MONASH University

Stem cell therapies for preterm brain injury and inflammation

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A thesis submitted for the degree of
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The Ritchie Centre, Hudson Institute of Medical Research
Obstetrics and Gynaecology, Medicine, Nursing and Health Sciences.

Supervisors: Suzanne Miller, Courtney McDonald, Graham Jenkin, Beth Allison & Michael Fahey

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This thesis is dedicated to my Mum, Yvette

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Acknowledgements

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Declarations

I hereby declare that this thesis 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.

This thesis includes 2 original papers published in peer reviewed journals, 1 submitted publications and one traditional chapter (*Chapter 3*). The core theme of the thesis is perinatal brain injury and treatment with cell therapies.

The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Department of Obstetrics and Gynaecology, Monash University, under the supervision of Suzanne Miller, Courtney McDonald, Graham Jenkin, Beth Allison and Michael Fahey.

Co-authors are included in all published work to acknowledge active collaboration between researchers and the nature of team-based research. Sections are renumbered and collated to generate a consistent presentation within this thesis.

THESIS CHAPTER	PUBLICATION TITLE	STATUS	NATURE AND % OF STUDENT CONTRIBUTION
CHAPTER 1	*Perinatal brain injury as a consequence of preterm birth and intrauterine inflammation*	***Accepted***	80% Writing, drafting and revisions of manuscript
CHAPTER 4	*Human umbilical cord blood therapy protects cerebral white matter from systemic LPS exposure in preterm fetal sheep*	***Accepted***	80% Performed all lab work, data analysis and writing of original manuscript drafts
CHAPTER 5	*Preclinical examination of the neuroprotective effects of umbilical cord blood cells versus mesenchymal stem cells for inflammation-induced preterm brain injury*	***Submitted***	80% Performed all lab work, data analysis and writing of original manuscript drafts

Madison Claire Badawy Paton, PhD Candidate
16th August, 2018

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Suzanne Lee Miller, Supervisor
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Thesis summary

In Australia, a baby is born with brain injury that underlies cerebral palsy (CP) every 15 hours. Almost half of all individuals with CP in Australia were born preterm (less than 37 weeks gestation). A large proportion of these preterm births have been caused or complicated by an infection or inflammation of the placenta and its membranes during development, termed chorioamnionitis. Exposure of the fetus to chronic inflammation increases the risk of a fetal inflammatory response whereby the brain and other vital organs become damaged. The brain is most vulnerable to injury during a critical period around 28-32 weeks of gestation when white matter cells or oligodendrocyte progenitors are migrating, differentiating and becoming functional.

Brain injury following exposure to infection, preterm delivery, or a combination of both can lead to life-long neurological, learning and behaviour deficits. Unfortunately, there is no treatment that can be applied soon after birth that targets and protects the brain when an infant is born preterm and subsequent to chorioamnionitis. This thesis investigates different cell therapies for brain injury associated with preterm brain inflammation. I have compared the effects of umbilical cord blood (UCB) therapy versus a potential *off the shelf* cell treatment of mesenchymal stem cells (MSCs) in a fetal ovine model of preterm brain inflammation, induced via lipopolysaccharide (LPS) administration to the fetus.

Firstly, I detail the methodology I developed for isolation and characterisation of human UCB along with the expansion of umbilical cord tissue MSCs, and my attempts to expand cord blood endothelial progenitor cells (EPCs). I showed that healthy term umbilical cord blood was easily collected and characterised, and had minimal MSC content. A subset of expanded umbilical cord tissue MSCs met all the criteria for defining this specific cell type, including quad-lineage differentiation, normal karyotype and flow analysis. Whilst an array of expansion techniques were used to grow EPCs from UCB, these were unsuccessful. Accordingly, I principally used and compared UCB and MSCs as cell therapies in the subsequent experimental chapters.

In preclinical experiments, I used UCB as a potential neuroprotective therapy for the preterm brain in our model of brain inflammation; around 91 days gestational age, pregnant ewes underwent surgery to implant catheters directly to the fetus. After a period of recovery, 150ng LPS was administered via the fetal jugular vein daily for 3 consecutive days. On the third day, 6 hours after the final LPS administration, 100 million UCB mononuclear cells were administered. Seven days later, the fetal brains were collected and the efficacy of UCB as a therapy to protect the white matter of the brain was assessed histologically. My results show that UCB cells can prevent loss of total oligodendrocytes, as well as mature myelinating oligodendrocytes. UCB also reduced brain cell death and inflammation, thus proving to be a comprehensive treatment for preterm brain injury.

Lastly, I directly compared these results from the UCB group with a further cohort of animals treated with 10 million umbilical cord tissue isolated and characterised MSCs. My results show that overall, MSCs profoundly dampened the neuroinflammatory response to LPS. However, unlike UCB, MSCs did not prevent cell death, or the loss of mature myelinating oligodendrocytes. Molecular analysis showed that 7 days after the final LPS dose, gene expression of many inflammatory and blood brain barrier proteins were not different to those in control animals. Of significance however, in the MSC group, I observed a consistent downregulation of both pro-and anti-inflammatory genes and dampening of many other immune and growth genes which may affect the brain's ability for repair. Here, I show for the first time the contrasting effects of UCB and MSC treatment in an inflammatory model of brain injury, and reveal that UCB is the superior therapy in this context.

Overall, this thesis demonstrates that both UCB and MSC therapies have a role in improving outcomes following inflammation-induced brain injury. The results I have obtained show that UCB proves to be a more comprehensive intervention for the perinatal brain in a large animal model of brain inflammation, not only modulating inflammation, but stopping the progression of programmed cell death and white matter cell loss. This injury is a common neuropathology in the pathogenesis of CP. Taken together, these studies indicate that UCB should be pursued as an intervention for babies born preterm at risk of brain injury from exposure to *in utero* inflammation.

Research Disseminations

Manuscripts arising from this thesis

Paton, M., McDonald, C., Allison, B., Fahey, M. Jenkin, G. and Miller, S. (2017). Perinatal brain injury as a consequence of preterm birth and intrauterine inflammation: Designing targeted stem cell therapies. *Frontiers in Neuroscience*, 11: 200, doi: 10.3389/fnins.2017.00200.

Paton, M., Allison, B., Fahey, M., Sutherland, A., Nitsos, I., Bischof, R., Dean, J., Moss, T., Polglase, G., Jenkin, G., McDonald, C. and Miller, S. (2018). Human umbilical cord blood therapy protects cerebral white matter from systemic LPS exposure in preterm fetal sheep. *Developmental Neuroscience, In-Press, (accepted June 2018).*

Paton, M., Allison, B., Fahey, M., Sutherland, A., Nitsos, I., Pham, Y., Bischof, R., Moss, T., Polglase, G., Jenkin, G., McDonald, C. and Miller, S. (2018). Examination of the neuroprotective effects of umbilical cord blood versus mesenchymal stem cells for inflammation-induced preterm brain injury in fetal sheep. *American Journal of Obstetrics and Gynaecology, Submitted.*

Manuscripts arising from projects not included in this thesis

McDonald, C., Penny, T., **Paton, M**., Sutherland, et al. (2018). Effects of umbilical cord blood cells, and subtypes, to reduce neuroinflammation following perinatal hypoxic-ischemic brain injury. *Neuroinflammation*, 17; 15 (1). *Doi* 10.1186/s12974-018-1089-5.

Li, J., Yawno, T., Sutherland, A., Gurung, S**.**, **Paton, M.,** et al. (2018). Preterm umbilical cord blood derived mesenchymal stem/stromal cells protect preterm white matter brain development against hypoxia-ischemia**.** *Experimental Neurology*, *308 (1), 120-131. Doi* 10.1016/j.expneurol.2018.07.006

Aridas, J., McDonald, C, **Paton, M.**, Yawno, T., et al. (2015). Cord blood mononuclear cells prevent neuronal apoptosis in response to perinatal asphyxia in the newborn lamb. *Journal of Physiology*, 1;594 (5). doi 10.113/JP271104.

Conference abstracts

2018

Paton, M., Li, J., Allison, B., Fahey, M., Sutherland, A., Yawno, T., Bischof, R., Nitsos, I., Moss, T., Polglase, G., Jenkin, G., Miller, S and McDonald, C. Optimising perinatal stem cell therapy- Comparative analysis of umbilical cord blood cells vs mesenchymal stromal cells for preterm brain injury. Hershey Conference on Developmental Brain Injury, California, USA, 2018. Oral.

Paton, M., Allison, B., Fahey, M., Sutherland, A., Nitsos, I., Bischof, R., Moss, T., Polglase, G., Jenkin, G., McDonald, C. and Miller, S.. Mesenchymal stem cells modulate brain inflammation after LPS administration in preterm fetal sheep. PSANZ, Auckland, New Zealand, 2018. Oral

Paton, M., Allison, B., Fahey, M., Sutherland, A., Nitsos, I., Bischof, R., Moss, T., Polglase, G., Jenkin, G., McDonald, C. and Miller, S.. Assessing the efficacy of human umbilical cord blood versus mesenchymal stem cell therapy for preterm brain inflammation. FNW, Queenstown, New Zealand, 2018. Oral

2017

Paton, M, McDonald, C.M., Allison B., Fahey M, Sutherland A., Pham, Y., Moss, T., Polglase, G., Jenkin, G. and Miller, S. Assessing human umbilical cord blood therapy as an early treatment for preterm brain inflammation in fetal sheep. Fetal and Neonatal Physiological Society, Osaka, Japan, 2017. Oral

Paton, M, McDonald, C.M., Allison B., Fahey M, Sutherland A., Mihelakis, J., Nitsos, I., Stanojkovic, D., Jenkin, G. and Miller, S. LPS-induced brain injury in preterm fetal sheep is modulated by umbilical cord blood therapy**.** PSANZ 2017, to be published. Poster

McDonald, C., Penny, T., Paton, M., Sutherland, A., Nekkanti, L., Castillo-Melendez, M., Fahey, M., Jones, N., Jenkin, G. and Miller, S.. What is so special about umbilical cord blood: understanding the role of different cells in preventing cerebral palsy. PSANZ 2017, to be published. Poster

Penny, T., Sutherland, A., Paton, M., Pham, Y., Jenkin, G., Miller, S. and McDonald, C. Umbilical cord blood cell therapy for neonatal hypoxic ischemic brain injury: Does it influence long-term behavioral outcomes? PSANZ 2017. Poster

2016

Paton, M., Jenkin, G., Miller, S., Alers, N., Mockler, J. and McDonald, C.. Investigating the clinical application of preterm versus term umbilical cord blood**.** Journal of Paediatrics and Child Health**,** Issue 52, Issue Supplement S2. PSANZ 2016. Oral and poster

Paton, M, McDonald, C.M., Allison B., Fahey M, Sutherland A., Nitsos, I., Stanojkovic, D., Jenkin, G. and Miller, S. Brain inflammation in preterm fetal sheep to examine the benefits of umbilical cord blood and cord tissue stem cell therapies. FNW 2017, Townsville, Australia. Oral

Paton, M, McDonald, C.M., Allison B., Fahey M, Sutherland A., Mihelakis, J., Nitsos, I., Stanojkovic, D., Jenkin, G. and Miller, S. Modelling preterm brain inflammation in fetal sheep to assess the benefits of stem cell therapies. Monash Health Research Week. Poster

Paton, M, McDonald, C.M., Allison B., Fahey M, Sutherland A., Mihelakis, J., Nitsos, I., Stanojkovic, D., Jenkin, G. and Miller, S. Modelling preterm brain inflammation in fetal sheep to assess the benefits of stem cell therapies. Students of Brain Research Symposium 2016. Oral

2015

Paton, M, McDonald, C.M., Aridas, J., Yawno, T., Fahey M, Catillo-Melendez, M., Jenkin, G. and Miller, S. Umbilical cord blood stem cells: a new line of defence against cerebral palsy. Monash Health Research Week. Oral

Paton, M, McDonald, C.M., Aridas, J., Yawno, T., Fahey M, Catillo-Melendez, M., Jenkin, G. and Miller, S. Umbilical cord blood stem cells: a new line of defence against cerebral palsy. Ritchie Centre Colloquium. Poster

Prizes, awards and grants

2018:

Monash Postgraduate Publication Award, July-September round ($4,624)

Fetal and Neonatal Workshop of Australia and New Zealand, *Senior investigator prize for best oral presentation* ($400)

Perinatal Society of Australia and New Zealand- *Ritchie Centre Award for Translational Research* ($1000)

2017:

Kahli Sargent Research Studentship ($50,000)

Hudson Institute Travel Grant ($2500)

Falling Walls Lab Australia Finalist, with travel award to Canberra ($250)

Fetal and Neonatal Perinatal Society*. Tania Gunn Best Student Oral* ($500)

3 Minute Thesis, Ritchie Centre Colloquium, *1st Place* ($250)

Monash Health Research Week, *1st place Neuroscience & Mental Health* ($500)

Monash Health Research Week, *Hudson Institute Representative and Student Finalist, 2nd place oral presentation* ($300 travel grant)

2016:

Cerebral Palsy Alliance Career Development Grant ($52,578)

Perinatal Society of Australia and New Zealand, *PSANZ Travel Award* ($500)

Monash Novartis Engagement Award (All expenses-paid work experience, Sydney)

RTP Stipend- Monash Scholarship ($26,682 annually Feb 2016-Aug 2018)

2015:

Monash Health Research Week, *Monash Health Young Investigator award* ($1500).

3 Minute Thesis, The Ritchie Centre, *1st Place, first year PhD*.

Abbreviations and symbols

°C	Degrees Celsius
µg	Microgram
µL	Microlitre
µm	Micrometre
%	Percent
±	Plus or Minus
AEC	Amnion epithelial cells
APC	Allophycocyanin
APC-cy7	Allophycocyanin complex Cy7
BBB	Blood Brain Barrier
BBB	Blood Brain Barrier
BDNF	Brain Derived Growth Factor
BSA	Bovine Serum Albumin
Cas-3	Caspase-3
CD-	Cluster of Differentiation
cDNA	Complimentary Deoxyribonucleic Acid
cm	Centimetre
CNS	Central Nervous System
CP	Cerebral Palsy
CSF	Cerebrospinal Fluid
C_t	Cycle Threshold
CXCR	Chemokine Receptor
D	Day
DAB	3,3'-Diaminobenzadine
dH_2O	Distilled Water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPX	Dibutylphthalate Polystyrene Xylene
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay

EPCs	Endothelial Progenitor Cells
FACS	Fluorescent Activated Cell Sorting
FIRS	Fetal Inflammatory Response Syndrome
FITC	Fluorescein isothiocyanate
GDNF	Glial-Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
GMH	Germinal Matrix Haemorrhage
h/hr	hour
Hb	Haemoglobin
HIE	Hypoxic Ischemic Encephalopathy
HIF	Hypoxia Inducibible Factor
H_2O_2	Hydrogen Peroxide
HR	Heart Rate
HREC	Human Research Ethics Committee
IA	Intra-Amniotic
ID	Inner Diameter
Ig	Immunoglobulin
IV	Intra-Venous
Iba-1	Ionized Calcium-Binding Adapter Molecule-1
IL-	Interleukin
IVH	Intraventricular Haemorrhage
kg	Kilogram
LPS	Lipopolysaccharide
M	Molar Mass
MBP	Myelin Basic Protein
mg	Milligram
MHC	Major Histocompatability Complex
mL	Millilitre
mm	Millimetres
mM	Millimolar
mmHg	Millimetres of Mercury
MNCs	Mononuclear Cells

MSCs	Mesenchymal Stem Cells
mRNA	Messenger Ribonucleic Acid
MPO	Myeloperoxidase
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
MW	Molecular Weight
NaCl	Sodium Chloride
NGS	Normal Goat Serum
NICU	Neonatal Intensive Care Unit
nm	nanometre
NRS	Normal Rabbit Serum
OD	Outer Diameter
ODD	Oxygen dependant domains
Olig-2	Oligodendrocyte transcription factor-2
OPC	Oligodendrocyte Progenitor Cell
P	Passage
PaCO2	Partial Pressure of Carbon Dioxide
PaO2	Partial Pressure of Oxygen
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PFA	Paraformaldehyde
PG	Prostaglandin
pH	Potential Hydrogen
PROM	Premature Rupture of Membranes
PRR	Pattern recognition receptors
PVWM	Periventricular White Matter
PVL	Periventricular Leukomalacia
qPCR	Quantitative Real Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
s	Seconds
SCWM	Subcortical White Matter
SaO2	Arterial Oxygen Saturation

SD	Standard Deviation
SDF-1	Stromal Cell-Derived Growth Factor-1
SEM	Standard Error of the Mean
Strep-HRP	Streptavidin Horseradish Peroxidase
SVZ	Subventricular Zone
TBS	Tris-Buffered Saline
TJP	Tight Junction Protein
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
UCB	Umbilical Cord Blood
VEGF	Vascular Endothelial Growth Factor
WM	White Matter

Chapter 1

Literature Review

This chapter is presented in its published manuscript form, from the journal *Frontiers in Neuroscience*, with updated current literature provided thereafter.

1.1 Published review, *Frontiers in Neuroscience*

Paton, M., McDonald, C., Allison, B., Fahey, M. Jenkin, G. and Miller, S. (2017). Perinatal brain injury as a consequence of preterm birth and intrauterine inflammation: Designing targeted stem cell therapies. *Frontiers in Neuroscience*, 11: 200, doi: 10.3389/fnins.2017.00200.

frontiers in Neuroscience

REVIEW
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Perinatal Brain Injury As a Consequence of Preterm Birth and Intrauterine Inflammation: Designing Targeted Stem Cell Therapies

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Chorioamnionitis is a major cause of preterm birth and brain injury. Bacterial invasion of the chorion and amnion, and/or the placenta, can lead to a fetal inflammatory response, which in turn has significant adverse consequences for the developing fetal brain. Accordingly, there is a strong causal link between chorioamnionitis, preterm brain injury and the pathogenesis of severe postnatal neurological deficits and cerebral palsy. Currently there are no treatments to protect or repair against brain injury in preterm infants born after pregnancy compromised by intrauterine infection. This review describes the injurious cascade of events in the preterm brain in response to a severe fetal inflammatory event. We will highlight specific periods of increased vulnerability, and the potential effects of therapeutic intervention with cell-based therapies. Many clinical trials are underway to investigate the efficacy of stem cells to treat patients with cerebral palsy. Stem cells, obtained from umbilical cord tissue and cord blood, normally discarded after birth, are emerging as a safe and potentially effective therapy. It is not yet known, however, which stem cell type(s) are the most efficacious for administration to preterm infants to treat brain injury-mediated inflammation. Individual stem cell populations found in cord blood and tissue, such as mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs), have a number of potential benefits that may specifically target preterm inflammatory-induced brain injury. MSCs have strong immunomodulatory potential, protecting against global and local neuroinflammatory cascades triggered during infection to the fetus. EPCs have angiogenic and vascular reparative qualities that make them ideal for neurovascular repair. A combined therapy using both MSCs and EPCs to target inflammation and promote angiogenesis for re-establishment of vital vessel networks is a treatment concept that warrants further investigation.

Keywords: preterm, brain, inflammation, chorioamnionitis, stem cells, endothelial progenitor cells, mesenchymal stem cells, cerebral palsy

INTRODUCTION

Nearly 10% of all births are preterm, <37 weeks completed gestation (Beck et al., 2010). The survival of very preterm infants (28–32 weeks gestation) and extremely preterm infants (<28 weeks gestation) has improved over the past several decades (Miyazaki et al., 2007; Tita and Andrews, 2010), however prematurity still accounts for up to 70% of perinatal deaths and, in survivors, adverse neurodevelopmental outcomes, and cerebral palsy (CP; Singh et al., 2011). After birth there are no clinical treatments to protect or repair the brain of preterm infants. Moreover, whilst survival rates for very/extremely preterm infants have improved, a recent review revealed that the rate of CP in this population remains static (Oskoui et al., 2013). It is known that 40–70% of cases of preterm birth are complicated by inflammation affecting the placenta and its membranes, termed *chorioamnionitis* (Tita and Andrews, 2010). Chorioamnionitis and preterm birth are strongly linked, with rates of chorioamnionitis inversely correlated to fetal gestational age and preterm birth (Galinsky et al., 2013). Chorioamnionitis is implicated in 66% of preterm births at 24 weeks or earlier, with this figure decreasing to 16% by 34 weeks gestation (Lahra and Jeffery, 2004). Independently, both preterm birth and chorioamnionitis contribute to brain injury in infants born preterm and the development of subsequent neurological deficits. However, when these occur simultaneously, the degree of brain injury is more severe.

Acute inflammation of the feto-placental environment, broadly describing chorioamnionitis, is a principal contributor to premature rupture of membranes (PROM) and spontaneous preterm birth (Romero and Mazor, 1988; Tita and Andrews, 2010). PROM does not have to be present for a diagnosis of chorioamnionitis—more than one third of patients that deliver preterm will have intact placental membranes—yet 13% of these patients will later be diagnosed as having chorioamnionitis (Goncalves et al., 2002). Nevertheless, PROM is the most easily identifiable factor for indicating chorioamnionitis, which may be accompanied by vaginal bacterial abnormalities, meconium stained amniotic fluid, prolonged labor and altered placental histopathology (Tita and Andrews, 2010).

Chorioamnionitis causes fetal inflammation and injury to the immature brain, increasing the likelihood of intraventricular hemorrhage (IVH) and diffuse white matter injury (Wu and Colford, 2000). In addition to the fetal inflammatory response, hypoxia also contributes to perinatal brain injury associated with chorioamnionitis and preterm birth (Khwaja and Volpe, 2008). Both fetal inflammation and hypoxia mediate neuropathology, acting to induce breakdown of the blood brain barrier (BBB), neuroinflammation, and oligodendrocyte cell damage (Malaeb and Dammann, 2009). With no specific treatments available to protect the brain injury caused by infection, most cases involving ruptured membranes require the infant to be delivered early to reduce the risk of further compromise. However, by the time of delivery, the infant has been exposed to inflammatory mediators that pose significant risks to the developing brain. The current standard of care following diagnosis of chorioamnionitis during pregnancy is focused on timely delivery of the infant. Accordingly, fetuses that have been exposed to chorioamnionitis are often further compromised by preterm birth (Guzick and Winn, 1985), and the subsequent risks for the immature brain. In particular, chorioamnionitis and preterm birth both have profound adverse effects on the developing white matter of the brain, which is the key etiology in the pathogenesis of CP (Kaukola et al., 2006).

Abbreviations: CP, Cerebral palsy; PROM, premature rupture of membranes; IVH, intraventricular hemorrhage; BBB, blood-brain barrier; FIRS, fetal inflammatory response syndrome; IL, interleukin; PVL, periventricular leukomalacia; GMH, germinal matrix hemorrhage; LPS, lipopolysaccharide; TLR, toll-like receptor; ROS, reactive oxygen species; HIF-1α, hypoxia-inducible factor 1α; OPCs, oligodendrocyte progenitor cells; PRR, pattern recognition receptor; HIE, hypoxic-ischemic encephalopathy; ODD, oxygen-dependant domains; UCB, umbilical cord blood; EPCs, endothelial progenitor cells; MSCs, mesenchymal stem cells; MHC, major histocompatibility complex; HE, hepatocyte growth factor; BDNF, brain derived neurotrophic factor; VEGF, vascular endothelial growth factor; GDNF, glial-derived neurotrophic factor; PGE2, prostaglandin; SDF-1, stromal cell-derived factor; IGF-1, insulin-like growth factor; CXCR-4, C-X-C chemokine receptor-4.

CHORIOAMNIONITIS

Chorioamnionitis is characterized as intra-amniotic infection where bacterial invasion results in acute inflammation of the placenta and/or fetal membranes (Tita and Andrews, 2010). The bacterial species *Mycoplasma* is the most common bacterial form present in cases of chorioamnionitis, however multiple microorganisms may be involved (Romero and Mazor, 1988; Czikk et al., 2011). These include *Streptococcus hominis*, *Fusobacterium*, *Gardnerella vaginalis*, and *Escherichia coli*. Up to 25% of all preterm births <37 weeks gestation are complicated by chorioamnionitis (Goncalves et al., 2002). In some cases, chorioamnionitis is diagnosed before labor through maternal symptoms (referred to as *clinical chorioamnionitis*), and may be confirmed after delivery by histologic analysis of the placenta. Most cases are termed *subclinical chorioamnionitis*, with no obvious symptoms during pregnancy, but placental histopathology after preterm birth revealing significant levels of activated neutrophils and macrophages, indicative of infection (Miyazaki et al., 2007; Gisslen et al., 2016).

Neonates who are born with clinical chorioamnionitis may show symptoms of infection, including increased heart rate, decreased blood pressure and impaired cardiac output (Yanowitz et al., 2002). However, subclinical and undiagnosed infection is also implicated in serious adverse perinatal outcomes (Gibbs, 2001). One population-based study involving >250,000 Australian births found an overall incidence of subclinical histologic chorioamnionitis of 22.6%, however less than half of these cases showed a fetal inflammatory response as evidenced by the presence of infection in the umbilical cord and/or chorion (Gordon et al., 2011). The presence of a fetal inflammatory response was strongly associated with spontaneous preterm labor, but chorioamnionitis in the absence of a fetal inflammatory response was correlated to unexplained fetal death (Gordon et al., 2011). These data demonstrate that chorioamnionitis is a significant contributor to stillbirth and adverse neonatal outcomes, often mediated by fetal inflammatory response syndrome.

Fetal Inflammatory Response Syndrome (FIRS)

FIRS describes inflammation of multiple fetal organs *in utero* as a result of systemic immune activation, and is associated with increased preterm perinatal morbidity and mortality (Romero and Mazor, 1988; Bashiri et al., 2006). FIRS is defined by elevated interleukin-6 (IL-6) fetal plasma concentration in the presence of chorioamnionitis, PROM or preterm delivery (Madsen-Bouterse et al., 2010). The adverse consequences of fetal exposure to inflammation can be significant for the developing brain. There is a strong association between elevated cord blood IL-6 and the risk of CP for the preterm neonate (Yoon et al., 1995).

FIRS is present in the majority of fetuses exposed to chorioamnionitis (Gomez et al., 1997) principally because the fetus is in contact with an infectious amniotic fluid and membrane environment. Pro-inflammatory cytokines, such as IL-6, may also be present in the uteroplacental circulation (Yoon et al., 2000; Andrews et al., 2006). Where chorioamnionitis is present, the fetal lungs, skin, and gut have been shown to initiate a local inflammatory response, which in turn can evolve into a systemic response and FIRS (Kallapur et al., 2014). In some cases, however, FIRS may also be stimulated by blood-borne maternal or placental microorganisms (Tita and Andrews, 2010). Whatever the primary source, FIRS, and in particular elevated fetal circulating IL-6 concentration, is closely associated with preterm birth and adverse neonatal outcomes (Hofer et al., 2013). Specifically, high levels of fetal IL-6 are linked to fetal cardiovascular dysfunctions, and lung and brain pathologies (Galinsky et al., 2013).

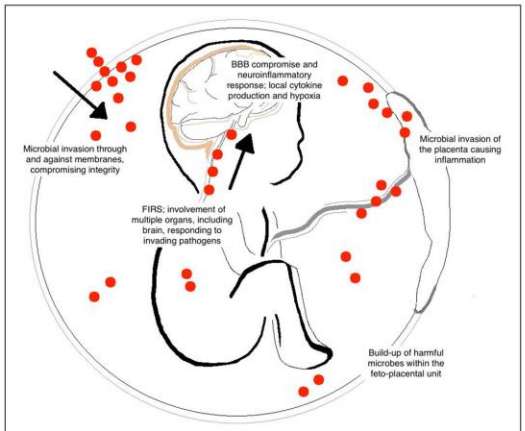

FIGURE 1 | Contributing factors of brain injury to the fetus following chorioamnionitis. Bacterial entry during fetal development can occur by many different routes including the placental circulation and through placental membranes (indicated by the red dots). The invasion of microorganisms results in inflammation of the feto-placental unit and the development of fetal inflammatory response syndrome (FIRS). Within the fetus, this can cause hypoxia, blood vessel damage, and blood brain barrier (BBB) compromise. These common responses lead to long-term alterations in white matter development within the fetal brain.

Brain Injury

Chorioamnionitis and FIRS are highly associated with neonatal brain injury and the subsequent diagnosis of CP (Shatrov et al., 2010; Kuypers et al., 2012). This is the result of many contributing factors and downstream inflammatory cascades that occur following microbial invasion of the feto-placental unit (**Figure 1**). Although primarily harmful, inflammatory cascades also involve the recruitment of immune cells and release of growth factors to areas of damage that stimulate endogenous brain repair and regeneration (Dammann and O'Shea, 2008). However, when brain inflammation becomes prolonged or severe, it can exacerbate damage through further influx of cytokines, chemokines, and other inflammatory mediators released from glial cells (Stoll et al., 2003).

Increased circulating pro-inflammatory cytokines, including IL-6, cross the BBB, increase BBB leakiness, and induce a neuroinflammatory response through the activation of microglia and astrocytes (McAdams and Juul, 2012; Schmidt et al., 2016). Pro-inflammatory cytokines that access the fetal brain then act upon vulnerable cell populations, disrupting normal maturation, and development of the white matter in particular (Dammann and Leviton, 1997). Accordingly, the most common neuropathology that occurs in human infants who were exposed to chorioamnionitis is white matter injury, diagnosed by cranial ultrasound, and MRI (Gaudet et al., 2009). The most severe white matter brain injury is generally observed in infants born between 24 and 32 weeks gestation (Volpe, 2009b). Either cystic or diffuse white matter injury bordering the ventricles of the brain, so called periventricular white matter lesions, or periventricular leukomalacia (PVL), is the most common form of brain injury detectable on MRI in patients born preterm and in those with CP (Volpe, 2001; Mann and Horber, 2007). Up to 90% of preterm babies who develop CP will have diffuse or cystic periventricular white matter injury (Krägeloh-Mann and Horber, 2007). It is well-described that this selective vulnerability of the developing white matter of the brain to an inflammatory stimulus is due to cell loss and/or interference with oligodendrocyte maturation (Back et al., 2007). The function of mature oligodendrocytes is to myelinate the developing axons, but impaired myelination is the key pathological feature of PVL. Mature, myelinating oligodendrocytes are present within the periventricular white matter region of the human brain from about 32 weeks gestation but, prior to this, pre-oligodendrocytes predominate (Back et al., 2001). Acute vulnerability of pre-oligodendrocytes is attributed to the predominance of these cells within the white matter (~90% of total oligodendrocytes) between 23 and 32 weeks gestation, with very few mature myelinating oligodendrocytes. If pre-oligodendrocyte development is disrupted, the normal maturation toward the myelinating cells is inhibited. In turn, hypomyelination and disorganization of the white matter is highly correlated with cognitive and functional impairments in children with CP (Hoon et al., 2009).

In addition to PVL, hemorrhage (germinal matrix or intraventricular hemorrhage, GMH/IVH) is a critical contributor

to poor neurodevelopmental outcome in preterm infants who were exposed to chorioamnionitis *in utero*. Within the preterm brain, the blood vessels have a relatively low density compared to the term brain, and are easily damaged by inflammatory stimuli which then increases the risk of bleeding and injury to surrounding white matter (Brew et al., 2014). The etiology of hemorrhage within the white matter of preterm infants is multifactorial, but is principally contributed by low vascular density, immaturity of cerebral vessels (with ongoing angiogenesis and vasculogenesis), together with blunted vasoreactivity (Brew et al., 2014). Pathological stimuli, including acute periods of hypoxia-ischemia, and influx of pro-inflammatory cytokines, contributes to brain bleeds with the immature white matter because of the inability of immature vessels to adequately adapt to changing perfusion pressure (Børch et al., 2010). Consequently, brain bleeds occur in up to 50% of infants born preterm at a very low birth weight and can contribute to PVL, with the white matter adjacent to the lateral ventricles becoming coagulated and necrotic (Volpe, 2009a). In contrast, in the very preterm period, the gray matter including cortical regions is highly vascularized and able to withstand injury or mild periods of hypoxia or inflammation (Brew et al., 2014).

Preterm birth and chorioamnionitis is characterized by periods of brain hypoxia, either from inflammation of the placenta disrupting blood flow to the fetus or as a result of inflammation (Baburamani et al., 2012; Stanek, 2013). In response to hypoxia, brain vasculogenesis is upregulated in an attempt to increase brain perfusion, such that capillary networks must sprout and develop to support oxygen and nutrient requirements of the brain (Baburamani et al., 2012). However, these new vessels tend to be more fragile and susceptible to pericytes detachment and cytokine damage. Periods of hypoxia and fluctuations in cerebral oxygenation are exacerbated after birth in preterm infants who require mechanical or assisted ventilation (Barton et al., 2014). This can cause brain damage through oxidative stress and inflammation (Polglase et al., 2012). Therefore, in preterm infants with the additional complication of chorioamnionitis, hypoxia as well as cerebral inflammation causes more severe brain injury (Adams Waldorf and McAdams, 2013).

EXPERIMENTAL ANIMAL MODELS TO EXAMINE CHORIOAMNIONITIS

Animal models of chorioamnionitis and fetal inflammation greatly assist the examination of mechanisms underlying neuroinflammation and the progression toward perinatal brain injury. Animal models are critical in assessing the therapeutic potential of various treatments, and lipopolysaccharide (LPS) is one of the most common experimental compounds used to mimic infection. LPS is a component of gram-negative bacterial wall and acts via toll-like receptors (TLRs, specifically TLR 4) to illicit a strong inflammatory response (Briscoe et al., 2006). Experimentally, sterile LPS can be administered during pregnancy into the amniotic cavity or directly to the fetus, to induce a fetal inflammatory response that mimics the inflammatory milieu and white matter brain injury that is observed in human chorioamnionitis (Grigsby et al., 2003; Duncan et al., 2006).

