were validated, genomic DNA primers required validation of their annealing temperature. As Phusion High Fidelity with 6% DMSO gave clean bands of interest after determination of primer concentration, these conditions were used for the validation of genomic primers.

![Agarose gel image](image_url)

*Figure 5: 1% agarose gels, run at 90Volts for 45 minutes. Ladder is GeneRuler 1KB ladder, 3000, 1500bp, 1000bp and 500bp bands highlighted by arrows. Genomic DNA Primers are: **CX3CR1Genomic_FWD** AAATGATGGACCCAATGCAC and **CX3CR1Genomic_Rev** AGCGAGCACTATTTGTGGTC, expected band size = 1596. Phusion High Fidelity Buffer with 6% DMSO Enzyme mix used. All temperatures used except for 58°C gave clear bands and had no non-specific binding.*
**TREM2 primer validation**

As for section 1, primers were designed using a combination of Snapgene software and Basic Local Alignment Search Tool (BLAST). To confirm primers could successfully bind within and detect the plasmid, primers were designed that would bind to the short homology arm and the PGK-promotor region (see Figure 6). Similarly, primers that were able to detect WT genomic DNA were designed which bound within the short genomic arm (see Figure 7). Table 12 lists the primers used for *TREM2* targeting, the expected band sizes, and PCR cycling conditions used.

**Table 12: *TREM2* Screening primers.**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5'-&gt; 3'</th>
<th>Primer Pairs</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>TREM2-Vec-Gen_FWD</td>
<td>GTAGAATTGACCTGCA GGGG</td>
<td>Confirmation Primers Vec-GenFWD &amp; Genomic _REV= 1647 bp</td>
<td>Phusion High Fidelity with 5% DMSO at 64 deg annealing</td>
</tr>
<tr>
<td>TREM2-Genomic_FWD</td>
<td>CACTTTGACATGTGG TTGG</td>
<td>Validate confirmation primers Vect-GenFWD &amp; Gen_Vec REV = 1502bp</td>
<td></td>
</tr>
<tr>
<td>TREM2-Gen-Vec_Rev</td>
<td>TGAACACTTGCTCTGT GCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TREM2-Genomic_Rev</td>
<td>TGGAGTCATAGGGGC AAGAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 6: A vector map of TREM2 targeting donor plasmid. The TREM2-Gen_Vec_REV and TREM2-VECGEN_FWD primers in purple text indicate binding within the PGK promotor and the TREM2 Short Homology arm.

Figure 7: Visualisation of the genomic primers and their binding sites within the TREM2 gene. Both the Genomic FWD and Genomic REV primers bind to the short homology arm.
Primers for TREM2 vector DNA were validated using plasmid DNA and by checking the annealing temperature using Phusion HF, with and without DMSO, as seen in Figure 8A/B. It was found that all conditions appeared to work equally well, and did not appear to form primer dimers or amplify non-specific bands. Erring on the side of caution, using Phusion High Fidelity supplemented with 6% DMSO for all future PCR was implemented.

Figure 8: 1% agarose gels, run at 90Volts for 45 minutes. Ladder is GeneRuler 1KB ladder, with 3000, 1500bp, 1000bp and 500bp bands highlighted by arrows. Vector DNA primers are: TREM2-Vec-Gen_FWD GTGAATTCCTGACAGGGG and TREM2-Gen-Vec_Rev TGAACACTTGCTCTGTGCAG, expected band size – 1520bp. Gel A is Phusion High Fidelity Buffer without DMSO, and Gel B is Phusion High Fidelity Buffer with 6% DMSO. Both conditions appear to strongly amplify the band of interest across all temperatures tested, but also have a strong secondary band of approximately 200-300bp at the bottom.
Once vector primers were validated, genomic DNA primers needed validation. *TREM2* genomic primers were confirmed to work at a range of temperatures when using Phusion High Fidelity with 6% DMSO, and increasing temperature reduced the prevalence of non-specific binding of a larger band, as seen in Figure 9.

Figure 9: 1% agarose gels, run at 90Volts for 45 minutes. Ladder is GeneRuler 1KB ladder, with 3000, 1500bp, 1000bp and 500bp bands highlighted by arrows. Genomic DNA Primers are: **TREM2-Genomic_FWD** CACTTTGAGCATGTGGTTGG and **TREM2-Genomic_Rev** TGGAGTCATAGGGCAAGAC, expected band size = 1471bp. Phusion High Fidelity Buffer with 6% DMSO Enzyme mix used. All temperatures used gave clear bands and had no non-specific binding.
**TMEM119 Primer Validation.**

As for *CX3CR1* targeting, primers were designed using a combination of Snapgene software and Basic Local Alignment Search Tool (BLAST). To confirm primers could successfully bind within and detect the plasmid, primers were designed that would bind to the short homology arm and the PGK-promotor region (see Figure 10). Similarly, primers that were able to detect WT genomic DNA were designed which bound within the short genomic arm (see Figure 11). Table 13 lists the primers used for *TMEM119* targeting, the expected band sizes, and PCR cycling conditions used.

![Figure 10: A vector map of TMEM119 targeting donor plasmid. The TMEM119-Gen-Vec_REV, TMEM119-VECGEN_FWD primers in purple text indicate binding within TMEM119 Short Homology arm.](image_url)
Figure 11: Visualisation of the genomic forward primer and binding sites within the TMEM119 gene. The Genomic FWD Primer binds to the short homology arm.

Table 13: TMEM119 Primers and PCR cycling conditions.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5'-&gt; 3'</th>
<th>Primer Pairs</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vec-Gen_FWD</td>
<td>CGACTCTAGCGATATCGAGC</td>
<td>Confirmation Primers</td>
<td>Phusion High Fidelity with 6% DMSO at 64 deg annealing</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>Vec-GenFWD &amp; Genomic_REV= 2157 bp</td>
<td></td>
</tr>
<tr>
<td>Genomic_FWD</td>
<td>TTCCTGACACTCCCTG</td>
<td>Validate confirmation primers</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>Vec-GenFWD &amp; Gen_Vec_REV = 1895bp</td>
<td></td>
</tr>
<tr>
<td>Gen-Veg_Rev</td>
<td>ACATGAGTTCCAGCAGG</td>
<td>Genomic_FWD &amp; Genomic_REV = 2042 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genomic_Rev</td>
<td>TGCCATCAAGACCAAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Primers for TMEM119 vector DNA were validated using plasmid DNA and by checking the annealing temperature using Phusion HF, with and without DMSO, as seen in Figure 12. It was found that although there appeared to be some non-specific binding occurring in the absence of DMSO (Figure 12 – A), addition of DMSO and increasing annealing temperature removed these non-specific bands (Figure 12 – B)
Figure 12: 1% agarose gels, run at 90Volts for 45 minutes. Ladder is GeneRuler 1KB ladder, with 3000, 1500bp and 1000bp bands highlighted by arrows. Vector DNA primers are: **TMEM119-Vec_Gen_FWD** CGACTCTAGCGATATCGAGC and **TMEM119-Gen-Vec_Rev** ACATGAGTCCAGCCCATTTC expected band size = 1895bp. Gel A is Phusion High Fidelity Buffer without DMSO, and Gel B is Phusion High Fidelity Buffer with 6% DMSO.(B) 64 NTC bands have non-specific binding due to contamination with unknown DNA, however the NTC in (A) is clean. Both conditions appear to strongly amplify the band of interest across all temperatures tested, but also have a strong secondary band of approximately 200-300bp at the bottom.
Figure 13: 1% agarose gels, run at 90Volts for 45 minutes. Ladder is GeneRuler 1KB ladder, with 3000, 2000bp and 1000bp bands highlighted by arrows. Genomic DNA Primers are: **TMEM119 Genomic FWD:** TTCCTGACACTCCCTCCTTG and **TMEM119-Genomic_Rev** TGCCATCAAGACCAAGG, expected band size = 2042bp. Phusion High Fidelity Buffer with 6% DMSO Enzyme mix used. All temperatures used gave clear bands and had no non-specific binding.
**PU.1 Targeting**

As for section 1, primers were designed using a combination of Snapgene software and Basic Local Alignment Search Tool (BLAST). To confirm primers could successfully bind within and detect the plasmid, primers were designed that would bind to both homology arms and the NeoKan Resistance Cassette (see Figure 14). Similarly, primers that were able to detect WT genomic DNA were designed which bound within the short genomic arm (see Figure 15). Table 14 lists the primers used for PU.1 targeting, the expected band sizes, and PCR cycling conditions used. Unlike primers used in CX3CR1/TREM2 and TMEM119 targeting, the melting point of these primers was too high for typical three-step cycling conditions. Therefore, the Phusion mastermixes with 2-step cycling conditions were used as per manufacturer’s recommendations.

**Table 14: PU.1 Screening Primers**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5'-&gt; 3'</th>
<th>Primer Pairs</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>PU.1 R arm Vec-Gen FWD</td>
<td>GCCCTCGAATCGTGGATCCACT</td>
<td>Confirmation Primers Vec-GenFWD &amp; Genomic REV = 1526 bp</td>
<td>Phusion GC-Rich buffer with 6% DMSO using the 2 step cycle (72 degrees annealing and extension combined)</td>
</tr>
<tr>
<td>PU.1 R arm Gen-Vel REV</td>
<td>AGTGAGCCATGTTCCGTGCACACT</td>
<td>Validate confirmation primers Vec-GenFWD &amp; Gen_Vel REV = 1393bp</td>
<td></td>
</tr>
<tr>
<td>PU.1 R arm Genomic FWD</td>
<td>GCTGGCATAGGATTAGGCCCTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PU.1 R arm Genomic REV</td>
<td>CCATTTCGCCCTTCTTGTGGCCCTG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 14: A vector map of PU.1 targeting donor plasmid. The PU.1-Gen-Vec_REV, PU.1-VEC-GEN_FWD primers in purple text indicate binding within PU.1 Short Homology arm, while the PU.1 LHA FWD, LHA-Genomic REV indicate binding within the Long Homology arm, and the NeoKan FWD primer indicates binding within the Neomycin/Kanamycin Resistance Cassette.

Figure 15: Visualisation of the genomic forward primer and binding sites within the PU.1 gene. PU.1 LHA FWD binds within the Long Homology Arm, while the PU.1 SHA FWD and PU.1 SHA REV both bind to the short homology arm.
As discussed above, the primers for PU.1 had a higher than average melting point, which made 3-step PCR cycling conditions redundant. Here, 2-step cycling conditions were used to confirm the appropriate buffer system for PCR. The clearest bands for Vector primers were obtained using the GC-rich buffer with added 5% DMSO (Figure 16).

Next, genomic primers were validated in the same way as vector primers. 2-step conditions were used to confirm the appropriate buffer system for PCR. Only one condition allowed Genomic primers to amplify, the GC-rich buffer with added 5% DMSO.
Figure 17: 1% agarose gels, run at 90Volts for 45 minutes. Ladder is GeneRuler 1KB ladder, with 1500bp, 1000bp and 500bp bands highlighted by arrows. Genomic DNA Primers are: **PU.1 Genomic FWD**: GCTGGCCATAGCATTAAGCCCTCG and **PU.1 Genomic REV**: CCATTGGCTTCTCTGTGGCCCTG, expected band size = 1414bp. HF = High Fidelity Master Mix, HFD = High Fidelity Master Mix + 5% DMSO, GC = GC Rich Buffer Master Mix, GCD = GC Rich Buffer Master Mix + 5% DMSO.
IRF8 Primer Validation

As for section 1, primers were designed using a combination of Snapgene software and Basic Local Alignment Search Tool (BLAST). To confirm primers could successfully bind within and detect the plasmid, primers were designed that would bind to both homology arms, the LacZ cassette and m-Cherry Regions (see Figure 18). Similarly, primers that were able to detect WT genomic DNA were designed which bound within the short genomic arm (see Figure 15). Table 145 lists the primers used for IRF8 targeting, the expected band sizes, and PCR cycling conditions used. Unlike primers used in CX3CR1/TREM2 and TMEM119 targeting, the melting point of these primers was too high for typical three-step cycling conditions. Therefore, the Phusion master mixes with 2-step cycling conditions were used as per manufacturer’s recommendations.

Table 15: IRF8 Screening Primers

<table>
<thead>
<tr>
<th>IRF8 Long arm GEN FWD</th>
<th>GGACCAGAGCTCTGCAGCTCTAG</th>
<th>Confirmation Primers</th>
<th>Phusion HF with 6% DMSO at 71 degree annealing temperature, 30sec/kb extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-Cherry IRF8 Reverse</td>
<td>CGGTCTGGGTGCCCTCGTAG</td>
<td>m-Cherry rev and IRF8 L arm Gen FWD = 4541 bp Validate confirmation primers</td>
<td></td>
</tr>
<tr>
<td>M-Cherry IRF8 FWD</td>
<td>CTACGAGGGCACCCAGACCG</td>
<td>mCherry FWD and LacZ TV REV =1014 bp L-Arm Gen FWD and R-arm GEN-Vect Vec REV = 5872 bp</td>
<td></td>
</tr>
<tr>
<td>Lac-Z REV TV</td>
<td>GTGAGCGAGTAACACCCGC GG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRF8 R-Arm GEN_VEC REV</td>
<td>GTGGAAGTTGCAGGGAGCCGA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 18: A vector map of IRF8 targeting donor plasmid. The IRF8-Gen-FWD2 primer binds within the Short Homology arm, IRF8 Long arm GEN FWD binds to the long homology arm, LacZ REV and m-Cherry FWD primers bind to the LacZ and mCHerry regions of the plasmid respectively.

First, the internal vector plasmids were validated to confirm detection of both the LacZ and m-cherry regions (Figure 21). It was possible to successfully amplify the 1014bp product under all testing conditions.

Figure 19: Visualisation of where the IRF8 Long arm GEN-FWD and IRF8 R-Arm GEN FWD 2 primers sit within the IRF8 genomic region.
Figure 20: 1% agarose gels, run at 90Volts for 45 minutes. Ladder is GeneRuler 1KB ladder, with 1500bp, 1000bp and 500bp bands highlighted by arrows. Vector DNA Primers are M-Cherry IRF8 FWD CTACGAGGGCACCCAGACCG and Lac-Z REV TV GTGAGCGAGTAACAACCCGTCG, expected band size = 1014bp. HF = High Fidelity Master Mix, HFD = High Fidelity Master Mix + 5% DMSO, GC = GC Rich Buffer Master Mix, GCD = GC Rich Buffer Master Mix + 5% DMSO.