Animal models used to induce chorioamnionitis and a fetal inflammatory response have identified three critical mechanistic components that mediate the progression of perinatal brain injury; breakdown of the BBB, neuroinflammation, and hypoxia, which together contribute to neuropathology (Duncan, 2002; Back and Rivkees, 2004; Schmidt et al., 2016).

Breakdown of the Blood Brain Barrier (BBB)

The BBB provides the structural interface between circulating blood and cerebral tissue/cerebral extracellular fluid via a cellular barrier composed of endothelial cells (Ballabh et al., 2004). The BBB is a selective barrier, predominantly regulated by gap junctions between endothelial cells, externally lined by perivascular pericytes, and astrocytes, which together form the neurovascular unit (Weiss et al., 2009). In its role of physical separation of the circulatory components from cells within the brain, the BBB maintains brain homeostasis by preventing peripheral toxins and cells from entering the brain, removing waste products from the brain and regulating fluid and metabolic balance (Abbott et al., 2006). Under pathological conditions the BBB may become compromised, and this is shown to be the case in response to a severe fetal inflammatory insult (Ballabh et al., 2004).

Deterioration of the BBB occurs when the tight junctions between endothelial cells are compromised, particularly when membrane proteins are damaged (Nitta et al., 2003; Weiss et al., 2009). BBB compromise can be caused by innate immune activation and by the release of free radicals, prostaglandins, interleukins, glutamate, histamine, and many other substrates (Abbott et al., 2006). Whilst it is not really understood how, or if, endotoxins such as LPS infiltrate the brain, they may elicit damage through direct binding of TLRs on microglia within the brain or via cerebral endothelial cells of the BBB.

Neuroinflammation

As the brain matures, it is able to counter invading pathogens through innate immunity involving the pattern recognition receptors (PRRs), TLRs (Chen and Nuñez, 2010). However, when activation of these receptors is sustained, BBB permeability is enhanced, and peripheral immune cells gain access to the developing brain (Hagberg et al., 2015). Ongoing TLR activation on immune cells is associated with increased immune cell infiltrates, microglial activation and cytokine release (Cai et al., 2000). It is suggested that, in particular, TLR3 protein expression in the brain is upregulated in cases of white matter injury and invading pathogens in the preterm brain (Vontell et al., 2013). TLR3 is implicated in normal neural cell differentiation and proliferation. When injury to the premature brain occurs, redistribution of TLR3 on microglia over neurons can result in morphological changes in the white matter through the inhibition of axonal growth (Cameron et al., 2007). This is

hypothesized to be one of the main reasons that the brain has such limited regenerative capacity following injury.

Microglia are the resident immune cells in the brain that rapidly respond to inflammation or changes in the brain microenvironment (Kreutzberg, 1996). When the BBB becomes compromised, invading blood compounds may activate microglia rapidly; potentially within 60 s (Davalos et al., 2005). Aggregates of activated microglia are indicative of neuroinflammation and ongoing tissue injury through cytokine release (Verney et al., 2012; Adams Waldorf and McAdams, 2013). Microglial activation and cytokine release is also brain region specific; in fetal preterm sheep, LPS injected intra-amniotically increases IL-1β in the fetal brain cortex, hippocampus and cerebellar regions within 2 days (Strackx et al., 2015). After 7 days, microglial and astrocyte proliferation in damaged regions was evident with apoptosis and reduced myelination occurring in the subcortical white matter and hippocampus.

The key cytokine IL-6, as well IL-1α, IL-1β, and IL-18 are released from activated immune cells such as microglia, mast cells, and neutrophils, which in turn stimulate the release of reactive oxygen species (ROS), TNF, and excitatory amino acid agonists including glutamate, that act together to initiate neural cell apoptosis (Bona et al., 1999; Hagberg et al., 2015). These substances can have direct effects on vulnerable oligodendrocyte progenitor cells (OPCs). Three changes occur to OPCs that directly affect white matter development in the premature brain following inflammation: depletion of actively proliferating OPCs, maturational arrest of OPCs, and death of OPCs (Billiards et al., 2008; Volpe et al., 2011). Pro-inflammatory cytokine release and binding of IL1-β and TNF-α to their respective receptors TNF-R_1 and IL-$1R_1$ situated on oligodendrocytes is a principal cause of damage to these cells (Deng et al., 2014). It is known that periods of hypoxia lead to upregulation of TNF-R1 and IL-1 R_1 in the brain. Neighbouring reactive astrocytes release IL1-β and TNF-α, with signaling through their receptors causing downstream activation of apoptotic pathways and proliferative inhibition of oligodendrocytes (Deng et al., 2014).

Hypoxia

Animal models of perinatal compromise have elucidated that a fetal inflammatory response directly causes brain hypoxia-ischemia, and also results in an oxygen-independent inflammatory hypoxia, driven via activation of the hypoxia-inducible factor 1α (HIF-1α) pathway (Huang et al., 1998; Duncan, 2002; Peebles et al., 2003). Independently, each of these may contribute to the progression of white matter injury. TLR and NF-κB on placental tissues are activated and cytokine release is upregulated within the placenta and membranes (Briscoe et al., 2006). Pro-inflammatory cytokines cause physical and functional changes in placental tissues, with arteries and veins in the umbilical cord and placenta becoming inflamed (Tita and Andrews, 2010). Inflammation of the cord and the placenta can restrict blood flow and affect placental function. This can lead to acute or prolonged periods of hypoxia-ischemia during fetal gestation.

Within the brain, oxygen-independent hypoxia can occur as a consequence of neuroinflammation due to pro-inflammatory cytokines, ROS, and excitotoxic substances that stimulate anaerobic metabolism. At least in part, this is attributable to the release of nitric oxide from astrocytes following neuroinflammation, causing mitochondrial failure (Ferriero et al., 1996). Hypoxia also inhibits the degradation of HIF-1α (Huang et al., 1998) which may have duel effects to promote repair but also to induce injury (Shi, 2009). For example, HIF-1α regulates genes that mediate cell proliferation and angiogenesis; each of these processes are essential to tissue repair following insult, but aberrant cell proliferation is associated with maturational arrest of oligodendrocytes, and immature angiogenic blood vessels are associated with hemorrhage (Segovia et al., 2008; Brew et al., 2014). Hypoxia is also associated with calcium influx after inflammation-induced glutamate release from immune cells, causing excitotoxicity and resulting in Bax (pro-apoptotic protein) translocation to the mitochondria on OPCs and release of cytochrome-c. These observations indicate that hypoxia has the ability to impair the development of OPCs via activation of cell death through enzymes caspase-9 and caspase-3 (Simonishvili et al., 2013).

It is difficult to tease apart the individual contribution to brain injury that breakdown of the BBB, neuroinflammation, and hypoxia each make. Nonetheless, novel discoveries in large animal models using LPS to stimulate a fetal inflammatory response have revealed that inflammation and hypoxia can independently result in significant white matter injury (Duncan, 2002; Duncan et al., 2006). However, it is most likely that, in preterm infants exposed to chorioamnionitis, both inflammation and hypoxia contribute to the pathogenesis of white matter injury.

There are no approved therapies that can be offered to infants born preterm following exposure to chorioamnionitis, despite our knowledge that these infants are at the greatest risk for brain injury.

STEM CELL THERAPIES

Stem cells have emerged as a promising potential neuroprotective and neuroreparative treatment. Stem cells have the potential for self-renewal as well as the ability to differentiate into mature cell lineages (Bruder et al., 1997; Shen et al., 2004): a feature which allows stem cells to be beneficial for repair, remodeling or new tissue growth after tissue and cell damage. Stem cells also function indirectly to modify endogenous cell responses and can secrete growth factors and cytokines that mediate tissue repair (Baraniak and McDevitt, 2010). Umbilical cord blood (UCB) stem cells, in particular, have a number of benefits that support their therapeutic use for infants after birth who are deemed at high risk for brain injury following antenatal or birth complications. Clinical use of UCB has been well-described since the first UCB cell transplantation in 1988 (M-Reboredo et al., 2000). UCB is a commonly used therapy in a range of hematological conditions like blood cancers as well as graft-vs. host disease. The use of

UCB and placental stem cells avoids issues that surround the use of bone marrow derived, and other forms of stem cells that necessitate invasive collection, as the umbilical cord and placenta are normally discarded at birth. Additionally, due to the primitive nature of these cells and a lack of immune recognition surface antigens present on most adult cells, the risk of graft vs. host disease is reduced (Gluckman et al., 1997). Therefore, the risks associated with other more mature types of tissue transplant are minimized.

UCB Clinical Trials for CP

Clinical trials have demonstrated the safety of UCB administration to children with established CP (Lee et al., 2012; Feng et al., 2015; Novak et al., 2016). Currently, however, it remains unclear how effective UCB could be in the setting of chorioamnionitis and preterm birth. No trials have been established to assess the efficacy of UCB or stem cell therapies for white matter injuries in preterm newborns. There are however, a number of trials in children and adults that suggest these cells may be an effective therapy.

Novak and colleagues recently reviewed the evidence for the use of stem cells in improving gross motor function in children with CP (Novak et al., 2016); four randomized clinical trials and one non-randomized clinical trial were included for analysis. Two of these trials involved the use of allogeneic UCB transplantation (Min et al., 2013; Kang et al., 2015). All trials reported improvements in motor function following stem cell therapy. However, the two trials using UCB found that treatment, either administered alone or with rehabilitation, resulted in greater motor function improvements than rehabilitation alone. Combining the two studies allowed for the calculation of an odds ratio of 2.62 in favor of UCB therapy for CP. All stem cell trials reported low adverse events (<3%); these included vomiting, breathing difficulties, skin, or eye reactions, with no reported differences in serious adverse events such as hospitalization and seizures. This meta-analysis concluded that stem cell treatment in patients with established CP made a significant short-term improvement in gross motor function after 6 months. We await the results from longer follow-up (>2 years) to ascertain the long-term benefit of stem cell treatment.

Information regarding benefits or risks associated with allogeneic UCB transplantation are pivotal to understanding the types of cell therapy that may be suitable for preterm newborns. In the case of treating those vulnerable to preterm brain injury following chorioamnionitis, allogeneic cell treatment may be the most appropriate. Preterm babies are not the best candidates for autologous UCB transplants for a number of reasons; (i) collection volumes of UCB are proportional to gestational age and preterm birth is therefore implicated in low collection volumes (Wen et al., 2012; Mazzoccoli et al., 2016), (ii) it is unknown how maternal and fetal complications, including gestational diabetes, intrauterine growth restriction, preeclampsia, and chorioamnionitis, predominant in preterm births, alter the relative proportion of stem and immune cells (Wen et al., 2012; Li et al., 2014) and (iii) it has been suggested that, in large clinical trials, such as the Duke University UCB trial (clinicaltrails.gov), recruitment of participants was significantly lower than anticipated (63 recruited vs. a planned recruitment of 120) due to the lack of autologous cord blood storage in children that go on to be diagnosed with CP (Duke Translational Medicine Institute Website, 2012).

DESIGNING TARGETED STEM CELL THERAPIES: MSCS AND EPCS

As described above, a main injurious cascade associated with chorioamnionitis and fetal brain injury is inflammation. The major mechanism of action of UCB cells is its anti-inflammatory capacity. This is attributable to the many immunomodulatory cells present in UCB, including mesenchymal stem cells (MSCs; Liao et al., 2013; Zhao et al., 2016). A further injurious cascade related to chorioamnionitis is hypoxia and blood vessel damage. A potent and well-described population of cells in UCB, namely endothelial progenitor cells (EPCs), function to promote neovascularization and vessel repair (Kalka et al., 2000; Murohara et al., 2000). Therefore, a targeted cell therapy, using the cell properties of EPCs and MSCs may be favorable for preterm infants exposed to chorioamnionitis *in utero* (Broxmeyer, 2005; Mei et al., 2010).

MSCs

MSCs are readily sourced from placental tissues, including the umbilical cord, as well as adult bone marrow and adipose tissue (Hass et al., 2011). The neuroprotective and reparative capacity of MSCs is primarily facilitated by their anti-inflammatory properties and their capacity to secrete neurotrophic and anti-apoptotic factors (Mahmood et al., 2004; van Velthoven et al., 2010). MSCs are well-tolerated due to their low expression of cell surface molecules responsible for the initiation of immune responses, such as low expression of human MHC class 1 and their lack of MHC class 2 molecules (Jacobs et al., 2013).

MSCs prevent inflammatory and apoptotic processes involved in brain injury by actively down-regulating pro-inflammatory cytokines (Ahn et al., 2013; MacFarlane et al., 2013). This was shown in a rodent study where intraventricular hemorrhage was induced immediately following birth and UCB-derived MSCs were transplanted into the brain ventricle (Ahn et al., 2013). Treatment with MSCs suppressed the upregulation of cytokines IL-1α, IL-1β, IL-6, and TNF-α within the CSF and periventricular brain region. This resulted in an attenuation of swelling and fluid formation around the brain and improved behavioral outcomes. The role of MSCs in altering a systemic immune response has been directly demonstrated in an experiment on mice with sepsis (Mei et al., 2010). Six hours after induced sepsis, mice were treated with MSCs derived from bone marrow or saline (control). Results showed that mice treated with MSCs had reduced overall levels of circulating cytokines, down-regulation of inflammatory mediators like IL-6, IL-1β and an up-regulation of pathways responsible for bacterial elimination like C-C chemokine ligand 5 and Fc receptor-mediated immune-cell homing and phagocytosis in macrophages and monocytes. This study supports the use of MSCs for a targeted therapy against infection and inflammation.

MSCs immunomodulatory function is multifaceted, demonstrating not only direct secretion of anti-inflammatory cytokines but also altering immune cell programming and proliferation. MSCs have been shown to modify immune function directly by impairing the differentiation of dendritic cells, the main antigen-presenting cell in human immunity, from monocytes (Ramasamy et al., 2007). MSCs also alter dendritic cell release of pro-inflammatory cytokine TNF-α (Aggarwal and Pittenger, 2005). This can exert long term immune inhibition peripherally, which could be protective in the setting of preterm brain inflammation. T-helper cell production of IFN-γ in the presence of MSCs is also decreased by 50% and IL-4 production increased by over 500% (Aggarwal and Pittenger, 2005). IL-4 release promotes STAT6 pathway activation in MSCs that leads to an increase in TGF-β production (Kyurkchiev et al., 2014). In turn, TGF-β release from MSCs mediates inhibition of inflammatory T-helper cell cytokines, inhibits T-cell proliferation and increases recruitment of T-regulatory cells which help maintain immune tolerance (Zheng et al., 2015). The stimulation of natural killer cells in the presence of MSCs showed a reduced level of IFN-γ secretion by more than 80%. This is important because reducing IFN-γ release from T and natural killer cells inhibits the direct activation of macrophages and stops the activation of pro-inflammatory transcription factors like NF-κβ (Mühl and Pfeilschifter, 2003). These results indicate that MSCs have the capacity to alter macrophage function in the setting of inflammation and brain injury, likely acting via a reduction in immune response.

MSCs also secrete and recruit anti-inflammatory cytokines, growth factors and neurotrophic factors, including hepatocyte growth factor (HGF), brain derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), glial derived neurotrophic factor (GDNF), and IL-10 (Salgado et al., 2015). This is important as exogenous MSCs can promote a more anti-inflammatory environment within the brain, favoring reparative mechanisms. It has been postulated that a key immunomodulatory factor released by MSCs is prostaglandin (PGE2), with one study showing that when PGE2 was inhibited, the immunomodulatory effects of MSCs was abolished (Aggarwal and Pittenger, 2005).

If MSCs are to be used in neonates, expansion of cell populations may be necessary to provide a therapeutic dose. MSC colonies are only found in ∼20–30% of all UCB samples collected from term births (Fan et al., 2009), but umbilical cord tissue is a more readily available source of MSCs (Schugar et al., 2009). One gram of umbilical cord tissue can yield up to 11 million cells, of which up to 50% are characterized as mesenchymal progenitors (Schugar et al., 2009). These cells maintain their stem-like phenotype over multiple passages and show high proliferative capability. It has been shown that MSCs passaged up to six times still maintained their stem cell-like phenotype with tri-lineage differentiation potential, maintained expression of typical MSC surface antigens and lack of myeloid or endothelial surface antigens (Ruan et al., 2014). Therefore, a promising source of MSCs for expansion and use therapeutically for preterm brain inflammation is umbilical cord tissue.

If MSCs can be administered at a relevant time point during peak inflammation, they may be able to reduce the pro-inflammatory environment and protect the brain following chorioamnionitis. A mouse model of chorioamnionitis induced via fetal LPS exposure showed that maternal administration of MSCs resulted in reduced IL-6 concentration in the fetal brain (Lei et al., 2015). This was also accompanied by a reduction in activated microglia and improved neurobehavioral outcomes in mice. By suppressing pro-inflammatory mediators and enhancing anti-inflammatory factors, MSCs also create a more supportive environment for repair following brain injury.

Whilst MSCs can be administered following chorioamnionitis to protect against inflammation-induced brain injury, MSCs could potentially be combined with EPCs to support other neuroreparative processes, including vascular repair. Interestingly, MSCs and MSC-like spindle shaped cells have also been shown to support angiogenesis in multiple studies *in-vitro* when co-cultured with EPCs and also *in vivo* in the treatment of experimental hindlimb ischemia (Bhang et al., 2012; Peters et al., 2015).

EPCs

EPCs can be readily obtained from adult peripheral blood and UCB. EPCs alone may be a promising source for the treatment of inflammation-induced brain injury, with a number of studies reporting that under ischemic conditions, EPCs are able to migrate to regions of tissue damage and/or hypoxia and assist in the neovascularization process (Kalka et al., 2000; Fan et al., 2010).

Endogenous EPCs are well-recognized for their role in vasculogenesis. Vascular remodeling involves the recruitment of stem cells for the formation of new blood vessel and capillary networks, and begins with EPCs becoming mobilized from their primary source, bone marrow (Liu et al., 2007). Mobilization is supported primarily by VEGF, matrix metalloproteinase-9 and erythropoietin (Asahara et al., 1999; Dimmeler, 2010). After this initial phase, endogenous EPCs migrate toward vessel damage by "adhesion rolling" in which the cells attach to surface molecules found on the blood vessel walls (Liu and Velazquez, 2008). VEGF and multiple adhesion molecules including β2 integrins, E-selectin, and P-selectin, assist in this process (Chavakis et al., 2005). EPCs are then able to integrate into the injured endothelial monolayer to support neovascularization and further release of vascular growth factors.

In vivo animal experiments support that therapeutically administered EPCs home to regions of tissue damage and participate in vascularization (Kalka et al., 2000; Werner et al., 2003). Neovascularization at sites of damaged brain tissue is vital for cell repair, especially following inflammation and hypoxia. In response to hypoxic injury, blood vessels proliferate and form new networks (Baburamani et al., 2012). This is functionally important for the rapid restoration of oxygen to the brain. However, these newly formed capillaries are unstable and are likely to be dangerous for brain function in the long term, increasing the risk of intracranial hemorrhage (Ballabh, 2010). This abnormal blood vessel development is attributed to the decreased availability of VEGF and reduced EPC mobilization

immediately following an insult, and is a phenomenon noted in chorioamnionitis (Kramer et al., 2005; Mooney et al., 2014). Thus, exogenous administration of EPCs in the preterm infant may aid in the establishment of strengthened blood vessel formation that creates more viable and long-lasting vascular networks.

In addition to mediating blood vessel repair, EPCs may have therapeutic benefits through their paracrine activity. EPCs release multiple growth factors and cytokines that contribute to the inhibition of cell death, increased cell proliferation, and recruitment of local stem cells to the site of injury (Tongers et al., 2010). Following an inflammatory or ischemic insult, in which blood flow is impaired, endogenous endothelial cells become activated and initially release nitric oxide, allowing for the expansion of arterioles to compensate for vessel damage. When this expansion is prolonged, blood vessels shunt blood around damaged tissue where vessels have become non-functional and collateral vasculature is formed (Schaper and Scholz, 2003). EPCs themselves are highly receptive to the collateral response whereby they sense stress and endothelial activation, upregulate VEGF receptors and activate Akt signaling which promotes EPC differentiation at sites of damage (Mogi et al., 2008; Tongers et al., 2010). EPCs also release VEGF, stromal cell derived factor (SDF-1), and insulin-like growth factor (IGF-1), which are reparatory soluble factors stimulated following ischemic conditions (Urbich and Dimmeler, 2004; Urbich et al., 2005), with each playing a complimentary role in blood vessel repair. VEGF release is important following brain injury, not only in the formation and repair of blood vessels, but for stimulating axonal outgrowth and the inhibition of hypoxic death of neurons (Sun et al., 2003). VEGF release by EPCs also promotes neurogenesis by direct differentiation and growth of neural precursors. SDF-1 is necessary under inflammatory or ischemic conditions to recruit endothelial progenitors to sites of injury via C-X-C chemokine receptor-4 (CXCR-4; Peplow, 2014). For example, when vessel damage or hypoxia is present in the brain, SDF-1 is released from blood vessels and stem cells, which signals the recruitment of EPCs to the site of injury to aid in repair. IGF-1 is also critical for vascular remodeling and tissue repair as its release into the brain promotes neuronal growth and survival (Madathil et al., 2010). IGF-1 also assists in oligodendrocyte maturation and myelination (Mason et al., 2003). In multiple animal studies of brain ischemia, IGF-1 administration has been shown to reduce neuronal cell death and stimulated glial cell proliferation (Liu et al., 2001; Lin et al., 2009).

These data indicate the significant role that EPCs could play in neuroprotection through the regeneration of strong and long lasting blood vessel networks in brain regions at risk of damage. Thus, while EPCs demonstrate multiple potential neuroprotective functions, it is principally their capacity for blood vessel regeneration that has great use in the setting of chorioamnionitis and neuroinflammation.

Umbilical cord tissue and UCB are excellent sources of MSCs and EPCs, respectively. It is important to elucidate the potential for these cells independently, or as a combined therapy where EPCs and MSCs could be co-administered as a potential targeted therapy for chorioamnionitis and damage to the developing brain.

CONCLUSION

Chorioamnionitis and preterm birth are significant contributors to perinatal injury, and often co-exists. The consequences of fetal exposure to infection are complex, mediated by downstream systemic and cerebral hypoxic and inflammatory events that contribute to brain injury. It is known that chorioamnionitis and preterm birth are primary causes of perinatal brain injury and the subsequent development of CP, but currently there are no treatments that could be administered after birth to protect or repair the immature brain. We propose that targeted cell therapy with UCB or cord tissue MSCs and EPCs could be a suitable and efficacious solution. Administration of MSCs soon after birth during the peak phase of neuroinflammation, followed by the subsequent administration of EPCs to induce vascular remodeling, may act to normalize brain development and/or repair the damage consequent to chorioamnionitis. A combined therapy of UCB endothelial progenitor cells and cord tissue MSCs warrants further investigation as a neonatal therapy to reduce the burden of perinatal brain injury and CP.

AUTHOR CONTRIBUTIONS

All authors contributed to the intellectual data presented in this manuscript, all authors contributed to the preparation of this manuscript, and all authors gave consent to the final submission.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

1.2 Additional and relevant literature for this thesis

The role of stem cell therapies in neurodevelopment and perinatal brain injury is a fast-moving field. Additional and important literature on this topic is discussed in this section which were outside the scope of our published review paper (above) or were published after April 2017.

1.2.1 Trending therapies for the preterm brain and chorioamnionitis

Alongside stem cell therapies, there has been excellent further progress of other treatments for brain injury associated with preterm birth and fetal inflammation. These include the use of IL-1 receptor antagonists, human amnion epithelial cells and melatonin, to name a few.

1.2.1.1 IL-1 receptor antagonist

As previously stated, the primary symptom of clinical chorioamnionitis is maternal fever, mediated by pyrogens synthesised and secreted by macrophages. In particular, the pyrogen interleukin-1 (IL-1) is implicated in chorioamnionitis, with higher IL-1 levels in amniotic fluid and placental tissues correlated with a more severe inflammatory profile in the fetus, and subsequent poor neurological outcome [1]. When released, IL-1 binds to its receptors and signals the production of the gene products IL-1 α and β. IL-1β mRNA is expressed in the lung, brain and fetal membrane in response to chorioamnionitis. It has been shown that IL-1 receptor antagonists show efficacy in rats and sheep following inflammation, preventing the biological effects of LPS [2, 3].

Currently, there are no trials that investigate the role of IL-1 receptor antagonists for brain injury in babies or children. However, stage II trials for adult ischemic stroke and traumatic brain injury are underway [4, 5]. Early results have shown that IL-1 receptor antagonists are safe, well tolerated, reach the brain and reduce neuroinflammatory responses.

1.2.1.2 Amnion epithelial cells

All evidence to date suggests that amnion epithelial cells (AECs) work similarly to UCB cells as a therapy in preventing brain damage associated with chorioamnionitis. AECs are capable of secreting strong anti-inflammatory mediators and also have the ability to differentiate into many different lineages including neural cells [6, 7]. However much like MSCs, AECs most likely exert therapeutic benefits through their immunomodulatory and paracrine abilities. A large animal study of human AECs administered to fetal sheep following LPS showed that AEC treatment reduced grey and white matter injury [8]. The study concluded that this change was most likely due to direct modulation by cells over the recruitment and activation of inflammatory cells to the brain.

As shown with UCB cells, AECs migrate to sites of injury. They reduce inflammatory cascades within the brain and prevent cell death via the inhibition of microglial activation [8]. They can work directly to supress the release of cytokines TNF-α, IL-8, IL-6 and IL-1 at sites of injury, preventing the build-up of neutrophils and macrophages that can damage the brain tissue. However, AECs are also known to exert more long-term changes towards the modulation of inflammation. This effect was demonstrated in a sheep model of LPS-induced lung injury, where AEC therapy did not improve inflammatory outcomes after an acute insult [9]. AECs have also been shown in vitro to differentiate into neurons, secreting dopamine, glutamic acid, neurotrophic factors and other neurotransmitters [10]. This may allow for enhanced regeneration and recovery of the injured brain following inflammation. However, this differentiation has not been replicated in vivo.

In the last year, there have been no published studies on clinical trials using AECs for any neonatal brain injury, only methods published for use in adult stroke in a dose-escalation safety [11]. To date, there have been few studied which directly investigate the neuroprotective effects of AECs, all of which have been carried out in the sheep [8, 12]. These have significantly advanced the understanding of how AECs function to protect the white matter of the brain, with one study demonstrating that AECs prevent neuronal injury and white matter dysregulation [13]. Whilst AECs have huge potential as a therapy for a variety of perinatal complications and draws many functional similarities to UCB, AECs are a single cell type, and we propose that UCB may have more therapeutic

potential in the setting of fetal inflammation and white matter injury due to its heterogeneity.

1.2.1.3 Melatonin

Melatonin has been studied as a neuroprotective agent for many decades. Melatonin is considered a potential therapy in many conditions due to its potent antioxidant and free radical scavenging abilities. Endogenously, the pineal gland produces the majority of melatonin that regulates our circadian rhythm [14]. However, administering melatonin above endogenous levels is shown to assist with regeneration and repair in response to compromise, primarily through melatonin's strong antioxidant capabilities, but it can also mediate cell growth and differentiation of neural stem cells [15]. Since these progenitors are known to differentiate into mature neurons and other neural support cells, melatonin as a treatment of neurodegeneration and brain injury is under investigation.

It has been known for nearly 20 years that the actions of melatonin make it a potential therapy for inflammation and inflammatory-induced tissue damage [16]. In particular, LPS induced brain damage is effectively treated with melatonin in neonatal rats, with a reduction in oxidative stress and microglial activation [17]. Melatonin's mechanism of action as a neuroprotective agent is primarily due to its free radical scavenging, anti-inflammatory and anti-apoptotic abilities. Melatonin works by detoxifying reactive oxygen species (ROS) by donating a single electron to electrophic compounds and in doing so scavenges hydroxyl radical groups [18]. Free radicals within the brain after hypoxic or inflammatory events degrade cell membrane lipoproteins and malondialdehydes are produced as a result of lipid peroxidation [14]. Melatonin also acts indirectly to reduce lipid peroxidation through stimulating the activity of enzymes responsible for the metabolism of harmful ROS [19]. A recent study in term sheep following birth asphyxia revealed that melatonin administered intravenously (i.v.) or transdermally prevented significant brain injury and cell death within white matter brain regions [20]. Another ovine asphyxia study demonstrated that prophylactic maternal melatonin reduced white matter neuroinflammation and improved myelinating white matter cell counts in fetuses [21].

Melatonin clinical trials for the preterm brain are ongoing, with initial pharmacokinetic studies complete, and dosing trials finishing across the UK with longer-term studies planned to improve functional MRI in neonatal intensive care units [22, 23].

1.2.2 Updates on relevant cell therapies and clinical trials for cerebral palsy, 2017-2018

Much optimism surrounds the potential of stem cells as neuroprotective agents in the setting of preterm brain injury. Proponents emphasise that stem cells do not have limited or single targets like drug interventions. Stem cells have the capacity to be administered and respond to the environment using various cues [24]; resulting in a potentially unique therapy for every patient. Since early 2017, there has only been one trial that has published results describing the use of UCB therapy for children with established CP [25]. This randomised, double-blinded, cross-over study enrolled children up to 6 years of age with varying CP diagnoses for treatment with their own stored UCB. Cell-treated participants received between 10-50 million cells/kg delivered in a single dose. Motor outcomes and MRI were assessed at 1 and 2 years after treatment. Results demonstrated that children receiving cell doses greater than 20 million/kg had the best motor improvement, and these clinical findings also correlated with a normalisation of brain connectivity on MRI. From this study, we may be able to derive an appropriate cell dose to improve brain and motor outcomes. These results also support a potential correlation between improved clinical outcomes and structural brain changes.

Since the publication of the above literature review, there have been several reports and updates for current clinical trials involving the use of MSCs for CP. A randomised and placebo-controlled trial of MSC therapy was tested in patients aged 3-12 with diagnosed CP [26]. MSCs derived from UCB were administered in 4 doses over 4 weeks (5×10^7 cells each), along with daily rehabilitation. MSCs were shown to be safe, with no severe adverse events. Overall, the cells significantly enhanced gross motor ability, even 2 years post-treatment. However, mean age at cell treatment was around 7 years. A further randomised-controlled study directly compared the efficacy of bone marrow-MSCs to bone marrow mononuclear cells (MNCs) for established CP [27]. Patients aged from 6-12.5 years received 4 doses of autologous cells, equivalent to 1×10^6 million/kg each. At

one-year follow-up, results demonstrated that MSCs were significantly better than MNCs at improving both gross and fine motor function.

While these studies provide updated information supporting the use of UCB and MSCs to improve clinical and radiological outcome measures, there remain questions on dosing and timing of therapy. There is a need to standardise cell type, dosing and timing of administration. No published randomised clinical trials assess the efficacy of early stem cell-based therapy whilst preclinical data supports this, and recent work reflects that earlier interventions are associated with improved neurological outcomes [28, 29]. We propose that treating established CP is not as ideal as preventing it altogether.

1.3 Summary and project aims

There are currently no therapies that specifically target neuroprotection of the preterm brain following exposure to *in utero* inflammation. Cells derived from UCB and umbilical cord tissue are well characterised and have shown potential as a neonatal therapy but have never been tested in ovine experimental models of preterm brain inflammation.

The overall aim of this thesis is to investigate different cell therapies for the preterm brain exposed to *in utero* inflammation and injury. In the first experimental chapter (*Chapter 3*), we explore the ability to collect, expand and characterise different cell types. Due to the ease of collection of human UCB as well as the expansion of MSCs, we subsequently used these cell types in experimental chapters. We initially aimed to also examine a potential neuroprotective role of EPCs, but as detailed in Chapter 3, the cell expansion of EPCs proved extremely difficult and time consuming to standardise and accordingly EPCs were not examined further.

Chapters 4 and *5* of this thesis focus on the application and comparison of UCB cells and cord tissue MSCs in an ovine model of preterm brain inflammation. We hypothesised that both UCB and MSCs would protect against the loss of white matter cells and modulate neuroinflammation. We envisage that information gained from this thesis will inform future clinical practice for the most efficacious and appropriate cell therapy for infants born preterm at risk of brain injury as a result of *in utero* inflammation.

Chapter 2

General Methods

2.1 Human sample collection, cell isolation and preparation

2.1.1 Ethics approval

UCB and cord tissue was collected from healthy caesarean section deliveries at Monash Health, Clayton, Victoria. Signed consents were obtained under Human Research Ethics Committee (HREC) project number 01067B, reference: The Ritchie Centre Human Tissue Bank (Gynaecology and Perinatal), with Monash Health HREC 12387B for the storage and retrieval of cells for scientific experiments.

2.1.2 Patient eligibility

Women delivering at Monash Health or Jessie McPherson Private Hospital were approached regarding recruitment. Eligible patients were deemed "healthy" via inclusion criteria (Appendix Table 8.1) with an intact placenta suitable for umbilical cord blood collection. Pregnant mothers were approached regardless of gravidity, parity or age. For inclusion, babies were required to be born at term (>37 weeks completed gestation) with no abnormal fetal movements, Doppler's, ultrasounds or blood pathology results. Women were excluded if they had pre-existing medical conditions or any pregnancy or placental complications such as pre-eclampsia, intrauterine growth restriction or gestational diabetes.

2.1.3 Recruitment and informed consent

Informed consent and study information was provided by MP to any eligible patient (Appendix Document 8.1). Patients were informed about the general study aims and outcomes involving placental donation and were also made aware of the absence of incentives, patient follow-up, diagnosis, or patient-specific results generated from the study. All patient involvement and information from the study was kept confidential. Placental donation and the attendance of MP in the delivery room for collection of the placenta was voluntary. Patients were free to withdraw from the study at any time without any affect to their medical care. Final patient inclusion occurred following written

informed consent, obtained by MP in the presence of a witness, normally a nurse, family member or partner to the woman delivering.