Once the Vector primers were validated, genomic primers were checked. Initially, primers designed for the IRF8 genomic regions failed, forcing a redesign to place the primers into the long homology arm. The nearly 6000bp product was, however, successfully amplified under all conditions tested (see Figure 21). Although the product was large, a typical 3-step PCR cycle with an annealing temperature of 68°C was sufficient to allow amplification.
Figure 21: 0.8% agarose gels, run at 90Volts for 1 hour and 45 minutes. Ladder is GeneRuler 1KB ladder, with 6000bp, 3000bp and 1000bp bands highlighted by arrows. IRF8 gDNA Primers are: IRF8 Long arm GEN FWD: GGACCAGAGCTTCTGCAGCTCTAG and IRF8 R-Arm GEN_VEC REV GAGAAAGTTCAGGAGGAGGCGA, expected band size = 5872bp. HF = High Fidelity Master Mix, HFD = High Fidelity Master Mix + 5% DMSO, GC = GC Rich Buffer Master Mix, GCD = GC Rich Buffer Master Mix + 5% DMSO.
Appendix III
Karyotyping results

Figure 22: G-banding karyogram for CX3CR1-H9-hES cells. Chromosome 12 (blue box) clearly displays trisomy. G-banding karyotyping was performed by Monash Pathology services
Figure 23: Karyotyping report from Monash Pathology for H9-CX3CR1 targeted cells
Figure 24: G-bandering karyogram for TMEM119-H9 cells. Chromosome 12 (blue box) displays clear trisomy. G-bandering karyotyping was performed by Monash Pathology Services.
Figure 25: Karyotyping report from Monash Pathology for H9-TMEM119-H9 targeted cells
Figure 26: RNA-seq comparison for chromosome 12 between CX3CR1 cells (here referred to as “Grubman” – n=3), and published data on stem-cell derived microglia (here referred to as Abud). All data cluster closely together and there are no significant differences between genes analysed (genes are identified by “G00000xxx”. Sequencing data obtained from GSE89189.
Figure 27: Karyogram for TREM2-H9 cells. Chromosome 12 (blue box) displays clear trisomy. G-bandining karyotyping was performed by Monash Pathology Services.
Figure 28: Karyotyping report from Monash Pathology for H9-TREM2 targeted cells
Figure 29: Karyogram for IRF8 targeted cells. Chromosome 12 (blue box) highlights supernumerary isochromosome presence.
Figure 30: Karyotype report from Monash Pathology for H9-IRF8 targeted cells.

**Figure 30: Karyotype report from Monash Pathology for H9-IRF8 targeted cells.**

**Figure 30: Karyotype report from Monash Pathology for H9-IRF8 targeted cells.**

**Figure 30: Karyotype report from Monash Pathology for H9-IRF8 targeted cells.**
Figure 31: Karyogram for WT untargeted H9 cells. Chromosome 12 (blue box) highlights supernumerary isochromosome presence.
Figure 32: Karyotype report from Monash Pathology for H9-WT.
Appendix IV
Calculations for movement of microglia during phagocytosis.

To calculate the distance moved, the centre of the soma in Figure 33, Frame A and Frame B were measured in Fiji for x,y coordinates. Addition of a straight line creates the hypotenuse of an imaginary triangle (see Figure 33 Frame C).

If $a^2 + b^2 = c^2$, and if x and y coordinates are described as “a” and “b”, then $c = \sqrt{((x_1 - x_2)^2 + (y_1 - y_2)^2)}$.

Here, the coordinates of microglial soma are (35.89, 22.65) and (45.26, 49.44) for A and B respectively.

$c = \sqrt{((45.26 - 35.89)^2 + (49.44 - 22.65)^2)}$

Therefore c (distance between soma in Frame A vs Frame B) = 28.38µm
Figure 33: stills from Figure 23 in main thesis body, frames (A) and (L) (here labelled (A) and (B)), to illustrate the motility of microglia and the directional movement. In both (A) and (B), the centre of the microglia is highlighted (white circle). In C, the distance between the two circles is highlighted by the red, double headed arrows. Scale bar = 20µm.
## Appendix V

Comparison of published stem cell derived microglia protocols

*Table 16: Published stem cell protocols and their differences.*

<table>
<thead>
<tr>
<th>Author</th>
<th>iPSC vs hESC</th>
<th>2d or 3d?</th>
<th>Presence of serum?</th>
<th>Differentiation Length</th>
<th>Was coculture performed?</th>
<th>What cells were used for coculture?</th>
<th>How was inflammation induced?</th>
<th>Cytokines measured?</th>
<th>How were cytokines measured?</th>
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<tbody>
<tr>
<td>Abud et al 2017</td>
<td>ipsc lines</td>
<td>2d</td>
<td>no</td>
<td>38 days</td>
<td>yes</td>
<td>rat hippocampal/cortical neurons / 3d brainoids</td>
<td>LPS/IFNγ/IL-1b</td>
<td>TNF-a, IL-6, IL-8, IL-10, IL-1a, CCL2, CCL3, CCL4, CXCL10, CCL17</td>
<td>ELISA</td>
</tr>
<tr>
<td>Amos et al 2017</td>
<td>iPSC/H1</td>
<td>3d EB's</td>
<td>FBS</td>
<td>40 days</td>
<td>no</td>
<td></td>
<td>LPS</td>
<td>IL-1b, CCL2, TNFAIP3</td>
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</tr>
<tr>
<td>Brownjohn et al 2017</td>
<td>iPSCs</td>
<td>3d EB's</td>
<td>FBS</td>
<td>25-35 days</td>
<td>yes</td>
<td>3d cortical brainoids</td>
<td>LPS</td>
<td>IL-1b, IL-6, TNFa</td>
<td>ELISA</td>
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<tr>
<td>Douvaras et al 2018</td>
<td>iPSC, RUES, H9</td>
<td>2d</td>
<td>no</td>
<td>60 days</td>
<td>No</td>
<td></td>
<td>IFNγ/LPS, IL-10, IL-13/IL-4</td>
<td>RANTES, I-TAC, BAFF, GR0-a, and MIP3a</td>
<td>Human XL Cytokine Array</td>
</tr>
<tr>
<td>Haensler et al 2018</td>
<td>iPSC</td>
<td>3d EB's</td>
<td>no</td>
<td>60-75 days</td>
<td>yes</td>
<td>cortical neurons</td>
<td>LPS/IFNγ</td>
<td>See Haenseler et al 2018 for full list.</td>
<td>Luminex multiplex assay</td>
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<tr>
<td>Study</td>
<td>Cell Line</td>
<td>Time Window</td>
<td>Treatment</td>
<td>Outcome</td>
<td>Model</td>
<td>Results</td>
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<tr>
<td>McQuade et al 2018</td>
<td>iPSC</td>
<td>2d</td>
<td>no</td>
<td>36-38 days</td>
<td>yes</td>
<td>xenotransplantation-compatible MITRG mice</td>
<td>not measured</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesci et al 2018</td>
<td>iPSC</td>
<td>2d</td>
<td>no</td>
<td>60 days</td>
<td>yes</td>
<td>neural precursor cells</td>
<td>LPS</td>
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<tr>
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<td>74 days</td>
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<td>forebrain</td>
<td>LPS/IFNγ</td>
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</table>

Results include cytokines and chemokines produced in response to treatment, measured using Cytometric Bead Array by BD Biosciences or Semiquantitative membrane assay.
<table>
<thead>
<tr>
<th>Study</th>
<th>Type</th>
<th>Treatment</th>
<th>Duration</th>
<th>Feeders</th>
<th>Days</th>
<th>Cytokines/Chemokines</th>
<th>Assay</th>
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<tr>
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<td>iPSCs</td>
<td>Yes with feeders</td>
<td>25 days</td>
<td>No</td>
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<td>IL21, IL27, IL32a, MIF, sERpE1, TNF, TREM1</td>
<td>Multiplex</td>
</tr>
<tr>
<td>Takata et al 2018</td>
<td>iPSCs</td>
<td>Yes with feeders</td>
<td>26 days</td>
<td>Yes</td>
<td>2d</td>
<td>IL-1a, IL-1b, IL-6, TNF, IL12p3, IL-10, IL-18, IL-1Ra, IL-18</td>
<td>Luminex</td>
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<tr>
<td>Xu et al 2019</td>
<td>iPSCs</td>
<td>No</td>
<td>37 days</td>
<td>No</td>
<td></td>
<td>IL-1b, IL-2, IL-6, TNF, RANTES, CXCL1, CXCL2, CCL17, CCL22</td>
<td>Multiplex</td>
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</table>
Appendix VI
Manuscript submitted to Stem Cell Reports.

A CX3CR1 reporter hESC line facilitates integrative analysis of in vitro derived microglia and reveals improvement of microglia identity upon neuron-astrocyte co-culture

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8These authors contributed equally to this work.

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Running title: a new reporter line to study microglia in vitro

Key words: microglia iPS differentiation protocol, microglia genetic reporter, synapse internalization, RNA-seq.

Highlights

- integrated molecular characterisation reveals differences in iMGL protocols
- a dual tdTomato and nanoluciferase reporter tool was generated to track iMGL via CX3CR1
• kinetics of surface marker expression during iMGL differentiation
• co-culture with astrocytes/neurons restores ex vivo microglial transcription factors

In this article, Jose Polo and colleagues molecularly compare existing microglia differentiation methods from stem cells, generate a new dual fluorescent and enzymatic reporter tool to study microglia and show that human astrocyte/neuron co-culture pushes the regulatory landscape of in vitro microglia-like cells to more closely resemble bona fide microglia.

Summary

Multiple protocols have been published for generation of iMGLs from hESCs/iPSCs. To date, there are no guides to assist researchers to determine the most appropriate methodology for microglial studies. In order to establish a framework which will facilitate future microglial studies, we first performed a comparative transcriptional analysis between iMGLs derived using multiple protocols, which allowed us to establish the baseline protocol that is most representative of bona fide human microglia. Secondly, using CRISPR to tag the classic microglial marker CX3CR1 with nanoluciferase and tdTomato, we generated and functionally validated a reporter ESC line. Finally, using this cell line, we demonstrated that co-culture of iMGL precursors with human astrocytes and neurons enhanced transcriptional resemblance of iMGLs to ex vivo microglia. Together our comprehensive molecular analysis and reporter cell line are a useful resource for neurobiologists seeking to use iMGLs for disease modelling and drug screening studies.

Introduction

Microglia, the resident macrophages of the central nervous system, are essential for brain development and function (Paolicelli et al. 2011, Thion et al. 2018), and have been genetically, epigenetically (Gjoneska et al. 2015) and transcriptionally (Zhang et al. 2013) shown to be directly involved in neurodegenerative diseases including Alzheimer’s disease and Multiple Sclerosis (Skene and Grant 2016).

iPSC/hESC derived cells are a useful research platform as they are potentially more representative of human development and systems compared to cell lines or animal models. Recently, multiple protocols describing differentiation of human embryonic or human induced pluripotent stem cells (hESCs or iPSCs, respectively) towards microglia-like cells (iMGLs) have been described (Abud et al. 2017, Brownjohn et al. 2018, Douvaras et al. 2017, Garcia-Reitboeck et al. 2018, Haenseler et al. 2017, Muffat et al. 2016, Ormel et al. 2018, Pandya et al. 2017, Takata et al. 2017). The differences between these protocols will inherently result in transcriptomic and functional variation between iMGLs generated, thus it is critical to understand which of these most closely resemble in vivo microglia.

Primary microglia were recently shown to rapidly downregulate key signature genes upon in vitro culture (Gosselin et al. 2017), indicating that growth factors that are currently utilised for in vitro culture
are insufficient for establishment or maintenance of microglial identity. To study microglia and examine their interactions with other cells, it is useful to track permanent reporter expression targeted onto a key microglial gene that is not downregulated during in vitro culture.

In this brief report, we have performed a molecular comparison of existing iMGL differentiation strategies to identify the baseline protocol with greatest molecular similarity to ex vivo microglia. Next, we used a dual CRISPR-Cas9-nickase system to selectively target one allele of the microglial marker CX3CR1 in the H9 hESC line, tagging the gene with a dual fluorescent/enzymatic construct, whilst ensuring physiological expression of CX3CR1 protein. We have functionally validated iMGLs derived from this reporter cell line, demonstrating expression of key microglial markers, functional cytokine responses and internalisation of synaptosome fragments. Finally, we demonstrated that co-culture of iMGLs with human astrocytes and neurons improves the transcriptional identity of iMGLs. Our reporter line and integrative transcriptional analysis can be utilised by researchers worldwide to further improve iMGL molecular signatures, with the ultimate aim of accurately recapitulating in vivo microglia for disease modelling and drug screening applications.

Results and Discussion

**Molecular comparison of existing microglia differentiation protocols**

Since the first description of a directed differentiation protocol yielding IBA1⁺CD11b⁺CD45⁺ cells from a hiPS or hES lineage in 2016 (Muffat et al. 2016), to date nearly 10 differentiation protocols have been described to generate iPSC-derived microglia like cells (iMGLs, Table 1) (Abud et al. 2017, Brownjohn et al. 2018, Douvaras et al. 2017, Garcia-Reitboeck et al. 2018, Haenseler et al. 2017, Muffat et al. 2016, Ormel et al. 2018, Pandya et al. 2017, Takata et al. 2017). However, the transcriptomes generated by these protocols have been only been compared to primary microglia cultured in vitro, and bona fide ex vivo microglia rapidly change identity upon in vitro culture resulting in ~6000 genes deregulated over 2-fold (Gosselin et al. 2017). Thus, there is a need for microglia researchers to determine which of these protocols to adopt or adapt for their own studies. The protocols differ primarily by the method used to generate microglial progenitors, with some methods relying on embryoid body formation to generate mesoderm (Brownjohn et al. 2018, Garcia-Reitboeck et al. 2018, Haenseler et al. 2017, Muffat et al. 2016, Takata et al. 2017), whereas others follow a 2D induction of mesoderm myeloid differentiation (Abud et al. 2017, Douvaras et al. 2017, Pandya et al. 2017), and some protocols purify intermediates by FACS (Abud et al. 2017, Douvaras et al. 2017, Takata et al. 2017) or MACS (Pandya et al. 2017). A recent study also detected native iMGL development within cerebral organoids (Ormel et al. 2018), previously found to be devoid of myeloid cells. The difficulty of comparing protocols is further confounded by the different, although partially overlapping, functional validation experiments used. We therefore utilised two recent landmark publications that for the first time transcriptionally profiled ex vivo FACS-isolated microglia from fresh post mortem or surgery-resected human brain (Galatro et al. 2017,
Gosselin et al. 2017), as a comparison for the *bona fide* microglial transcriptional signature. In our analysis, we included all studies containing hiMGLs that were profiled by RNA-seq, and that contained at least on common group with any other dataset included, for the purpose of cross-study normalisation (Abud et al. 2017, Douvaras et al. 2017, Muffat et al. 2016) (Table S1). Thus, we excluded datasets with only microarray data (Haenseler et al. 2017, Pandya et al. 2017), no RNA-seq for hiMGLs (Garcia-Reitboeck et al. 2018, Takata et al. 2017), and the dataset containing no additional sequencing group other than the iMGLs generated in that study (Brownjohn et al. 2018). Our results revealed that *ex vivo* microglia clustered close together irrespective of the study or fresh post mortem compared to surgery resected origin of the cells, providing confidence in the method used for normalisation (Figure 1A). Similarly, the brain lysate groups sequenced in both studies clustered together. Our results indicate that the first MDS dimension was dominated by the transition from non-myeloid to myeloid cells, and that the second dimension represented the differences in environment *ex vivo* to *in vivo*. The third dimension separated cells present in the brain from peripheral cells, as *ex vivo* monocytes and dendritic cells separated from *ex vivo* microglia primarily in this dimension (Figure 1B). These results show that there is a component of environment, and particularly of brain environment, in addition to the myeloid lineage that needs to be faithfully recapitulated for a molecularly representative model of microglia. Of the iMGL protocols compared in this study, the protocol of Abud et al (2017) most closely resembled *ex vivo* microglia transcriptionally, and clustered with *bona fide* microglia following at least 24h *in vitro* culture (Fig 1A-B). The additional iMGL protocols examined here clustered more closely with *in vitro* cultured fetal microglia (pFMGLs), and thus may require further maturation. Furthermore, the higher internal variability of differentiations in (Muffat et al. 2016) compared to other protocols may suggest that either embryoid body formation or multiple sequential collections of progenitors over several weeks may inherently generate more variability than multi-step synchronised directed differentiation or FACS isolation of pure target populations.