2.1.4 Umbilical cord blood collection and MNC isolation

At caesarean section delivery, after clamping of the cord and immediate removal of the placenta, 100-150 ml of UCB was collected via the umbilical vein into a cord blood collection bag (Macopharma, NSW, Australia, 200 ml cord blood collection bag with 21 ml citrate phosphate dextrose with 8 ml line rinsing pouch). Collected blood was centrifuged at 1000 g for 12 minutes (acceleration 9, deceleration 0). The mononuclear cell (MNC) layer was collected via aspiration with a plastic pasture/transfer pipette (ThermoFisher Scientific, VIC, Australia) and rinsed with PBS (1 x, pH 7.4, ThermoFisher Scientific, VIC, Australia) before undergoing a red blood cell lysis (500 ml MilliQ water, 4.15 g NH_4Cl, 0.5 g $KHCO_3$ and 16.6 mg EDTA, 3 minutes). The reaction was stopped with excess media (16.5% fetal bovine serum [FBS] in DMEM:F12; ThermoFisher Scientific, VIC, Australia). A sample of the cells was aliquoted for cell count and viability assessment using trypan blue exclusion dye (Gibco, Waltham, MA, USA). Cells of a known concentration were cryopreserved in 10% dimethyl sulfoxide (DMSO) in FBS and stored in liquid nitrogen until cells were required.

UCB MNCs contains many different stem/progenitor cells. Initially, we planned to examine two principal cell types, endothelial progenitor cells (EPCs) from UCB and mesenchymal stem cells (MSCs) from cord tissue for neuroprotective potential. To do this, we first had to isolate and expand these cells from primary tissue and blood sources.

2.1.5 EPC isolation and expansion

After fresh MNC isolation (see section 2.1.4), cells underwent a series of magnetic column separations for CD133+ or CD34+ cell populations. CD133+ or CD34+ cells were isolated using a MicrobeadKit (Miltenyi Biotec, NSW, Australia) according to manufacturer's instructions. Briefly, an Fc receptor blocking buffer was applied to the MNCs along with CD133+ or CD34+ microbeads. Cells were incubated for 30 minutes, then washed with a rinsing solution (PBS with 0.5% bovine serum albumin (BSA, Sigma-

Aldrich, Missouri, USA) and 2 mM EDTA (Sigma-Aldrich, Missouri, USA) and centrifuged at 300 g for 5 minutes. Cells were then resuspended in 2ml of buffer and added onto a 3 ml magnetic column (LS 3ml Miltenyi Biotec, NSW, Australia). CD133- and CD34- flow-through was discarded, with the captured positive population eluted by flushing the demagnetised column. The positive population was then counted and cryopreserved in 10% DMSO in FBS, and stored in liquid nitrogen until required. Cells positive for CD133 were now referred to as "EPCs" used in this thesis.

For expansion experiments, frozen EPC aliquots were thawed, initially seeded into 96-well plates at either 10,000, 20,000 or 50,000 cells/well. Cells were plated in a specific endothelial media (endothelial cell growth medium-2, EGM-II, Lonza, VIC, Australia) suspended in serum-free expansion medium-2 (SFEM-II, StemCell Technologies, Australia) and supplemented in increasing growth factor concentrations according to well number. The wells contained a combination of vascular endothelial growth factor (VEGF), stromal cell-derived factor 1a (SDF), human stem cell factor (SCF), thrombopoietin (TPO), FMS-like tyrosine kinase (FLT), IL (interleukin) -6 and IL-3 with the combinations and concentrations dependent on the experiment (described in detail, *Chapter 3*).

2.1.5.1 MTS assays

For colorimetric quantification of cell number of EPCs after expansion experiments, the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay was used. The test plates were aspirated so all media was removed. In the case of cells which were semi-adherent, media was aspirated, centrifuged and the cell pellet was replaced in the wells. The MTS solution (CellTiter 96, Aqueous One Solution Cell Proliferation Assay, Promega, VIC, Australia), was thawed and diluted in EGM-II. The MTS assay solution was then added to the wells and left to incubate at 37°C, with the plate being read at 490 nm every hour (SpectraMax i3, Molecular Devices, Australian Biosearch, WA, Australia).

2.1.6 MSC isolation and expansion

After clamping of the cord and immediate delivery of the placenta, 10 cm sections of umbilical cord were cut and placed in a sterile dry container at room temperature. Umbilical cord tissue was immediately processed in a tissue culture facility. Umbilical cord tissue was rinsed with PBS and dried with sterile gauze. A specialty tissue mincing blade (kindly donated by Cytomatrix, Australia) was used to remove and discard umbilical cord segment ends. Remaining umbilical cord tissue was sectioned into 1cm pieces. Each 1cm piece was placed into a 10cm tissue culture plate, with the tissue then minced for 3-5 minutes. Each tissue culture plate was filled with 10ml media (16.5% FBS in Gibco DMEM:F12; ThermoFisher Scientific, VIC, Australia) and placed in an incubator at 37°C with 21% oxygen. Media was changed every 2-3 days. On day 7, cells were trypsinised from the plate (0.05% trypsin, 3 minutes at 37°C) and manually counted (trypan blue exclusion dye, Gibco, Waltham, MA, USA).

After 2 weeks of cell growth, plates with homogenous cell populations with fibroblast morphology were referred to as "MSCs". MSCs were reseeded at a density of 2000 cells/cm^2 in duplicate into tissue culture flasks (T175, Thermo Fisher Scientific, VIC, Australia). The remainder of the cells were cryopreserved in 10% DMSO (Merck Millipore, Vic, Australia) in FBS and stored in liquid nitrogen. MSCs were subcultured/passaged (P) when confluency reached 80%. At P2, cells were cryopreserved until required. 10 days prior to fresh cell infusion, P2 cells were rapid thawed and plated in T175 flasks at P3.

2.1.7 Flow cytometry cell verification

Flow cytometry was carried out on UCB, MSC and EPC samples. A sample of 10,000-100,000 cells was suspended in buffer (PBS and 10% FBS in distilled water) and placed into fluorescence activated cell sorting (FACS) tubes (BD, VIC, Australia). The tubes were centrifuged (300 g, 5 minutes) and a primary antibody cocktail was added to the tubes (Table 2.1) to determine specific target population surface marker expression. Samples were run alongside compensation controls (CompBeads, BD Biosciences, NSW, Australia) for the fluorescence fluorescein isothiocyanate (FITC), allophycocyanin (APC), allophycocyanin complex Cy7 (APC Cy7) and phycoerythrin (PE) for UCB and

FITC, APC, APC Cy7, peridinin-chlorophyll-protein complex cy5.5 (Percp cy5.5), V500 and PE for MSCs, prepared according to manufacturer's instructions.

Antibody cocktails or individual antibodies for each cell type are listed in Table 2.1. For UCB, monocytes were identified via gating of CD14+ cells from CD45+. Haematopoietic stem cells were identified from a population of CD34+/CD45low, CD45+ cells. Endothelial cells were gated from CD133+, CD45+ cells. MSCs were identified from CD45-, Stro-1+ cells. T-regulatory cells were gated from CD4+, CD25+ and Foxp3+ cell populations. MSCs were individually screened for populations of CD90+, CD105+, CD73+, CD44+, Stro-1+, HLA-ABC+, CD45-, CD34- and HLA-dr. Expanded EPCs from CD34+ of CD133+ UCB cells were incubated individually and gated for CD45+, CD133+, CD4+, VEGF+, and CD31+. Cell population values were recorded. Positive and negative cell populations as % from total living cells gated according to size and complexity, were recorded.

Table 2.1: Flow Cytometry Markers

Sample analysis	Antibody cocktail	Target
UCB MNCs	#1 CD14 (eBiosciences), CD34 (eBiosciences), CD133 (Miltenyi Biotech), CD45 (Miltenyi Biotech)	Monocytes, HSCs, EPCs
	#2 CD45 (Miltenyi Biotech), Stro-1	MSCs
	#3 CD4, CD25, Foxp3 (eBiosciences)	T-regulatory cells
Cord tissue MSCs P3	CD90+ (Bioss) CD105+ (Bioss) CD73+ (BD Biosciences)	Non-haematopoietic
	CD44+ (Miltenyi Biotech)	Mesenchymal
	Stro-1 + (BioLegend)	Mesenchymal/Endothelial
	CD45- (Miltenyi Biotech)	Leuokocytes
	CD34- (eBiosciences)	Non-haematopoetic
	HLA-ABC- (eBiosciences)	MHC class-1 antigen
	HLA-DR- (eBiosciences)	MHC class-2 antigen
UCB EPCs	CD45+ (Miltenyi Biotech)	General lymphocyte marker
	CD133+ (Miltenyi Biotech)	Endothelial progenitors
	CD34+ (eBiosciences)	Haematopoietic progenitors
	VEGF+ (R&D Systems)	Angiogenic/mature vascular marker
	CD31+ (BD Biosciences)	Endothelial adhesion molecule

2.2 Animal Surgery

Aseptic surgery was performed on pregnant Border-Leister cross Merino ewes carrying a single fetus at 90-92 days gestation (term = 147 days gestation). Firstly, pregnant ewes of known mating date were obtained from Gippsland Field Station, Monash University (Churchill, VIC) and acclimatised at Monash Animal Research Precinct before being transported for experimentation at Monash Medical Centre in Building Block F Animal House holding facility, at least one week prior to surgery. Ewes were fed twice daily with a mixture of Lucerne chaff and had free access to drinking water. Ewes were maintained in a constant 12 hr light/dark cycle (8am to 8pm) with an ambient temperature of ~20°C.

2.2.1 Pre-surgical preparation and anaesthesia

Animals were fasted 18-24 hours prior to surgery, although they had access to fresh drinking water. Anaesthesia was induced via an i.v. bolus injection of 20 mg/kg sodium thiopentone (Pentothal, Boehringer Ingelheim, Australia) into the maternal jugular vein. The ewe was intubated with a size 8 endotracheal tube (Portex Ltd., Kent, England) and anaesthesia was maintained using 1-2.5% isoflurane (Isoflow, Abott Pty. Ltd., Australia) in oxygen/nitrous oxide (O_2 2-3L : N_2O <1L). The ewe was continuously mechanically ventilated using a Campbell anaesthetic ventilator (UICI Engineering, Australia) for the duration of surgery.

At the time of anaesthesia, the ewe received a prophylactic dose of i.v. antibiotics with a combination of ampicillin (1 g, Austrapen, CSL Ltd., Parkville, Australia) and oxytetracycline (0.1 mg/kg; Engemycin, MSC Animal Health, New Zealand).

Once sedated, the ewe was placed in a supine position and the neck, abdomen and groin was shorn. The sites of incision were cleaned thoroughly using aqueous solution of chlorhexidine (gluconate 0.5% w/v in H_2O; Hibiclens, ICI Pharamaceuticals, Australia) and 3-4 applications of Betadine antiseptic scrub (10% w/v povidine-iodine; Flaudings, SA, Australia). This was followed by a final exterior covering of Betadine antiseptic solution (10% w/v povidine-iodine, Fauldings, SA, Australia). The ewe was moved onto

the surgical table and the incision sites were rinsed with chlorhexidine (gluconate 4% w/v; Hibiclens, ICI Pharmaceutics, Australia) in isopropyl alcohol (4% w/v).

Surgical equipment including drapes, towels and gowns were sterilised by autoclave. All maternal and fetal catheters were sterilised by ethylene oxide gas. The surgical procedures outlined were performed using strict aseptic technique. Surgeons scrubbed their hands and arms with chlorhexidine (gluconate 4% w/v isopropyl alcohol 4% w/v; Hibiclens, ICI Pharmaceutics, Australia) and wore caps, masks, sterile surgical gowns and gloves.

2.2.2 Fetal surgery

On the operating table, the ewe was covered with a sterile plastic sheet and drape. The incision site was exposed and a 15 cm incision just lateral to the abdominal midline was performed, ensuring that the superficial mammary veins were avoided. The incision extended from the umbilicus to the margin of the udders. The subcutaneous tissue was blunt dissected so that the linea alba was visible. A second incision was made through the linea alba, exposing the abdominal cavity and uterus. The uterine horn was then palpated to confirm a singleton pregnancy and the position of the fetus. The uterus overlying the fetal head was exteriorised through the abdominal incision, and a small incision as made to the uterine wall and fetal membranes to expose the head of the fetus. To minimise amniotic fluid loss, the uterine wall and fetal membranes were clamped to the skin of the fetal neck.

2.2.2.1 Fetal jugular vein catheterisation

With the fetal head exposed, a small incision was made to the left side of the neck where the jugular vein was then identified by blunt dissection. Two silk ties were placed around the vessel; the distal silk was tied to occlude the vessel and the proximal tie was held taught, occluding blood flow. A small incision was made to the vessel and a polyvinyl catheter (inner diameter 0.86 mm, outer diameter 1.52 mm; Dural Plastics, Australia) filled with heparinised saline (25,000 IU Heparin; Multiparin Heparin, Fisons Pty Ltd., Australia; in 1L saline 0.9% NaCl, Baxter Healthcare Pty. Ltd., Australia) was inserted into the vessel lumen. The catheter was then secured using the proximal tie placed around the vessel. The patency of the catheter was tested by flushing saline into the vessel. The

incision was closed using a continuous suture with a silk tie. The fetus was returned back into the uterus and the uterus closed. The uterus and its membranes were closed in two steps; Polysorb Suture 4-0 (Medtronic, NSW, Australia) using an interlocking mattress suture for the first layer, and internalising the first stitches on the second layer.

2.2.2.2 Fetal femoral artery catheterisation

The hindquarters of the fetus was exposed. An incision was made to the left inner hind-leg and the femoral artery was identified then isolated by blunt dissection. Two silk ties were placed around the vessel; the distal silk was tied to occlude the vessels and the proximal tie was held taught, occluding blood flow. A small incision was made to the vessel and a polyvinyl catheter (inner diameter 0.50 mm, outer diameter 1.00 mm; Dural Plastics, Australia) filled with heparinised saline (25,000 IU Heparin; Multiparin Heparin, Fisons Pty Ltd., Australia; in 1L saline 0.9% NaCl, Baxter Healthcare Pty. Ltd., Australia) was inserted into the vessel lumen. The catheter was then secured using the proximal tie placed around the vessel. The patency of the catheter was tested by flushing saline into the vessel. The incision was closed using a continuous suture with a silk tie.

2.2.2.3 Amniotic fluid catheter insertion

A third catheter (inner diameter 1.5 mm, outer diameter 2.7 mm; Dural Plastics, Australia) was secured onto the skin of the fetal abdomen for amniotic fluid sampling and pressure monitoring. This catheter was filled with heparinised saline (25,000 IU Heparin; Multiparin Heparin, Fisons Pty Ltd., Australia; in 1 L saline 0.9% NaCl, Baxter Healthcare Pty. Ltd., Australia) and secured to ensure that the end of the catheter would remain in the amniotic cavity once the fetus was returned to the uterus. Four small holes were cut in the secured end of the amniotic catheter to minimise the likelihood of being blocked by fetal membranes.

All catheters were then secured to the back of the fetus using a silk tie threaded through the skin, 1 cm from the tail, to ensure that the catheters did not become knotted or twist around the fetus. The fetus was returned into the uterus and the uterus closed as described above.

The catheters were then exteriorised through a small incision made in the right side of the ewe. The muscle and skin was then sutured to prevent leaking of any abdominal fluid using a continuous stitch (Vetafil Bengen; A.E. Stansen and Co., Australia). The linea alba was sutured with absorbable suture (Maxon Microfilament Absorbable Suture, Medtronic, NSW, Australia) using an interrupted pattern. The subcutaneous tissues including the adipose layer were closed using a continuous suture (Maxon Microfilament Absorbable Suture) followed by a simple continuous stitch using a synthetic suture (Vetafil Bengen; A.E. Stansen and Co., Australia) to close the skin.

2.2.3 Maternal Surgery

Whilst the fetal surgery was being undertaken and the ewe was in a supine position, an additional surgeon isolated the maternal jugular vein through palpation. An incision was made in the right side of the neck, to expose the jugular vein through blunt dissection. The vein was occluded on one side using a silk tie, with a small incision being made to the vessel wall so that a polyvinyl catheter (inner diameter 1.5 mm, outside diameter 2.5 mm) could be inserted 15-20 cm in the direction of the heart. The catheter was tied into place using silk ties and checked for patency. The skin was then closed using a simple continuous stitch with a silk suture (Vetafil Bengen).

When all catheters were surgically instrumented to both ewe and fetus, each catheter was fitted with a three-way stopcock and once again flushed with sterile heparinised saline (25,000 IU Heparin; Multiparin Heparin, Fisons Pty Ltd., Australia; in 1L saline 0.9% NaCl, Baxter Healthcare Pty. Ltd., Australia).

When the surgery was near completion, isoflurane was gradually withdrawn from the ventilation gas. This allowed for the ewe to slowly recover from anaesthesia. Each incision site was sprayed with Betadine antiseptic solution (10% w/v povidine-iodine, Fauldings, SA, Australia) and an abdominal sterile pad was placed on the abdominal incision. The ewe was fitted with elasticised tubular netting (size 7, Surgifix, Biersdorf Australia Pty Ltd., Australia) to provide post-surgical support. Once the ewe showed spontaneous breathing the mechanical ventilator was disconnected from the endotracheal tube. The ewe was monitored to ensure that continuous spontaneous breathing occurred,

and the endotracheal tube was removed when the swallowing reflex of the ewe was established.

2.2.4 Surgical aftercare

The ewe was administered a transdermal Fentanyl patch (Sandoz, NSW, Australia, 1mg/hr) on the inner left leg for pain relief. The ewe was then returned to a mobile cage with free access to Lucerne chaff. Once the ewe regained full consciousness with good neck control the ewe was also allowed free access to water. During the initial post-operative period (12 hours), the animal was intensely monitored to ensure that the animal was improving in condition including ability to move, stand and eat. Over the next 4 days, the ewes were allowed to recover from surgery without experimental intervention. During these 4 days, fetal arterial and maternal blood samples were collected to assess wellbeing (see section 2.3 below). All catheters were flushed daily to maintain patency. Ewes and fetuses were monitored daily with particular care given to the ewes' eating and drinking habits as well as wound healing.

Animals received antibiotics for three consecutive days post-surgery. Antibiotic cover was provided via a combination of ampicillin (500 mg) and engemycin (500 mg) i.v. to the ewe, ampicillin i.v. to the fetus (200 mg) and ampicillin into the amniotic fluid via the amniotic fluid catheter (300 mg).

2.2.5 Allocation of animals into treatment groups

Animals were randomly assigned to one of 4 groups, either; control: saline; LPS: 150 ng LPS (055:B5, University of Queensland); LPS+UCB: 150 ng LPS and 100 million UCB mononuclear cells; or LPS+MSCs: 150 ng LPS and 10 million human umbilical cord tissue MSCs (Figure 2.1).

2.3 Animal wellbeing, monitoring and maintenance

2.3.1 Recording of fetal haemodynamics

Following 4 days of recovery, fetal femoral arterial and amniotic fluid pressures were measured using pressure transducers (Becton, Dickson and Co., USA) and recorded

digitally using a computerised data acquisition system (PowerLab ADInstruments, NSW, Australia). Catheters were connected to pressure transducers and the signals amplified before they were recorded digitally. Mean femoral arterial pressure and heart rate were derived electronically from the femoral arterial pressure. All data were recorded and analysed using a computer software package (Chart v8, PowerLab ASInstruments, NSW, Australia).

2.3.2 Fetal blood sampling

The fetal sampling regimen of each study is shown in Figure 2.2. Fetal arterial blood gases were collected 1 hour prior to infusion (LPS/saline; -1 hour) on Day 1 (D1) then +1, 3, 6 and 12 hours after LPS/saline. This regimen was repeated on D2-4. From D5-10 arterial blood gases were taken daily. Amniotic fluid was collected daily from each animal in the control and LPS groups only.

Fetal arterial blood samples (0.1-0.2 ml) were collected for immediate analysis of blood gases, including partial pressure of oxygen (PaO_2; mmHg) and carbon dioxide ($PaCO_2$; mmHg), oxygen saturation (SaO_2; %), pH, haemoglobin (Hb; g/dL), glucose (mmol/L), lactate (mmol/L) and haematocrit (Hct; %). Analysis was made using an ABL Blood Analyser (ABL700 Blood Gas Analyser, Radiometer, Denmark). These samples were collected pre-LPS/saline on D1 then 1, 3, 6 and 12 hours after LPS or saline (Figure 2.2). This was repeated up until D4 where samples were collected once per day thereafter.

Fetal arterial blood samples (1 ml) were collected and transferred into an ethylene diamene tetra-acetic (EDTA) tube (BD Biosciences, NSW, Australia) to prevent coagulation. The blood was centrifuged (4000 rpm, 10 minutes) and the plasma was aliquoted into 3 samples for storage at -20°C.

One aliquot was transferred for long-term storage at -80°C for later cytokine analysis. These samples were collected pre-LPS/saline on D1 then 3, 6 and 12 hours after LPS or saline (Figure 2.2). This sampling regime was carried out each day until the end of D4, where daily samples were collected thereafter.

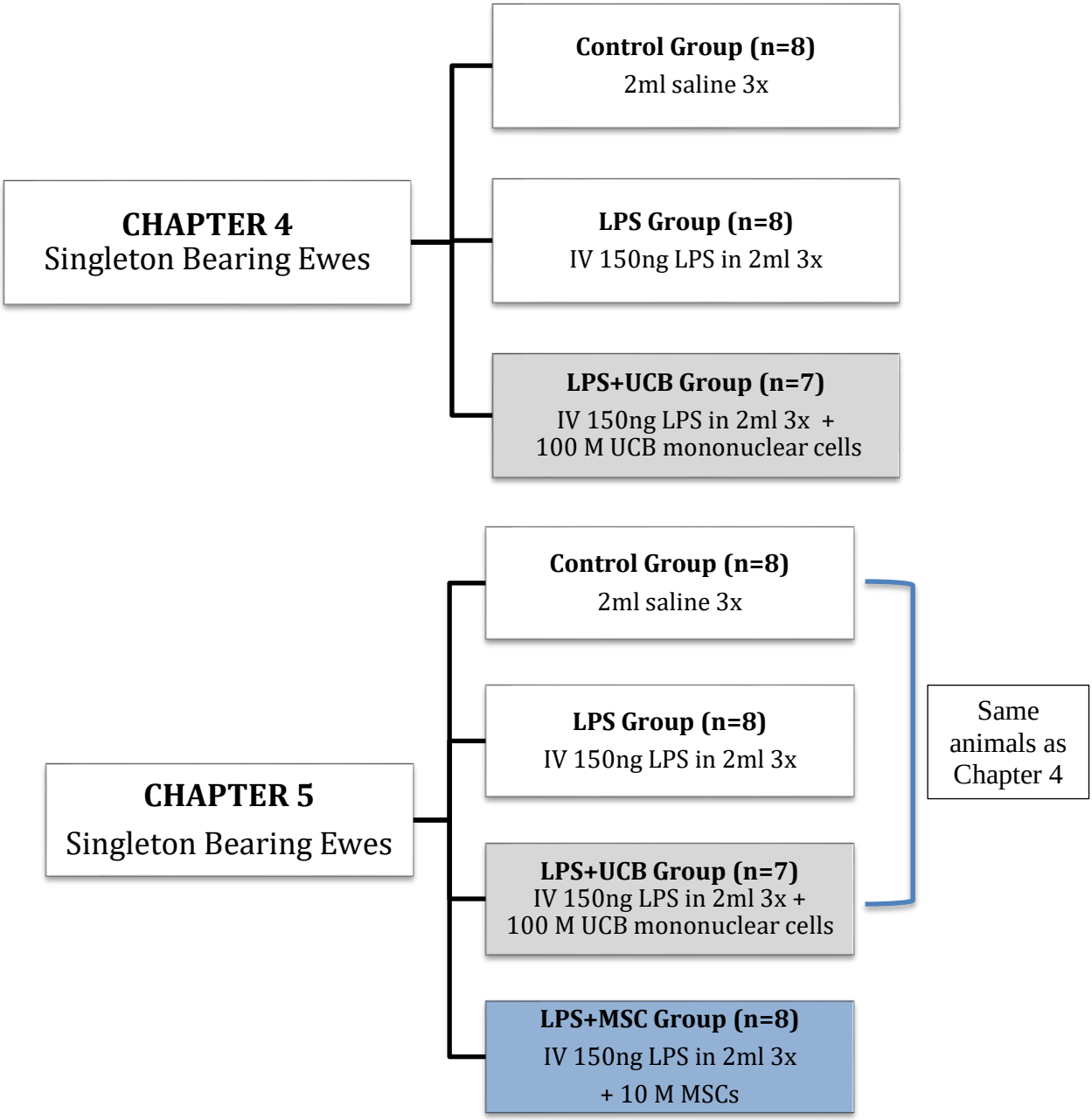
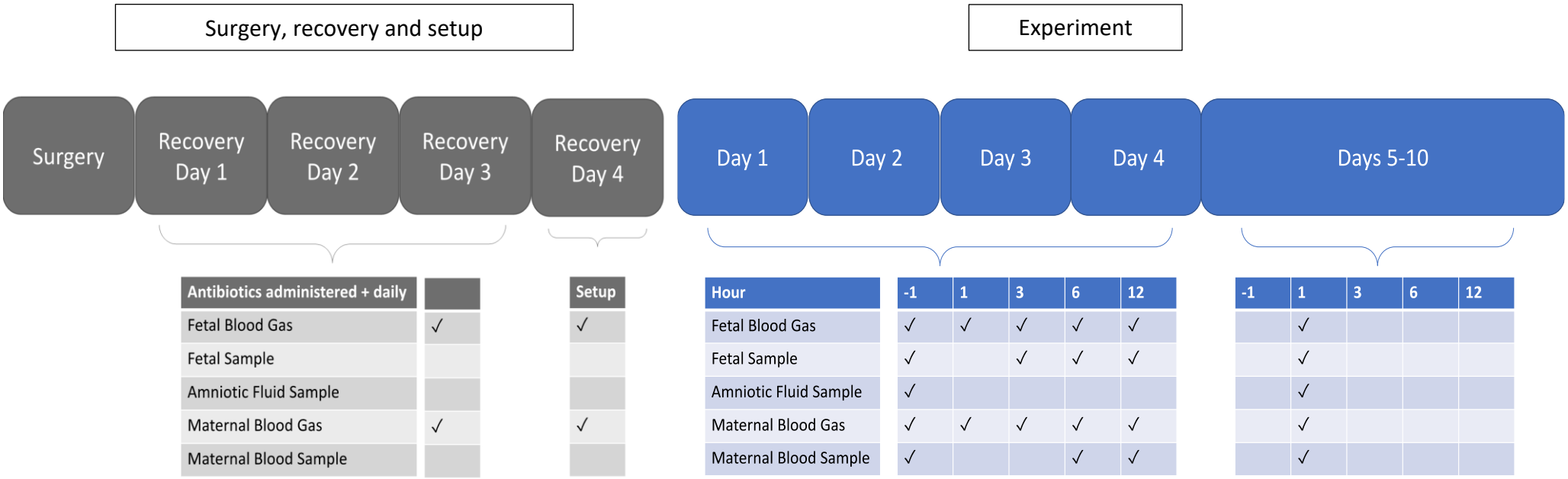


Figure 2.1: Experimental Animals for Each Chapter



Antibiotics administered + daily		Setup
Fetal Blood Gas	✓	✓
Fetal Sample		
Amniotic Fluid Sample		
Maternal Blood Gas	✓	✓
Maternal Blood Sample		

Hour	-1	1	3	6	12
Fetal Blood Gas	✓	✓	✓	✓	✓
Fetal Sample	✓		✓	✓	✓
Amniotic Fluid Sample	✓				
Maternal Blood Gas	✓	✓	✓	✓	✓
Maternal Blood Sample	✓			✓	✓

-1	1	3	6	12
	✓			
	✓			
	✓			
	✓			
	✓			

Figure 1.2: Experimental Timeline and Sampling Regime

2.3.3 Maternal blood collection

Maternal blood sampling was initially undertaken in animals assigned to the control and LPS alone groups. Maternal venous blood gas samples (0.2 ml) were collected for immediate analysis of blood gases. This included partial pressure of oxygen (PaO_2; mmHg) and carbon dioxide ($PaCO_2$; mmHg), oxygen saturation (SaO_2; %), pH, haemoglobin (Hb; g/dL), glucose (mmol/L), lactate (mmol/L) and haematocrit (Hct; %). Analysis was made using an ABL Blood Analyser (ABL700 Blood Gas Analyser, Radiometer, Denmark). These blood gases were collected pre-LPS/saline on D1 then 1, 3, 6 and 12 hours after LPS or saline (Figure 2.2). This was repeated up until D4 where samples were collected once per day thereafter.

Maternal venous samples (1 ml) were collected and transferred into an EDTA tube. The sample was centrifuged (4000 rpm, 10 minutes) and the plasma was aliquoted into 3 samples for storage at -20°C. One aliquot was transferred for long-term storage at -80°C for cytokine analysis. These samples were collected pre-LPS/saline, then at 6 and 12 hours after LPS or saline (Figure 2.2). From days 4-10, samples were then collected daily.

2.4 LPS and saline administration

On experimental Day 1, 2 and 3 (around 95-97 days gestation) fetuses received either saline (1.5 ml) or LPS (LPS; 055:B5, provided and manufactured by Philip Bird, University of Queensland, 150 ng in 1.5 ml sterile water) i.v. followed by a 2 ml saline flush. Beat to beat changes in fetal and maternal blood pressure and heart were recorded throughout the administration and subsequent recovery of LPS/saline injections.

2.5 Preparation and administration of cell treatment

On experimental Day 3, fetuses randomly allocated to receive stem cell treatment were administered 100 million UCB MNCs or 10 million MSCs. Treatment was given 6 hours after the final LPS/saline dose. Prior to cell administration, cells were incubated with carboxyfluorescein diacetate succinimidyl ester (CFSE) at 37°C for 15 minutes. The cells were then washed and resuspended in 2 ml PBS, transferred into a syringe and infused i.v. followed by a 2 ml saline flush.

See specific chapters (*Chapter 4 and 5*) for cell preparation prior to infusion.

2.6 Post mortem

On experimental Day 10, ewes and their fetuses were humanely killed by a lethal bolus dose of sodium pentobarbitone (100 mg/kg; Lethabarb Virbac Pty. Ltd., Peakhurst, Australia) injected via the maternal jugular vein catheter. The ewe was confirmed dead following absence of a papillary and somatic reflex. The fetus was removed from the uterus to undergo post mortem and tissue collection. Fetal weight, sex and general condition were noted and the presence of meconium staining was recorded.

2.6.1 Collection of CSF

The fetus was positioned so that the epidural space could be accessed. Cerebrospinal fluid (CSF) was collected via a 26-gauge needle-linked syringe. If CSF was contaminated by any blood matter, the samples was centrifuged (4000 rpm, 10 minutes). CSF was aliquoted and stored at -80°C.

2.6.2 Collection of fetal brain

The fetal brain was removed from the cranial cavity via a midline incision between the eyes to the top of the spine. The skull was removed to expose the brain and the spinal cord, the connective tissue and nerves were severed at cervical vertebrae 1. The brain was then collected and weighed. The 2 hemispheres were separated.

2.6.3 Right hemisphere brain preparation for histology

The entire right side of the brain was immersion fixed in 4% PFA, for later processing and use in histological analysis of a section labelled C1 (Figure 2.3).

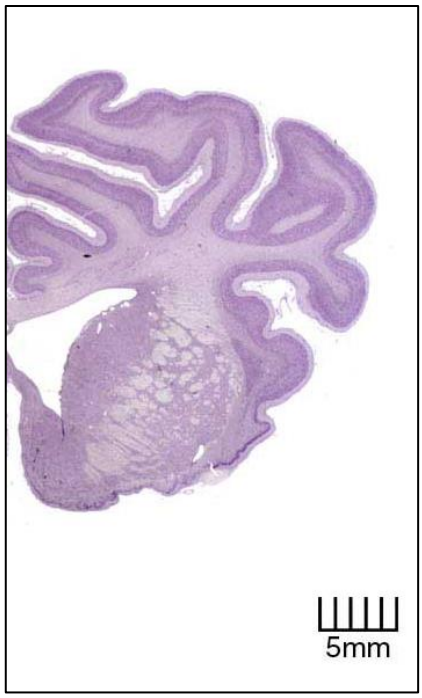


Figure 2.3: Representative image of sheep brain at the level of the lateral ventricles and full exposure of periventricular white matter, termed C1. Specimen 61-693, section 640 Welker Wisconsin Collection, image adapted from: https://msu.edu/~brains/brains/sheep/scans/0640/image1.html

2.6.4 Left hemisphere brain and organ collection for molecular analysis

The left hemisphere choroid plexus was collected and immediately snap-frozen in liquid nitrogen before being moved into a cryostorage vial (Cryo.s, PP, with screw cap, sterile; Greiner Bio-One, Frickenhausen, Germany). Left hemisphere brain tissue was dissected on ice for use in molecular analysis. A 0.5 cm segment was cut coronally at the level where white matter and midbrain were identifiable with identification of the lateral ventricles (Figure 2.3). A section of the most posterior 0.5 cm of the brain, was also collected. Both of these brain segments were embedded in optimum cutting temperature gel (OCT Compound, Sakura Finetek, CA, USA) and frozen on dry ice before being moved for storage at -80°C.

The spinal cord, pons and medulla were separated. All of the white matter was then dissected out of the brain, followed by the hippocampus and midbrain, thalamus. These brain and spinal cord samples were each individually homogenised by scalpel, snap frozen directly in liquid nitrogen, then placed for storage into cryostorage vials (Cryo.s, PP, with screw cap, sterile; Greiner Bio-One, Frickenhausen, Germany) and stored at -80°C.