We first examined whether we could generate iMGLs that molecularly and functionally resembled iMGLs in our hands, and thus whether the protocol of (Abud et al. 2017) was robust to be adopted in multiple different labs. As the astrocyte-derived factors necessary for microglial survival and ramification *in vitro* were IL-34, CSF1, TGFβ and cholesterol (Bohlen et al. 2017), and the iMGL maturation media described in (Abud et al. 2017) contained all but cholesterol, we also added cholesterol to the differentiation protocol. We showed that in our hands in two independent labs, the differentiation stages morphologically followed those initially described (Figure 1C) and was consistent for iMGLs generated from 8 iPS cell lines tested comprising 3 control lines, 3 AD patient lines and 2 CRISPR-corrected lines (Oksanen et al. 2017). We showed that d12 HPCs lost TRA-1-60 and gained CD43 expression and that at d38, iMGLs uniformly expressed CD45 and CD11b (Figure S1A). iMGLs also expressed IBA1 as shown by IF (Figure S1B). Moreover, iMGLs phagocyted pHrodo-red labelled *E. coli*, with enhanced phagocytosis (Figure 1D) and elevated cytokine responses (Figure S1C) in response to LPS stimulation. These data together suggest that the differentiation protocol described by (Abud et al. 2017)
was readily adaptable in multiple labs. Thus, using this protocol as a baseline, we designed an approach to generate a tool for the microglial community that would facilitate established and emerging microglial researchers alike to examine microglial identity and functions *in vitro* and *in vivo*, in the context of physiology and disease (Fig 1E).

**CRISPR generation and validation of CX3CR1<sup>tdTomato</sup> H9 ES cells for tracking microglial differentiation**

We reasoned that a microglial reporter line would be a useful tool for the derivation of microglia and to allow efficient identification and live cell tracking of microglial cells *in vitro* and *in vivo* allowing rapid cell sorting without the need for additional labelling. Thus, we used a CRISPR-Cas9 derived method to facilitate insertion of a dual fluorescent (*tdTomato*) and enzymatic (nanoLuc) reporter into H9 cells. We chose to use a CRISPR system previously described (Mali et al. 2013, Ran et al. 2013), which utilises dual Cas9-nickase constructs, to reduce the incidence of off-target double strand breaks. Our donor vector contained two selection cassettes, a diphtheria toxin A (DTA) cassette, and a neomycin/kanamycin resistance cassette as well as two homology arms for the *CX3CR1* gene (Figure 2A). The donor vector enabled replacement of the stop codon of the *CX3CR1* open reading frame with an IRES-*tdTomato*-T2A-Nanoluc-polyA-FRT-Neo-FRT construct. The DTA cassette was designed so that correct insertion of the donor vector into the host *CX3CR1* locus resulted in excision of the DTA cassette. PCR testing indicated that of the 24 clones picked, up to 14 were correctly targeted, a success rate of approximately 58%. Clone 6.8 was clearly correctly targeted according to PCR (Figure S2A), and when analysed by Southern blot (Figure S2B), was shown to be a heterozygous clone. This line was confirmed to be pluripotent by teratoma assay (Figure S2C) and has been used in all studies described herein, without excision of the neomycin expression cassette.

To verify that the reporter construct was functional and not silenced in iMGLs, we generated iMGLs from the targeted H9 reporter cell line and examined the expression of the fluorescent reporter compared to endogenous CX3CR1 by FACS. *tdTomato* expression correlated with expression of CX3CR1 by FACS, and insertion of the reporter did not interfere with expression of CD11b, CD45 or TREM2, and iMGLs remained negative for the macrophage-specific marker CCR2 (Figure 2B). We confirmed that H9-CX3CR1<sup>tdTomato</sup> iMGLs expressed P2RY12 and TREM2, as well as the *tdTomato* reporter (Figure 2C). To validate that the nanoluciferase reporter was functional, we measured nanoluciferase enzymatic activity in media harvested from H9 and H9-CX3CR1<sup>tdTomato</sup> iMGLs at different time points throughout differentiation. Together our data indicate that both the fluorescent and enzymatic reporters are functional in this cell line and can be used to track iMGL differentiation via CX3CR1 expression in cells and media using FACS, IF or luminescence approaches.

We next used this line to track the kinetics of surface marker expression changes during the differentiation
process and to determine the order of changes occurring during iPS differentiation to iMGL. Thus, we followed the loss of the pluripotency marker TRA-1-60, which was lost in over 50% of cells from d4, and was entirely absent from non-adherent iHPCs by d12 of differentiation (Figure 2E, Figure S3A). Loss of TRA-1-60 preceded acquisition of primitive lymphoid/myeloid marker CD43, which was present on 98% of non-adherent (iHPCs) by d12. 14.9% of iHPCs also co-expressed CD11b and CD45, and most of these cells (84.6%) were also positive for the CX3CR1<sup>tdTomato</sup> reporter (Figure 2E, Figure S3A). A proportion of CD43<sup>+</sup> cells at d12 (30.9%) also upregulated CD235a independently of CX3CR1 expression (Figure S3A). Of the microglial markers CD11b, CD45, CX3CR1 and TREM2, CD11b and CX3CR1 were upregulated first, with ~90% cells expressing both markers by d18, whereas ~90% of cells expressed CD45 only by d30 (Figure 2F, Figure S3B-C). Of the surface markers we examined, TREM2 was the last to be upregulated, even at d32, showing a progressive increase, from 22.3% of iMGLs expressing TREM2 at d18 to >90% by d32 (Figure 2F, Figure S3B-C). Together our data show the order and kinetics of surface marker expression during iMGL differentiation and demonstrate how the reporter cell line generated in this study can be used to track differentiation kinetics in response to different stimuli or for drug screening.

**Functional testing of H9 CX3CR1<sup>tdTomato</sup> derived iMGLs**

For our reporter line to be a useful tool for microglia, neuroscience and drug discovery researchers, it must perform robustly in a variety of experimental setups, including imaging, co-culture and functional assays, as well as for a variety of readouts including those that require either cells or media for analysis. Thus, we sought to validate our line in these settings. Cytokine responses and phagocytosis of cells expressing damage or danger signals, as well as interactions with synapses are critical in vivo microglial functions. We confirmed that H9 CX3CR1<sup>tdTomato</sup> secrete the appropriate range of cytokines and chemokines in response to LPS (Figure 3A) and phagocytose fluorescently labelled synaptosomes isolated from human brain (Figure 3B). We also showed that H9 CX3CR1<sup>tdTomato</sup> iMGLs can internalise native synaptic material immunostained with PSD95, when co-cultured with human ReN cell cultures containing neurons and astrocytes (Figure 3C). Together these data show that CX3CR1<sup>tdTomato</sup> derived iMGLs possess the functional properties of microglia in vitro.

**Co-culture of iMGLs with human neurons and astrocytes shifts transcriptional state towards ex vivo microglial identity**

It is becoming clear that the brain niche specifies an independent component of microglial cell fate (Gosselin et al. 2014). To attempt to mimic the context-specific functions and cell identity of microglia, to date iMGLs have been co-cultured with rat hippocampal neurons (Abud et al. 2017), hiPS-derived neurons (Takata et al. 2017), NPC conditioned medium (Muffat et al. 2016), and cerebral organoids (Abud et al. 2017, Brownjohn et al. 2018, Ormel et al. 2018), or astrocytes (Pandya et al. 2017), which were each reported to partially improve functions and morphology of iMGLs. To further characterise this, we included
transcriptional data from iMGLs co-cultured with rat hippocampal neurons (iMGL_rat_neuron; (Abud et al. 2017), or NPC conditioned medium (iMGL_muffat+NCM; (Muffat et al. 2016), which interestingly did not significantly enhance the similarity of iMGLs to ex vivo microglia (Figure 4A-B, Figure S4A). This data suggests that signals provided by direct contact with rat hippocampal neurons or NPC conditioned medium are not sufficient to significantly shift the transcriptional signature of iMGLs towards ex vivo microglia (Figure 4A-B), and this may be attributed either to the species differences between rodent and human microglia (Galatro et al. 2017, Gosselin et al. 2017, Smith and Dragunow 2014), or to the absence of astrocyte-derived signals requisite for microglial maturation (Bohlen et al. 2017). Similarly, our data show that an additional astrocyte-derived metabolite required by microglia (cholesterol) was unable to push the iMGL molecular identity towards ex vivo microglia as shown by their overlapping position on the MDS plot (Figure 4A-B). We therefore hypothesised that co-culture of iMGLs or their precursors with human astrocytes and neurons may recapitulate the components of the microglial transcriptional network that are controlled through cell- cell contacts. For this, we utilised ReN VM cells which are immortalised human NPCs, shown to be electrophysiologically active upon differentiation to a neuron-astrocyte culture (Choi et al. 2014). Indeed, the resultant transcriptomes of iMGLs co-cultured with ReN-derived neurons and astrocytes were shifted in the second MDS component towards ex vivo microglia, suggesting acquisition of aspects of the ex vivo transcriptional signature (Figure 4A-B, Figure S4A-B). Similarly, when we integrated transcriptional data from a recent publication showing innate development of microglia in cerebral organoids (Ormel et al. 2018), the iMGL signatures were also shifted in the second MDS component towards an ex vivo like state, further highlighting the capacity of direct contact with niche cells to influence microglial molecular identity. Interestingly, oMGL, both at 38 and 52 days of culture were not as shifted along the first MDS component trajectory between iHPC and iMGL/microglia, suggestive of incomplete maturation towards the microglial lineage as iMGL_abud. These results are consistent with the role of the developmental trajectory with initial epigenetic priming outside the brain or brain- like environment in establishment of microglial lineage identity, followed by the requirement of niche signals for tissue imprinting (Amit et al. 2016).

To investigate the nature of the molecular transition induced in iMGLs by contact with human astrocytes and neurons further, we focused on changes to transcription factors shown to be deregulated in microglia in vitro. Transcription factor landscapes govern cell identity transitions and are the ultimate master regulators of signals transduced from the environment; this is particularly evident in the adaptations of macrophage subpopulations to their environment (Bennett et al. 2018, Gosselin et al. 2014). We therefore reasoned that early or partial cell fate changes could be captured by examining whether the transcription factor network that is deregulated upon in vitro culture may be restored by co-culture with human neurons and astrocytes. We thus examined whether the expression levels of the 63 TFs associated with ex vivo specific super enhancers (ATAC-seq open chromatin regions also carrying a H3K27ac mark) or ex vivo enriched motifs (Gosselin et al. 2017) were restored to ex vivo levels. We first performed a
clustering and correlation analysis of all iMGL transcriptomes based on expression levels of these 63 critical microglial TFs. This analysis showed that iMGLs cultured using the Abud protocol and co-cultured with neurons/astrocytes clustered with bona fide microglia, and the expression of microglial TFs was highly correlated between these samples (Figure 4C, Figure S4C). Similarly, the transcriptomes from oMGL that innately develop within organoids (Ormel et al. 2018), also clustered together with ex vivo microglia in this analysis. Importantly, although microglial TFs were unchanged in iMGLs cultured in cholesterol (Figure 4D), 12 microglial TFs were significantly upregulated in iMGL_abud+ReN, including multiple TFs from the JUN, FOS, EGR and KLF families (Figure 4E). Together this data shows that direct interactions of iMGLs with human neurons and astrocytes leads to a shift in the transcriptional program of iMGLs towards a more ex vivo like state.

Nonetheless, there remain important differences between iMGLs and ex vivo microglia, even in the presence of human neurons and astrocytes – indeed 1967 DEGs remain, although this is a significant improvement on the 4461 DEGs between ex vivo microglia and iMGL_abud (adj. p<0.05; Tables S2-3). As ReN cells are derived from fetal ventral mesencephalon, it is possible that co-culture with adult neurons and astrocytes may provide additional maturation signals. Moreover, microglial transcriptomes are known to be regionally heterogeneous, at least within the mouse (Grabert et al. 2016), which may be an epigenetically-controlled function of microenvironment and neuronal turnover rates (Ayata et al. 2018), thus co-culture with cells from a particular region of interest may also yield increased molecular resemblance to bona fide microglia. Together, our data provide a framework as well as laboratory and transcriptional tools. These tools allow the comparison and integration of existing and newly generated datasets, and a pluripotent microglia reporter line that can be used to track iMGLs and their media alone or in co-culture systems for a variety of molecular and functional assays or drug screening approaches.

**Experimental Procedures**

**Differentiation of iPS/ES cells to microglia-like cells**

8 iPS cell lines and their culture conditions are as described before (Oksanen et al. 2017). These cells were used for validation of the protocol and the data is presented in Figure 1 and Figure S1. The generation of H9 CX3CR1^tdTomato^ ESCs is described below in “Gene editing”.

**iHPC Differentiation Base Medium:** IMDM (50% v/v, 12440053, ThermoFisher Scientific), F12 (50% v/v 11765054, ThermoFisher Scientific), ITS-X (2% v/v, 51500056, ThermoFisher Scientific), L- ascorbic acid 2-Phosphate magnesium (64 μg/ml, A8960, Sigma), monothioglycerol (400 μM, M6145, Sigma), PVA (10 μg/ml, P8136, Sigma), Glutamax (1X, 35050061, ThermoFisher Scientific), chemically-defined lipid concentrate (1X, 11905031, ThermoFisher Scientific), non-essential amino acids (NEAA, 1X, 11140050, ThermoFisher Scientific), Penicillin/Streptomycin (P/S, 1% v/v, 15140122, ThermoFisher Scientific). Use 0.22 μm filter.
**iMGLs Differentiation Medium:** phenol red-free DMEM/F12 (1:1, 11039021, ThermoFisher Scientific), ITS-G, (2% v/v, 41400045, ThermoFisher Scientific), B27 (2% v/v, 17504044, ThermoFisher Scientific), N2 (0.5% v/v, 17502048, ThermoFisher Scientific), monothioglycerol (200 μM, M6145, Sigma), Glutamax (1X, 35050061, ThermoFisher Scientific), NEAA (1X, 11140050, ThermoFisher Scientific), and additional insulin (5 μg/ml, I2643, Sigma), filtered through a 0.22 μm filter; supplemented with M-CSF (25 ng/ml, 130-096-492, Miltenyi biotec), IL-34 (100 ng/ml, 130-108-977, Miltenyi biotec), and TGFβ-1 (50 ng/ml, 130-108-969, Miltenyi biotec) and cholesterol (1.5 μg/ml, 700000P, Avanti Polar Lipids).

The protocol for iMGL derivation was adapted from Abud et al. with modifications from the StemDiff Hematopoietic Kit (05310, Stem Cell Technologies) similar to (McQuade et al. 2018). H9 CX3CR1tdTomato cells were cultured on vitronectin (A14700, ThermoFisher Scientific)-coated T25 flasks in E8 medium (A1517001, ThermoFisher Scientific). 2 days prior to differentiation, cells were detached in 0.5mM EDTA and 40-80 colonies /well were seeded into a 12 well plate in E8 medium. On day 0, E8 medium was exchanged for 1 mL of supplemented iHPC Differentiation Base Medium per well. iHPC Differentiation Base Medium supplemented with FGF2 (50 ng/ml, 130-093-564, Miltenyi biotec), BMP4 (50 ng/ml, 130-111-165, Miltenyi biotec), Activin-A (12.5 ng/ml, 130-115-010, Miltenyi biotec), ROCKi (1 μM, 130-103-922, Miltenyi biotec) and LiCl (2 mM, L7026, Sigma), and incubated in a hypoxic incubator. On day 2, medium was changed to 1 mL of iHPC Differentiation Base Medium supplemented with FGF2 (50 ng/ml) and VEGF (50 ng/ml, 130-109-385) and incubated in a hypoxic incubator. On day 4, medium was changed to 1 mL iHPC Differentiation Base medium containing human FGF2 (50 ng/ml), VEGF (50 ng/ml), TPO (50 ng/ml, 130-095-752, Miltenyi biotec), SCF (10 ng/ml, 130-096-695, Miltenyi biotec), IL-6 (50 ng/ml, PHC0061, ThermoFisher Scientific), and IL-3 (10 ng/ml, PHC0031, ThermoFisher Scientific) and incubated under normoxia. Half the medium was replaced on days 5 and 7 with the same medium as day 4. On day 10, the supernatant containing the HPCs was collected, centrifuged (300 x g for 5 min at room temperature; RT), then 0.5ml cell-containing medium was replaced and supplemented with 0.5ml fresh medium. On day 12, the supernatant containing HPCs was collected and plated onto matrigel (1:40, 354277, hESC-qualified Matrix, LDEV-Free, Falcon)-coated 12 well plates at 1x10^5 cells/well in iMGL complete differentiation medium. Every two days, each well was supplemented with 0.5 ml per well of complete differentiation medium, and at day 22 a 50% media change was performed. From day 35, iMGLs were cultured in complete iMGL differentiation medium supplemented with human CD200 (100 ng/ml, C311, Novoprotein) and CX3CL1 (100 ng/ml, 300-31, Peprotech) for an additional three days. iMGLs were stimulated with LPS for 24h prior to RNA isolation or collection of culture supernatant.
Gene Editing

For gene targeting, H9-Wild Type hESCs were co-cultured on 6 cm dishes with Mouse Embryonic Fibroblasts (MEFs) until 80% confluent in hESC media (20% (v/v) Knock-Out Serum Replacement (KSR), 1% Non-Essential Amino Acids (NEAA), 0.5% Glutamax I, 1% (v/v) P/S, 0.625% (v/v) β-mercaptoethanol in DMEM/F12 + Glutamax I (all ThermoFisher), replaced daily. On reaching confluency, for targeting, cells were dissociated from the plate using 1 ml Accutase, collected into a 15 mL Falcon, centrifuged (160 xg, 4 min, RT), and depleted of MEFs for 1 hr by seeding onto an uncoated 6cm dish with hESC media. All culture for nucleofection and selection occurs on MEFs at a density of 12,000 /cm² unless specifically stated otherwise. Nucleofection was achieved by following manufacturer’s instructions for the LONZA Amaza Primary P3 kit (LONZA, cat #V4XP-3024). On the day of targeting, cells were dissociated as described above and one million cells/1 µg Vector DNA/1 µg sgRNA were resuspended in 100 µl of LONZA Amaza Primary P3 nucleofection solution. Nucleofection was performed using the LONZA Nucleofection cuvette and the CB-156 setting. Cells were immediately replated onto a 6 cm² dish in hESC media supplemented with 10 µM ROCKi, and 20 ng/ml FGF2. 72 h post nucleofection, cells were incubated in hESC media containing G418 at 50 ng/ml (ThermoFisher). Media containing G418 was replaced daily for 10 d. Surviving colonies were manually picked using a dissecting microscope, and seeded into individual wells of a 12 well plate. Clones were grown to confluency, expanded into 6 well plates, and either frozen down at -20°C as pellets for gDNA extraction and PCR screening, or frozen down for stocks in Freeze Mix (10% (v/v) DMSO, 50% (v/v) FBS, 40% (v/v) hESC media).

Confirmation of targeting PCR

Clones were screened using FWD primer: CATGCTCCAGACTGCCTTG, REV primer: GTCTGGACGGGTGAATACAG (expected band size of 1806 bp for integrated construct), using Phusion High Fidelity 2x MasterMix, on a 3-step cycle (98 °C 30 sec initial denaturation, 35 cycles of [5 sec at 98 °C, 30 sec at 66 °C, 30 sec at 72 °C], 72 °C final extension of 10 min as per manufacturer’s instructions (ThermoFisher). These primers indicate correct vector and endogenous upstream/downstream DNA integration. PCR products were run on 1% (w/v) agarose gel, 90 V, 45 min, and visualised using the Gel Doc apparatus. Clones indicated as successfully targeted were then prepared for Southern Blot analysis.

Southern Blotting

gDNA was extracted using the BIOLINE Isolate II gDNA extraction kit as per manufacturer’s instructions. A minimum of 20 µg was digested O/N at 37 °C using BsrGI or SacI (NEB, R0575S, R0156S), and then run for 16 h on a 1% (w/v) agarose gel, at 24 V. gDNA was transferred to Hybond N+ polyvinyl membrane O/N, through capillary transfer using Whatmann paper, 20x SSC buffer (3 M NaCl, 300 mM sodium citrate, pH to 7.0 with HCl) and weighted Perspex sheets. Membrane was then denatured, rinsed and dried in an 80
℃ oven for a minimum of 20 h, prior to being stored between Whatmann paper and wrapped in foil. Probes were prepared for plasmids through restriction endonuclease digest (EcoRI-HF, NEB), separation on a 1% (w/v) agarose gel, and isolated using the BIOLINE Isolate II PCR/GEL Kit as per manufacturer’s instructions. Probes were hybridised O/N with α-ATP-[P³²] (Perkin Elmer), rinsed with 20x SSC, and hybridised to the membrane O/N, rinsed again with 2x SSC, and visualised using the Amersham Typhoon imaging system.

**Teratoma Assay**

Teratoma assays were adapted from (Polanco et al. 2013) and were performed in accordance with national and institutional guidelines (Ethics number MARP-2017-063). H9.CX3CR1tdTomato cells were washed in PBS, harvested in 0.5 mM EDTA for 5 min 37 ℃, washed in 5 ml PBS, centrifuged (300xg, 5 min RT), and gently resuspended in E8 media containing 33% (v/v) Matrigel (Corning 354277) to 2x10⁶ cells/200 µl. 1x10⁶ cells were injected subcutaneously into each flank of NOD.Cg-Prkdc<sup>scid</sup>Il2rg<sup>tm1Wjl</sup>/SzJ (NSG) mice using a 26G needle. Mice were monitored weekly post-transplantations and euthanized at 12 weeks. The teratoma and surrounding tissue were removed, fixed with 4% (v/v) PFA in Sorenson’s buffer, and paraffin sections (5 µm) were prepared for hematoxylin and eosin (H & E) staining and histological examination under an Olympus BX51 microscope for assessment of the human tissue types generated.

**Cytometric bead array**

CBA was carried out using the BD (New Jersey, USA) CBA human flexi kit using a protocol modified from the manufacturer’s protocol. 5 µl of each standard (highest concentration at 5000 pg/ml in assay diluent) and samples were incubated with 5 µl of capture bead mix (containing 0.1 µl of each cytokine Capture Bead diluted in Capture Bead Diluent) for 1 h in a 96 well V-bottom assay plate. This was followed by the addition and incubation with 5 µl PE detection reagent mix (containing 0.1 µl of each cytokine PE Reagent) diluted in Detection Reagent Diluent for 1 h in the dark. Each well was then washed once with 200 µl of Wash Buffer, and beads were resuspended in 80 µl of Wash Buffer for analysis by FACS using the LSRFortessa X-20 (BD Biosciences). At least 200 single bead events from each cytokine population were collected. Results obtained were analysed using the FCAP Array Software Version 3.0 (BD).

**Phagocytosis assay**

The phagocytosis assay was performed using the pHrodo-red or pHrodo-green E. coli bio-particles (P35361 or P35366, ThermoFisher Scientific) as per manufacturer’s instructions. In brief, particles were resuspended in 2 mL of iMGL differentiation media, sonicated for 50 seconds, then vortexed for at least 30 seconds. Microglia were grown on 12 well plates at 100,000 cells per well in complete iMGL media. 24 h prior to phagocytosis assay, half the wells were treated with LPS (100ng/ml, L4391, Sigma). 1 hour prior to assay, half of the LPS and non-LPS wells were treated with cytochalasin D (C8273, Sigma) at 10 µM to inhibit
phagocytosis, and incubated for 1 hour at 37°C. Immediately prior to the phagocytosis assay, particles were vortexed for at least 30 seconds, and diluted at a 1:60 concentration. Particles were added to wells, and incubated for 1 hour at 37°C, in the dark. Following incubation with bio-particles, microglia were collected in a 15 mL tube, washed in PBS 2x, centrifuged at 300 xg for 5 min between washes. Microglia were resuspended in 100 μl of FACS buffer (PBS with 2% (v/v) FBS, 0.05 mM EDTA) prior to FACS analysis.

**Synaptosome isolation, labelling and phagocytosis**

Synaptosomes were isolated from human brain tissue (obtained from Victorian Brain Bank, Ethics Approval: MUHREC 2016-0554) according to Syn-PER Synaptic Protein Extraction Reagent (87793) protocol. The protein concentration was measured by nanodrop, and synaptosomes were labelled with blue fluorescent 2.0 μm FluoSpheres™ Carboxylate-Modified Microspheres (Life tech, F8824) according to manufacturer instructions. Briefly, 5.5 mg synaptosomes were resuspended at 5 mg/ml in MES buffer (1.1 ml) for 15 min at RT and labelled following the addition of 7.6 mg EDAC for 2 h at RT, then O/N at 4 °C. Conjugates were sonicated for 2 sets of 10 cycles (20 sec on, 30sec off), then 16.2 mg glycine was added to quench the reaction. After washing, conjugates were resuspended in 1 ml 1% (w/v) BSA with 2 mM sodium azide, and stored at 4 °C prior to addition to cells. Following 22.5 h LPS treatment, iMGLs were incubated with conjugated-synaptosomes (3.44 μg/ml) for a further 1.5 h. iMGLs were collected as above and analysed by FACS for internalisation of synaptosome labelled particles.

**Nanoluciferase assay**

Nanoluciferase activity in iMGL supernatants was assessed using the Nano-Glo® Luciferase Assay System (N1110, Promega) according to manufacturer’s instructions. Briefly, Nanoluciferase Assay Reagent was prepared immediately prior to the assay with Nano-Glo® Luciferase Assay Substrate diluted 1:51 in Nano-Glo® Luciferase Assay Buffer. 50 μl assay reagent was mixed with 50 μl cell- conditioned culture media for 3 min and nanoluciferase activity was measured using the luminescence detection mode on a FLUOstar omega microplate reader (BMG Labtech). The average signal per well over 10 min, beginning 3 min after substrate addition, was used as the final reading.

**Culture and differentiation of ReN neural progenitor cells**

ReN cells (Millipore) were maintained and differentiated as previously described (Choi et al. 2014). Briefly, cells were maintained on Matrigel-coated flasks (BD Biosciences, San Jose, CA, USA) in DMEM/F12 (11320033, ThermoFisher Scientific) supplemented with 2 μg/ml heparin (07980, StemCell Technologies, 07980), 2% (v/v) B27, 20 μg/ml EGF (130-097-749, Miltenyi biotec), 20 μg/ml bFGF and 1% (v/v) P/S. Neuronal and glial differentiation was achieved by growth factor withdrawal, with twice-weekly half media changes. For co-culture assays, iMGLs were added to 21 d differentiated ReN cells for a further 21 d as a ratio of (1:10), either in 6 well plate or 48 well plate on coverslips, for FACS and immunofluorescence,
respectively.

Flow cytometry and cell sorting

Cells were stained with antibodies to microglial and macrophage cell surface markers (CX3CR1-BV786, 1:20, 744489, BD Biosciences; CD11b-BV650, 1:100, 101259, Biolegend; CCR2-BV421, 1:20, 564067, BD Biosciences; TREM2-APC, 1:10, FAB17291A R&D Systems; CD45-APC.Cy7, 1:200, 25-0459-T100, Tonbo Biosciences; CD235a, PE-cy7, 1:200, 349112, Biolegend). Zombie violet (1:200) or propidium iodide (1:500) were used to discriminate live/dead cells, as appropriate for the antibody panel. For co-culture RNA-seq experiments, the CX3CR1<sup>tdTomato</sup> reporter was used for isolation of iMGLs from astrocyte-neuron co-cultures using the FACSria™ III cell sorter.

Immunofluorescence

iMGLs were grown alone or in co-culture with ReN cells, on 8mm glass coverslips (72296-08 PD25, Emgrid Australia) coated with matrigel. Cells were initially fixed with 4% (v/v) paraformaldehyde (PFA) for 1 min, added directly to media. Media and PFA was replaced with pre-warmed 4% (v/v) PFA for 10 min at RT. Cells were washed 3x with PBS, blocked for 1 h with 10% (v/v) donkey serum or normal goat serum, then stained with the following primary antibodies overnight (O/N) at 4 °C: P2RY12 (1:400, HPA013796, Sigma), TREM2 (1:200, AF1828-SP, R &D), IBA1 (1:500, 019-19741, Novachem), MAP2 (1:500, MAB3418, Millipore), GFAP (1:500, Z0334, DAKO), Synapsin I (1:1500, 574777, Millipore), PSD95 (1:500, ab12093 Abcam). After O/N incubation, cells were washed 3x with PBST, then incubated rocking for 2 h at RT with either Alexa-fluor 488 donkey anti goat (1:800, A-11055, ThermoFisher Scientific), Alexa-fluor 488 goat anti rabbit (1:800, A-11008, ThermoFisher Scientific) or Alexa-fluor 488 goat anti mouse (1:800, A-11001, ThermoFisher Scientific) secondary antibodies, followed by DAPI (1 μg/ml, D1306, ThermoFisher Scientific). Coverslips were washed 2x with PBST, then mounted on slides with Dako Fluorescence Mounting Medium (S3023, DAKO). Slides from ReN-iMGL co-cultures were imaged on a Nikon C1 confocal microscope using a 40x oil 1.4 NA objective and 1x zoom with 1024x1024 resolution, resulting in a pixel size of 90 nm. Slides for iMGL monocultures were imaged on a Leica SP8 confocal microscope using a 40x oil 1.24 NA objective and 0.75x zoom with 2048x2048 resolution, resulting in a pixel size of 188 nm.