2.6.5 Collection of other organs and tissue (not included in analysis)

The lungs, liver, kidneys, adrenals, spleen, thymus (left, upper) and heart were removed and weighed. The lung was perfusion fixed in formalin. The right side of the spleen was formalin fixed. A placental lobe, segment of the lower left lung lobe, spleen, thymus and adrenals were individually homogenised by scalpel, snap frozen directly in liquid nitrogen, then placed for storage into cryo-storage vials (Cryo.s, PP, with screw cap, sterile; Greiner Bio-One, Frickenhausen, Germany) and stored at -80°C.

2.7 Histological analysis

2.7.1.1 Paraffin embedding and tissue sectioning of the fetal brain

After 4 days in fixing solutions, the right side of the brain was prepared for embedding by placing segments into cassettes (Leica Biosystems, VIC, Australia). The tissue was then returned to fresh fixing solutions for a further 3 days. The cassettes were processed using an automatic tissue processor (Histokinette, Hendrey Pelays and Electric Equipment Ltd., U.K.). This process involved dehydration in 70%, 90% then 100% ethanol, followed by another 2 x 100% ethanol immersions. The tissues were then placed into two repeated incubations of 100% chloroform and then xylene, then placed into paraffin wax for 2 hours under vacuum to ensure complete penetration of the tissue.

Brain sections were cut at a thickness of 10 μm and transferred into a 40°C water bath before being mounted onto glass slides (Superfrost plus+, Menzel Glaser, Saarbruckner, Germany). The slides were baked in an oven at 60°C for one hour.

2.7.1.2 Immunohistochemistry procedure

Immunohistochemistry is the process of visually identifying proteins by binding to specific surface antigens. These experiments utilised primary and secondary antibodies

in combination. Specific protocols for each antibody is listed in Table 2.2. Firstly, the brain sections were cleared in a series of xylene and ethanol washes. Then, antigen retrieval was used to permeabilise tissue; the slides were heated in citric acid buffer (pH 6) for 20 minutes in a microwave. The tissue was left in the buffer for a further 20-30 minutes to cool. To increase permeability and overcome the high lipid content of the brain, further detergent steps were sometimes necessary through the use of Triton X-100 (Sigma-Aldrich, Missouri, USA) and Tween-20 (Sigma-Aldrich, Missouri, USA). These detergents were used independently and in combination depending on the primary selected and the individual protocols. Blocking procedures to account for any non-specific binding was then carried out using solutions containing BSA, normal goat serum (NGS), normal rabbit serum (NRS) or a general Dako Protein Block (Agilent Technologies, VIC, Australia). The primary antibody was then applied to the slides and left overnight.

The secondary antibody was then applied to the sections and left to incubate. The secondary antibody binds to a streptavidin-horseradish peroxidase (Strep-HRP). So that this binding can be visualised, a final step applying diaminobenzadine (DAB) in combination with H_2O_2 is used to activate the Strep-HRP when bound. All positively bound primary will show as brown staining on the section.

2.7.1.3 Additional fluorescent procedures

In the instance where antigens were targeted with a fluorescent label instead of DAB, after incubating with primary antibodies a secondary with a bound fluorophore was added to the slides. This step was followed by rinses in distilled water to remove excess fluorescence and then binding was visualised by fluorescent microscopy.

2.7.2 Quantitative analysis of immunohistochemistry

Section C1 (Figure 2.3) was analysed in duplicate for each animal. Each brain region was imaged in triplicate (Olympus Microscope, Japan), generating 6 images per animal, per region. Regions of interest included periventricular white matter, subventricular white matter, subventricular zone and cortex. Cell counts were undertaken using Fiji/Image J

software. The cell counts were averaged for each animal and a mean was calculated for each group.

2.7.2.1 Analysis of Caspase-3, Olig-2 and MBP

Caspase-3 was used to identify apoptotic cells undergoing cell death. Olig-2 was used to analyse oligodendrocytes across the entire lineage, whilst MBP specifically labelled the mature and myelinating oligodendrocytes. The proportion of positively stained cells within the periventricular white matter, subventricular white matter, subventricular zone and cortex were assessed at 400x magnification. Cells per image were counted using ImageJ software (NIH Image, Bethesda, Maryland, USA). Mean counts were calculated across each animal, and averaged per group.

Table 2.2: Antibody-specific immunohistochemical protocol

Antibody	Target	Antigen Retrieval	Protein Block	Primary Antibody	[Primary Antibody]	Secondary Antibody	[Secondary Antibody]	Strep-HRP and DAB	[Strep-HRP] [DAB]
Caspase-3 (Cas-3)	Apoptotic cells	0.01M citric acid buffer (pH 6) heated for 15 mins then left to cool in buffer for 30 mins	5% NGS and 1% BSA in 0.3% TX-PBS	Mouse anti-Caspase-3, R&D Systems	1:1000	Goat anti-mouse, biotinylated IgG	1:200	Streptavidin horseradish peroxidase Metal enhanced DAB	1:200 1 DAB tablet in 10ml H2O + 3ul H2O2
Oligodendrocyte transcription factor-2 (Olig-2)	Oligodendrocytes (all lineage stages)	0.01M citric acid buffer (pH 6) heated for 15 mins then left to cool in buffer for 20 mins	5% NGS and 1% BSA in 0.3% TX-PBS	Mouse anti-Olig-2, Millipore	1:1000	Goat anti-mouse, biotinylated IgG	1:200	Streptavidin horseradish peroxidase Metal enhanced DAB i	1:200 1 DAB tablet in 10ml H2O + 3µl H2O2
Myelin basic protein (MBP)	Myelin (mature)	0.01M citric acid buffer (pH 6) heated for 15 mins then left to cool in buffer for 45 mins	10% NGS in PBS	Rat anti-MBP	1:500	Goat anti-rat	1:500	Streptavidin horseradish peroxidase Metal enhanced DAB	1:200 1 DAB tablet in 10ml H2O + 3µl H2O2
Albumin	Albumin protein in blood	Nil	5% NRS and 1% BSA in 0.3% TX-PBS	Rabbit anti-albumin	1:2000	Goat anti-rabbit	1:200	Streptavidin horseradish peroxidase Metal enhanced DAB	1:200 1 DAB tablet in 10ml H2O + 3µl H2O2

Glial Fibrillary Astrocytic Protein (GFAP)	Reactive astrocytes	0.01M citric acid buffer (pH 6) heated for 15 mins then left to cool in buffer for 30 mins	DAKO Protein Block	Mouse-anti GFAP	1:200	Goat anti-mouse, biotinylated IgG	1:200	Streptavidin horseradish peroxidase Metal enhanced DAB	1:200 1 DAB tablet in 10ml H2O + 3µl H2O2
O1 or O4	O1 – late oligodendrocyte progenitor O4 – early oligodendrocyte progenitor	Permealise sections with 0.1% T20 for 10 mins	DAKO Protein Block for 40 mins RT	Mouse-anti O4 OR Mouse-anti O1	1:200	Goat anti mouse alexa fluor 594	1:1000	Nil	Nil
Myeloperoxidase (MPO)	Perivascular neutrophils	1/10 proteinase K in PBS for 30 mins in humid chamber then 20 mins at RT	DAKO Protein Block for 30 mins RT	Rabbit anti-MPO	1:500	Goat anti-rabbit	1:200	Streptavidin horseradish peroxidase Metal enhanced DAB	1:200 1 DAB tablet in 10ml H2O + 3µl H2O2
Lectin	Microglia and macrophages	0.01M citric acid buffer (pH 6) heated for 15 mins then left to cool in buffer for 20 mins	5% NGS and 2% BSA in 0.1% TX-PBS for 30 mins	Peroxidase labelled lectin	1:200	Nil	Nil	Nil	1 DAB tablet in 10ml H2O + 3µl H2O2

2.7.2.2 Analysis of GFAP

GFAP was used as a marker for activated astrocytes/astrogliosis. The % coverage of positive cells within the periventricular white matter, subventricular white matter, subventricular zone and cortex were assessed at 400 x magnification. To calculate total area coverage within a selected image, an intensity threshold was set using ImageJ and batch analysis. Mean results were calculated across each animal, and averaged per group.

2.7.2.3 Analysis of lectin

Lectin immunohistochemistry labelled all microglia and macrophages. Lectin staining was analysed through selection of areas which contained easily distinguishable cell aggregates at 100 x magnification. Inflammatory cell aggregates were then captured at 200 x magnification to assess morphology. Animals were scored depending on the presence of a visualised aggregate in each brain region, as well as % coverage of total lectin aggregate relative to white matter region size, calculated using Aperio Digital Imaging software (Leica Biosystems).

2.7.2.4 Analysis of MPO

MPO was used to identify neutrophils associated with blood vessels within the brain. Blood vessels were identified within the periventricular white matter, subventricular white matter and subventricular zone. Blood vessels were selected at random and imaged at 400 x magnification. Vessels were quantified per field of view, and then also counted for the amount of positive MPO cells/vessel.

2.7.2.5 Analysis of fluorescent O1 and O4

O1 fluorescence was used to detect myelinating immature and mature oligodendrocytes, whilst O4 labelled the pre-myelinating immature and mature white matter cells. O1 and O4 was visualised through the subcortical white matter tracts of the brain using filter to view red fluorescence on a 594 antibody. Images were captured at 100 x and 200 x.

2.7.3 CSF and plasma cytokine analysis

Plasma collected on day 1 (prior to LPS), 2, 3, 6, 8 and 10 as well as CSF collected on day 10 were analysed for pro-inflammatory IL-1β and anti-inflammatory IL-10 using recombinant cytokines via capture enzyme-linked immunosorbent assay (ELISA) techniques on ovine-specific monoclonal antibodies, as previously reported [30, 31]. Briefly, well-plates were coated with antibodies (1:200 in coating buffer (0.05 M bicarbonate, pH 9.6) for IL-10 and 1:250 for IL-1β 1:250 for 1 hour. The plate was then blocked with 1% BSA (Sigma-Aldrich, Missouri, USA) in PBS for 1 hour. Samples of plasma or CSF were then applied to the wells and incubated for one hour, then detecting antibodies were added and incubated for 1 hour (1:4000 in diluting buffer (PBS with 0.05% Tween 20 (Sigma-Aldrich, Missouri, USA)., 0.1% BSA) for IL-10 and 1:500 for L-1β). Tetramethylbenzidinesubstrate solution (ThermoFisher Scientific, VIC, Australia) was added to each well for approximately 20 minutes, with the colour-change reaction stopped by adding 0.5 M H_2SO_4. The plate was then read on a plate reader (SpectraMax i3, Molecular Devices, Australian Biosearch, WA, Australia) at 570 nm. Recombinant ovine IL-1β and bovine IL-10 were used for standard curves [32].

2.7.3.1 Calculating cytokine concentrations

The concentrations of cytokines were determined relative to a standard curve. A limit of detection is determined for each plate using ([detection limit]/sqrt(2)). Values are expressed as ng/ml of sample tested.

2.7.4 Molecular analysis

2.7.4.1 RNA extraction and DNAase

Approximately 60-100 mg of snap-frozen tissue was weighed for RNA extraction and placed into an RNA free tube.

2.7.4.2 White matter RNA extraction

Purelink RNA Mini Kits (Thermo Fisher Scientific) were used for RNA extractions following manufacturer's instructions. Briefly, white matter underwent lysis using kit

made lysis buffer and 2-mercaptoethanol followed by homogenisation (Ultra-Turraz T-25; Janke & Kunkel, IKA-Laboritechnik, Germany). The tissue lysate was centrifuged (1600 rpm for 10 minutes) with the supernatant being collected and combined with 70% ethanol. The sample was shaken vigorously then immediately applied to the Purelink column. The columns were then centrifuged (1000 rpm for 2 minutes) with flow through discarded. On column DNase treatment was then applied as per section 2.7.4.4.

2.7.4.3 Spleen RNA extraction

Spleen samples underwent homogenisation with addition of TRIzol reagent (Thermo Fisher Scientific, VIC, Australia) to deactivate any RNA-degrading enzymes. The tissue lysate was applied to the Purelink RNA Mini Kit's column (Thermo Fisher Scientific, VIC, Australia), and manufacturer's instructions was followed, as described above. On column DNase treatment was then used as per section 2.7.4.4, below.

2.7.4.4 DNase treatment

DNase treatment (Purelink DNase, Thermo Fisher Scientific, VIC, Australia) was applied to both white matter and spleen samples after the on-column steps were complete to remove any DNA contamination. Wash buffer was applied to the column with 80 μl DNAse mixture (10 x DNAse I reaction buffer, with resuspended DNAse and water according to manufacturer's instructions) and incubated for 15 minutes at room temperature. Wash buffer was added to the column and samples centrifuged (1000 rpm for 2 minutes). A second buffer was added containing 50% ethanol and the columns were centrifuged (1000 rpm for 2 minutes). Finally, the sample RNA was eluted in 100 µl RNAse free water.

2.7.4.5 Nanodrop and gel electrophoresis

The RNA samples were immediately tested on Nanodrop (Nanodrop One, ThermoFisher Scientific, Vic, Australia) to check for purity and yield. 1 µl of each sample was tested for yield (ug/µl), absorbance at 260/230 and 260/230 ratio peaks of nucleic acid purity. Following determination of RNA yield, samples were prepared for gel electrophoresis. 1% agarose gel (0.5 g agarose (ThermoFisher Scientific, Vic, Australia), 5 µl gel red, 2 µl loading buffer in 50 ml 1x Tris-acetate EDTA solution was made and cooled. 2 μl

MilliQ water was added to 2 μl RNA. The sample was incubated at 65°C for 5 minutes then 2 μl gel loading dye (ThermoFisher Scientific, Vic, Australia) was added to the samples, then loaded into the gel. Electrodes were turned on and the gel was run at 100 volts for 30 minutes. The gel was then UV imaged (Molecular Imager Chemidoc XRS System, USA) for visualisation of rRNA bands.

2.7.4.6 cDNA synthesis

To make cDNA, manufacturer's instructions were followed using Superscript III First Strand kit (Invitrogen, ThermoFisher Scientific, Vic, Australia). Briefly, 1 μg of RNA was added with 1 μl random hexamers and 1 μl distilled nucleoside triphosphate mix, and combined with added water to a total of 11 μl. This mixture was heated to 65°C for 5 minutes, then incubated on ice for 1minute. 6 µl of dithiothritol and RNAseOUT buffer along with 1 μl Superscript III RT was added to the tubes and incubated for 5 minutes at 25°C, then 50°C for one hour. Samples were incubated at 70°C for 15 minutes, then 5µl of each sample was plated in a 96 well PCR plate (Sigma-Aldrich, Missouri, USA). Remaining cDNA was stored at -80°C.

2.7.4.7 PCR/Taqman

5µl of cDNA sample were prepared and plated onto a 96 well PCR plate (ThermoFisher Scientific, Vic, Australia) on ice and submitted to the Monash Health Translational Precinct Genomics Facility, Hudson Institute of Medical Research. Quality tests of the cDNA was performed using Sybr chemistry primers for housekeeping genes (ABI 7900 HT qPCR). This was followed by preamplification, then Taqman analysis of the 50 genes listed in Table 2.3 (96.96 Dynamic Array integrated fluidic circuit, Fluidigm). Primer sequences targeted were *ovis aries* specific from ThermoFisher Scientific (Vic, Australia).

Table 2.3: Ovis Aries probes, Taqman Gene Array (ThermoFisher Scientific)

Gene/probe	Accession #	Gene/probe	Accession #
18s (Housekeeping)	Oa4906333_g1	***RPL32*** (Housekeeping)	Oa04893129_g1
16s (Housekeeping)	Oa03225223_m1	***GAPDH*** (Housekeeping)	Oa04876321_m1
OAZ1 (Housekeeping)	Oa03220471_g1		
IL-1alpha	Oa04658682_m1	***MPO***	Oa04654413_g1
IL-1beta	Oa04656322_m1	***CAS-1***	Oa04775045_m1
IL-6	Oa04656315_m1	***FOXp3***	Oa03233950_g1
IL-10	Oa03212724_m1	***IL-18***	Oa04658606_m1
VEGF-R1	Oa04694159_m1	***TLR-4***	Oa04656419_m1
TNF	Oa04656867_g1	***NFkb1***	Oa04837805_m1
IFN-gamma	Oa04657363_m1	***MMP9***	Oa03215996_g1
GPX1	Oa04911462_g1	***MMP12***	Oa04744924_g1
OCLN	Oa04728972_m1	***TGF-B***	Oa04259484_m1
CASP3	Oa04817361_m1	***CCL2***	Oa04677078_m1
TJP-3	Oa04693836_g1	***CLDN1***	Oa03217991_m1
CXCL10	Oa04655788_g1	***CLDN7***	Oa03229690_g1
CXCL2/MCP-2	Oa04677078_m1	***TP53***	Oa03223218_g1
TNFa	Oa04655425_g1	***ANGIOGEN-1***	Oa04757067_m1
VEGFA	Oa04653812_m1	***HIF-1A***	Oa04877334_m1
TGFB1	Oa04259484_m1	***GDNF***	Oa04753601_m1
PDGFb	Oa04657355_m1	***CXCL12/SDF-1***	Oa04903471_m1
PECAM-1	Oa04677168_m1	***IGF-1***	Oa04657098_m1
IL-8	Bt03211906_m1	***NOX-1***	Oa04709255_g1
IL-4	Oa04927178_s1	***NOX-2***	Oa04793417_m1
1IL-2r	Oa04656586_m1	***NOX-4***	Oa04824941_g1
VCAM	Oa04918369_sH	***ICAM-1***	Oa04658651_m1

2.7.4.8 Molecular data analysis

Raw cycle threshold (C_t) values and amplification efficiencies of all target and housekeeping genes were imported into qBase^{Plus} Software in table format from Taqman generated output. The calibrated normalised relative quantities (CNRQ) of each gene was automatically calculated once housekeeping genes and tests were specified, and these values were exported for statistical analysis. Gene expression was expressed as a fold-change relative to controls, calculated from C_t values normalised to the 3 housekeeping genes, presented as CNRQ.

2.7.5 Statistical analysis

Cell counts and genes were analysed between groups via a one-way ANOVA (GraphPad Prism 7) with Tukey's post-hoc analysis. All physiological data was analysed via two-way repeated measures ANOVA with Tukey's post-hoc analysis, where HR and BP was compared between groups over time. Data is presented as mean +/- SEM. Statistical significance was set at $p<0.05$ for chapters in this thesis.

Chapter 3

Stem cell isolation, expansion and characterisation

This chapter has not been published or presented elsewhere and has been formatted as a traditional thesis chapter.

3.1 Abstract

Stem cells have great therapeutic potential as they can modulate the immune system and promote cell repair, making them treatment candidates for many conditions, including for neurodegenerative diseases. Stem cells can be isolated from a wide range of different sources, including the placenta and umbilical cord. The placenta and cord are discarded at birth, and therefore the harvesting of useful stem cells is very appealing, and large numbers of cells can be extracted. A single human umbilical cord blood (UCB) sample can yield ~500 million mononuclear cells (MNCs) [33], which can be used as a primary source of cells and in this case, requires minimal manipulation. Alternatively, individual stem and progenitor cell populations can be isolated from cord blood and cord tissue, such as endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs), but these must first be expanded in order to obtain clinical doses prior to therapeutic use.

In this study, we aimed to isolate and generate enough cells needed for our large animal studies, which reflect similar equivalent clinical doses required for human clinical trials. All cells were isolated from consented pregnant women with healthy, term, caesarean section placentas. UCB mononuclear cells were isolated in sufficient numbers to not require expansion, therefore full characterisation was performed on freshly isolated cells. MSCs from cord tissue and EPCs from UCB required expansion to increase the overall number of cells. Sufficient numbers of MSCs were expanded from umbilical cord tissue obtained from 2 donors and, upon characterisation, these donor cells met all requirements for the definition of an MSC according to international guidelines [34]. However, EPCs from UCB were not successfully expanded and did not yield enough cells for sufficient clinical doses, despite trying 11 different expansion trials.

In summary, this chapter describes our work to collect, isolate and expand cells from cord blood and cord tissue. UCB and cord tissue MSCs were effectively expanded to reach sufficient clinical doses required for human trials, however EPCs from cord blood were not able to be expanded using the protocols tested in this study. This is an important consideration when translating cell therapies for clinical use.

3.2 Introduction

Stem cells are a promising therapy for the prevention and treatment of cerebral palsy (CP). However, sufficient numbers of cells (the therapeutic dose) must be available for preclinical study and, in turn, clinical translation of this therapy. High cell yield is easily obtained from autologous sources such as human amnion epithelial cells (hAECs), bone marrow and umbilical cord blood (UCB) [35], where doses of >100 million cells can be achieved from single donor isolations. However, for other cell therapies where cells are not isolated in abundance, expansion may be required from either autologous or allogeneic sources.

Placental tissues are favoured as a source of cellular therapies as there are no ethical problems around their collection and use; placentas are routinely disposed of after delivery as medical waste. Also, due to the fetal origin of the tissues, cells from the placenta and UCB are considered primitive and have decreased expression of adult surface markers that would normally initiate immune responses in recipients [36]. Therefore, the risk of complications such as graft versus host disease and host rejection are reduced. The placenta is a particularly rich source of a variety of different stem and progenitor cells that make it an attractive source of cells for interventions to protect the developing brain. With respect to UCB, many new clinical trials are being undertaken to demonstrate safety and efficacy in established CP [37, 38]. Additionally, the use of term UCB is feasible with one donor sample potentially yielding over 500 million mononuclear cells (MNCs) [39].

It is important to highlight that autologous cell therapies in preterm populations are often hampered by low collection volume and yield [40]. Recent literature supports that UCB administered at doses above 20 million cells/kilogram contribute to improved neurological and motor outcomes [25]. Therefore, autologous UCB as a first line therapy for perinatal preterm brain injury may not be possible. Furthermore, sourcing donors and HLA-matching samples within the first few hours of life is difficult and not always feasible. In these instances, an *off-the-shelf* therapy that does not require donor matching and is pre-processed and stored may be favoured. This *off the shelf* therapy could potentially come from a universal donor which has been extensively characterised and

has cells with known potency. This is in contrast to primary isolated cells and UCB that are confounded by high donor variability in cell content, viability and potency.

Both endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) have potential at being able to target specific injurious cascades that occur following exposure to chorioamnionitis and preterm birth [41]. Therefore, both these cell types are proposed as being candidates as an *off the shelf* therapy for preterm brain injury. UCB is an ideal source of EPCs, in particular endothelial CD133+ cells. CD133+ cells are enriched with endothelial progenitors and this marker is also expressed in haematopoietic cells [42]. A protocol for isolation of this cell population from UCB using magnetic bead separation has already been published; this paper states that one human UCB sample can routinely provide around one million CD133+ cells [43]. However, expansion would be required to reach doses sufficient for clinical use as (~10 million cells [44, 45]), without expansion, multiple donor CD133+ fractions would have to be pooled (>5 donors/treatment) and this would not be feasible due to cost and time. There is extensive literature exploring the effect of growth factors and oxygen requirements for the expansion of a similar cell type, CD34+ haematopoietic stem cells [46, 47]. Published protocols can achieve an 80-fold cell expansion of these cells over 3 weeks [46]. Ideally, by optimising the combination of growth factors, a similar significant expansion of CD133+ cells would be possible.

Similar to EPCs, MSCs from UCB also require expansion to reach a cell number sufficient for clinical application; current clinical trials for a range of conditions administer 10^6 MSCs/ kilogram [48]. However, MSCs colonies can only be harvested from around 30% of all term human UCB samples [49], and in some studies, UCB MSCs could not be isolated at all [50, 51]. Further, even when MSCs are present in term UCB samples, the proportion of these cells is extremely low - less than 0.01% of all nucleated cells are MSCs [52]. Therefore, UCB as a primary source of MSCs is not highly feasible. An alternative source of fetal MSCs, obtained from umbilical cord tissue is considered a better option. MSCs isolated from cord tissue have well defined and characterised growth potential; the calculated MSC outgrowth from cord tissue when plated initially after isolation is over 3 million cells per gram of cord tissue sample [53]. Cord tissue MSCs also have an enhanced ability to differentiate, expand, avoid senescence and express

fewer mature cell markers compared to adult sources of MSCs [54, 55]. In this study, we sought to isolate and generate clinically relevant cell doses for each of our cells of interest, namely UCB, cord tissue derived MSCs and UCB derived EPCs.

3.3 Methods

Methods for this chapter can be found in *Chapter 2, General Methods*. Any specific cell culture methods with variations in standard growth factor combinations are restated in the results for clarification.

3.4 Results

3.4.1 UCB collection and analysis

In total, 6 donor UCB samples from term human pregnancies were collected and the MNCs isolated and stored using the method described in *Chapter 2*. On average, each donor yielded 92.20 ± 18.24 ml of blood with a total mononuclear cell count of 627.60 ± 22.10 million (Table 3.1).

Table 3.1: UCB collected for animal studies in Chapter 4 and 5

Donor #	**Collection volume (ml)**	**Cell count (millions)**
1601262	157	1335
1603185	-	-
1503272	105	600
1503271	80	147
160916	59	800
150429	60	256
Mean ± SEM	**92.20 ± 18.24**	**627.60 ± 212.10**

UCB has known donor variability in the proportion of progenitor and stem cells, and therefore to reduce the impact of donor variation in our in vivo studies, we decided to pool samples from 3 donors per treatment dose. This resulted in a total of 4 donor pools of cells after thawing, for use in the *LPS+UCB* cohort (Table 3.2). Three out of 4 of these donor pools were screened using flow cytometry for cell content of monocytes, HSCs, EPCs, MSCs and T-regulatory cells (Table 3.2). When analysing the donor pools as a

group, on average (±SEM), UCB contained 8.46% ±1.45 monocytes, 2.28% ±1.43 haematopoietic stem cells (HSCs), 2.10% ±1.51 EPCs, 0.068% ±0.02 MSCs and 14.18% ±10.86 T-regulatory cells (Figure 3.1). From the flow cytometry analysis, we could establish the cellular composition of each UCB donor pool used in our experiments. This revealed that all samples consistently showed low numbers of MSCs, while monocytes were the most abundant cell type of interest.

Table 3.2: Donor UCB pooled for animal studies in Chapter 4

Donor pooling combination for use in animal experiments		**Viability after thawing/pooling**	**Donor profile, flow cytometry (%)**				
			Monocytes	**HSCs**	**EPCs**	**MSCs**	**Tregs**
Donor pool #1	1601262 1603185 1503272	83%	8.84	1.12	0.65	0.05	N/A (Could not collect data)
Donor pool #2	1601262 1503271 1503272	78%	N/A (Could not collect data)	N/A (Could not collect data)	N/A (Could not collect data)	N/A (Could not collect data)	N/A (Could not collect data)
Donor pool #3	160916 150429 1503271	92%	5.78	0.63	0.53	0.04	25.03
Donor pool #4	1601262 1603185 1503271	80%	10.75	5.12	5.12	0.12	3.32

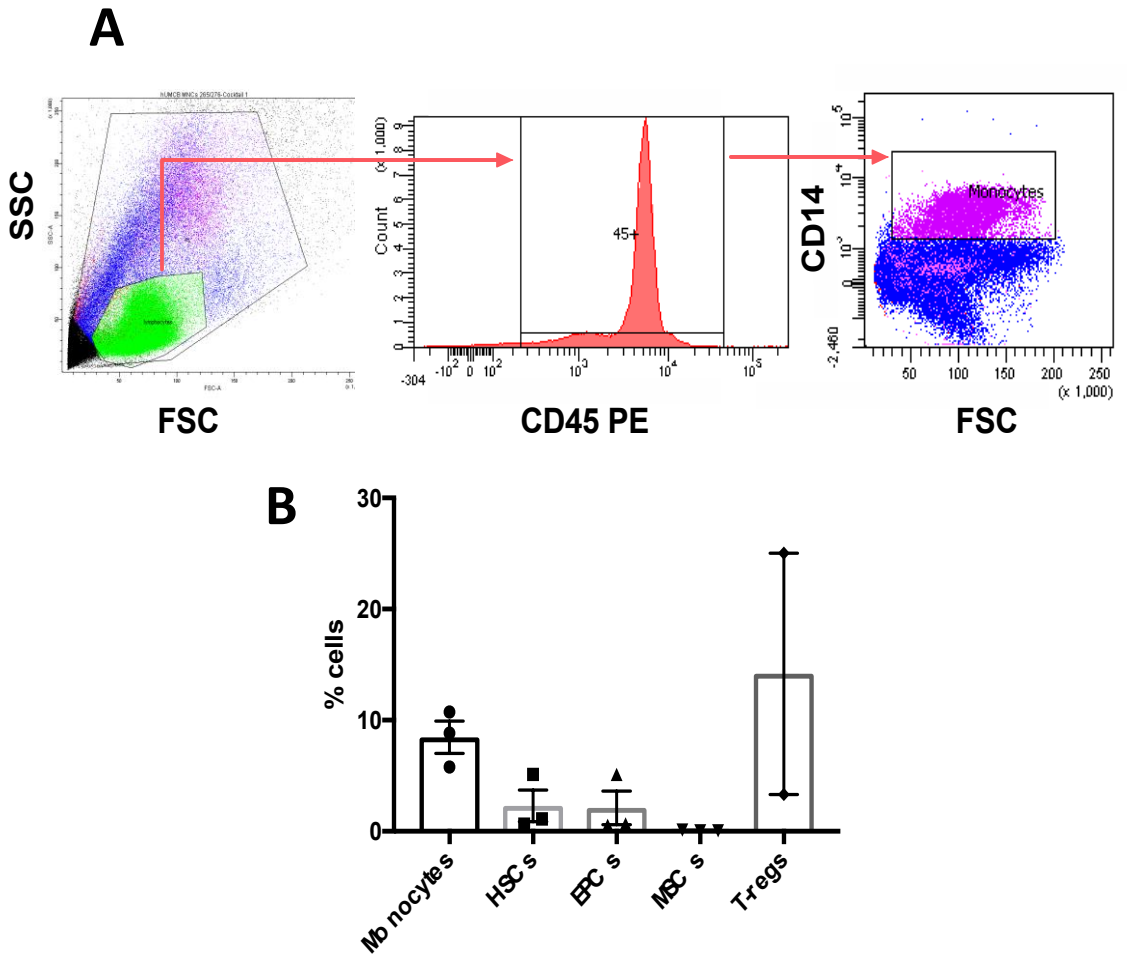


Figure 3.1: Flow cytometry of pooled UCB MNCs. (A) Representative image of gating strategy (monocytes as example) used for flow cytometry analysis of cell types in UCB. (B) Mean percentage of each cell type found in UCB, for monocytes (CD14+ from CD45+), HSCs (CD34+/45low lymphocytes), EPCs (CD133+ from CD45+), MSCs (CD45+ and Stro-1+) and T-regs (CD4+ and CD25/Foxp3+). SSC, side scatter; FSC, forward scatter; HSCs, haematopoietic stem cells, EPCs; endothelial progenitor cells, MSCs; mesenchymal stem cells, T-regs; T-regulatory cells.

3.4.2 MSC isolation, expansion and characterisation

Four umbilical cord tissue donor samples from healthy uncomplicated caesarean section deliveries were selected for isolation of MSCs. Cells were isolated and expanded using an established protocol detailed in *Chapter 2*. Donors 1-3 had normal and rapid cell growth to P3 in vitro with normal cell morphology (Figure 3.2); characterised as fibroblast-like, spindle- shape cells with homogenous cell growth stemming from colony formation. Donor 4 demonstrated slow growth after cryopreservation at P2, thawing and multiple passages, therefore it was excluded from further use. Thus, three out of 4 human samples (samples 1-3) had regular growth and further cell characterisation was performed.

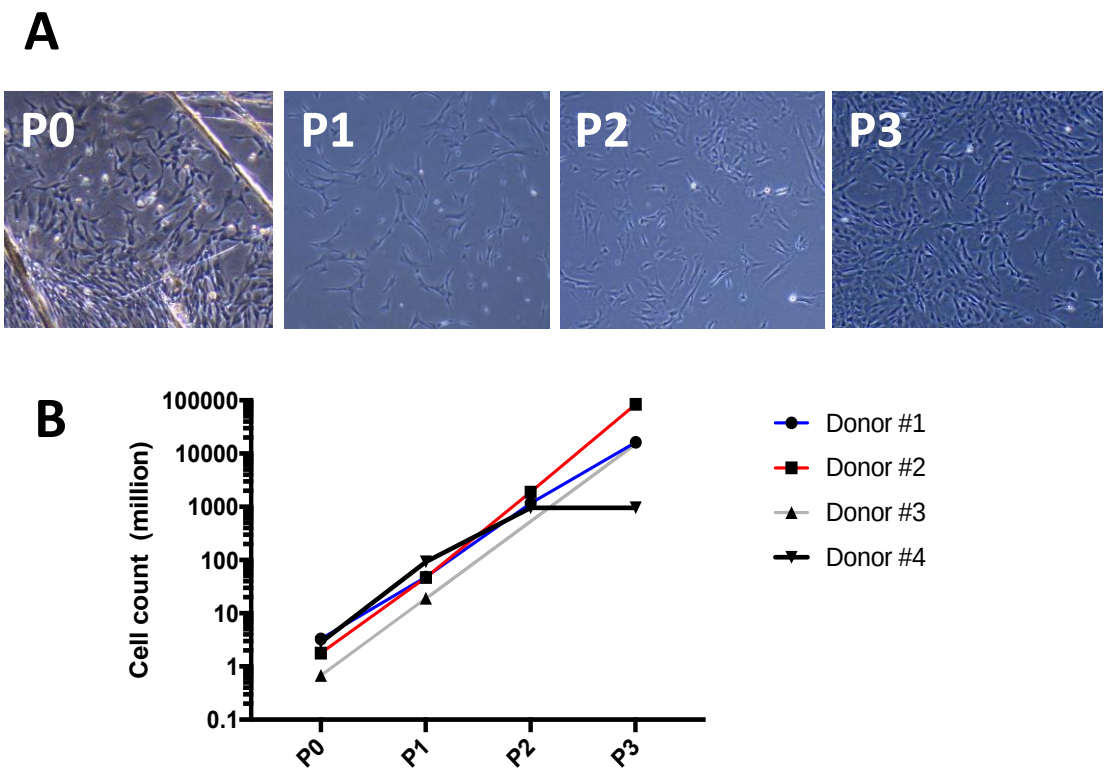


Figure 3.2: MSC growth in culture. (A) Representative phase-contrast image of MSCs at ~80% confluency at P1-P3, displaying homogenous, spindle-shaped morphology. Magnification is 200 x. (B) Growth curve of MSC donors 1-4 across P1-3. MSCs; mesenchymal stem cells and P3; passage 3.

Next, we performed MSC mesodermal differentiation assays for donors 1-3. All donor MSCs were able to successfully differentiate into osteoblasts (Figure 3.3 A, E, I), chondrocytes (Figure 3.3 B, F, J) and myocytes (Figure 3.3 C, G, K), however only cells from donor 1 and 2 differentiated into adipocytes with lipid droplet formation (Figure 3.3 D, H). Donor 3 MSCs lacked this ability (Figure 3.3 L). It is important to note however, that quad-lineage differentiation potential for adequate characterisation of MSCs, particularly adipocyte formation, is not necessarily required as it is known that immature MSCs sourced from fetal tissues have a lower capacity to differentiate into adipocytes [49].

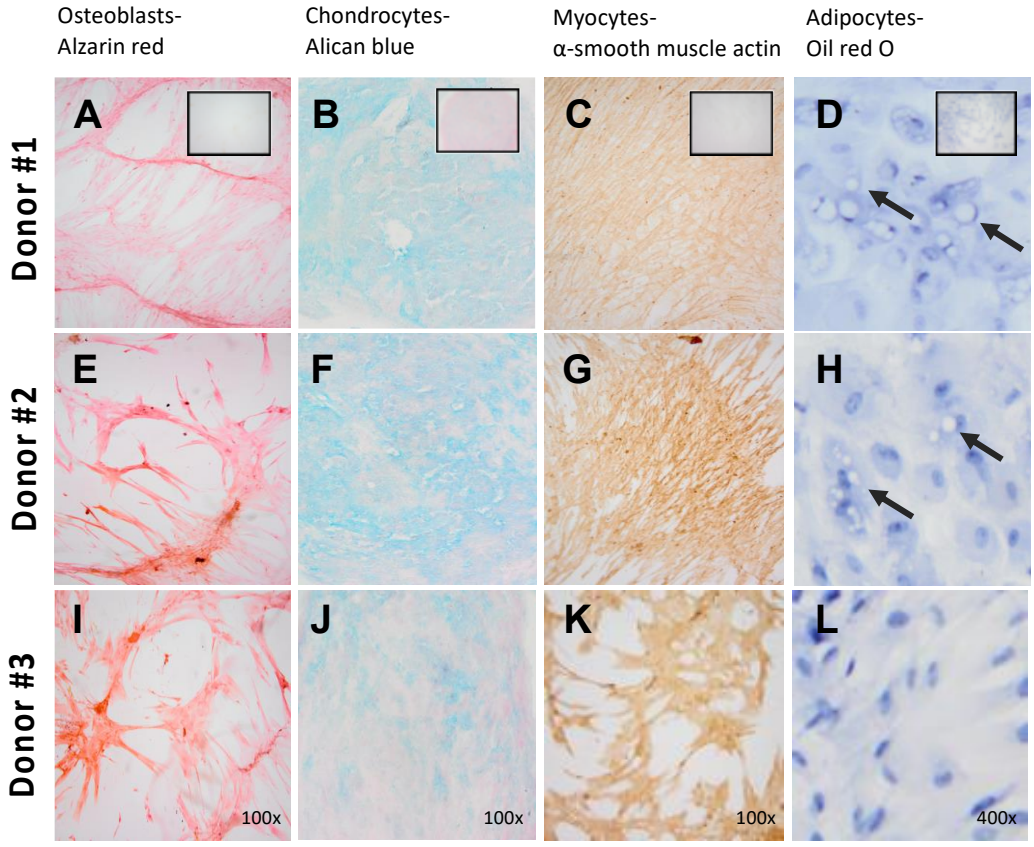


Figure 3.3: Mesodermal differentiation potential of different MSC donors. Images of quad-lineage differentiation potential of MSCs at P3 for growth into osteoblasts (100x magnification), chondrocytes (100x magnification), myocytes (100x magnification) and adipocytes (400x magnification), with negative controls overlayed in the first row. Representative images from two independent experiments per donor, performed in triplicate.

Next, karyotyping of cells obtained from 3 donor MSCs was performed at passage 3, revealing that cells from donors 1-2 had a regular, female 46 XX karyotype (Figure 3.4). Cells from donor 3 had an abnormal, male karyotype, with a trisomy on chromosome 2 (highlighted by the red circle). Donor 3 cells were therefore excluded from further analysis.

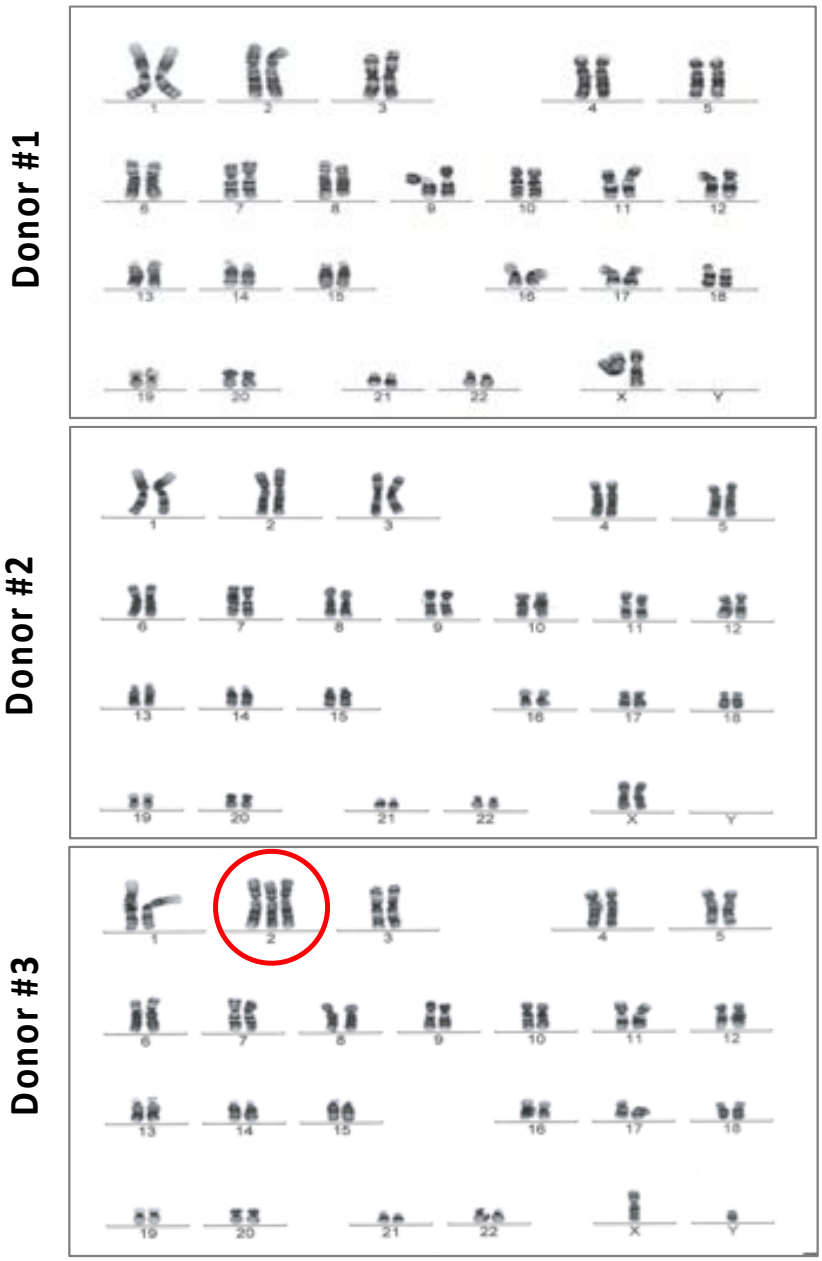


Figure 3.4 Karyotype analysis of different MSCs donors. Karyogram visualising G-banding of chromosomes from MSCs at P3. A chromosomal irregularity is highlighted by the red circle in donor #3. MSCs; mesenchymal stem cells and P3; passage 3.

Finally, the cell surface markers expressed on MSCs isolated from Donor 1 and 2 were analysed by flow cytometry. MSCs from both donors showed positive expression of CD73, CD90, CD105, HLA-ABC and CD44, and negative staining for CD34, CD45, HLA-DR and Stro-1 (Figure 3.5)

In conclusion, MSCs could be easily isolated from cord tissue and easily expanded in most cases, there were some issues with slow growth and abnormal karyotype in samples obtained from 2 donors. However our final two donor MSC samples (Donor #1, #2) met the classical International Society for Cellular Therapy (ISCT) requirements [34] of normal spindle cell morphology, quad-lineage differentiation, normal karyotype and relevant cell surface marker expression for cells to be considered "MSCs" for use in these studies.

3.4.3 EPC isolation and expansion

Using magnetic bead separation, CD133+ and CD34+ cells were isolated from UCB and stored according to methods detailed in *Chapter 2*. In order to measure proliferation of live cells in these studies, it was necessary to first identify an assay that could accurately detect differences in cell numbers in vitro. To do this we chose an MTS assay, which is a colorimetric assay where absorbance increases as the chemical is metabolised. Therefore, the absorbance is purported to be directly proportional to the cell number. However, because cells have different metabolic rates it was necessary to firstly establish what was the optimal time for MTS to be added to detect differences in the number of cells present. We initially confirmed the basal metabolic rate of CD133+ cells using a MTS assay on unstimulated CD133+ cells (Figure 3.6). Unstimulated CD133+ cells showed clear differences in absorbance depending on cell density when MTS reagent was added for more than 4 hours. From this initial test, we chose 8 hours after the addition of MTS as an optimal time to assess differences in cell number and proliferation.

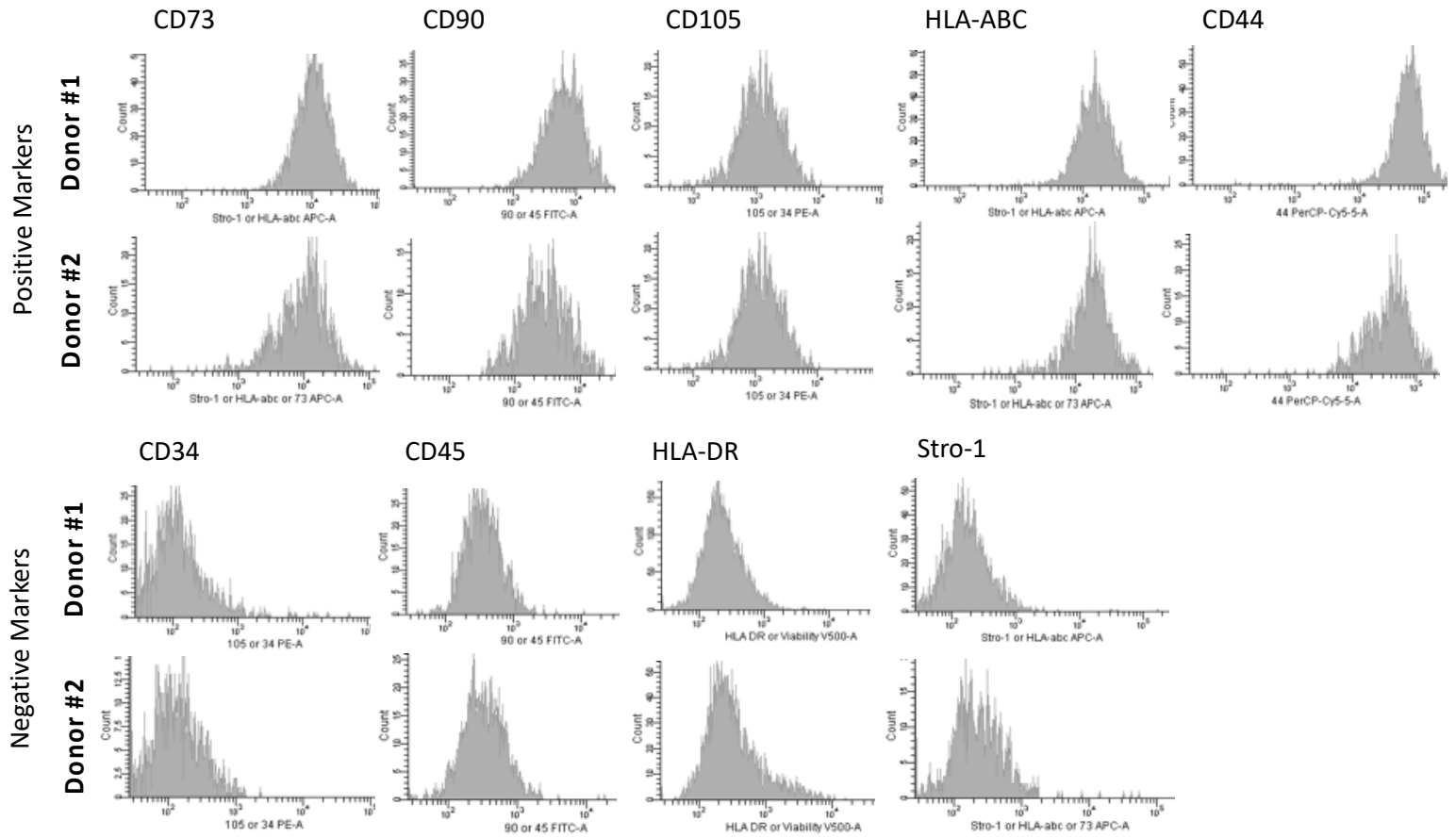


Figure 3.5: Flow cytometry analysis of different MSC donors. MSCs at P3 with positive staining for CD73, CD90, CD105, HLA-ABC and CD44 with negative staining for CD34, CD45, HLAD-DR and Stro-1. MSCs; mesenchymal stem cells and P3; passage 3.

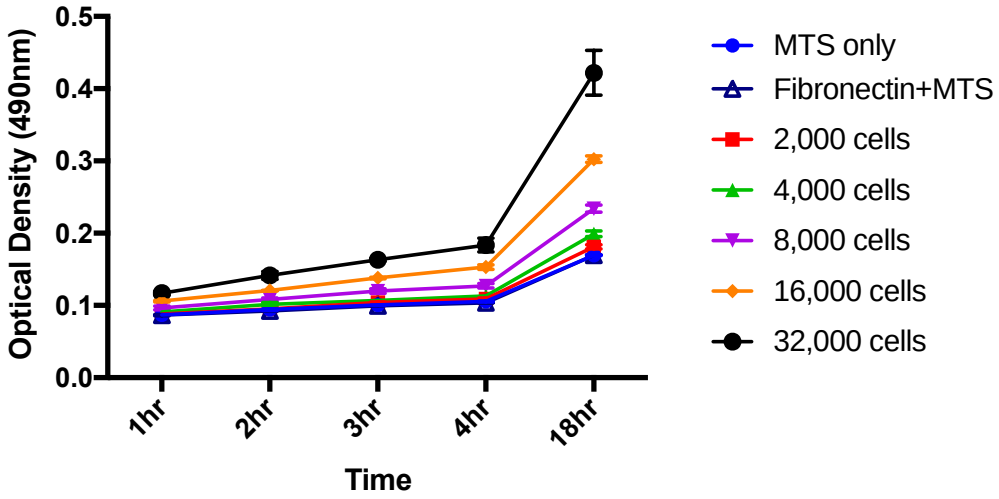


Figure 3.6: Basal metabolic activity of unstimulated UCB CD133+ cells. Using an MTS assay, CD133+ cells were seeded at varying densities (2,000- 32,000) with absorbance measured at 1, 2, 3, 4 and 18 hours to establish growth trajectory. OD; optical density. Performed in duplicate on 2 different donors.

A total of 11 expansion trials were carried out to attempt to increase cell populations of EPCs. Growth factors were added to cells and stimulated for 28 days and then proliferation was assessed using a MTS assay. CD133+ cells were initially tested, then trials of CD34+, in the presence of different growth factor combinations.

Initially, five independent experiments testing the expansion potential of three growth factors; vascular endothelial growth factor (VEGF), stromal derived growth factor 1 (SDF-1a) and stem cell factor (SCF) for CD133+ cells were performed. As part of this protocol, a number of different concentrations of VEGF and SCF were tested (shown below the graph; Figure 3.7). There were no differences in the proliferation of CD133+ using any of the growth factor cocktails compared to unstimulated controls (no growth factors present). This is contrasted to an experiment using CD34+ cells that are easily expanded under the same conditions (representative growth for 50ng/ml VEGF and 200ng/ml SCF), with nearly a two fold increase in the optical density.

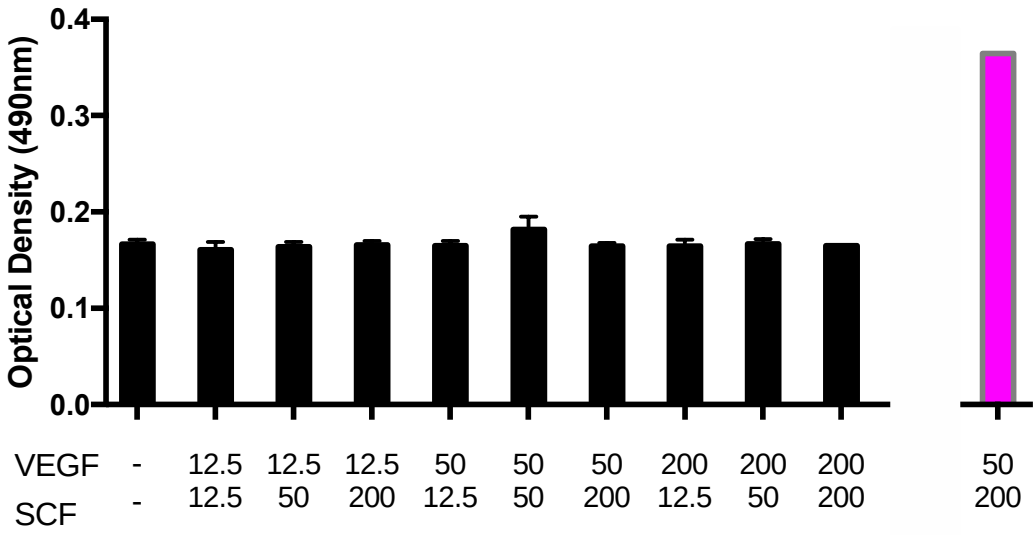


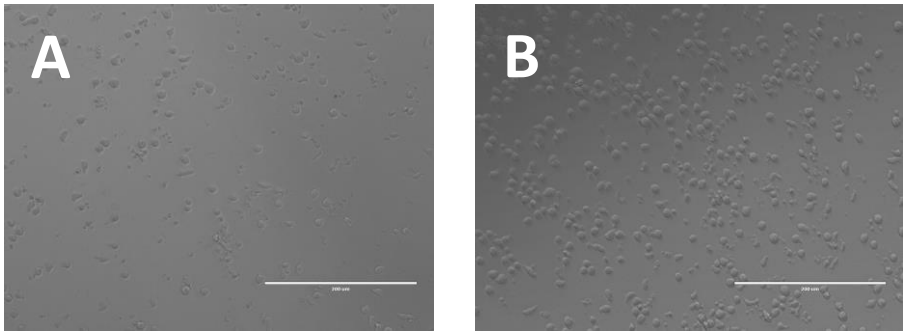
Figure 3.7: Proliferation of CD133+ cells stimulated with different concentrations of VEGF and SCF using a MTS assay. Average optical density of a MTS assay from 5 independent CD133+ expansion experiments using VEGF/SDF-1a +/- SCF (in ng) with MTS assay measured on cells after 4 weeks of expansion. This is contrasted to CD34+ cells stimulated with VEGF/SDF-1a +/- SCF after 4 weeks (shown in pink). OD; optical density, vascular endothelial growth factor; VEGF, stromal cell-derived factor 1a; SDF, human stem cell factor; SCF.

To determine whether it was the growth factor mix itself that did not promote cell proliferation, or if the CD133+ cells themselves failed to proliferate, the same growth factor combinations were tested on CD34+ cells. CD34+ cells are known to expand in published standard expansion media [46] that includes SCF, thrombopoietin (TPO), FMS-like tyrosine kinase (FLT3), interleukin (IL)-6 and IL-3. CD34+ cells were tested in 2 independent experiments with different combinations of VEGF, SDF-1a in combination with the standard mix of SCF, TPO, FLT3, IL-6 and IL-3 mix. Cells tested with these additional growth factors had rapid growth, even after only 1 week compared to just VEGF and SDF-1a (Figure 3.8, A versus B). When proliferation was quantified after 4 weeks of stimulation, using a MTS assay, we saw that CD34+ cells, in the presence of the growth factor mix, regardless of the additional SDF, VEGF and SCF, showed a high degree of expansion (Figure 3.8 C).

CD34+ cells clearly proliferated to a much higher degree when in the presence of SCF, TPO, FLT3, IL6 and IL3 when compared to initial expansion experiments of CD133+ cells. The addition of other endothelial growth factors such as VEGF and SDF did not provide any additive proliferation. However, these growth factors may have assisted with maintaining progenitor cell markers and avoiding differentiation after expansion. This suggestion was investigated using flow cytometry. We saw that the profiles of CD34+ cells, when grown in the presence of VEGF and SDF, also showed an overall increase in CD45, as well as an increase in CD34 expression when exposed to increasing concentrations of VEGF. This suggests that high VEGF concentrations may help retain and increase CD34, which indicates the stem progenitor potential of the cells (Figure 3.8 D). However, this higher VEGF exposure in culture, also increased the expression of VEGF protein which may indicate that over the experiment cells may be maturing, but given CD31 expression (mature endothelial cells marker) decreased, this is unlikely.

Using the same expansion cocktail that was shown to effectively expand CD34+ cells (Figure 3.8), we tested it using CD133+ cells to elucidate if the growth factor mix could also successfully expand this progenitor cell type. After 10 days of expansion using this new growth factor cocktail we were able to show that the CD133+ cells were able to grow more rapidly compared to unstimulated CD133+ cells (Figure 3.9 A). Further, different growth factor mixes were not correlated with proliferation, and this growth was still too slow to use for large-scale CD133+ expansion. For example, when the cells were counted manually, we observed only a 2-fold increase in cell number over the 10 day period (Figure 3.9 B).

Lastly, an additional published protocol was tested for its ability to expand CD133+ cells, specifically the non-adherent population [56]. Cells were seeded at 1 million cells, supplemented with IGF-1, ascorbic acid, VEGF and basic FGF. The non-adherent cells were then collected, counted and replated (D2, D6 and D10, Figure 3.10). However, results showed that the cells still did not expand and viability was negatively affected; on day 2 and 6 of expansion, on average cells had 74% viability and by day 10, the cell count was on average only 142,000 cells per well, with the viability falling to 6.5%. These cells were not sufficiently viable for flow analysis.



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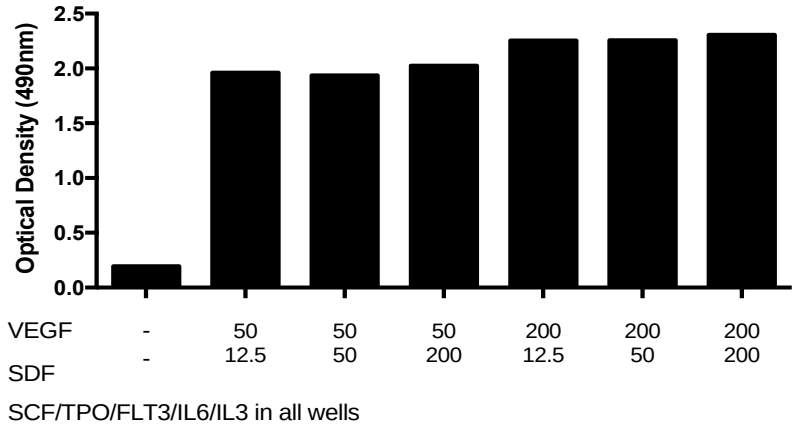


D

[VEGF/SDF]	CD45	VEGF	CD133	CD34	CD31
Media alone	**20.8**	**0.7**	**28.5**	**16.2**	**14.4**
12.5/50	51.3	4.9	18.1	32	2.8
12.5/200	52.2	5.0	26.9	45.4	3.2
50/50	47.4	3.2	18.8	35.5	1.5
50/200	51.3	5.2	24.6	40.4	2.3
200/50	48.7	3.9	14.5	17.9	1.9
200/200	53.7	5.1	23.3	36.6	2.3

Figure 3.8: CD34+ expansion with additional growth factors. Phase contrast images of CD34+ cell growth at 1 week, in media with (A) 200ng SDF/VEGF or (B) 200ng SDF/VEGF plus additional growth factors (SCF, TPO, FLT3, IL6 and IL3). Images are at 200x magnification. (C) MTS assay readings from single CD34+ expansion experiment using VEGF/SDF + SCF, TPO, FLT3, IL6 and IL3 (in ng) with MTS assay measured at 8 hours. (D) Flow cytometry analysis CD34+ cell expression of CD45, VEGF, CD133, CD34, and CD31 after 4 weeks. OD; optical density, vascular endothelial growth factor; VEGF, stromal cell-derived factor 1a; SDF, human stem cell factor; SCF, thrombopoietin; TPO, FMS-like tyrosine kinase; FLT, IL; interleukin.

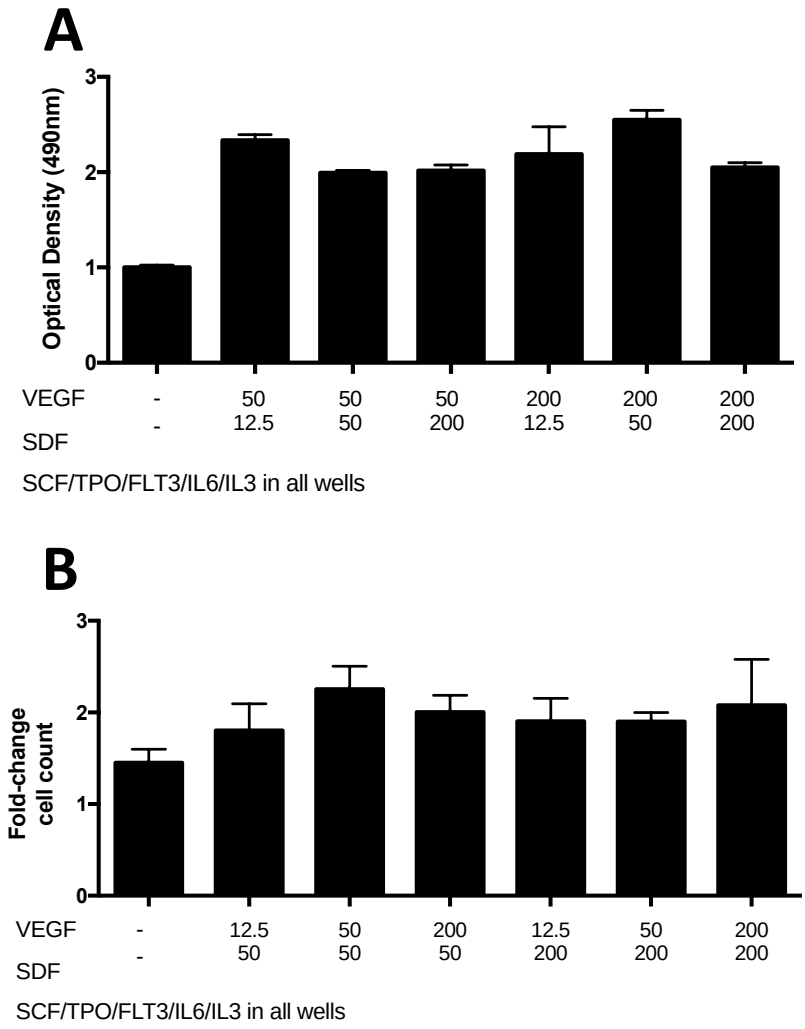


Figure 3.9: Proliferation and cell count of CD133+ cells after a 10 day expansion with additional growth factors. (A) MTS results after attempted expansion of CD133+ cells stimulated with SCF, TPO, FLT3, IL6 and IL3 with varying concentrations of VEGF and SDF (in ng, shown below) after 10 days. (B) Fold-change in cell number for each condition compared to control media of SCF, TPO, FLT3, IL6 and IL3 only (first column). OD; optical density, vascular endothelial growth factor; VEGF, stromal cell-derived factor 1a; SDF, human stem cell factor; SCF, thrombopoietin; TPO, FMS-like tyrosine kinase; FLT, IL; interleukin. Results from 2 independent donor experiments performed in duplicate.

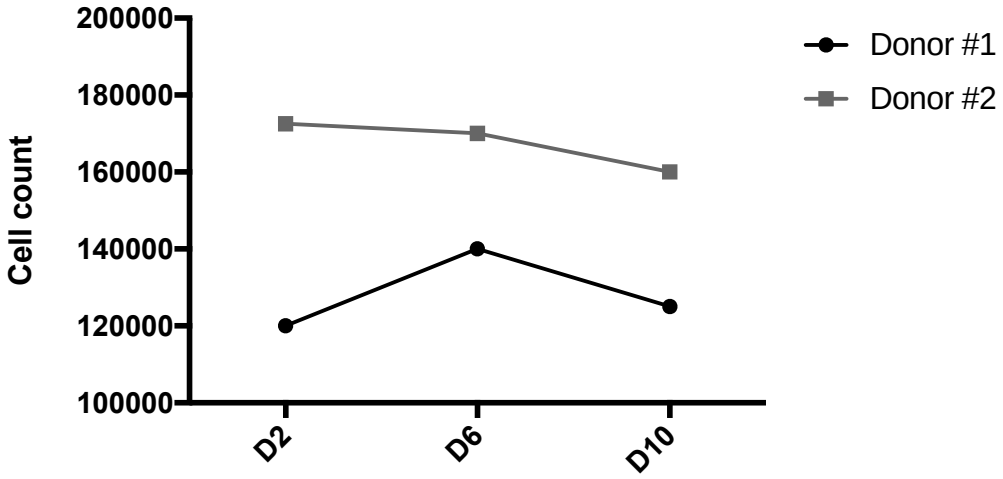


Figure 3.10: CD133+ cell count following expansion test using Cockshell & Bonder protocol. Manual cell counts of 2 donor CD133+ samples counted and replated at 2, 6 and 10 following supplementation with growth factors IGF-1, ascorbic acid, VEGF and basic FGF.

Upon the completion of over 11 expansion experiments (summarised in Figure 3.10), we were unable to develop a protocol to yield sufficient CD133+ endothelial cells to achieve clinical doses needed for our large animal studies. Our maximal expansion was only 2.3-fold (Figure 3.8) and this would not have been adequate, cost or time-effective enough to reach doses necessary for fetal sheep studies. The use of EPCs for the animal studies described in this thesis was not investigated any further.

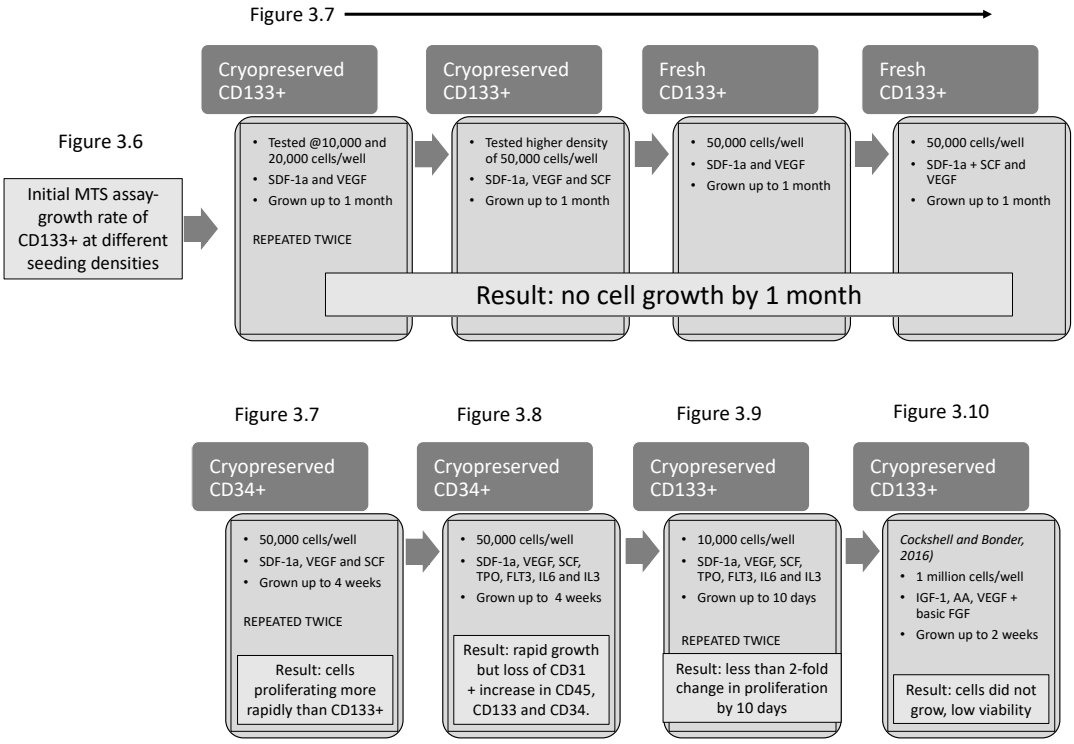


Figure 3.10: Timeline of EPC expansion experiments. Eleven expansion trials of CD133+ and CD34+ with different seeding densities and growth factor combinations. Vascular endothelial growth factor; VEGF, stromal cell-derived factor 1a; SDF, human stem cell factor; SCF, thrombopoietin; TPO, FMS-like tyrosine kinase; FLT, IL-6; interleukin-6 and IL-3; interleukin-3.