RNA sequencing

RNA-seq Library construction and sequencing

RNA extraction from 1-10 x10<sup>4</sup> FACS-sorted CX3CR1<sup>+</sup> iMGLs or from 2 wells of iMGLs harvested directly from 12 well plates, was performed on the QIAcube (Qiagen) using the RNeasy Micro Kit (Qiagen, Hilden, Germany) and RNA quality was assessed using the Bioanalyser (Agilent RNA 6000 Pico kit). The
libraries were prepared using 0.5-2 ng RNA with RIN value ≥ 8. An 8 bp sample index (Table S4) and a 10 bp unique molecular identifier (UMI) were added during initial poly(A) priming and pooled samples were amplified using a template switching oligonucleotide. The Illumina P5 (5’ AAT GAT ACG GCG ACC ACC GA 3’) and P7 (5’ CAA GCA GAA GAC GGC ATA CGA GAT 3’) sequences were added by PCR and Nextera transposase, respectively. The library was designed so that the forward read (R1) utilizes a custom primer (5’ GCC TGT CCG CGG AAG CAG TGG TAT CGC AGA GTA C 3’) to sequence directly into the index and then the 10 bp UMI. The reverse read (R2) uses the standard R2 primer to sequence the cDNA in the sense direction for transcript identification. Sequencing was performed on the NextSeq550 (Illumina), using the V2 High output kit (Illumina) in accordance with the Illumina Protocol 15046563 v02, generating 2 reads per cluster composed of a 19 bp R1 and a 72 bp R2.

Demultiplexing and Mapping

Sequencing reads were processed using an in house pipeline consisting of sabre tools (https://github.com/serine/sabre) and RNAsik (Tsyganov et al. 2018). Samples were demultiplexed with a fork of sabre tools with the commands below, using sequence barcodes in Table S4. After demultiplexing, raw-data was processed with RNAsik pipeline to generate QC metrics, including percentage of reads mapped and assigned to the reference genome and duplication rates, and raw read counts for differential expression analysis. Demultiplexed UMI tagged sequencing reads were aligned to the human genome (Ensembl GRCh38 primary assembly) using RNAsik. Read deduplication based on UMIs was performed with Je markdupes in RNAsik and transcript read counts calculated with featureCounts(Liao et al. 2014).

```
sabre pe -f ${RAW_DATA}/MultiplexRNASeq_S1_R1_001.fastq.gz)
   -r ${RAW_DATA}/MultiplexRNASeq_S1_R2_001.fastq.gz
   -b ${BARCODE} \
   --combine \
   --umi \
   --max-mismatch 1\n   --min-umi-len 9\n   --max-5prime-crop 2\n   --stats demultiplex.stats\n   --no-comment
```

`combine` - merges R1 and R2 since R1 only holds "metadata" i.e. sample identity
`umi` - append umi into FASTQ header
`min-umi-len` - trim longer umis to 9 bases, discard umi (reads) that are shorter than 9
`max-5prime-crop` - if matching barcode cannot be found at 5’ of R1, remove 1 base, with maximum bases allowed to be removed set to 2

Normalisation and integration of existing microglia RNA-seq datasets

For the purpose of comparing the maturation of different microglia-like cells as well as the difference of \textit{in vitro} cultivation to \textit{ex vivo} isolation, a range of publicly available data-sets (below) were integrated.

<table>
<thead>
<tr>
<th>Set number</th>
<th>Authors</th>
<th>GEO accession</th>
<th>Number of samples (used for this comparison)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Muffat et al.</td>
<td>GSE85839</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>Douvaras et al.</td>
<td>GSE97744.</td>
<td>24 (22)</td>
</tr>
<tr>
<td>3</td>
<td>Abud et al.</td>
<td>GSE89189.</td>
<td>43 (40)</td>
</tr>
<tr>
<td>4</td>
<td>Gosselin et al.</td>
<td>GSE89960</td>
<td>64</td>
</tr>
<tr>
<td>5</td>
<td>Galatro et al.</td>
<td>GSE99074</td>
<td>65</td>
</tr>
<tr>
<td>6</td>
<td>Ormel et al.</td>
<td>GSE102335</td>
<td>16 (13)</td>
</tr>
<tr>
<td>7</td>
<td>Grubman et al.</td>
<td>-</td>
<td>16 (14)</td>
</tr>
<tr>
<td>Combined</td>
<td></td>
<td></td>
<td>244 (234)</td>
</tr>
</tbody>
</table>

The processed RNA-seq data was used where possible. Data sets 1-3 are available as FPKM tables. Data sets 4, 5, and 6 as counts. Read counts tables were transformed to FPKM with the edgeR (Robinson et al. 2010) (version 3.22.3) \texttt{rpkm} function and using average transcript length as gene length. Data was normalised with the \texttt{removeBatchEffect} function specifying the data set numbers as batch and common groups where possible. The shared groups include brain samples, monocytes, microglia, and iPSCs. The normalised data was log-transformed and used as input for \texttt{plotMDS} to generate the multiple dimensional scaling analysis. For the heat maps to visualise clustering of samples and genes, normalised data was used as described above. Samples pertaining to the \textit{ex vivo} and \textit{in vitro} microglia cells were selected (a total of 98 samples). The genes of interest (63 TFs from Gosselin et al. 2017) are selected from the expression data. Genes that were not widely expressed with an RPKM value of >0.5 in more than 10 samples including \textit{ex vivo} microglia were filtered out. For Figure 4C, Pearson correlation was computed for all samples and visualised with \texttt{heatmap.2} (using "ward.D2" clustering method) from the gplots package (version 3.0.1). For Figure S4 the RPKM values are shown and \texttt{heatmap.2} was used to cluster samples using ward.D2.
Data availability

All sequencing data have been deposited to GEO under accession number xxx

Author Contributions


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Disclosure of Potential Conflicts of Interest

The authors disclose no conflicts of interest.

Figure legends

Figure 1. Comparison of existing differentiation protocols and differentiation of iPS cells to iMGLs.

A - B. Multidimensional scaling analysis of the integrated datasets, presented as MDS dimension 1 v 2 (A) and 2 v 3 (B) showing separation of ex vivo microglia (exMGL), fetal or adult primary microglia – pFMGL or pAMGL cultured without, or in the presence of serum; +serum), iMGLs generated using various published protocols (abud, _douvaras, _muffat), and ex vivo brain lysate (exBrain). For comparison and normalisation,
we also included various *in vitro*-generated stem cell transcriptomes (iPS, iHPC, iNPC) and primary peripheral myeloid cells (pPBMC; *ex vivo* monocytes, exM, and *ex vivo* dendritic cells (exDC). C. Bright field micrographs showing colony and cell morphology at various points throughout the differentiation process, representative of >30 independent differentiations using 8 independent iPS lines. Scale bar = 100 µm. D. iMGLs phagocytose pHrodo particles basally and following LPS stimulation, representative of *n*=3 independent experiments. Scale bar = 50 µm. E. Schematic of microglia differentiation protocol (adapted from Abud et al 2017) and microglia reporter validation strategy used in this study. See also Figure S1.

**Figure 2. Generation of a dual microglia reporter ESC cell line and kinetics of differentiation to iMGLs.** A. Schematic illustrating the CRISPR vector used for insertion of the tdTomato and nanoluciferase gene into the genome. Long and short homology arms (LHA) for CX3CR1 were designed, using an IRES linker for tdTomato expression. cDNAs encoding tdTomato and nanoluciferase were linked with a T2A fragment, allowing translation of both proteins. A Neomycin/Kanamycin resistance cassette under the control of a PGK promoter was included for positive selection of correctly targeted clones. A DTA coding cassette was also included for negative selection of cells that do not correctly integrate the donor vector. B. Expression of CX3CR1 on iMGLs corresponds to tdTomato expression, as demonstrated by FACS. H9 CX3CR1*tdTomato* iMGLs express CD11b, CD45, and TREM2 but not CCR2 as determined by FACS. C. iMGLs uniformly express P2RY12, TREM2, as well as the CX3CR1 tdTomato Scale bar = 50 µm). D. Detection of luciferase secretion by iMGLs following co-culture of iHPCs with mouse OBSCs for the number of days indicated. E-F River plot showing the kinetics of cell identity transitions during differentiation of iPS to iHPC. (E) and iHPC to iMGL (F), as measured by expression of the markers TRA-1-60, CD43, CD11b, CD45, CX3CR1-tdTomato and TREM2. Population proportions are presented as gated on the markers shown. Ungated FACS plots of the live cell populations are in Figure S3A-B. See also Figures S2-3.

**Figure 3. H9.CX3CR1*tdTomato* iMGLs secrete cytokine, internalise native synaptic material and can be readily tracked in co-cultures and brain slices.** A. Cytometric bead array for the cytokines and chemokines shown in H9.CX3CR1*tdTomato* iMGLs basally (open circles), or stimulated for 24h with LPS (closed circles, 100 ng/ml). Points represent the average of 3 individual wells from independent differentiations and are expressed as mean ± SEM. B. Histograms showing the percentage of live tdTomato⁺ iMGLs phagocytosing one, two or more synaptosome-conjugated fluorescent blue carboxylate 2.0 µm microspheres after 1.5 h co-incubation as determined by FACS. Cells were pre- treated for 22.5 h with LPS (100 ng/ml). Histograms are representative of 3 independent experiments performed in triplicate wells. C. iMGLs at 21 DIV were co-cultured for 21 d with ReN-cell derived mixture of human astrocytes and neurons, stained with MAP2 and GFAP (i), MAP2 and IBA1 (ii) and synapsin and PSD95 (iii), and visualised using confocal microscopy. Scale bars = 50 µm.
Figure 4. Co-culture with ReN human astrocytes/neurons, but not cholesterol alone, shifts iMGL transcriptional profile towards an *ex vivo* cell state.  

A - B. Multidimensional scaling analysis of the integrated datasets as in Fig 1A-B presented as MDS dimension 1 v 2 (A) and 2 v 3 (B), including the RNA-seq datasets generated in this study. iMGL_abud group includes the original data from Abud *et al* 2017 and data from this study, generated using the same protocol. As in Fig 1A-B, *ex vivo* microglia (exMGL) and iMGL_abud are included for comparison. Datasets include iMGLs co-cultured with rat hippocampal neurons, cholesterol or ReN cells (iMGL_rat_neuron, (Abud *et al*. 2017); iMGL_abud+cholesterol, this study; iMGL_abud+ReN, this study), or NPC conditioned medium (iMGL_muffat+NCM; (Muffat *et al*. 2016)), organoid microglia at d38 or 52 (oMGL38_ormel and oMGL52_ormel, (Ormel *et al*. 2018)). 

C. Cluster dendrogram (ward.D2) and correlation analysis (Pearson) of TF expression in *ex vivo* microglia and various *in vitro* iMGL protocols. D. Bar plot showing gene expression changes in *ex vivo* super enhancer or motif TFs with abs(LFC)>1 between iMGL_abud+cholesterol and iMGL_abud protocols. (LFC>0 represents upregulation in iMGL_abud+cholesterol). The colour of the bars depicts whether the direction of change for gene expression in iMGL_abud+cholesterol is the same as that for *ex vivo* microglia (teal) or opposite to *ex vivo* microglia (salmon). E. Bar plot as for D, depicting TF gene expression changes between iMGL_abud+ReN and iMGL_abud protocols. * adj. *p*<0.05; ** adj. *p*<0.001; *** adj. *p*<0.0001. See also Figure S4.

References


Figure 2

A

B

C

D

E

F
Figure 3

A

B

CX3CR1+

CX3CR1+ + LPS

Count

Synaptosome-beads

Synaptosome-beads

C

hMG+ ReN hAsto/hNeuron

ReN hAsto/hNeuron

MAP2/α-tub/Tomato

MAP2/α-tub/Tomato

REDGE/SynPASS/hTomato

REDGE/SynPASS/hTomato

C

C

C
Figure S1

A

B

C
Figure S2

A

B

C

Ectoderm

Mesoderm

Endoderm
Figure S3
Figure S4

A

B

C

27
Appendix VII
Preliminary Cytometric Bead Array Data

First basal signalling levels of key cytokines was performed, then investigations into microglia incubated with LPS (100ng/mL), TNF (100ng/mL), IFN\(\gamma\) (20ng/mL) or \(\alpha\)-synuclein were performed. As this was a preliminary investigation, \(n = 1\) for all data presented within this appendix. However, detailed investigations of cytokine secretions are continued in Chapters 4 and 5. It was uncertain whether CX3CL1 and TGF\(\beta\)1 should be included as they are known to have both anti- and pro-inflammatory properties depending on context. Therefore, CX3CR1 microglia were cultured in both the presence and absence of these two cytokines. Additionally, there was concern that removal of CX3CL1 from culture media during treatment with inflammatory mediators may result in culture deterioration, as alterations to CX3CL1/CX3CR1 signalling in vivo has been documented to impact microglial function(26-28).

Scales are specific to the cytokine being interrogated, and figures are not designed to be compared to each other.

Basal microglial secretion in the presence of CX3CL1 and TGF\(\beta\)1

Basal secretion of IL-1\(\alpha\), IL-1\(\beta\), IL-4, IL-6, IL-8, IL-10, MIP1\(\alpha\), TNF, IFN\(\gamma\), VEGF and CX3CL1 were confirmed to occur in microglia in vitro in the presence of CX3CL1 and TGF\(\beta\)1. As expected, the levels of most of these cytokines (except for IL-8, IL-10, MIP1\(\alpha\), TNF and CX3CL1, see Figure 34 and 35 below) are generally quite low across the 2- to 48-hour period of measurement. Further studies to clarify the range of basal cytokine secretion under these culture conditions would be helpful to clarify the range of basal secretion.
Figure 34: Basal interleukin secretion of known inflammatory (IL-1α, IL-1β, IL-6, IL-8) and anti-inflammatory (IL-4 and IL-10) factors when microglial CX3CR1-reporter cells are cultured in the presence of CX3CL1 and TGFβ1. IL-8 and IL-10 appear to be more strongly expressed over time than IL-1α, IL-1β, IL-4 and IL-6. n=1, no error bars.
Figure 35: Basal non-interleukin cytokine secretion when CX3CR1-reporter microglial cells are cultured in the presence of CX3CL1 and TGFβ1. TNF, CX3CL1 and MIP1α appear to have greater levels of secretion than IFNγ and VEGF. N=1, no error bars.
Basal Microglial secretion in the absence of CX3CL1 and TGFβ1
Basal signalling of of IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, MIP1α, TNF, IFNγ, VEGF and CX3CL1 is present in CX3CR1-microglia cultured in the absence of CX3CL1 and TGFβ1 (see Figures 36 and 37). As expected, detected levels of CX3CL1 were much lower in CX3CR1-microglia cultured in the absence of CX3CL1. Although, CX3CL1 was still clearly observed to be decreasing over time, this may be due to residual presence of CX3CL1 from culture maintenance. TNF basal secretion in microglia cultured in the absence of CX3CL1 and TGFβ1 appeared to be potentially greater than in microglial cultured in the presence of CX3CL1 and TGFβ1, but this requires further studies for confirmation. Generally, basal secretion of IL-1α, IL-1β, IL-4, IFNγ and VEGF was very low over all timepoints measured, whereas IL-6, IL-8, IL-10, MIP1α, TNF and CX3CL1 were present at much higher levels. As n=1 for culture with and without CX3CL1 and TGFβ1, there can be no statistical analysis performed to determine whether there are significant differences in secretion over time.
Figure 36: Basal secretion of interleukins when microglial CX3CR1-reporter cells are cultured in the absence of CX3CL1 and TGFβ1. N=1, no error bars.
Figure 37: Basal secretion of non-interleukins when microglial CX3CR1-reporter cells are cultured in the absence of CX3CL1 and TGFβ1. N=1, no error bars.
Stimulated microglial secretion in the presence of CX3CL1 and TGFβ1

Preliminary investigations of CX3CR1-microglia revealed microglia can and do have alterations to cytokine secretions when stimulated with one of LPS, TNF, IFNγ or α-synuclein. IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, MIP1α, TNF, IFNγ, VEGF and CX3CL1 were all secreted by CX3CR1-microglia when cultured in the presence of CX3CL1 and TGFβ1 (see Figures 38-48). As this work was strictly proof of principle, there can be no conclusions drawn regarding significant secretory differences between basal and inflammatory treatments, however, this would be easily rectified through experimental repetition.

These results confirm microglial responses to LPS, TNF, IFNγ and α-synuclein.

Figure 38: IL-1α secretion in microglial monoculture in the presence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. N=1. Expression is low across all timepoints (less than 10pg/mL).
Figure 39: IL-1β secretion in microglial monoculture in the presence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. N=1. LPS expression appears to be strongest.

Figure 40: IL-4 secretion in microglial monoculture in the presence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. N=1. Expression is minimal across all time points (less than 5pg/mL).
Figure 41: IL-6 secretion in microglial monoculture in the presence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. N=1. LPS appears to elicit the strongest secretion of IL-6 over time compared to TNF, IFNγ and α-synuclein.

Figure 42: IL-8 secretion in microglial monoculture in the presence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. N=1. TNF and LPS treatments seem to elicit stronger secretory responses compared to control, while IFNγ and α-synuclein seem to have a reduced response of IL-8 secretion compared to control (see Figure 36, IL-8 graph for comparison).
Figure 43: IL-10 secretion in microglial monoculture in the presence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. N=1. LPS and TNF treatment appear to cause the strongest secretions of IL-10 over time compared to control, whereas IFNγ treatment seems to reduce IL-10 secretion compared to control.

Figure 44: MIP1α secretion in microglial monoculture in the presence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. N=1. LPS appears to induce the strongest response for MIP1α secretion over time compared to control, while IFNγ and α-synuclein both appear to have reduced MIP1α secretion compared to control.
Figure 45: TNF secretion in microglial monoculture in the presence of CX3CL1 and TGFβ1, when stimulated by LPS, IFNγ or α-synuclein. N=1. LPS appears to cause microglia to secrete the largest amount of TNF compared to control. Secretion of TNF when microglia are treated with IFNγ or α-synuclein appears to be less than when treated with LPS.

Figure 46: IFNγ secretion in microglial monoculture in the presence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF or α-synuclein. N=1. Expression appears to be minimal (<5pg/mL) across all timepoints, although LPS and TNF treatment do appear to stimulate an increase in IFNγ secretion at 48 hours compared to control.
Figure 47: VEGF secretion in microglial monoculture in the presence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF or α-synuclein. N=1. Expression appears to be generally low (<10 pg/mL) across all timepoints, except for LPS, which at 4 hours is >20 pg/mL. All four treatments appear to produce an initial increase in VEGF secretion, peaking at 4 hours, when dropping away over the next 36 hours.
**Figure 48:** (Previous page) CX3CL1 secretion in microglial monoculture in the presence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. N=1. Although media is spiked with CX3CL1, all four treatments (and the control, see Figure 37) appear to show a rapid decline in the level of CX3CL1 over time.

**Stimulated microglial secretion in the absence of CX3CL1 and TGFβ1**

Here, CX3CR1-microglia cultured in the absence of external CX3CL1 and TGFβ1 are able to respond to known inflammatory stimulants LPS, TNF, IFNγ and α-synuclein (see Figures 49-59). All cytokines observed in were observed, and it appears that LPS may have again stimulated the strongest cytokine release. Confirmation of this would require further repetitions of these experiments.

![Graphs of LPS, TNF, IFNγ, and α-synuclein secretion](image)

**Figure 49:** IL-1α secretion in microglial monoculture in the absence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. Expression is generally low (<10pg/mL) across all timepoints measured. N=1
Figure 50: IL-1β secretion in microglial monoculture in the absence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. Expression is generally low (<20pg/mL) across all timepoints measured for IFNγ and α-synuclein treated microglia, while LPS and TNF treatments appear to stimulate greater secretion of IL-1β. N=1

Figure 51: IL-4 secretion in microglial monoculture in the absence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. Expression is generally minimal (<5pg/mL) across all timepoints measured. N=1
Figure 52: IL-6 secretion in microglial monoculture in the absence of CX3C11 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. LPS appears to stimulate the greatest secretion of IL-6 over time, while TNF and α-synuclein also appear to show a small change in IL-6 secretion over time. IFNγ treatment appears to have little impact on IL-6 secretion. N=1

Figure 53: IL-8 secretion in microglial monoculture in the absence of CX3C11 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. LPS and TNF appear to stimulate the greatest secretion of IL-8 over time. α-synuclein and IFNγ appear to have a reduced secretory response relative to LPS or TNF treatments. LPS and TNF appear to generate roughly equal secretion of IL-8 over time. N=1
Figure 54: IL-10 secretion in microglial monoculture in the absence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. LPS appears to stimulate the greatest level of secretion over time. N=1

Figure 55: MIP1α secretion in microglial monoculture in the absence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. LPS and TNF treatment both appear to cause peak secretion of MIP1α at 24 hours, while IFNγ and α-synuclein appear to peak at 8 hours. n=1.
Figure 56: TNF secretion in microglial monoculture in the absence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. TNF appears to be most strongly secreted in response to LPS or IFNγ. α-synuclein may have a lesser secretory response. N=1

Figure 57: IFNγ secretion in microglial monoculture in the absence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. Expression appears to be minimal (<5pg/mL) across all timepoints measured and treatments. N=1
Figure 58: VEGF secretion in microglial monoculture in the absence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. Secretion appears to be minimal (<10pg/mL) at all timepoints measured and in all treatments. N=1.

Figure 59: CX3CL1 secretion in microglial monoculture in the absence of CX3CL1 and TGFβ1, when stimulated by LPS, TNF, IFNγ or α-synuclein. All treatment groups display a drop in CX3CL1 secretion over time. n=1.
Appendix VIII
Comparison of Midbrain Co-culture and Midbrain monoculture VEGF secretion

Figure 60: Comparison of VEGF secretion between Neuron monocultures and Co-culture systems incubated with IFNγ (20ng/mL). Microglial monoculture not included due to cell number restrictions. Two-way ANOVA with post-hoc Tukey’s test for significance was performed. N=3 ***p<0.0005
Appendix IX
Supplementary forebrain neuron monoculture and forebrain neuron-microglia co-culture data.

Microglial-forebrain co-culture

**Basal Co-culture secretion**
All data unless otherwise stated in this section is n=1. Therefore, no statistical conclusions can be drawn. Due to experimental constraints, only a 24 hour period was measured instead of 48 hours as per *Chapters 3 and 4*.

![Figure 61: Basal secretion of interleukins in microglial-forebrain co-culture.](image-url)
Figure 62: Basal secretion of non-interleukins in microglial-forebrain co-culture.

Stimulated Co-culture secretion
Once basal secretion of cytokines was confirmed, the next step was to confirm stimulated secretion of cytokines by microglial-forebrain co-cultures. n=1, no error bars, no statistical significance can be drawn.
Figure 63: Secretion of IL-1α by microglial-forebrain co-cultures. Expression of IL-1α is low across all stimuli groups.

Figure 64: IL-18 secretion by microglial-forebrain co-cultures. Secretion is not increased by TNF, IFNγ or Amyloid-β at any time point. LPS appears to increase secretion, peaking at 4 hours post stimuli.
Figure 65: IL-6 secretion by microglial-forebrain co-cultures. Secretion appears increased by TNF treatment, minimally by IFNγ and not altered by LPS or Amyloid-β.

Figure 66: IL-8 secretion by microglial-forebrain cocultures. TNF strongly increases IL-8 secretion over time, LPS and IFNγ appear to peak at 8 hours, and Amyloid-β appears to peak at 24 hours. LPS, IFNγ and Amyloid-β secretion appear to be greatly reduced when comparing to TNF.
Figure 67: IL-10 secretion in microglial-forebrain co-cultures. Expression is low across all treatment groups, but appears to be greatest in cells treated with TNF at 24 hours.

Figure 68: MIP1α secretion in microglial-forebrain co-culture. No expression was observed when co-cultures were treated with IFNγ or Amyloid-β. Expression was minimal when stimulated with LPS. Stimulation with TNF caused secretion which peaked at 8 hours.
Figure 69: TNF secretion by microglial-forebrain co-cultures. No expression was observed when co-cultures were treated with IFNγ or Amyloid-β. TNF was only secreted when cultures were stimulated with LPS, and peaked at 8 hours.

Figure 70: IFNγ secretion by microglial-forebrain co-cultures. Stimulation with LPS or TNF did not increase expression. Stimulation with Amyloid-β appears to have strongly increased IFNγ secretion, peaking at 8 hours.
Figure 71: VEGF secretion by microglial-forebrain co-cultures. All treatments appear to elicit a response which peaks at 4 hours, prior to dropping by 8, then slowly increasing at 24 hours. TNF, IFNγ and Amyloid-β all appear to induce stronger responses than LPS.

Figure 72: CX3CL1 secretion by microglial-forebrain co-cultures. LPS, TNF and IFNγ treatments appear to drop away, while Amyloid-β appears to increase over the first eight hours before dropping away.
Summary of microglia-forebrain co-culture secretions

CX3CR1-microglia in co-culture with forebrain neural cultures are able to secrete cytokines, both basally and during stimulation with known inflammatory factors. It appears IL-1α, IL-1β, IL-10, MIP1α, TNF and IFNγ secretion tend to be minimally secreted (<5pg/mL) at all timepoints, whether being measured basally or when stimulated with LPS, TNF, IFNγ or Amyloid-β. IL-6 appears to be strongly secreted in response to TNF. VEGF secretion appears to be bi-modal (peaking at four hours, dropping at 8, then increasing at the 24-hour mark), and seems to be strongly secreted during all four stimulatory conditions. CX3CL1 appears to be secreted least during incubation with Amyloid-β, most strongly during incubation with TNF.

Forebrain neural monoculture.

Basal Forebrain secretion

Initial results of forebrain monoculture secretions indicate that during basal conditions, VEGF and CX3CL1 are present in much greater concentrations than IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, MIP1α, TNF and IFNγ. All graphs are n=2, no error bars, and no statistical conclusions can be drawn.
Figure 73: Basal secretion of interleukins by forebrain neural monocultures.
Figure 74: Basal secretion of non-interleukins by forebrain neural monocultures.
Stimulated forebrain secretion. Once basal secretion of cytokines was confirmed in forebrain neural monoculture, the next step was investigation of stimulated cytokine release. n=2, no error bars, no statistical significance can be drawn.

Figure 75: IL-1α secretion in forebrain neural monoculture. LPS, TNF, IFNγ and Amyloid-β all appear to stimulate low levels of IL-1α secretion.
Figure 76: IL-1β secretion by forebrain neural monocultures. Secretion is very low when stimulated by LPS, TNF or IFNγ, and is non-existent when stimulated by Amyloid-β.

Figure 77: Secretion of IL-4 by forebrain neural monocultures. Secretion is minimal at all time-points, regardless of treatment.
Figure 78: Secretion of IL-6 by forebrain neural monocultures. Stimulation with TNF appears to have had the greatest increase in IL-6 secretion. Secretion of IL-6 when stimulated by LPS or IFNγ appears to be minimal at all timepoints measured.

Figure 79: IL-8 secretion by forebrain neural monocultures. IFNγ stimulation does not appear to have altered IL-8 secretion at any timepoint.
Figure 80: IL-10 secretion by forebrain neural monoculture. Secretion is minimal at all time points and across all treatments.

Figure 81: MIP1α secretion by forebrain neural monoculture. Secretion is minimal (<5pg/mL) at all time-points. Treatment with LPS or TNF does appear to increase MIP1α over time, peaking at 48-hours.
Figure 82: TNF secretion by forebrain neural monoculture. Secretion is low when stimulated by LPS (<20pg/mL), and minimal (<5pg/mL) when stimulated with IFNγ or Amyloid-β. TNF secretion appears to peak at 48 hours when stimulated with LPS.

Figure 83: IFNγ secretion by forebrain neural monoculture. Secretion is minimal across all time-points and by all stimuli (<5pg/mL).
Figure 84: VEGF secretion by forebrain neural monocultures. LPS stimulus appears to most strongly influence VEGF secretion. There is some secretion when stimulated by IFNγ and TNF (<50pg/mL at peak) and minimal secretion when stimulated with Amyloid-β.