3.5 Discussion

The placenta is a significant source of many stem/progenitor cells which could serve as potential cell therapies for brain inflammation in a preterm cohort. UCB is easy to obtain and, from the results of this study, we have shown that each UCB sample can yield a large number of cells (over 100 million MNCs). We also demonstrated that only 0.068% of cells per UCB sample were potential MSCs. Therefore, we investigated cord tissue as a more viable source of MSCs for this study. We obtained a high yield of MSCs from cord tissue that were readily expandable and were fully characterised. We also demonstrated that EPCs from UCB were not able to be expanded to a high enough cell number to achieve clinical doses required. Therefore, UCB MNCs and MSCs from cord tissue were better sources of cells for use in large animal studies and for clinical translation. Given the lack of expansion potential of EPCs, this raises important questions about the ability of these cells to be clinically translated.

Using the ISCT guidelines for the minimal requirements to define a "MSC" [34], alongside use of additional flow cytometry markers and karyotyping, we were able to characterise our population of MSCs selected from 4 initial donor samples of cord tissue. Whilst the ISCT method of defining MSCs is useful, we coupled this characterisation with other robust markers of MSC cell surface markers (Stro-1+ and CD44+) and growth integrity. Demonstration of tri-lineage differentiation potential of cells, as stated in the ISCT method of characterisation, is still relevant, and often necessary, due to the similarity between MSCs and other cell types; for example, fibroblasts and MSCs are very similar not only in their general morphology but also their ability to differentiate [57, 58]. Accordingly, it is important to be able to distinguish between these cell types for use in preclinical experimental studies by looking at myocyte differentiation and flow cytometry cell surface markers. Coupling this method with karyotyping of the cells also assists in identifying any mutations in cell growth as we were expanding these cells in culture over a number of months.

A trisomy was uncovered when carrying out the karytotyping on P3 MSCs from one of our donors. Trisomy 3 from umbilical cord tissue is a rare mutation that is likely to have been the result of cell expansion and ongoing growth in culture. When a trisomy is found

in placental tissues in a clinical scenario, more fetal tissues are often collected alongside amniotic fluid to assess the extent of the mutation; it is the size of the mutation on the chromosome which will often indicate severity of any fetal abnormalities [59, 60]. However, this is an extremely unlikely fetal congenital abnormality on chromosome 3 as there are very few reported cases of this condition [61]. It is, thus, more likely to be an acquired mutation arising during long term culture of the cells which is not uncommon when expanding MSCs [62]. Therefore, it is important that when characterising cells for clinical use, strict acceptance criteria should be used and karyotyping of cells should be considered when designing these acceptance criteria.

Although *in vitro* characterisation of MSCs according to the criteria adopted in this thesis is the current "gold standard", there may still be variability of *in vivo* functionality and efficacy [63]. This was of interest to us and as a result, functional assessment of our expanded MSCs as a therapy for preterm brain inflammation was undertaken in fetal sheep (reported in *Chapter 5).*

For this study, we aimed to isolate EPCs from CD133+ UCB cells. Most research using EPCs as a cellular therapy have sourced CD133+ cells from late outgrowth cell cultures [64-66]. This requires replating, altering media growth factors and many weeks of culture before initial cell isolation. However, in our study, we selected CD133+ cells from UCB MNCs using magnetic bead separation. This cell population has been well studied and characterised previously and is postulated to be the best cell type to protect the neonatal brain in other preclinical models of brain injury [67]. Additionally, these cells can be easily and quickly isolated from their primary source and are, thus, potentially the best cell candidate for attempted expansion. However, given the difficulty with expanding CD133+ cells in this study, they may not be the ideal source of EPCs for clinical use.

In our hands, we found that EPCs were difficult to expand, and we could not achieve growth greater than 3-fold using a variety of growth factor combinations. Published work on EPC expansion using CD133+ cells shows that multiple methodologies may be required to isolate a population of semi-adherent CD133+ cells, including multiple on-column purification steps as the cells expand [68]. Often a variety of different growth

factor combinations are then changed over a number of weeks in order to maintain endothelial marker expression and also to enhance cell number [68, 69]. Some studies estimate that, from one UCB sample, the feasible expansion is around 6 million CD133+ cells over a short expansion period of 8 days [70]. In contrast, we could only expand cells ~2.5 fold, up to 1 million cells in 10 days. Our expansion was significantly more limited and may be attributed to our selected growth factor mixes, cell culture preparation or the fragile nature of semi-adherent cell populations. In previous studies using rodent models of ischemic injury, much smaller total number of EPCs were required (e.g. 500,000 cells administered per mouse [68]). In contrast, our study in fetal sheep requires approximately 10 million cells/kg, which is in line with what might be required clinically, and we were not able to achieve adequate numbers of EPCs with our expansion techniques.

In summary, both UCB and MSCs from cord tissue were effectively isolated, with MSCs expanded to reach sufficient doses for use in animal studies which would reflect relevant clinical doses. However, using a variety of different protocols, EPCs from cord blood were not able to be expanded. This is an important consideration when translating cell therapies for both research and clinical use.

Chapter 4

Umbilical cord blood stem cells for preterm brain inflammation

This chapter is *In Press* in *Developmental Neuroscience* and has been reformatted for consistency in this thesis.

Paton, M., Allison, B., Li, J., Fahey, M., Sutherland, A., Nitsos, I., Bischof, R., Dean., J., Moss, T., Polglase, G., Jenkin, G., *McDonald, C. and *Miller, S.. **Human umbilical cord blood therapy protects cerebral white matter from systemic LPS exposure in preterm fetal sheep.** *Developmental Neuroscience (In Press*).
*joint senior authorship

4.1 Abstract

Infants born preterm following exposure to *in utero* inflammation/chorioamnionitis are at high risk of brain injury and life-long neurological deficits. In this study, we assessed the efficacy of early intervention umbilical cord blood (UCB) cell therapy in a large animal model of preterm brain inflammation and injury. We hypothesised that UCB treatment would be neuroprotective for the preterm brain following subclinical fetal inflammation.

Chronically instrumented fetal sheep at 0.65 gestation were administered lipopolysaccharide (LPS, 150ng, 055:B5) intravenously over 3 consecutive days, followed by 100 million human UCB mononuclear cells 6 hours after the final LPS dose. Controls were administered saline instead of LPS and cells. Ten days after the first LPS dose, the fetal brain and cerebrospinal fluid were collected for analysis of subcortical and periventricular white matter injury and inflammation.

LPS administration increased microglial aggregate size, neutrophil recruitment, astrogliosis and cell death compared with controls. LPS also reduced total oligodendrocyte count and decreased mature myelinating oligodendrocytes. UCB cell therapy attenuated cell death and inflammation, and recovered total and mature oligodendrocytes, compared with LPS.

UCB cell treatment following inflammation reduces preterm white matter brain injury, likely mediated via anti-inflammatory actions.

4.2 Introduction

Preterm birth is the most significant contributor to neonatal morbidity and mortality [71]. Infants who survive preterm birth, particularly those born very preterm (< 32 weeks) or extremely preterm (< 28 weeks), are at high risk for neurodevelopmental deficits [72]. *In utero* inflammation of the fetal membranes and/or placenta, termed chorioamnionitis, is the principal underlying cause of preterm birth [73]. Chorioamnionitis affects up to 70% of extremely preterm births [74], with fetal clinical condition influenced by gestational age and whether the infection is clinical or subclinical [75]. Subclinical chorioamnionitis is the most common presentation, in which there are no overt maternal or fetal symptoms of infection, but manifests as preterm labour or premature rupture of membranes, and subsequent histological analysis of the placenta reveals compromise [74]. There are currently no treatment strategies to improve neonatal wellbeing or brain development associated with subclinical chorioamnionitis, principally because it is challenging to diagnose until delivery and assessment of the placenta and/or membranes [74]. However, subclinical chorioamnionitis is associated with adverse short and long-term outcomes including subtle but clinically important brain injury [76]. Overall, the likelihood of developing cerebral palsy (CP) is increased 3.6-fold when an infant is born preterm after chorioamnionitis compared with spontaneous preterm birth alone [77].

Chorioamnionitis increases the risk of fetal and neonatal adverse events via upregulation of fetal systemic inflammation, and subsequent major organ and brain injury. Chorioamnionitis, fetal inflammation, and preterm birth are strongly associated with neuroinflammation and subsequent perinatal brain injury [41]. Neuroinflammation can be characterised by inflammatory cell infiltration into the brain and activation of resident microglia and astrocytes, which can then lead to cell death and impaired white matter development [78, 79]. Magnetic resonance imaging (MRI) studies indicate that cerebral microstructure is altered in preterm infants exposed to chorioamnionitis compared to age-matched controls, at term equivalent follow-up [80]. This includes disorganised white matter reflected in altered fibre density and complexity compared to those infants not exposed to chorioamnionitis [80]. These subtle changes to white matter microstructure contribute to life-long learning and behavioural deficits [81]. In turn, it is well described that white matter injury underlies most cases of CP [81].

In modern cohorts of preterm infants, white matter pathology most commonly takes the form of non-cystic diffuse periventricular leukomalacia [82], wherein cerebral injury is microscopic and isolated to the central white matter with evidence of glial scarring, but without large cystic formations [81]. With advancing knowledge of the relationship between chorioamnionitis, fetal inflammation, preterm birth, and poor neurological outcome, treatment strategies must target cerebral inflammation to protect against injury to the developing brain following chorioamnionitis.

There is growing interest in the use of human umbilical cord blood (UCB) cells as a neuroprotective/neuroregenerative therapy for neurological disorders, in both paediatric and adult medicine [37]. Indeed, there are a number of clinical trials for the examination of UCB in infants and children with CP [83], and results to date support that UCB transplantation in children with established CP is associated with improved motor function with a low risk of adverse events [84]. A significant benefit for the use of UCB cells as a therapeutic intervention reflects the heterogeneous mix of stem cells and progenitor cells, which migrate to sites of injury, promote regeneration, and modulate inflammation [85].

The efficacy of UCB is mainly attributed to anti-inflammatory and reparative mechanisms, as evidenced in experimental studies showing reduced immune cell recruitment and activation in the brain, anti-oxidant effects, and increased concentrations of circulating anti-inflammatory cytokines and growth factors [28, 86]. However, to date the majority of studies have used early intervention UCB therapy in experimental models of perinatal hypoxic-ischemic brain injury at term [87], and therefore the mechanisms of injury, cell vulnerability and regions of interest may be quite different to cerebral injury associated with intrauterine inflammation. Infants exposed to chorioamnionitis, and born preterm, have a high risk for neurodevelopmental impairment and long-term motor and behavioural deficits [76]. Accordingly, preterm infants exposed to chorioamnionitis are a cohort of high-risk infants who could be identified soon after birth as candidates for neuroprotective/neuroregenerative therapy. Early intervention, within the first few days

after birth, presents a time for optimal benefit to reduce the progression of neonatal brain injury [28, 86].

The aim of the present study was to identify the neuroprotective effect of UCB cell therapy in a preterm-equivalent sheep model of diffuse white matter injury following subclinical chorioamnionitis. We modified a previously reported lipopolysaccharide (LPS) regimen [88] to initiate a fetal inflammatory insult and to produce a clinically relevant subtle and diffuse white matter injury. A cohort of LPS-treated fetal sheep were then administered human UCB mononuclear cells to assess the efficacy of UCB cells as a neuroprotective therapy for inflammation-induced preterm white matter injury. We hypothesised that UCB cells would protect the preterm brain from *in utero* inflammation by reducing cerebral cell death, neuroinflammation and subsequent white matter injury.

4.3 Materials and Methods

4.3.1 Ethical approval

This study was performed with human ethics approval from Monash Health Human Ethics Committee and animal ethics approval from Monash Medical Centre Animal Ethics Committee A. All experiments were performed in accordance with guidelines established by the National Health and Medical Research Council of Australia.

4.3.2 Animal Surgery and LPS administration

Pregnant Border Leicester-Merino cross ewes with a single fetus underwent sterile surgery at 91.4±0.9 days (mean±SD) gestation (term is ~147 days). Under isofluorane (Isoflo, Abbott, Sydney, Australia) general anaesthesia, a laparotomy was performed, the fetal head and neck were exposed, and a catheter was inserted into a jugular vein (inner diameter (ID) 0.86 mm, outer diameter (OD) 1.52 mm; Dural Plastics, Australia; used for LPS and cell therapy administration). The hind-limbs were exposed, and a catheter was inserted into a fetal femoral artery (ID 0.50 mm, OD 1.00 mm; used for blood sampling, blood pressure, and heart rate monitoring), and the amniotic cavity (ID 1.50 mm, OD 2.70 mm; for antibiotic administration).

After returning the fetus to the uterus, the catheters were exteriorised using an incision through the ewe's flank. A catheter was inserted into a maternal jugular vein (ID 1.50 mm, OD 2.70 mm), and antibiotics were administered post-surgery, and then for the next 3 days with 0.1mg/kg oxytetracycline (Engemycin; MSA Animal Health, Wellington, New Zealand) and 500mg of ampicillin (Austrapen; CSL Ltd., Parkville, Australia). The animals then had a rest day with no intervention, in which the fetal arterial and amniotic catheters were connected to transducers (Powerlab SP; ADInstruments) for baseline blood pressure and heart rate monitoring. Due to the (small-bore) fetal catheters, with a tendency to lose patency over long durations, not all groups had sufficient data to compare time-points after D4.

At 95 days of gestation, fetuses were randomised to one of 3 groups: (i) control: intravenous (i.v.) fetal injection of saline on experimental days 1, 2 and 3; (ii) LPS: fetuses administered 150 ng i.v. LPS (from *E.Coli* 055:B5; courtesy of Dr Phillip Bird, University of Queensland) on experimental days 1, 2 and 3; and (iii) LPS+UCB: fetuses administered LPS on experiment days 1, 2 and 3, plus 100 x 10^6 human UCB stem cells via the jugular vein on experimental day 3, at 6 hours after the final LPS administration (see Supplementary Fig S1). LPS and/or cell treatment was administered over a 5-minute period and the catheter was cleared with a subsequent 2 ml saline flush over 2 minutes. Blood samples (1 ml) were collected via the fetal femoral artery catheter prior to each saline, LPS or cell administration, at 3, 6 and 12 hours afterwards and then daily from days 4 to 10, with 0.1 ml of blood used to measure blood gas parameters (ABL Blood Gas Analyser, Radiometer, Denmark).

On day 10, animals were humanely killed via a lethal overdose of pentobarbitone (Lethabarb, Virbac, Australia). Cerebrospinal fluid (CSF) was collected from the fetus by inserting an 18g needle between the first 2 cervical vertebrae, and the fetal brain was removed and weighed, with the right side of the brain then immersion fixed in 4% paraformaldehyde before paraffin embedding, and the left side cut coronally into 5mm sections and frozen at -80° in optimal cutting temperature compound (Tissue-Tek OCT; Sakura, USA). The brain was coronally sectioned at 10μm with both fixed and frozen tissues mounted on SuperFrost Plus (Thermo Fisher Scientific, Scoresby, Australia) glass slides.

4.3.3 UCB isolation and administration

Human term UCB samples were obtained from women with uncomplicated pregnancies undergoing elective caesarean section at term (>37 weeks gestation). Women gave written, informed consent for the collection and use of UCB. After clamping of the cord and delivery of the placenta, UCB was collected via the umbilical vein. The blood was centrifuged at 1000 g with the mononuclear cell layer being collected and resuspended in phosphate buffered saline (PBS; Gibco, Waltham, MA, USA), before undergoing red blood cell lysis by combining the cells with buffer (ammonium chloride, potassium

bicarbonbate and EDTA in MilliQ water). The lysis was stopped with excess media (16.5% fetal bovine serum [FBS] in DMEM:F12; Gibco). Cells were manually counted and viability assessed using trypan blue exclusion dye (Gibco). Cells were cryopreserved in dimethyl sulfoxide (10% DMSO, Sigma in FBS) and stored in liquid nitrogen.

On the day of cell infusion (D3, 6 hours after the last LPS dose), cells were rapidly thawed, centrifuged and resuspended in media, with a sample counted. Each animal received UCB pooled from 3 donor samples to the sum of 100 x 10^6 mononuclear cells resuspended in 2 ml PBS.

Cells were administered directly to the fetus via slow infusion into the jugular vein catheter over 5 minutes, ensuring that the line was cleared with a subsequent flush of 2 ml saline over 2 minutes. Fetal haemodynamics were monitored for any acute changes in heart rate or blood pressure which may indicate a fetal response to cell administration or embolism.

4.3.4 CSF cytokine analysis

CSF, collected at post mortem on day 10, was analysed for pro-inflammatory cytokine IL-1β using recombinant cytokines via ELISA, as previously reported [31].

4.3.5 Immunostaining and analysis of neuropathology

Stained brain sections were analysed in duplicate sections, with each region of interest imaged across 3 fields of view per slide. The brain regions of interest included the sub-cortical white matter (SCWM) and periventricular white matter (PVWM).

4.3.5.1 3,3'-diaminobenzidine immunohistochemistry

Cellular apoptosis was quantified in the brain via immunostaining on fixed tissue with activated caspase-3 (cas-3, 1:1000; R&D Systems, Minneapolis, USA). Astrocytes were visualised using glial fibrillary acidic protein (GFAP, 1:800, Sigma)

immunohistochemistry. Brain microglia and macrophages were visualised via immunohistochemistry with peroxidase labelled lectin (1:200, Sigma), while neutrophil accumulation was visualised using myeloid peroxidase (MPO, 1:500, Dako). Total number of oligodendrocytes were visualised by staining for oligodendrocyte transcription factor 2 (Olig-2, 1:1000; Millipore, Billerica, USA), while myelin basic protein (MBP, 1:500; Millipore) was used for visualisation of mature myelinating oligodendrocytes.

Briefly, for each immunohistochemical stain, brain sections were dewaxed through xylene-alcohol series. Antigen retrieval was performed via heating of the sections in citric acid buffer, followed by incubation with 0.3% hydrogen peroxide in 50% methanol for 20-30 minutes to block endogenous peroxidases. The sections were then rinsed and blocked with animal serum (cas-3, lectin and Olig-2: 5% normal goat serum with 1% bovine serum albumin; GFAP: 5% normal rabbit serum; MPO, Dako Protein Block; MPB: 10% normal goat serum) in PBS-Tween 20 (Sigma Aldrich, Australia) for 30-45 minutes. The slides were then incubated overnight in their appropriate primary antibody solution at 4°C. The secondary antibody (1:200 caspase-3 and MPO: biotinylated goat anti-rabbit IgG antibody; Vector Laboratories, Bulingame, USA; 1:200 GFAP: biotinylated rabbit anti-mouse IgG antibody; Wake Pure Chemical Industries; 1:200 Olig-2 and 1:500 MBP: goat anti-mouse IgG) was then incubated on the sections for one hour. Lectin immunohistochemistry did not require a secondary label. Staining was visualised with 3,3'-diaminobenzidine (DAB, Pierce Biotechnology, Rockford, USA) as per the manufacturers instructions. The slides were then cleared and cover-slipped with aqueous mounting medium (DPX; Merck, Kilsyth, Australia). After allowing the slides to dry, immunopositive cells were visualised via light microscopy (Olympus, Tokyo, Japan). Cell counts were performed using Image J (NIH, Bethesda, USA) at 400x magnification, with densitometry performed using threshold coverage measurements, that reads percentage coverage of staining as grey levels in contrast to background staining.

4.3.5.2 Fluorescence immunohistochemistry

For O4 and O1 (oligodendrocyte marker-4; marker of pre-myelinating, immature and mature oligodendrocytes, and oligodendrocyte marker-1; marker for myelinating immature/ mature oligodendrocytes [89]) fluorescence immunohistochemistry, frozen

brain tissues were dried on slides for 20 minutes, incubated in detergent solution (PBS-Tween 20), and then blocked for endogenous peroxidases using hydrogen peroxide. Sections were then blocked (Dako Protein Block), incubated with O4 or O1 primary antibody for 72 hours at 4°C (mouse monoclonal IgM, 1:200; O4 and O1 antibodies generated in-house by JMD; hybridoma cell lines gifted from Dr Ben Emery, OHSU, Portland, USA), followed by anti-mouse Alexa 594 secondary antibody (1:1000; goat anti-mouse Alexa 594; Invitrogen, Mount Waverley, Australia) for 1 hour. The slides were rinsed in water, dried, then mounted in medium for analysis. Images were captured at 200x magnification.

4.3.6 Statistics and analysis

In total, 23 fetal sheep were studied; n=8 *Control*, n=8 *LPS*, and n=7 *LPS+UCB*. Results were averaged across animals in each group.

Lectin aggregates were analysed at 100x magnification, then the stained area was traced using Image J analysis to calculate the size of lectin coverage. Lectin positive cells were assessed for their size and morphology as a qualitative indicator of the cell types present (that is, macrophages or microglia) at 400x magnification. MPO analysis was conducted via selection of 6 fields of view per region containing blood vessels. All analysis was conducted with the assessor blinded to the group.

For maturational changes in oligodendrocyte lineage cells, a semi-quantitative analysis (in n=3 animals per group) of the expression of single-labelled O4 and O1 antigens was undertaken. Animals included for this analysis reflected average expression of Cas-3 and GFAP and therefore were representative for the overall neuropathology of their cohort. Both O4 and O1 expression was quantified via cell counts of positive expression at 20x magnification in the SCWM.

Data was presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6 Software (GraphPad Software, San Diego, USA). Physiological recordings were analysed using a two-way repeated measures ANOVA

with Tukey's post-hoc analysis. Parametric histological and cytokine data were analysed using a one-way ANOVA with Tukey's post-hoc analysis. Non-parametric data were analysed using a Kruskal-Wallis test followed by Dunn's multiple comparisons post-hoc analysis. Statistical significance was set at $p \leq 0.05$.

4.4 Results

4.4.1 Fetal arterial blood gas and physiological parameters

There were no significant differences between control, LPS or LPS+UCB arterial blood gas parameters including pH, lactate, pO_2 or pCO_2 across the experimental period, including the immediate period following LPS (Table 4.1). Fetal heart rate and blood pressure throughout the experiment in all groups remained unchanged (Table 4.1).

4.4.2 Post mortem body and organ weight results

Body weight at the time of post mortem (~105 days) was not different between groups (Table 4.2). Organ:body weight ratios for brain, heart, lung and kidney weights were not significantly different between groups. Organ:body weight ratios for spleen and liver in the LPS group were significantly higher than controls ($p=0.01$ and $p=0.02$, respectively), and total adrenal weight:body weight tended to be higher in LPS animals ($p=0.08$). The adrenal weight:body weight of LPS+UCB animals was significantly reduced compared to the LPS group ($p=0.05$). Organ:body weight ratios of spleen and liver in LPS+UCB animals did not differ from either control or LPS groups (Table 4.2).

Table 4.1: Arterial blood gas, heart rate and blood pressure variability

		Baseline D1 Pre-LPS	3h	6h	12h	D2 Pre-LPS	12h	D3 Pre-LPS	12h	D4 Pre-LPS	12h	D10
pH	Control	7.399 ±0.01	7.40 ±0.01	7.40 ±0.06	7.388 ±0.02	7.37 ±0.01	7.38 ±0.01	7.38 ±0.01	7.39 ±0.01	7.37 ±0.02	7.39 ±0.01	7.37 ±0.01
	LPS	7.36 ±0.01	7.38 ±0.01	7.38 ±0.01	7.38 ±0.01	7.38 ±0.01	7.38 ±0.01	7.38 ±0.01	7.39 ±0.01	7.39 ±0.01	7.40 ±0.01	7.37 ±0.02
	LPS+ UCB	7.38 ±0.00	7.37 ±0.01	7.38 ±0.01	7.38 ±0.00	7.37 ±0.01	7.37 ±0.01	7.37 ±0.01	7.37 ±0.01	7.36 ±0.01	7.38 ±0.01	7.35 ±0.01
Lactate (mmol)	Control	1.00 ±0.05	0.97 ±0.03	1.10 ±0.03	1.27 ±0.21	1.00 ±0.05	1.02 ±0.06	1.08 ±0.05	1.10 ±0.07	1.12 ±0.06	0.98 ±0.05	1.17 ±0.07
	LPS	1.10 ±0.15	0.97 ±0.12	1.23 ±0.12	1.11 ±0.09	1.07 ±0.05	1.00 ±0.04	0.99 ±0.07	0.97 ±0.04	1.00 ± 0.03	1.03 ±0.03	0.93 ±0.08
	LPS+ UCB	1.22 ±0.10	1.12 ±0.09	1.19 ±0.10	1.24 ±0.11	1.20 ±0.10	1.13 ±0.08	1.10 ±0.08	1.16 ±0.08	0.98 ±0.13	1.03 ±0.07	0.98 ±0.07
pO_2 (mmHg)	Control	26.60 ±1.51	23.55 ±1.01	28.44 ±2.89	24.18 ±01.03	25.00 ±1.18	26.48 ±1.78	25.48 ±1.03	24.58 ±1.08	27.02 ±1.11	25.23 ±0.93	26.97 ±1.28
	LPS	30.71 ±0.79	25.69 ±0.98	30.42 ±0.78	27.50 ±0.66	30.75 ±1.24	26.33 ±1.08	28.87 ±1.66	29.70 ±2.03	30.18 ±0.96	27.90 ±0.83	30.93 ±2.43
	LPS+ UCB	27.27 ±0.80	25.71 ±0.70	26.46 ±1.84	25.49 ±0.59	25.03 ±0.05	23.56 ±0.51	25.50 ±0.72	24.60 ±0.64	27.68 ±0.97	28.50 ±1.99	26.30 ±0.64
pCO_2 (mmHg)	Control	46.38 ±1.13	41.74 ±1.43	44.91 ±1.27	47.55 ±1.86	47.43 ±1.33	47.53 ±2.15	48.18 ±1.31	48.02 ±1.42	50.60 ±0.44	48.07 ±1.60	49.43 ±3.46
	LPS	42.45 ±1.23	42.20 ±1.40	45.53 ±2.44	46.67 ±1.37	46.60 ±0.78	47.26 ±1.03	46.36 ±0.94	44.89 ±1.46	45.96 ±0.88	47.06 ±0.65	47.10 ±2.94
	LPS+ UCB	46.88 ±1.02	45.95 ±1.16	45.63 ±1.16	47.39 ±1.34	46.26 ±0.90	47.26 ±2.14	47.09 ±0.73	47.60 ±10.53	48.15 ±1.54	46.97 ±1.49	48.62 ±1.05
Blood pressure (% Δ from baseline, mmHg)	Control			0.06 ±1.16	-0.15 ±1.30		-2.55 ±0.59		-1.64 ±1.40		-0.31 ±0.58	-1.72 ±0.55
	LPS			-1.35 ±0.33	-1.68 ±0.26		1.56 ±0.42		0.54 ±0.54		1.05 ±0.31	0.69 ±0.23
	LPS+ UCB			-2.21 ±0.34	-0.71 ±0.25		-0.65 ±0.12		-0.93 ±0.20		1.96 ±0.42	1.98 ±0.35
Heart rate (% Δ from baseline, BPM)	Control			-8.79 ±1.64	-8.26 ±1.88		3.39 ±2.94		0.24 ±1.32		-1.48 ±1.85	-2.81 ±1.66
	LPS			6.68 ±3.20	-0.89 ±1.85		9.37 ±5.33		2.07 ±1.92		-5.29 ±2.35	-5.65 ±1.94
	LPS+ UCB			-4.42 ±1.73	-7.46 ±2.36		-1.47 ±3.43		1.31 ±1.51		-	-

Arterial blood gas parameters over the experiment, and blood pressure and heart rate variation as % change from daily baseline (each time point taken from mean of 3 x 1 minute epochs, averaged per time-point). The dashed line indicates when UCB was administered in the LPS+UCB group. Data expressed as mean ± SEM. pO_2, arterial partial pressure of oxygen; pCO_2, arterial partial pressure of carbon dioxide; BPM, beats per minute; mm/Hg, millimetres of mercury. Two-way repeated measures ANOVA, $*p \leq 0.05$.

Table 4.2: Post mortem body weight and organ:body weight

	Control	LPS	LPS + UCB	P-value
Body weight (kg)	**1.63 ±0.06**	**1.501 ±0.09**	**1.593 ±0.09**	**0.55**
Brain weight: Body weight	19.43 ±1.06	20.10 ±1.16	19.43 ±0.30	0.85
Total adrenal: Body weight	0.16 ±0.00^{a}	0.21 ±0.00^{b}	0.14 ±0.00^{a}	0.04
Spleen: Body weight	1.74 ±0.00^{a}	2.49 ±0.00^{b}	2.04 ±0.00^{ab}	>0.01
Heart: Body weight	8.81 ±0.79	8.19 ±0.21	8.04 ±0.21	0.14
Lung: Body weight	43.78 ±1.84	41.21 ±0.99	42.47 ±1.98	0.54
Liver: Body weight	46.30 ±2.25^{a}	56.66 ±2.96^{b}	50.34 ±1.96^{ab}	0.02
Total kidney: Body weight	10.77 ±0.74	10.29 ±0.52	9.944 ±0.52	0.64

Post mortem body weight (kg) and body:organ weights (mg kg^{-1}). Data expressed as mean ± SEM, p-value is ANOVA p-value summary. One-way ANOVA, different letters indicate $*p \leq 0.05$.

4.4.3 Cerebral inflammation

There was no statistical difference observed in CSF IL-1β between controls and LPS-treated animals (Fig 1A). However, IL-1β in CSF was significantly reduced in LPS+UCB fetuses compared to LPS alone ($p=0.03$).

The presence of lectin aggregates within all brains was observed at 100x magnification (Fig 1B). The aggregates viewed at 400x magnification were principally comprised microglia with an activated/ameboid morphology (Fig 1C) [8]. However, in the SCWM of the LPS group, the area of the activated microglial cell aggregates was increased compared to controls (a > 2-fold increase, but $p > 0.05$), while a similar increase was not observed in the LPS+UCB group (Fig 1D). There were no differences in aggregate size between groups in the PVWM (Fig 1E), but it should also be noted that there was very high variability in aggregate sizes between groups.

MPO positive neutrophils were significantly increased within the blood vessels of LPS animals in the SCWM, compared with controls ($p=0.02$, Fig 1H). There was no significant difference in neutrophil accumulation within blood vessels in the PVWM and SCWM in the LPS+UCB group, compared with controls. Neutrophil extravasation from the blood vessel lumen (Fig 1G) was found in sites where MPO-positive neutrophils were present in the blood vessels.

4.4.4 Apoptosis-mediated cerebral cell death and astrogliosis

Caspase-3 positive apoptotic cell counts within the SCWM were significantly increased in LPS animals compared with controls ($p=0.02$; Fig 2A), but there was no change in the PVWM (Fig 2B). Expression of caspase-3 in LPS+UCB animals was not different to controls.

The density of astrocyte coverage (GFAP-positive staining) was significantly increased in the SCWM of LPS animals compared to controls ($p=0.05$, Fig 2C). However, GFAP coverage was similar in the PVWM across all groups. Astrocyte morphology did not vary between groups.