Figure 85: CX3CL1 levels in forebrain neural monocultures. Stimulation with LPS appears to produce the greatest levels of CX3CL1, whereas TNF, IFNγ and Amyloid-β all appear to result in reduced levels of CX3CL1.
Summary of forebrain monoculture secretions.
Due to insufficient n’s, no statistically significant conclusions can be drawn. However, IL-1α, IL-β, IL-4, IL-10, MIP1α and IFNγ appear to be minimally expressed across all timepoints measured, and during stimulation by LPS, TNF, IFNγ and Amyloid-β. IL-6 appears to be substantially greater in secretion during stimulation with TNF at 8-hours compared to LPS, IFNγ and Amyloid-β treatment. Similarly, IL-8 is strongly secreted at approximately 48-hours when stimulated with LPS, but appears to be almost doubled in concentration when stimulated with LPS. VEGF appears to be most strongly secreted during treatment with LPS, and is otherwise not secreted in great concentrations when stimulated with TNF, IFNγ or Amyloid-β. Finally, CX3CL1 appears to be most strongly secreted when stimulated with LPS, and least secreted with treated with Amyloid-β. These results will need further replications before concrete conclusions and comparisons between datasets can be drawn.
## Appendix X

Media composition for Forebrain and Midbrain neurons

### Table 17: Comparison of reagents used for forebrain and midbrain media.

<table>
<thead>
<tr>
<th>Common reagents</th>
<th>Forebrain media only</th>
<th>Midbrain media only</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Penicillin/streptomycin</td>
<td>50µM D-Serine</td>
<td>20ng/mL GDNF</td>
</tr>
<tr>
<td>1% Glutamax I</td>
<td></td>
<td>2.5µM DAPT</td>
</tr>
<tr>
<td>2% B27 supplement</td>
<td></td>
<td>1ng/mL TGF-β3</td>
</tr>
<tr>
<td>Neurobasal Medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20ng/mL BDNF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5mM dibutyrylcyclicAMP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200µM Ascorbic Acid</td>
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<td></td>
</tr>
</tbody>
</table>
Appendix XI

Alternative data analysis of cytometric bead array results, using One-Way ANOVA

Microglia Monocultures in midbrain media

One potential criticism of the data analysis in this thesis is that the method of analysis (a Two-Way ANOVA with post-hoc Tukey’s test) is very conservative, and only the very extreme changes were determined as significant changes over time. Using a less conservative method of data analysis (a One-way ANOVA), results in a greater number of significant events being recorded, as summarised in Tables 18 and 19.

Detailed data analysis for each cytokine can be found in Figures 85-95. Each figure includes a column graph of the secreted cytokine, an ANOVA summary table, and a short conclusion statement. The One-Way ANOVA performed analysed changes over time. Each graph is n=3.

Table 18: Cytokine secretion over time by microglia monoculture in midbrain media using One-Way ANOVA

<table>
<thead>
<tr>
<th></th>
<th>VEHICLE</th>
<th>LPS</th>
<th>TNF</th>
<th>A-SYNUCLEIN</th>
<th>NUMBER OF SIGNIFICANT CHANGES OVER TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>y</td>
<td>y</td>
<td>y</td>
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<tr>
<td>IL-6</td>
<td>n</td>
<td>n/d</td>
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<td>n</td>
<td>0</td>
</tr>
<tr>
<td>IL-8</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>0</td>
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<tr>
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<td>n</td>
<td>1</td>
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<tr>
<td>TNF</td>
<td>n</td>
<td>n</td>
<td>n/m</td>
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<tr>
<td>IFNγ</td>
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<td>y</td>
<td>n</td>
<td>y</td>
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<tr>
<td>VEGF</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>y</td>
<td>1</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>y</td>
<td>y</td>
<td>n</td>
<td>y</td>
<td>3</td>
</tr>
</tbody>
</table>

n/d = not detected, n/m = not measured, n = Not significant, y = yes, significant change over time
Table 19: Cytokine secretion over time by microglia monoculture in midbrain media using Two-Way ANOVA with post-hoc Tukey’s test

<table>
<thead>
<tr>
<th></th>
<th>VEHICLE</th>
<th>LPS</th>
<th>TNF</th>
<th>A-SYNUCLEIN</th>
<th>NUMBER OF SIGNIFICANT CHANGES OVER TIME</th>
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<tbody>
<tr>
<td>IL-1A</td>
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<td>IL-1B</td>
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<tr>
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<tr>
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<td>n</td>
<td>n</td>
<td>n</td>
<td>0</td>
</tr>
<tr>
<td>TNF</td>
<td>n</td>
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<td>n/m</td>
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<td>y</td>
<td>y</td>
<td>n</td>
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n/m = not measured  n = Not significant,  y = yes, significant change over time
**Conclusion:** Significant change over time during incubation with Vehicle

<table>
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<th>pg/mL</th>
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<tbody>
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<td>24 hours</td>
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<tr>
<td>48 hours</td>
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</tbody>
</table>

ANOVA summary

- **F** value: 4.354
- **P** value: 0.0058
- Significant diff. among means (P < 0.05)? Yes
- **R** square: 0.3323

**Conclusion:** Significant change over time during incubation with LPS

<table>
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<th>Timepoint</th>
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<td>24 hours</td>
<td></td>
</tr>
<tr>
<td>48 hours</td>
<td></td>
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</tbody>
</table>

ANOVA summary

- **F** value: 5.655
- **P** value: 0.0010
- Significant diff. among means (P < 0.05)? Yes
- **R** square: 0.5385

**Conclusion:** Significant change over time during incubation with TNF

<table>
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<th>Timepoint</th>
<th>pg/mL</th>
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<td>24 hours</td>
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<tr>
<td>48 hours</td>
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ANOVA summary

- **F** value: 4.435
- **P** value: 0.0256
- Significant diff. among means (P < 0.05)? Yes
- **R** square: 0.6395

**Conclusion:** Significant change over time during incubation with α-synuclein

<table>
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<tr>
<th>Timepoint</th>
<th>pg/mL</th>
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<tbody>
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<td>2 Hours</td>
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<td>24 hours</td>
<td></td>
</tr>
<tr>
<td>48 hours</td>
<td></td>
</tr>
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</table>

ANOVA summary

- **F** value: 13.28
- **P** value: 0.0008
- Significant diff. among means (P < 0.05)? Yes
- **R** square: 0.5551

**Figure 86:** Secretion of IL-1α over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. Vehicle, LPS, TNF and α-synuclein incubation all alter secretion over time.
**Vehicle**

<table>
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<td>24 hours</td>
<td>25</td>
</tr>
<tr>
<td>48 hours</td>
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</tr>
</tbody>
</table>

ANOVA summary

- **F**: 1.864
- **P value**: 0.1387
- **P value summary**: ns
- **Significant diff. among means (P < 0.05)**: No
- **R square**: 0.1756

**Conclusion**: No significant change over time during incubation with Vehicle.

**LPS**

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<th>pg/mL</th>
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</thead>
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<td>24 hours</td>
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</tr>
<tr>
<td>48 hours</td>
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</tbody>
</table>

ANOVA summary

- **F**: 1.962
- **P value**: 0.1327
- **P value summary**: ns
- **Significant diff. among means (P < 0.05)**: No
- **R square**: 0.2464

**Conclusion**: No significant change over time during incubation with LPS.

**TNF**

<table>
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</thead>
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<td>4 hours</td>
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<tr>
<td>8 hours</td>
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</tr>
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<td>24 hours</td>
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</tr>
<tr>
<td>48 hours</td>
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</table>

ANOVA summary

- **F**: 4.34
- **P value**: 0.0239
- **P value summary**: *
- **Significant diff. among means (P < 0.05)**: Yes
- **R square**: 0.6121

**Conclusion**: Significant change over time during incubation with TNF.

**α-Synuclein**

<table>
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<tr>
<th>Time point</th>
<th>pg/mL</th>
</tr>
</thead>
<tbody>
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<td>0.5</td>
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<td>24 hours</td>
<td>2</td>
</tr>
<tr>
<td>48 hours</td>
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</tr>
</tbody>
</table>

ANOVA summary

- **F**: 1.501
- **P value**: 0.2809
- **P value summary**: ns
- **Significant diff. among means (P < 0.05)**: No
- **R square**: 0.4001

**Conclusion**: No significant change over time during incubation with α-synuclein.

*Figure 87: Secretion of IL-1β over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. TNF (but not Vehicle, LPS, or α-synuclein) incubation significantly alters secretion over time.*
Figure 88: IL-4 secretion in microglial monoculture over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. No significant alterations to IL-4 secretion observed. IL-4 not detected during LPS incubation (data not shown).
Figure 89: IL-6 secretion in microglial monoculture over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. No significant alterations to IL-6 observed. IL-6 not detected during LPS incubation (data not shown).
**Figure 90: IL-8 secretion in microglial monoculture over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. No significant alterations to secretion observed.**
Figure 91: IL-10 secretion in microglial monoculture over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. LPS and α-synuclein (but not Vehicle or TNF) incubation significantly alter secretion over time.
**Vehicle**

ANOVA summary

<table>
<thead>
<tr>
<th>F</th>
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<tbody>
<tr>
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<td>0.5836</td>
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<td>P value summary</td>
<td>ns</td>
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<td>No</td>
</tr>
<tr>
<td>R square</td>
<td>0.761</td>
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</tbody>
</table>

Conclusion: No significant change over time during incubation with Vehicle.

**LPS**

ANOVA summary

<table>
<thead>
<tr>
<th>F</th>
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</tr>
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<tbody>
<tr>
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<td>0.0003</td>
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<tr>
<td>P value summary</td>
<td>***</td>
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<td>Significant diff. among means (P &lt; 0.05)?</td>
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</tr>
<tr>
<td>R square</td>
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Conclusion: Significant difference over time during incubation with LPS.

**TNF**

ANOVA summary

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<tr>
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<td>P value summary</td>
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<tr>
<td>Significant diff. among means (P &lt; 0.05)?</td>
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<td>R square</td>
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Conclusion: No significant change over time during incubation with TNF.

**α-Synuclein**

ANOVA summary

<table>
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</tr>
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<td>Significant diff. among means (P &lt; 0.05)?</td>
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<tr>
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</tbody>
</table>

Conclusion: No significant change over time during incubation with α-synuclein.

**Figure 92:** MIP-1α secretion in microglial monoculture over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. LPS (but not Vehicle, TNF or α-synuclein) incubation significantly alters secretion over time.
### Vehicle

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>pg/mL</th>
</tr>
</thead>
<tbody>
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<td>2 Hours</td>
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<tr>
<td>4 hours</td>
<td></td>
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<td>8 hours</td>
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</tr>
<tr>
<td>24 hours</td>
<td></td>
</tr>
<tr>
<td>48 hours</td>
<td></td>
</tr>
</tbody>
</table>

ANOVA summary

<table>
<thead>
<tr>
<th>F</th>
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</tr>
</thead>
<tbody>
<tr>
<td>1.18</td>
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</tr>
</tbody>
</table>

*P value summary*: ns

Significant diff. among means (P < 0.05)? No

R square: 0.1182

**Conclusion**: No significant change over time during incubation with Vehicle.

### LPS

<table>
<thead>
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<th>Timepoint</th>
<th>pg/mL</th>
</tr>
</thead>
<tbody>
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ANOVA summary

<table>
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<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.324</td>
<td>0.2898</td>
</tr>
</tbody>
</table>

*P value summary*: ns

Significant diff. among means (P < 0.05)? No

R square: 0.1807

**Conclusion**: No significant difference over time during incubation with LPS.

### α-Synuclein

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Hours</td>
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<td>4 hours</td>
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<td>8 hours</td>
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</tr>
<tr>
<td>24 hours</td>
<td></td>
</tr>
<tr>
<td>48 hours</td>
<td></td>
</tr>
</tbody>
</table>

ANOVA summary

<table>
<thead>
<tr>
<th>F</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.269</td>
<td>0.0182</td>
</tr>
</tbody>
</table>

*P value summary*: *<br>Significant diff. among means (P < 0.05)? Yes

R square: 0.7008

**Conclusion**: Significant change over time during incubation with α-synuclein.

---

**Figure 93**: TNF secretion in microglial monoculture over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. α-synuclein (but not Vehicle or LPS) incubation significantly alters secretion over time.
Figure 94: IFNγ secretion microglia monoculture over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. LPS and α-synuclein incubation (but not Vehicle or TNF incubation) significantly alter secretion over time.
Conclusion: No significant change over time during incubation with Vehicle

Conclusion: No significant difference over time during incubation with LPS

Conclusion: No significant change over time during incubation with TNF

Conclusion: Significant change over time during incubation with α-Synuclein

Figure 95: VEGF secretion in microglial monoculture over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. α-Synuclein (but not Vehicle, LPS or TNF) incubation significantly altered secretion over time.
Figure 96: CX3CL1 concentration over time in microglial monocyte over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. Vehicle, LPS, and α-synuclein incubation all alter secretion over time. TNF does not alter CX3CL1 concentration over time.
Midbrain monocultures in midbrain media

Using a less conservative method of data analysis (a One-way ANOVA), results in a greater number of significant events being recorded for midbrain monocultures in vitro, as summarised in Tables 20 and 21.

Detailed data can be observed in Figures 96-106.

Table 20: Cytokine secretion over time by midbrain monoculture using One-Way ANOVA

<table>
<thead>
<tr>
<th>VEHICLE</th>
<th>LPS</th>
<th>TNF</th>
<th>A-SYNUCLEIN</th>
<th>IFNγ</th>
<th>NUMBER OF SIGNIFICANT CHANGES OVER TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1A</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>IL-1B</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>IL-4</td>
<td>y</td>
<td>n</td>
<td>n</td>
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<tr>
<td>IL-6</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>n</td>
<td>3</td>
</tr>
<tr>
<td>IL-8</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>4</td>
</tr>
<tr>
<td>IL-10</td>
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<td>n</td>
<td>n</td>
<td>n</td>
<td>0</td>
</tr>
<tr>
<td>MIP-1A</td>
<td>n</td>
<td>n</td>
<td>y</td>
<td>n</td>
<td>1</td>
</tr>
<tr>
<td>TNF</td>
<td>n</td>
<td>n</td>
<td>n/m</td>
<td>n</td>
<td>0</td>
</tr>
<tr>
<td>IFNγ</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n/m</td>
<td>0</td>
</tr>
<tr>
<td>VEGF</td>
<td>n</td>
<td>n</td>
<td>y</td>
<td>n</td>
<td>1</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>0</td>
</tr>
</tbody>
</table>

n/m = not measured, n= no significant changes, y = yes significant changes over time

Table 21: Cytokine secretion over time by midbrain monoculture using Two-Way ANOVA with post-hoc Tukey’s Test

<table>
<thead>
<tr>
<th>VEHICLE</th>
<th>LPS</th>
<th>TNF</th>
<th>A-SYNUCLEIN</th>
<th>IFNγ</th>
<th>NUMBER OF SIGNIFICANT CHANGES OVER TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1A</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>0</td>
</tr>
<tr>
<td>IL-1B</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>0</td>
</tr>
<tr>
<td>IL-4</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>0</td>
</tr>
<tr>
<td>IL-6</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>0</td>
</tr>
<tr>
<td>IL-8</td>
<td>n</td>
<td>n</td>
<td>y</td>
<td>n</td>
<td>1</td>
</tr>
<tr>
<td>IL-10</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>0</td>
</tr>
<tr>
<td>MIP-1A</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>0</td>
</tr>
<tr>
<td>TNF</td>
<td>n</td>
<td>y</td>
<td>n/m</td>
<td>n</td>
<td>0</td>
</tr>
<tr>
<td>IFNγ</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n/m</td>
<td>0</td>
</tr>
<tr>
<td>VEGF</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>0</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>0</td>
</tr>
</tbody>
</table>

n/m = not measured, n= no significant changes, y = yes significant changes over time
Conclusions:

Vehicle:

- No significant change over time during incubation.

LPS:

- No significant change over time during incubation.

TNF:

- No significant change over time during incubation.

IFN-γ:

- No significant change over time during incubation.

α-Synuclein:

- No significant change over time during incubation.

Figure 97: Midbrain monoculture secretion of IL-1α over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. No significant alterations to secretion observed.
Figure 98: Midbrain monoculture secretion of IL-18 over time by incubation treatment. N=3. Data analysed using One-Way ANOVA.

No significant alterations to secretion observed
Figure 99: Midbrain monoculture secretion of IL-4 over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. Incubation with Vehicle significantly altered secretion over time.
Figure 100: Midbrain monoculture secretion of IL-6 over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. Vehicle, LPS and TNF all significantly alter IL-6 secretion over time.
Figure 101: Midbrain monoculture secretion of IL-8 over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. Vehicle, LPS, TNF and α-synuclein all significantly alter IL-6 secretion over time.
Figure 102: Midbrain monoculture secretion of IL-10 over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. No significant alterations to secretion observed.
Figure 103: Midbrain monoculture secretion of MIP-1α over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. Incubation with TNF significantly altered secretion over time.
Figure 104: Midbrain monculture secretion of TNF over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. No significant alterations to secretion were observed.
Figure 105: Midbrain monolayer secretion of IFN-γ over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. No significant alterations to secretion were observed.
Figure 106: Midbrain monoculture secretion of VEGF over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. Incubation with TNF significantly altered secretion over time.
Figure 107: Midbrain monoculture secretion of VEGF over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. No significant alterations over time observed.
Co-cultures of midbrain neurons with microglia in midbrain media

Using a less conservative method of data analysis (a One-way ANOVA), results in a greater number of significant events being recorded for co-cultures of midbrain neurons with microglia in vitro, as summarised in Tables 22 and 23. Detailed data can be observed in Figures 107-117.

Table 22: Cytokine secretion over time in co-cultures using One-Way ANOVA

<table>
<thead>
<tr>
<th>VEHICLE</th>
<th>LPS</th>
<th>TNF</th>
<th>A-SYNUCLEIN</th>
<th>IFNγ</th>
<th>NUMBER OF SIGNIFICANT CHANGES OVER TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1A</td>
<td>n</td>
<td>n</td>
<td>y</td>
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</tr>
<tr>
<td>IL-1B</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n/d</td>
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</tr>
<tr>
<td>IL-4</td>
<td>n</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
<td>0</td>
</tr>
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<td>n</td>
<td>y</td>
<td>n</td>
<td>y</td>
<td>2</td>
</tr>
<tr>
<td>IL-8</td>
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<td>n</td>
<td>y</td>
<td>n</td>
<td>1</td>
</tr>
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<td>y</td>
<td>n</td>
<td>n</td>
<td>1</td>
</tr>
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<td>MIP-1A</td>
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<td>y</td>
<td>y</td>
<td>n</td>
<td>3</td>
</tr>
<tr>
<td>TNF</td>
<td>n</td>
<td>n</td>
<td>n/m</td>
<td>y</td>
<td>1</td>
</tr>
<tr>
<td>IFNγ</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n/m</td>
<td>0</td>
</tr>
<tr>
<td>VEGF</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td>y</td>
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<td>CX3CL1</td>
<td>n</td>
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<td>n</td>
<td>0</td>
</tr>
</tbody>
</table>

n/d = not detected, n/m = not measured, n= no significant changes, y = yes significant changes over time

Table 23: Cytokine secretion over time in co-cultures using Two-Way ANOVA with post-hoc Tukey’s Test

<table>
<thead>
<tr>
<th>VEHICLE</th>
<th>LPS</th>
<th>TNF</th>
<th>A-SYNUCLEIN</th>
<th>IFNγ</th>
<th>NUMBER OF SIGNIFICANT CHANGES OVER TIME</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0</td>
</tr>
<tr>
<td>IL-1B</td>
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<td>n</td>
<td>n</td>
<td>n</td>
<td>0</td>
</tr>
<tr>
<td>IL-4</td>
<td>y</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>1</td>
</tr>
<tr>
<td>IL-6</td>
<td>n</td>
<td>n</td>
<td>y</td>
<td>n</td>
<td>1</td>
</tr>
<tr>
<td>IL-8</td>
<td>n</td>
<td>n</td>
<td>y</td>
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<td>n</td>
<td>1</td>
</tr>
<tr>
<td>TNF</td>
<td>n</td>
<td>n</td>
<td>n/m</td>
<td>n</td>
<td>0</td>
</tr>
<tr>
<td>IFNγ</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n/m</td>
<td>0</td>
</tr>
<tr>
<td>VEGF</td>
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<td>n</td>
<td>y</td>
<td>y</td>
<td>2</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>0</td>
</tr>
</tbody>
</table>

n/d = not detected, n/m = not measured, n= no significant changes, y = yes significant changes over time
### ANOVA Summary

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>P value</th>
<th>P value summary</th>
<th>Significant diff. among means (P &lt; 0.05)?</th>
<th>R square</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vehicle</strong></td>
<td>0.9036</td>
<td>0.4782</td>
<td>NS</td>
<td>No</td>
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</tr>
</tbody>
</table>

**Conclusion:** No significant change over time during incubation with Vehicle

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>P value</th>
<th>P value summary</th>
<th>Significant diff. among means (P &lt; 0.05)?</th>
<th>R square</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LPS</strong></td>
<td>0.9339</td>
<td>0.4854</td>
<td>NS</td>
<td>No</td>
<td>0.13</td>
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</tbody>
</table>

**Conclusion:** No significant difference over time during incubation with LPS

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>P value</th>
<th>P value summary</th>
<th>Significant diff. among means (P &lt; 0.05)?</th>
<th>R square</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TNF</strong></td>
<td>3.887</td>
<td>0.0143</td>
<td>*</td>
<td>Yes</td>
<td>0.3931</td>
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</table>

**Conclusion:** Significant change over time during incubation with TNF

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>P value</th>
<th>P value summary</th>
<th>Significant diff. among means (P &lt; 0.05)?</th>
<th>R square</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IFNγ</strong></td>
<td>0.07</td>
<td>0.8271</td>
<td>NS</td>
<td>No</td>
<td>0.1084</td>
</tr>
</tbody>
</table>

**Conclusion:** No significant change over time during incubation with IFN γ

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>P value</th>
<th>P value summary</th>
<th>Significant diff. among means (P &lt; 0.05)?</th>
<th>R square</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>α-Synuclein</strong></td>
<td>1.35</td>
<td>0.2470</td>
<td>NS</td>
<td>No</td>
<td>0.1884</td>
</tr>
</tbody>
</table>

**Conclusion:** No significant change over time during incubation with α-synuclein

---

**Figure 108:** Co-culture secretion of IL-1α over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. Secretion of IL-1α significantly altered over time during incubation with TNF.
Figure 109: Co-culture secretion of IL-1α over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. No significant alterations over time observed. Secretion during incubation with IFNγ not detected at any point (data not shown).
Figure 110: Co-culture secretion of IL-4 over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. No significant alterations over time observed. Secretion during incubation with LPS, TNF or IFNγ not detected at any point (data not shown).
Figure 11: Co-culture secretion of IL-6 over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. Secretion significantly altered over time during incubation with LPS and α-synuclein.
Figure 112: Co-culture secretion of IL-8 over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. Secretion significantly altered over time during incubation with TNF.
**Figure 113:** Co-culture secretion of IL-10 over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. Secretion significantly altered over time during incubation with TNF.
Figure 114: Co-culture secretion of MIP-1a over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. Secretion significantly altered over time during Vehicle, LPS and TNF incubation.
**Figure 115:** Co-culture secretion of TNF over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. Secretion significantly altered over time during incubation with α-synuclein.
Figure 116: Co-culture secretion of IFNγ over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. No significant alterations to secretion over time were observed.
Figure 117: Co-culture secretion of VEGF over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. Secretion significantly altered over time during all incubation conditions.
Figure 118: Co-culture secretion of CX3CL1 over time by incubation treatment. N=3. Data analysed using One-Way ANOVA. No significant alterations to secretion over time observed.
Microglia monoculture in forebrain media.

Using a less conservative method of data analysis (a One-way ANOVA), results in a greater number of significant events being recorded for microglia monocultures in forebrain media, as summarised in Tables 24 and 25. Detailed data can be observed in Figures 118-128.

Table 24: Cytokine secretion over time by microglia monoculture in forebrain media using One-Way ANOVA

<table>
<thead>
<tr>
<th>VEHICLE</th>
<th>LPS</th>
<th>TNF</th>
<th>AMYLOID-β</th>
<th>IFNγ</th>
<th>NUMBER OF SIGNIFICANT CHANGES OVER TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1A</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>y</td>
</tr>
<tr>
<td>IL-1B</td>
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<td>y</td>
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<td>TNF</td>
<td>n</td>
<td>y</td>
<td>n/m</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>IFNγ</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n/m</td>
<td>n</td>
</tr>
<tr>
<td>VEGF</td>
<td>y</td>
<td>y</td>
<td>n</td>
<td>n/m</td>
<td>n/m</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>y</td>
<td>y</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
</tbody>
</table>

n/m = not measured, n= no significant changes, y = yes significant changes over time

Table 25: Cytokine secretion over time by microglia monoculture in forebrain media using Two-Way ANOVA with post-hoc Tukey’s Test

<table>
<thead>
<tr>
<th>VEHICLE</th>
<th>LPS</th>
<th>TNF</th>
<th>AMYLOID-β</th>
<th>IFNγ</th>
<th>NUMBER OF SIGNIFICANT CHANGES OVER TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1A</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>IL-1B</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>IL-6</td>
<td>n</td>
<td>y</td>
<td>n</td>
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<td>n</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
</tbody>
</table>

n/m = not measured, n= no significant changes, y = yes significant changes over time

**measured,** **n=** **no significant changes,** **y =** **yes significant changes over time**
Figure 119: Secretion of IL-1α over time in microglia monocultures in FB media, by incubation treatment. N=3. Data analysed using One-Way ANOVA. Significant alteration over time during incubation with IFNγ.
Figure 120: Secretion of IL-1β over time in microglia monocultures in FB media, by incubation treatment. N=3. Data analysed using One-Way ANOVA. Significant alteration over time during LPS, TNF and IFNγ incubations.
**Figure 121:** Secretion of IL-6 over time in microglia monocultures in FB media, by incubation treatment. N=3. Data analysed using One-Way ANOVA. Significant alteration over time during LPS, and IFNγ incubations.
<table>
<thead>
<tr>
<th>Time Point</th>
<th>pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Hours</td>
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</tr>
<tr>
<td>4 Hours</td>
<td></td>
</tr>
<tr>
<td>8 Hours</td>
<td></td>
</tr>
<tr>
<td>24 Hours</td>
<td></td>
</tr>
<tr>
<td>48 Hours</td>
<td></td>
</tr>
</tbody>
</table>

**Vehicle**

<table>
<thead>
<tr>
<th>ANOVA Summary</th>
<th>F</th>
<th>P value</th>
<th>P value summary</th>
<th>Significant diff. among means (P &lt; 0.05)?</th>
<th>R square</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.436</td>
<td>0.0608</td>
<td>ns</td>
<td>No</td>
<td>0.178</td>
</tr>
</tbody>
</table>

**Conclusion:** No significant change over time during incubation with Vehicle.

---

**LPS**

<table>
<thead>
<tr>
<th>ANOVA Summary</th>
<th>F</th>
<th>P value</th>
<th>P value summary</th>
<th>Significant diff. among means (P &lt; 0.05)?</th>
<th>R square</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12.05</td>
<td>&lt;0.0001</td>
<td>****</td>
<td>Yes</td>
<td>0.5172</td>
</tr>
</tbody>
</table>

**Conclusion:** Significant change over time during incubation with LPS.

---

**TNF**

<table>
<thead>
<tr>
<th>ANOVA Summary</th>
<th>F</th>
<th>P value</th>
<th>P value summary</th>
<th>Significant diff. among means (P &lt; 0.05)?</th>
<th>R square</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10.89</td>
<td>&lt;0.0001</td>
<td>****</td>
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<td>0.5152</td>
</tr>
</tbody>
</table>

**Conclusion:** Significant change over time during incubation with TNF.

---

**IFNγ**

<table>
<thead>
<tr>
<th>ANOVA Summary</th>
<th>F</th>
<th>P value</th>
<th>P value summary</th>
<th>Significant diff. among means (P &lt; 0.05)?</th>
<th>R square</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.912</td>
<td>0.0417</td>
<td>ns</td>
<td>No</td>
<td>0.3178</td>
</tr>
</tbody>
</table>

**Conclusion:** Significant change over time during incubation with IFNγ.

---

**Amyloid-β**

<table>
<thead>
<tr>
<th>ANOVA Summary</th>
<th>F</th>
<th>P value</th>
<th>P value summary</th>
<th>Significant diff. among means (P &lt; 0.05)?</th>
<th>R square</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.016</td>
<td>0.1229</td>
<td>ns</td>
<td>No</td>
<td>0.2429</td>
</tr>
</tbody>
</table>

**Conclusion:** No significant change over time during incubation with Amyloid-β.

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Figure 122: Secretion of IL-8 over time in microglia monocultures in FB media, by incubation treatment. N=3. Data analysed using One-Way ANOVA. Significant alteration over time during LPS, TNF and IFNγ incubations.
Figure 123: Secretion of IL-8 over time in microglia monocultures in FB media, by incubation treatment. N=3. Data analysed using One-Way ANOVA. Significant alteration over time incubation with LPS.

Conclusion: Significant change over time during incubation with LPS.
Figure 124: Secretion of IL-10 over time in microglia monocultures in FB media, by incubation treatment. N=3. Data analysed using One-Way ANOVA. Significant alteration over time incubation with LPS.
Figure 125: Secretion of MIP-1α over time in microglia monocultures in FB media, by incubation treatment. N=3. Data analysed using One-Way ANOVA. Significant alteration over time during all incubation conditions.
Figure 126: Secretion of TNF over time in microglia monocultures in FB media, by incubation treatment. N=3. Data analysed using One-Way ANOVA. Significant alteration over time during incubation with LPS.
## Conclusions

- **Vehicle**: No significant change over time during incubation with Vehicle.
- **LPS**: No significant change over time during incubation with LPS.
- **TNF**: No significant change over time during incubation with TNF.
- **Amyloid-β**: No significant change over time during incubation with Amyloid-β.

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**Figure 127**: Secretion of IFNγ over time in microglia monocultures in FB media, by incubation treatment. N=3. Data analysed using One-Way ANOVA. No significant alterations to secretion observed.
Figure 128: Secretion of VEGF over time in microglia monocultures in FB media, by incubation treatment. N=3. Data analysed using One-Way ANOVA. Significant alteration over time during Vehicle and LPS incubations.
Figure 129: Concentration of CX3CL1 over time in microglia monocultures in FB media, by incubation treatment. N=3. Data analysed using One-Way ANOVA. Significant alteration over time during Vehicle and LPS incubations.