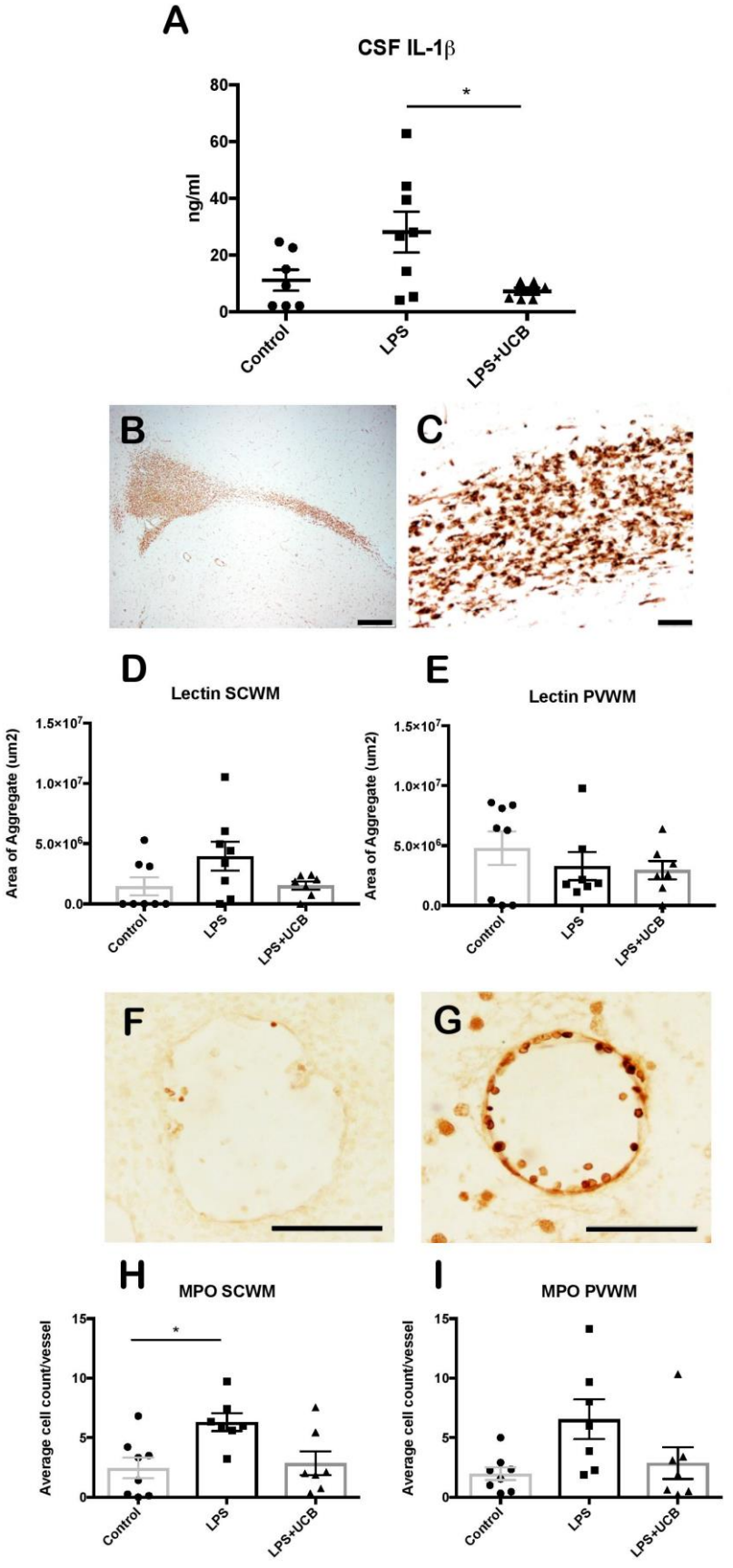

A
CSF IL-1β
ng/ml
80
60
40
20
0
*
Control
LPS
LPS+UCB
B
C
D
Lectin SCWM
Area of Aggregate (um2)
1.5×10^7
1.0×10^7
5.0×10^6
0.0
Control
LPS
LPS+UCB
E
Lectin PVWM
Area of Aggregate (um2)
1.5×10^7
1.0×10^7
5.0×10^6
0.0
Control
LPS
LPS+UCB
F
G
H
MPO SCWM
Average cell count/vessel
15
10
5
0
*
Control
LPS
LPS+UCB
I
MPO PVWM
Average cell count/vessel
15
10
5
0
Control
LPS
LPS+UCB

Figure 4.1: Neuroinflammation within white matter. (A) IL-1β concentration (ng/ml) in CSF at post mortem. (B-E) Immunohistochemical staining showing lectin accumulation in the subcortical white matter (SCWM) and periventricular white matter (PVWM) (B; scale bar is 500μm, C; scale bar 50μm). Quantification of lectin staining in (D) SCWM and (E) PVWM in control, LPS and LPS+UCB groups. (F-I) Myeloid peroxidase (MPO) expression within blood vessels of the brain. (F) Control vessels with low MPO expression in blood vessels of white matter (representative image of SCWM). (G) MPO expression in LPS animal blood vessels of white matter, with neutrophil morphology and MPO extravasation in the vessel lumen (scale bar 50μm). Quantification of MPO expression within blood vessels of (H) SCWM and (I) PVWM regions in control, LPS and LPS+UCB brains. Data expressed as mean ± SEM, n=6-8, one-way ANOVA, $*p \leq 0.05$.

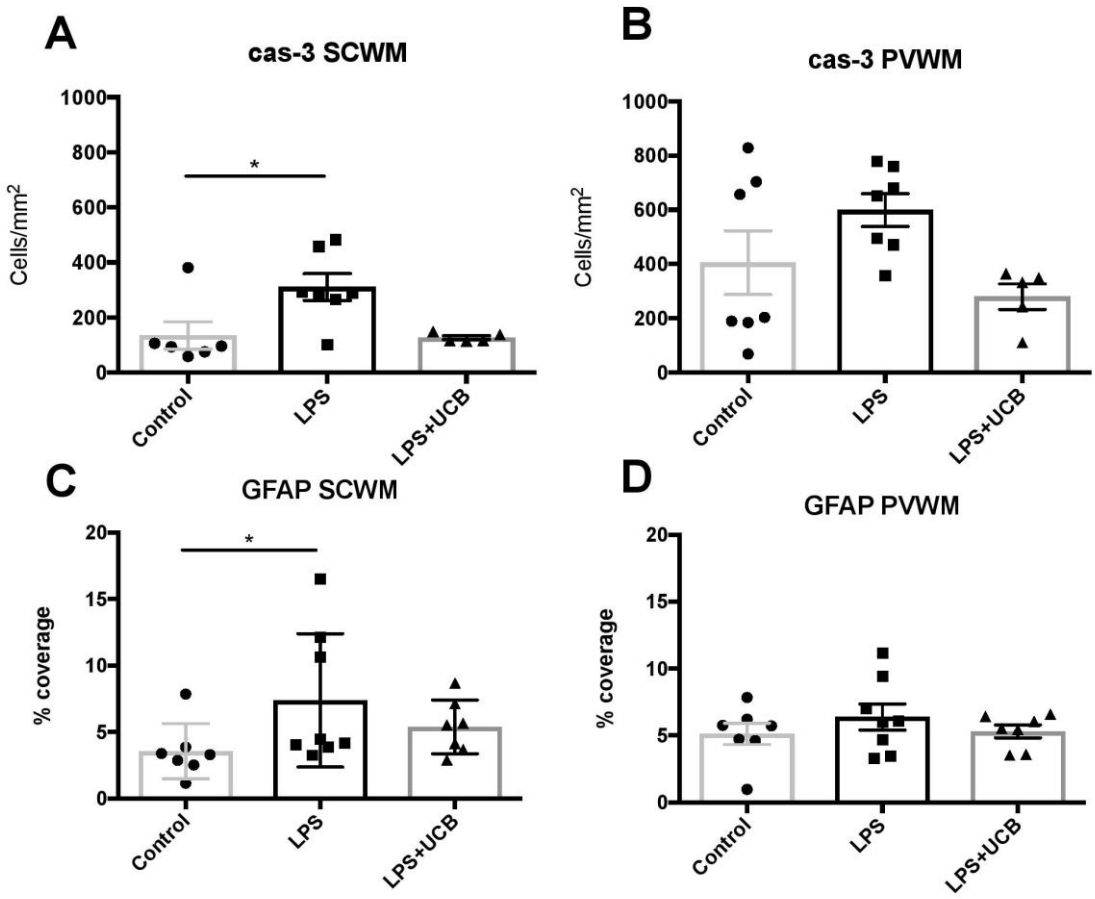


Figure 4.2: White matter cell death and astrogliosis. Expression of caspase-3 (cas-3) in the (A) subcortical white matter (SCWM) and (B) periventricular white matter (PVWM). Expression of glial astrocytic protein (GFAP) in the (C) SCWM and (D) PVWM (% coverage of staining). Data expressed as mean ± SEM, n=5-8, one-way ANOVA, *$p \leq 0.05$.

4.4.5 Cerebral white matter integrity

Olig-2 counts were significantly reduced in PVWM and SCWM of LPS treated animals, compared with controls (p=0.02 and p=0.05, respectively, Fig 3A, B), indicative of a decreased number of oligodendrocyte lineage cells. MBP-positive (mature) oligodendrocytes were significantly decreased within the SCWM in response to LPS administration (P=0.047 vs control; Fig 3C). The administration of UCB prevented loss of oligodendrocytes and promoted cell maturation, with no significant difference between control and LPS+UCB for Olig-2 and MBP cell counts. Further, the LPS+UCB group demonstrated a significant increase in the number of MBP positive mature oligodendrocytes within the PVWM compared to the LPS group (Fig 3D; p=0.046).

Cellular staining for both O4 and O1 were observed within the SCWM and PVWM of all brains. Within control brains, there was a higher proportion of O1-positive cells than O4 positive cells in the SCWM, indicative of a more mature phenotype of oligodendrocytes within control brains at ~105 days (Fig 4A, D). However, in the LPS group, there was a greater proportion of O4 cells than O1, suggesting that oligodendrocyte maturation had stalled (Fig 4B, D). LPS+UCB animals showed a lower proportion of O4 cells in the SCWM than LPS alone, with a similar expression of O1 to that in LPS animals (Fig 4C-D).

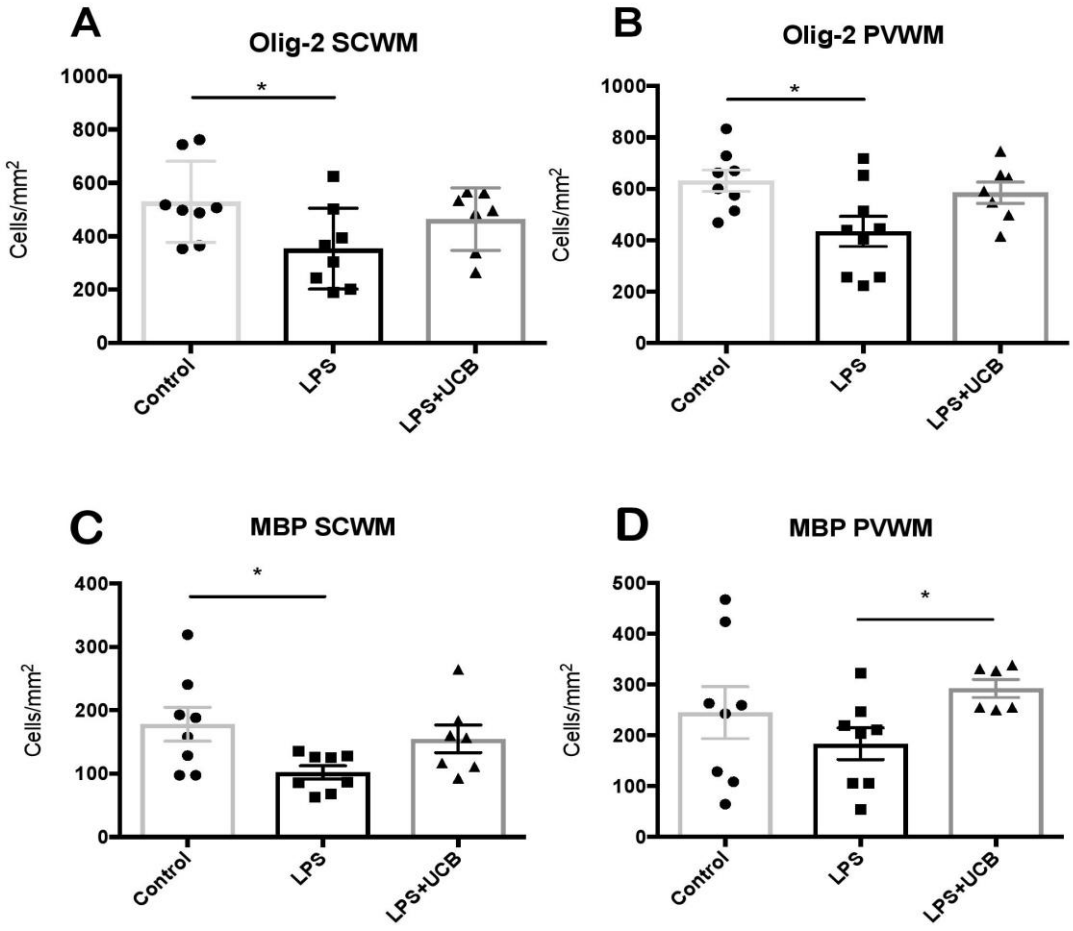
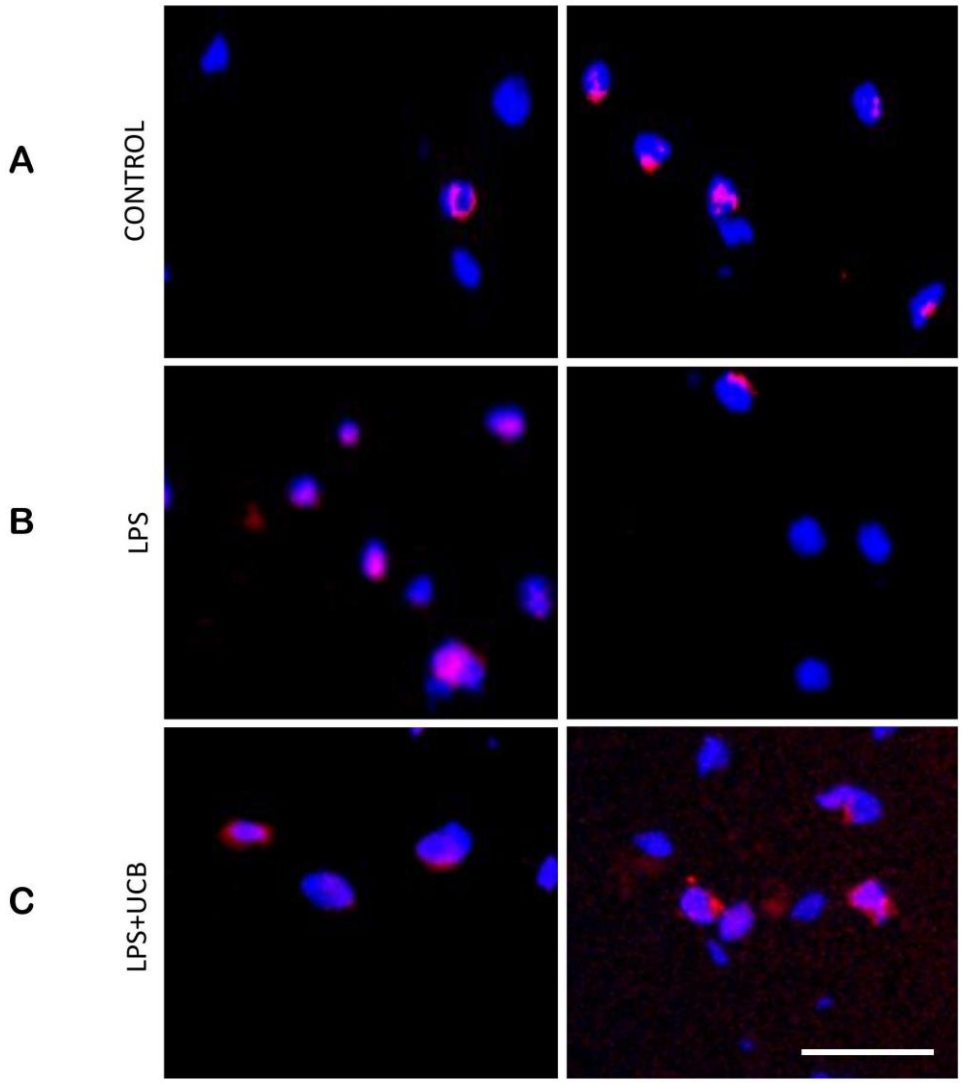


Figure 4.3: White matter integrity. Expression of oligodendrocyte transcription factor (olig-2+ cells) in the (A) subcortical white matter (SCWM) and (B) periventricular white matter (PVWM). Myelin basic protein (MBP) expression in the (C) SCWM and (D) PVWM. Data expressed as mean ± SEM, n=6-8, one-way ANOVA, $*p \leq 0.05$.


A
CONTROL
B
LPS
C
LPS+UCB

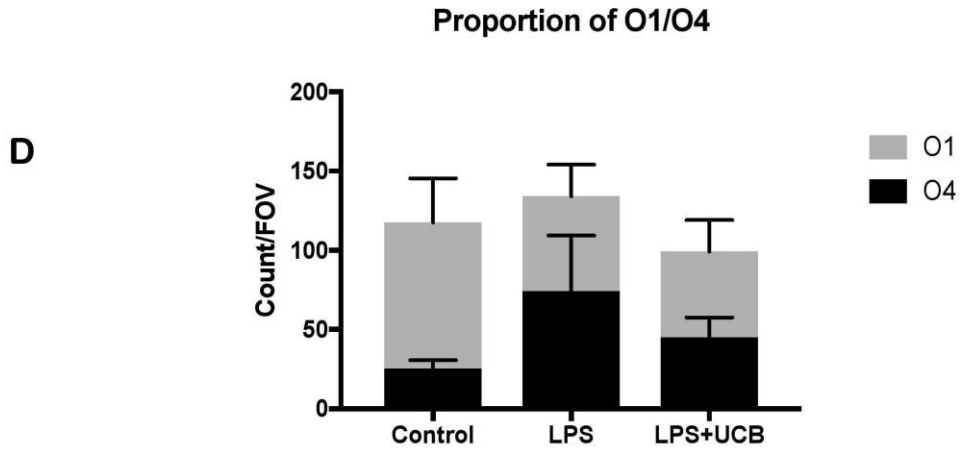

D
Proportion of O1/O4
Count/FOV
200
150
100
50
0
Control
LPS
LPS+UCB
O1
O4

Figure 4.4: O4 and O1 fluorescence immunohistochemical analysis. Representative images of O4 and O1 immunohistochemistry (red) with Hoescht nuclear stain (blue) in (A) control, (B) LPS and (C) LPS+UCB animals (scale bar 200μm). (D) Counts for O1 and O4 cell expression in subcortical white matter. Data expressed as mean ± SEM, n=3, one-way ANOVA, $*p \leq 0.05$.

4.5 Discussion

Here we show for the first time that human UCB cells can restore white matter development following a neuroinflammatory insult initiated by LPS administration. UCB cells reduced cerebral pro-inflammatory cytokine expression in the CSF, microglial aggregation and neutrophil recruitment in cerebral white matter, and ameliorated apoptosis-mediated cell death. UCB cells prevented loss of oligodendrocyte lineage cells, and normalised oligodendrocyte maturation as evidenced by improved MBP cell expression and restoration of the maturation of pre-oligodendrocytes into myelinating immature/mature oligodendrocytes. There are no current therapies that can be applied after preterm birth to protect and normalise brain development, despite significant knowledge to show that infants born preterm, particularly those born extremely preterm, are at considerable risk for neurodevelopmental deficits in motor and cognitive function [72]. In a high proportion of very preterm births, the infant has been exposed to the effects of chorioamnionitis, and in the long-term, chorioamnionitis is associated with MRI evidence of white matter brain injury [90]. Chorioamnionitis most commonly lacks profound fetal systemic inflammation and clinical markers of fetal distress, and in so doing is said to be subclinical, but nonetheless remains associated with white matter damage to the developing brain [74, 80].

UCB cells are safe and feasible as a therapeutic option in children with CP, and a recent meta-analysis investigating the efficacy of UCB across randomised controlled trials has demonstrated that UCB, alongside rehabilitation, is more effective at improving motor deficits than rehabilitation alone [91]. However, this preclinical animal study is the first to show that human UCB cells are protective for white matter development specifically in response to an inflammatory insult. Human UCB contains a heterogeneous mix of stem and progenitor cells which together, and separately, act in an immunomodulatory, angiogenic, anti-apoptotic and trophic manner for neuroprotective benefits [41, 83, 87]. The cell types present in UCB include mesenchymal stem cells (MSCs), haematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs), T regulatory cells, and monocyte-derived suppressor cells [85], with each of these cell types potentially moderating the effects of different injurious cascades, both systemically and in the brain. Cells found within UCB can secrete factors such as IL-10, monocyte chemoattractant protein-1,

angiogenin, vascular endothelial growth factor, brain derived neurotrophic factor and platelet-derived growth factors, all of which reduce neuroinflammation and promote neuronal repair [92, 93]. Therefore, these properties make UCB an excellent candidate treatment in the setting of *in utero* and/or perinatal inflammation or infection.

Neuroinflammation is a principal pathological feature present in the preterm brain [94], and microglial activation and aggregation, and release of pro-inflammatory cytokines are fundamental mechanisms that contribute to cell degeneration and interruption of brain development [94, 95]. In the present study, neuroinflammation following fetal LPS exposure was histologically evident as increased microglial and macrophage aggregations, and increased neutrophil recruitment within cerebral blood vessels, together with an, albeit not significant, elevated IL-1β in cerebrospinal fluid 10 days after the first LPS administration. That IL-1β was not profoundly increased likely reflects the 10 day period between LPS administration and tissue collection, together with the well characterised tolerance which can develop over repeated LPS exposure [96]. The observation of MPO-positive neutrophils in association with cerebral blood vessels is important, as neutrophils accumulate within the vasculature prior to infiltrating brain tissue, where they are shown to contribute to brain injury in the neonate, mediated via endothelial dysfunction, nitric oxide production and infiltration of neutrophils into the brain [97]. Neutrophil recruitment is modulated by an upregulation of chemokine ligand receptor signalling (particularly chemokine ligand-1 binding to its receptor chemokine receptor-2), a key mediator of inflammatory cell recruitment associated with exposure to chorioamnionitis [98]. Neutrophil localisation around the cerebral blood vessels contribute to inflammatory neonatal brain injury [97], and this is the first study to show that UCB cell treatment can modify this cerebrovascular response. Further, in the current study, we observed that UCB therapy was associated with significantly reduced CSF IL-1β levels compared to LPS alone animals. There is strong epidemiological evidence to link an upregulation of systemic and cerebral pro-inflammatory cytokines with neonatal brain injury [99], and increases in IL-1β in placental tissues are correlated with poor neurological outcome and CP [100]. Activated microglial aggregates within the brain are associated with oligodendrocyte death via inflammatory cytokine secretion and oxidative stress [101, 102]. The results from this study indicate that the anti-inflammatory actions

of UCB cells are mediating neuroprotective benefit, as shown previously in hypoxia-ischemia mediated preterm and term perinatal brain injury [28, 86].

In the preclinical animal model presented here, we aimed to mimic the intrauterine/ fetal environment associated with subclinical chorioamnionitis that most frequently occurs in human pregnancy [74]. While subtle, this subclinical fetal inflammation contributes to diffuse white matter injury [80], which is now the most commonly reported neuropathology associated with CP [81]. To mimic the most common fetal response to chorioamnionitis we caused a relatively subtle fetal inflammatory insult, which did not cause a profound fetal haemodynamic or systemic inflammatory response. Indeed LPS and/or stem cell treatment was not associated with changes in fetal blood gas parameters and were within the normal range for fetal sheep at this preterm gestational age [88]. LPS administration did result in increased spleen and liver weights compared to control animals, indicative of an immune-modulated response to LPS exposure [103], and we found this a useful indicator that LPS exposure was having an effect on the fetus in the absence of profound haemodynamic changes. An increase in spleen and liver weight is likely caused by an increase in T- and B-cell proliferation in response to immune activation, which would be expected to increase local release of cytokines [104, 105]. This change in organ weight was not seen in the LPS+UCB group, thereby demonstrating that UCB moderates the peripheral response to such stimuli, which may in turn contribute to the neuroprotective benefits of the cells. This finding is supported by previously published work showing that maternally administered MSCs are neuroprotective for the fetus in response to LPS exposure in pregnant mice, principally via actions at the placenta [106]. Combined, these results suggest that UCB/MSC cells target inflammation-regulatory sites, rather than the brain, to exert a neuroprotective benefit on the fetus.

White matter injury was present within the subcortical and periventricular regions of the fetal brains exposed to LPS alone. We did not observe any areas of gross cystic lesions in our brain regions of interest in response to low dose LPS, but found decreased numbers of oligodendrocyte lineage cells within the white matter (~30% fewer cells than controls), and specifically, a significant decrease in the number of mature MBP-positive oligodendrocytes in the SCWM (~45% fewer cells than control). The level of apoptosis

was also more than double in the SCWM of LPS animals, compared to controls. Together, these results are indicative that programmed cell death (apoptosis) affects oligodendrocyte lineage cells within the white matter, preventing maturation into myelinating oligodendrocytes. Normal oligodendrocyte development involves maturation of pre-oligodendrocytes into immature and then mature myelin producing oligodendrocytes. Disruption or arrest of this maturational process results in a total reduction of mature myelinating oligodendrocytes and subsequent hypomyelination [89]. This maturational progress can be examined by investigating the proportion of O4 and O1-positive oligodendrocytes [107], which principally stain pre-myelinating pre-oligodendrocytes, and myelinating immature/mature oligodendrocytes, respectively. We observed that the white matter regions of control brains exhibited more mature (O1) oligodendrocytes than immature (O4) cells, while LPS treatment resulted in a higher proportion of immature cells, suggesting that LPS was disrupting the maturation of oligodendrocytes. Notably, UCB therapy appears to provide a level of protection for oligodendrocyte maturation. While this requires further confirmation using a range of oligodendrocyte lineage markers (as per Back [89]), the present study illustrates the potential for UCB therapy to be adopted as a clinical therapy for the protection of normal white matter brain development in preterm neonates.

A limitation of the results presented here is that we did not observe a profound or consistent neuropathological profile in the LPS-treated group. In part, this is because we aimed to induce a relatively mild fetal inflammatory response, as often occurs clinically, but in turn we found a large variability as seen with the spread of individual data points within each group (Figures 4.1 to 4.4). This could be attributed to the well characterised selective vulnerability of white matter regions and cells as gestation progresses [108]. Whilst the white matter regions were selected based on expected regional vulnerability near the lateral ventricles, each animal may be developmentally different; the process of maturation of oligodendrocyte lineage cells demonstrates a high degree of developmental plasticity over this highly dynamic period for white matter development [82]. In some cases we were also limited by a small sample size for tissue analysis; that is, due to the nature of the fragile preterm tissue, analysis often could only be conducted in 5-6 animals per group (see Figure legends). However, taken together, our results showed that LPS

induced a significant region-specific neuroinflammatory response, apoptosis-mediated cell death, and reduced number of oligodendrocytes. These were all modified by administration of UCB cells. Critically, this experiment provide proof-of-concept that UCB therapy can elicit beneficial effects in the setting of preterm brain inflammation following a systemic inflammatory insult. Our results provide the first necessary steps towards translational insight into the usefulness of UCB in the setting of neuroinflammation. The neuropathology observed in this study reflects what is commonly noted where chorioamnionitis is diagnosed in human infants, predominantly non-cystic, subtle and diffuse white matter injury which is then highly correlated poor neurological outcome [109]. Future studies are now needed to assess the benefits of UCB cells administered soon after preterm delivery, to determine whether these cells can not only mediate microstructural repair, but also reduced neuropathology evident on MRI and follow-up motor development, behaviour, and long-term neurological outcome.

We have previously shown that both autologous and allogeneic sheep UCB cells are efficacious at reducing term and preterm hypoxic-ischemic brain injury [28, 86]. We have now expanded our characterisation of the potential benefits of UCB and understand that human UCB cells reduce neuroinflammation, cell death and improve white matter development. While we were able to demonstrate that UCB therapy is neuroprotective, we can only speculate as to which cells are having a beneficial effect; UCB contains a heterogeneous cell mix with variation amongst donors [110]. Further studies are warranted to better understand the potential role of constituent cells, and to better understand if one cell type is better than a mixed population of whole UCB for treatment of this condition.

4.5.1 Conclusion

This study is the first to show that human UCB therapy is anti-inflammatory and protects white matter brain development in a large animal model of chorioamnionitis. There are no current neuroprotective interventions for infants who are born preterm, and have been exposed to chorioamnionitis *in utero*. However, our results strongly support that UCB therapy could be utilised in this population of high-risk neonates. The results of this study

lay the foundation for the therapeutic use of UCB cells after preterm birth to decrease brain injury and restore normal white matter development. UCB is a promising therapy to restore white matter development in infants exposed to chorioamnionitis and neuroinflammation.

4.6 Acknowledgements

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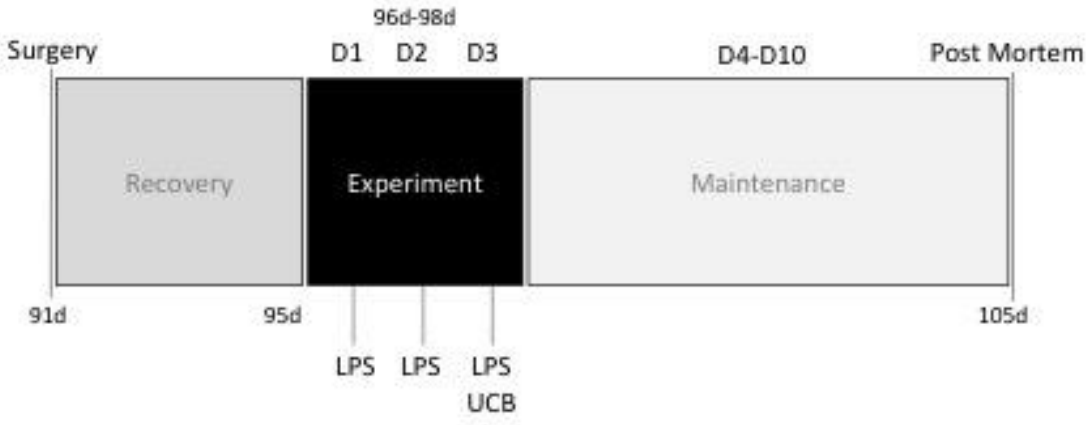
4.7 Supplementary Figures



Supplementary Figure 4.1: Experimental timeline. Surgery was performed at ~91 days fetal gestational age, followed by a 4-day recovery period. On days 1, 2 and 3 (D1-3), LPS animals received LPS (or saline for controls). LPS+UCB animals received cells 6 hours after the last LPS dose of D3. The animals were monitored until day 10 (D10), where a post mortem for collection of tissue samples (brain, CSF, kidney, heart, liver, spleen, lung and adrenals) was carried out.

Chapter 5

Umbilical cord blood versus mesenchymal stem cells for preterm brain inflammation and injury

This chapter has been submitted to *American Journal of Obstetrics and Gynaecology* and has been reformatted for consistency in this thesis.

Paton, M., Allison, B., Fahey, M., Li, J., Sutherland, A., Nitsos, I., Bischof, R., Dean., J., Moss, T., Polglase, G., Jenkin, G., *McDonald, C. and *Miller, S.. **Examination of the neuroprotective effects of umbilical cord blood cells versis mesenchymal stem cells for inflammation-induced preterm brian injury in fetal sheep.** *American Journal of Obsetrcis and Gynaecology (Submitted, 16th August*).
*joint senior authorship

5.1 Abstract

Chorioamnionitis and fetal inflammation are principal causes of neuropathology detected after birth, particularly in very preterm infants. Umbilical cord blood (UCB) cells are neuroprotective in preclinical models of perinatal brain injury, but it is uncertain if allogeneic UCB cells are a feasible early intervention for preterm infants. In contrast, mesenchymal stem cells (MSCs) are more readily accessible and also show strong anti-inflammatory benefits.

We aimed to compare the neuroprotective benefits of UCB cells versus MSCs in a large animal model of inflammation-induced preterm brain injury. We hypothesised that mesenchymal stem cells would provide superior neuroprotection, via their anti-inflammatory properties.

Chronically instrumented fetal sheep at 0.65 gestation received intravenous lipopolysaccharide (LPS, 150ng, 055:B5, n=8) over 3 consecutive days to produce a mild-moderate neuroinflammation; or saline for controls (n=8). Cell-treated animals received 10^8 UCB mononuclear cells (n=7) or 10^7 human umbilical cord MSCs (n=8), intravenously, 6 h after the final LPS dose. Seven days later, cerebrospinal fluid was collected, brain white matter from the left hemisphere was snap frozen for molecular analysis, and the right hemisphere was fixed for histology.

LPS induced neuroinflammation and apoptosis, and reduced the number of mature oligodendrocytes in the subcortical white matter. MSCs reduced white matter astrogliosis, but umbilical cord blood cells did not have the same effect. UCB significantly decreased cerebral apoptosis and protected mature myelinating oligodendrocytes, but MSCs did not.

UCB is superior to MSCs for reducing inflammation-induced preterm brain injury in fetal sheep. UCB may provide a clinically viable cell therapy that prevents cell death, protects vulnerable oligodendrocytes, and promotes brain repair in a preterm cohort.

5.2 Introduction

Preterm birth is strongly associated with brain injury and life-long disability [111]. Chorioamnionitis affects up to 70% of infants born preterm [112] and produces a fetal inflammatory response, that may be accompanied by neuropathological processes including blood brain barrier breakdown, neuroinflammation, loss of oligodendrocytes and subsequent white matter injury [80]. Accordingly, chorioamnionitis is associated with developmental deficits at school age [112]. There are currently no neuroprotective or reparative therapies for preterm infants born after chorioamnionitis.

Umbilical cord blood (UCB) contains a rich mix of stem and progenitor cells [113]. UCB therapy is being trialled currently in term neonatal encephalopathy and established neurological disorders including cerebral palsy (CP) [83]. UCB is easily accessible, has low immunogenicity and has proven safety and neuroprotective efficacy [25, 114]. Preclinical models of preterm white matter injury show UCB suppresses inflammation and normalises oligodendrocyte development [37, 41, 87]. However, autologous UCB therapy may not be feasible in preterm infants due to low yield, and UCB cell composition may be compromised by antenatal complications [28, 40] leaving patients to rely on allogeneic UCB [87], which present temporal and human leukocyte antigen (HLA)-matching barriers [41].

Mesenchymal stem cells (MSCs) also have neuroprotective properties and are readily isolated from healthy umbilical cord tissue [115, 116]. Currently, >450 clinical trials are using MSCs for various conditions (https://clinicaltrials/gov/), but none target preterm brain injury. MSCs secrete cytokines and soluble factors in response to inflammation and regulate immune processes and tissue repair[87]. Engraftment of MSCs within tissues is not necessary [117, 118]. MSCs are easily sourced, do not require HLA-matching, have an established safety profile, and tolerate long-term cryopreservation without compromising function [57, 119].

Mild and diffuse brain injury consistent with the neuropathology identified in modern cohorts of infants with chorioamnionitis and CP [120-122], can be induced in fetal sheep by lipopolysaccharide (LPS) administration. We aim to compare the anti-inflammatory

and neuroprotective benefits of MSCs or UCB, in preterm fetal sheep with LPS-induced neuropathology. We hypothesise that MSCs would reduce neuroinflammation and protect white matter development to a greater extent than UCB.

5.3 Materials and methods

5.3.1 Human tissue collection

Ethics approval was obtained from the Monash Health Human Ethics Committee. UCB and cord samples were obtained from consenting women at term (>37 weeks gestation) undergoing elective caesarean sections.

After clamping of the cord and placental delivery, <140ml of venous UCB was collected into bags (Macopharma, Australia) and 5cm of umbilical cord was collected into a sterile container.

5.3.1.1 Umbilical cord blood

UCB mononuclear cells were isolated as previously described [123]. Briefly, blood was centrifuged, the buffy coat collected and cells counted using trypan blue exclusion (Gibco), before cryopreservation in dimethyl sulfoxide (10% DMSO, Sigma in FBS), and storage in liquid nitrogen.

For intravenous (i.v.) administration, cells were thawed, washed and counted. Cells were fluorescently labelled with carboxyfluorescein succinimidyl ester (CFSE) before administration. Each animal received 10^8 mononuclear cells (pooled from 3 donors) in 2ml phosphate buffered saline (PBS).

5.3.1.2 MSCs

Umbilical cord tissue was processed immediately for MSC isolation as previously described [124]. Briefly, a 1-2cm section of the cord was minced manually (>3min), media was added (16.5% fetal bovine serum [FBS] in DMEM:F12; Gibco), and incubated (37°C, 21% oxygen, 5% CO_2). Media was changed every 3 days and passaged at 80% confluency. Cells were cryopreserved after passage 2 (10% DMSO in FBS). Cells were rapid-thawed 10 days prior to use and plated at passage 3. On administration day, MSCs were counted and fluorescently labelled with CSFE, and an aliquot (3 million cells) retained for karyotyping (G-banding, Cytogenetics Laboratory, Victoria). Each animal received i.v. 10^7 MSCs (2 donors) in 2ml PBS.

5.3.2 Flow cytometry analysis

MSCs and UCB were incubated with primary antibodies for flow cytometry analysis following manufacturer's instructions. Cells were assessed using BD FACSCantoII and analyzed with FACS Diva software (BD Biosciences). Antibodies used for UCB included CD14 (eBiosciences), CD34 (eBiosciences), CD133 (Miltenyi Biotech), CD45 (Miltenyi Biotech), Stro-1 (BioLegend), CD4 (eBiosciences) and FoxP3/CD25/CD4 (eBiosciences). Antibodies used for MSCs included CD73 (BD Biosciences), CD90 (Bioss), CD105 (Bioss), CD44 (Miltenyi Biotech), CD34, CD45, Stro-1, HLA-DR (eBiosciences) and HLA-ABC (eBiosciences).

5.3.3 Animal Surgery and LPS administration

Animal experiments were undertaken with approval from Monash Medical Centre Animal Ethics Committee and conducted following National Health and Medical Research Council of Australia guidelines.

Pregnant Border Leicester-Merino ewes with a single fetus underwent sterile surgery at 91.1±0.2d (mean±SD) gestation (term is ~150 days) as previously described [123]. Briefly, ewes were placed under general anaesthesia and catheters inserted into the fetal jugular vein, femoral artery and amniotic cavity. The ewe recovered for 4 days prior to experimental intervention.

At 95d gestation (experimental d1) fetuses were assigned to; (i) control: saline; (ii) LPS: i.v. 150ng LPS (055:B5, University of Queensland); (iii) LPS+UCB: i.v. 150ng LPS and 10^8 UCB mononuclear cells; or LPS+MSCs: i.v. 150ng LPS and 10^7 cord tissue MSCs. LPS/saline administration occurred daily from experimental d1-3. UCB/MSC administration occurred on d3, 6hrs after the final dose of LPS.

On experimental d10 animals were humanely killed via pentobarbitone overdose (Lethabarb, Virbac, Australia). Fetal cerebrospinal fluid (CSF) was collected, and the brain removed and weighed. The right brain hemisphere was immersion fixed in 4% paraformaldehyde. After paraffin embedding, the brain was coronally sectioned at 10μm

and mounted on SuperFrost Plus (Thermo Fisher Scientific, Australia) slides. The left side was snap frozen for PCR.

5.3.4 CSF cytokine analysis

CSF concentrations of IL-1β were measured by ELISA [31].

5.3.5 Neuropathological assessment

Neuropathology was assessed using cresyl violet acid fusion staining. Cellular apoptosis and astrogliosis/reactive astrocytes were quantified in the brain after immunostaining using activated caspase-3 (cas-3, 1:1000, R&D Systems) and glial fibrillary acidic protein (GFAP, 1:800, Sigma), respectively. Mature myelinating oligodendrocytes were stained with myelin basic protein (MBP, 1:500, Millipore). Immunostaining was revealed using 3,3-diaminobenzidine (DAB; Pierce Biotechnology, USA).

For analysis, images were acquired at 400x magnification under light microscopy (Olympus BX-41, Australia). Image J (NIH, USA) was used to perform densitometry using threshold coverage measurements. The analysis was blinded (MCBP), with 3 non-overlapping regions selected in the sub-cortical white matter (SCWM) and periventricular white matter (PVWM).

5.3.6 Quantitative real-time PCR

mRNA was extracted from snap-frozen white matter, following the manufacturer's instructions (PureLink RNA Mini Kit, Thermo Fisher, Australia), with DNase I treatment (Thermo Fisher, Australia). Complimentary DNA was synthesised from mRNA (SuperScript III Reverse Transcriptase; Thermo Fisher, Australia), and custom Taqman gene primers (Applied Biosystems) were used for quantitative RT-PCR (7300 RT-PCR, Applied Biosystems). Housekeeping control primers were RPL32, OAZ1 and 18s. For analysis, raw Ct values and amplification efficiencies of all target and housekeeping genes were imported into qBase^{Plus} Software and calibrated normalised relative quantities

(CNRQ) of each gene were calculated and exported for analysis. Gene expression is expressed as fold-change relative to calculated Ct values normalised to geometric mean of housekeeping genes.

5.3.7 Statistics

Analysis was performed using GraphPad Prism 6 (GraphPad Software, USA). Histological and molecular data were analysed using a one-way ANOVA with post-hoc individual t-test or Tukey's. Z For the z-scores of neuroinflammation, outcomes from GFAP and lectin were pooled then analysed using a two-way ANOVA with Tukey's post-hoc. Data are presented as mean ± standard error of the mean (SEM), and statistical significance set at $p<0.05$.

5.4 Results

In total 31 fetuses were studied; control n=8, LPS n=8, LPS+UCB n=7 and LPS+MSCs n=8. We previously presented some physiological and histological outcomes from these subjects [123]. Fetal characteristics were not different between groups (Table 5.1).

Table 5.1: cohort characteristics and white matter neuropathology

Variable	**Control**	**LPS**	**LPS+UCB**	**LPS+MSCs**
Animals, *n*	8	8	7	8
Gestational (days)	91.17 ± 0.31	91.50 ± 0.38	91.14 ± 0.14	90.25 ± 0.16
Body weight (kg)	1.60 ± 0.06	1.50 ± 0.09	1.51 ± 0.04	1.59 ± 0.08
Brain weight (g)	30.78 ± 0.91	28.24 ± 0.52	29.33 ± 0.86	30.41 ± 0.59
Sex ratio (m/f)	4/4	3/5	4/4	6/2
Rates of white matter neuropathology with cresyl violet acid fusion				
	Control (n=5)	**LPS (n=8)**	**LPS+UCB (n=7)**	**LPS+MSCs (n=7)**
Cystic lesions	0/5	2/8	1/7	0/7
Red blood cell extravasation	0/5	1/8	2/7	0/7
Inflammatory cell aggregates	1/5	2/8	2/7	0/7

Characteristics of each animal cohort used in this study including *n*-number, gestation, body weight, brain weight and the sex ratio as well as rates of neuropathology using cresyl violet acid fusion. n=5-8, reported as number of animals per group with abnormality.

5.4.1 UCB and cord tissue-MSC characterization

Flow cytometry of UCB mononuclear cells revealed 0.07±0.02% were MSCs (CD45-, Stro-1+). From the CD45+ population, 8.6±1.4% were monocytes (CD14+) and 2.1±1.5% were endothelial cells (CD133+). Gated from the lymphocytic populations, 2.3±1.4% were haematopoietic (CD34+/45low) and 14.2±10.9% were T-regulatory cells (CD25+/Foxp3, CD4+). Isolated MSCs met standard MSC criteria[31] and karyotyping (Sup Fig 5.1).

5.4.2 Neuropathology and Neuroinflammation

Control animals did not show any white matter abnormalities (Table 5.1). Cystic white matter lesions, extravasated red blood cells within the cerebral tissue (indicative of intraparenchymal hemorrhage) and inflammatory cell aggregates were evident in some LPS and LPS+UCB brains (Fig 5.1). MSC fetuses did not display white matter lesions, inflammatory aggregates or hemorrhage.

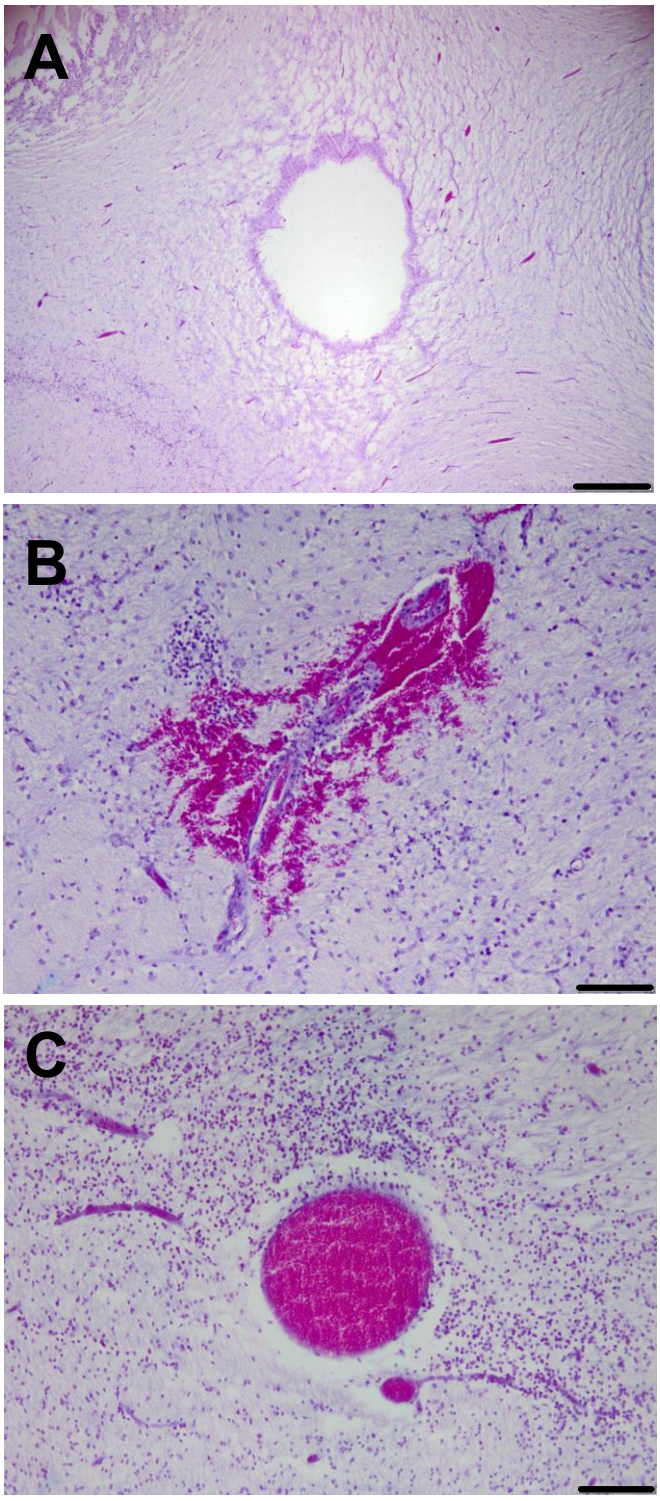


Figure 5.1: Representative images of neuropathology. (A) gross lesion, (B) red blood cell infiltration and (C) inflammatory cell aggregates around enlarged blood vessels in the white matter using cresyl violet acid fusion. Scale bar for (A) is 500um, and (B-C) is 100μm.

CSF concentrations of IL-1β were higher than control in LPS animals (control:11.1±3.7 *vs* LPS:28.1±7.2ng/ml, p=0.06). IL-1β concentrations in CSF were lower in LPS+UCB (7.23±1.24ng/ml, p=0.03) and LPS+MSC (12.41±1.63ng/ml, p=0.06) animals, compared to the LPS group.

Astrocyte activation (GFAP coverage, Sup Fig1) was higher in LPS animals than control (Fig 2A, p=0.02). Astrocyte activation was lower in MSC animals compared to LPS (p=0.001) and UCB (p=0.005) animals. In the PVWM, astrocyte activation was lower in LPS+MSC fetuses compared to LPS (Fig 2B, p=0.003). Microglial and macrophage cell aggregates (lectin+; Sup Fig 2) in SCWM was lower in MSC animals than LPS (Fig 2C, p=0.01) or UCB animals (p=0.02). Lectin expression was not different in the PVWM (Fig 2D).

We generated neuroinflammatory z-scores by combining astrocyte and microglial assessments. In SCWM, neuroinflammatory z-scores were higher in LPS animals than control (SCWM, Fig 2E, p=0.03). The MSC animals had lower neuroinflammatory z-scores than LPS animals (p=0.04) but UCB animals did not. No significant differences were detected in the PVWM (Fig 2F).

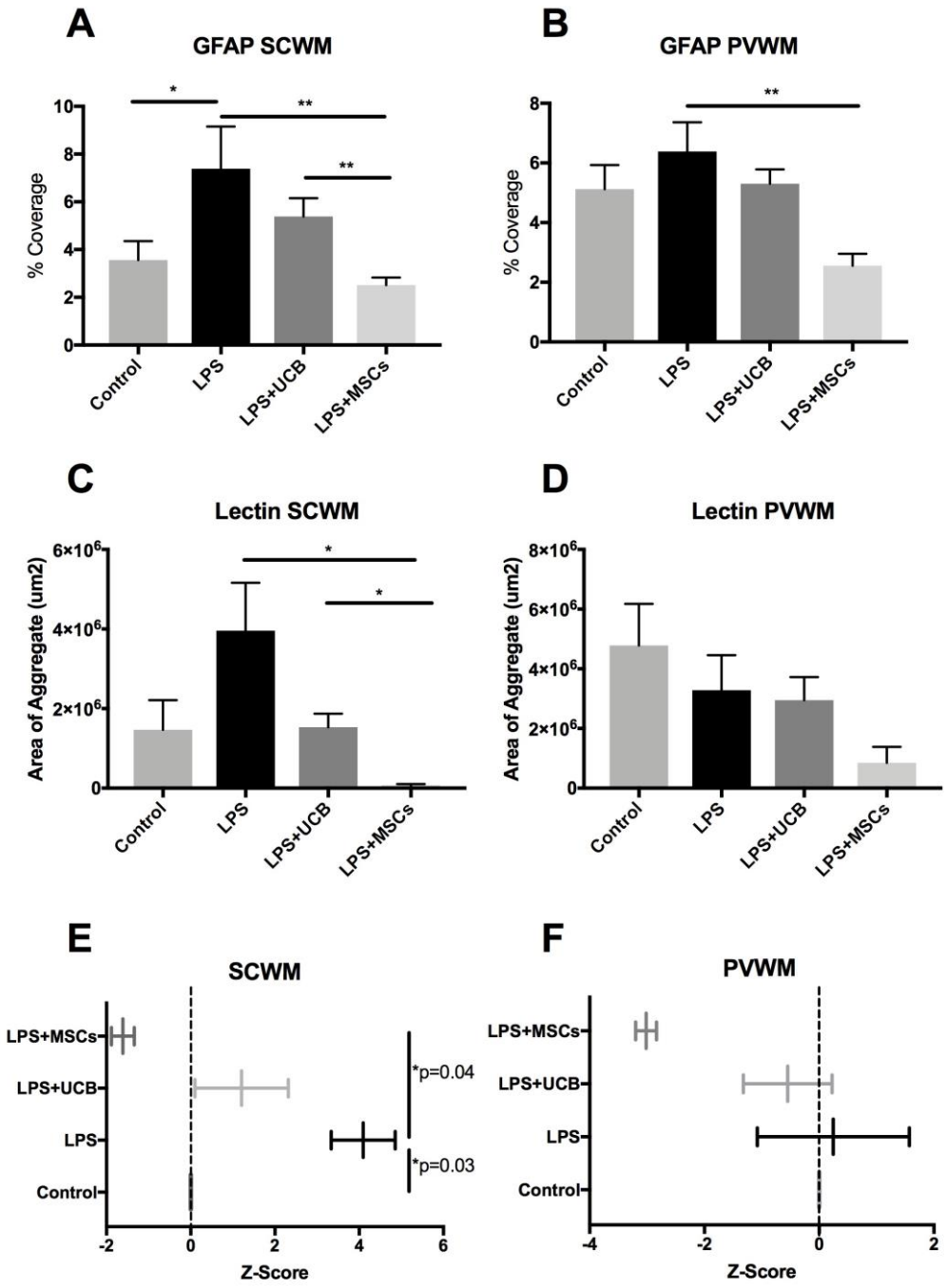


Figure 5.2: Neuroinflammation in the white matter. (A-B) glial fibrillary astrocytic protein coverage, (C-D) area of lectin aggregates and (E-F) Z-scores of neuroinflammation derived from glial fibrillary astrocytic protein and lectin in the subcortical white matter and periventricular white matter. (A-D) One way-ANOVA, with post-hoc t-tests (mean±SEM). (E-F) Two-way ANOVA with Tukey's post-hoc (mean ± min/max), n=6-8.

5.4.3 Cell death and white matter integrity

Activated caspase-3 in the SCWM was higher in LPS fetuses than control (Fig 3A & Sup Fig 2, p=0.04) and UCB animals (p=0.048), but not MSC animals. Cell death was higher in MSC animals compared to UCB animals (p=0.02). Cell death in the PVWM was not different between groups.

The number of mature myelinating oligodendrocytes in SCWM was lower in LPS animals than control (Fig 3C & Sup Fig 2, p=0.02). Oligodendrocyte number was higher in UCB animals compared to LPS (p=0.04) but not MSC animals. Mature oligodendrocyte cell counts in PVWM were higher in UCB animals compared to LPS (Fig 3D, p=0.004) but not MSC animals.

Sparse CSFE-labelled cells were observed within the brains of animals treated with UCB, but not MSCs.

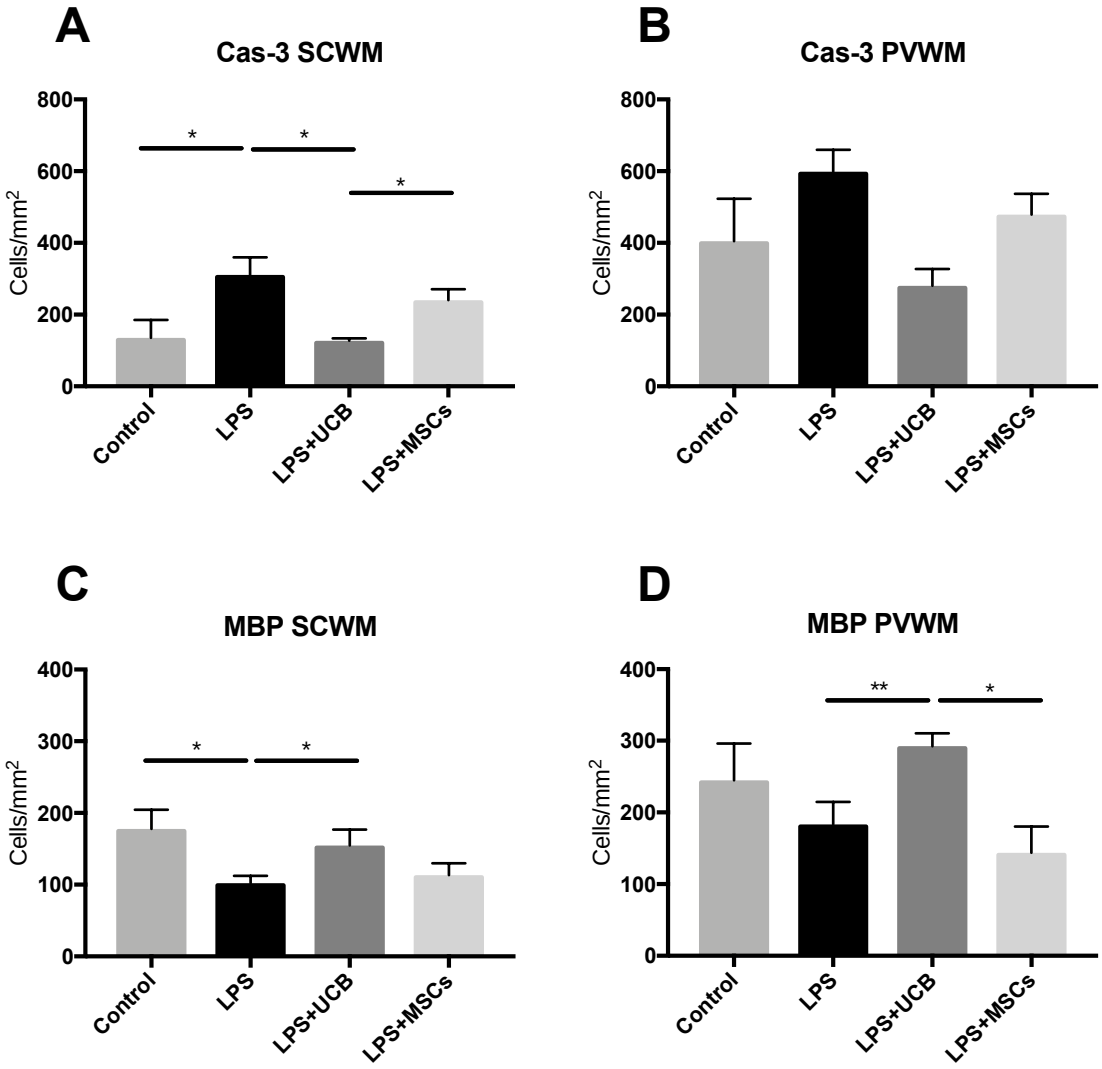


Figure 5.3: Cell death and white matter integrity. (A-B) activated caspase-3 and (C-D) myelin basic protein cell counts in the subcortical white matter and periventricular white matter. One-way ANOVA, with post-hoc t-tests, n=6-8.

5.4.4 Mechanisms of cell action

5.4.4.1 Inflammatory genes

White matter mRNA levels for interleukin (*IL)-1β*, *IL-6*, *IL-18*, *forkhead box p3* (*Foxp3*), *IL-4*, *nuclear factor kβ subunit-1* (*NFkβ1*) and *toll-like receptor-4* (*TLR-4*) were not different between control and LPS animals (Fig 4A-C, E-H, respectively). *Transcription growth factor-β1* (*TGF-β1*) mRNA levels tended higher in LPS animals than controls (3.72-fold increase, Fig 4D).

White matter mRNA levels for inflammatory proteins was not different between LPS and UCB animals.

White matter mRNA levels for *Foxp3* were lower in MSC lambs than controls (p=0.03, Fig 4E) and LPS (p=0.04) lambs. mRNA levels for *IL-1β, IL-6, IL-18, TGF-β1*, *IL-4* (p=0.06), *NFKβ1* and *TLR-4* tended lower in MSC animals than LPS animals.

5.4.4.2 Blood brain barrier and regulatory growth genes

White matter mRNA levels for tight junction protein *(TJP)-2*, *TJP-3*, *occludin* (*OCLN*), *myeloperoxidase gene* (*MPO*), *tumour protein gene 53* (*TP53*) and *insulin growth factor-1* (*IGF-1*) were similar for control and LPS animals (Fig 5A-F). UCB and MSC administration increased expression of levels of mRNA for *TJP-3* were higher in UCB (p=0.02) and MSC animals than controls (p=0.008, Fig 5B). MSC animals had lower *MPO* and *IGF-1* mRNA levels (p=0.03 and 0.02, Fig 5D-E) than controls.

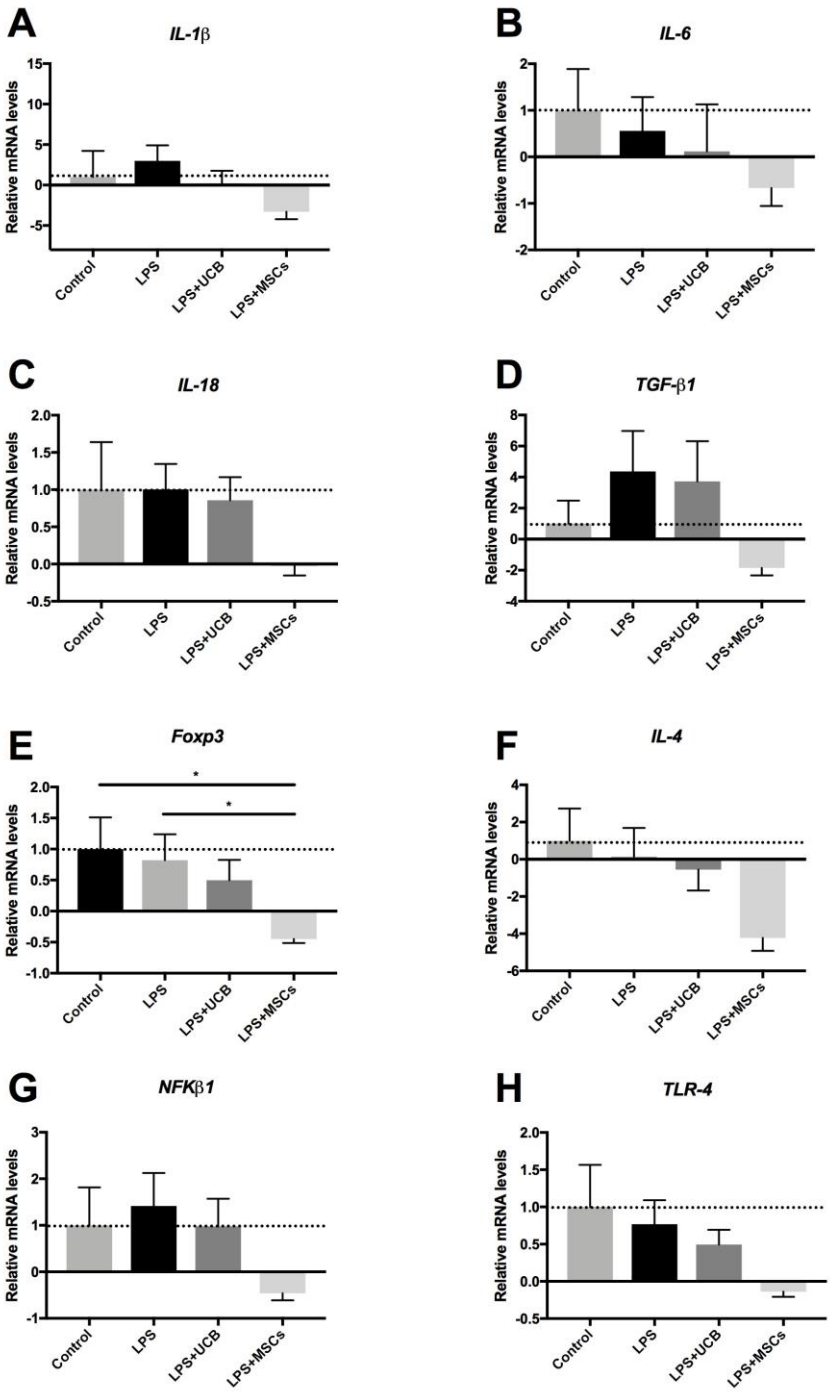


Figure 5.4: Inflammatory genes in the white matter. (A) interleukin-1β, (B) interleukin-6, (C) interleukin-18, (D) transforming growth factor β1, (E) forkhead box p3, (F) interleukin-4, (G) nuclear factor κβ subunit 1 and (H) toll like receptor-4 gene expression shown as relative mRNA change from controls, normalized from the calibrated normalised relative quantities from 3 housekeeping genes. One-way ANOVA (mean ± SEM), n=7-8.

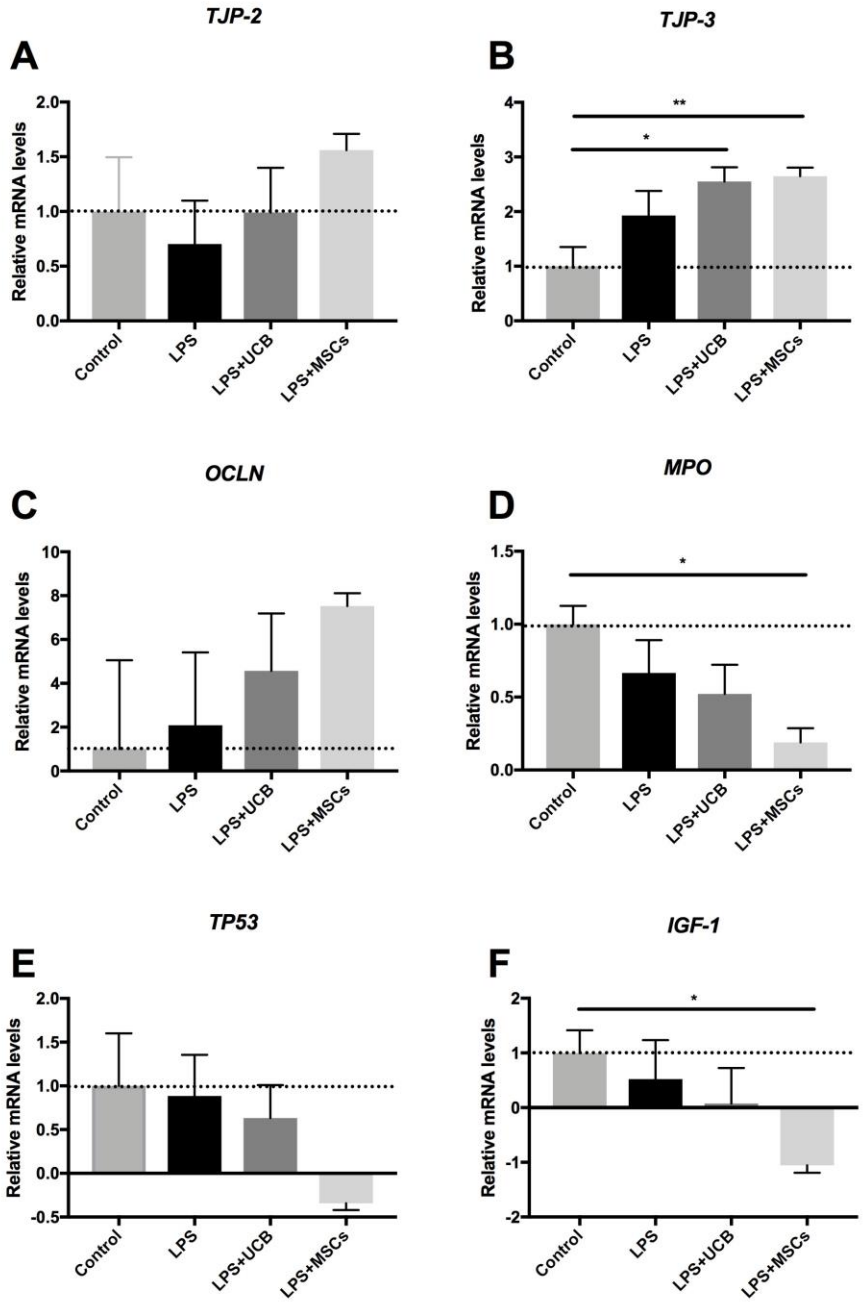


Figure 5.5: Regulatory blood brain barrier and growth genes in the white matter. (A) *tight junction protein-2*, (B) *tight junction protein-3*, (C) *occludin*, (D) *myeloperoxidase*, (E) *tumour protein 53* and (F) *insulin growth factor-1 gene* expression shown as relative mRNA change from controls, normalised from the calibrated normalized relative quantities from 3 housekeeping genes. One-way ANOVA (mean ± SEM), n=7-8.

5.5 Discussion

5.5.1 Principal findings

This study is the first to compare the neuroprotective efficacy of UCB cells versus MSCs for inflammatory preterm brain injury. Both UCB cells and MSCs have protective benefits for the preterm brain, but their effects on white matter are different. MSCs were strongly anti-inflammatory, dampening all indices of brain inflammation at the cellular and gene level. In contrast, UCB cells showed a reduced ability to mediate neuroinflammation, but importantly, prevented apoptosis-mediated cell death and protected mature myelinating oligodendrocytes. These differential effects of UCB and MSCs are likely due to specific actions of an isolated cell population (MSCs) versus a stem/progenitor cell mix (UCB). Indeed, we found that MSCs comprised less than 0.1% of the total mononuclear cells within cord blood samples used for this study, and UCB efficacy is likely attributable to the remaining cell populations.

5.5.2 Clinical importance

Chorioamnionitis and preterm birth are causal factors for neurological deficits and subsequent life-long disability, learning and behaviour dysfunctions [75]. Globally, preterm birth rates are high and may be increasing [73, 76], and neurological dysfunctions are increased at all levels of prematurity [125]. Here, fetal exposure to LPS in preterm sheep induced small cystic lesions in two animals, diffuse intraparenchymal hemorrhage, and subtle changes in white matter microstructure [123]. These subtle changes in white matter can lead to devastating neurological outcomes, as found clinically, with diffuse white matter injury being the most common neuropathology underlying CP [126]. Infants born preterm and with evidence of clinical or subclinical inflammation are at very high risk of poor neurological outcomes, but no targeted intervention currently exists.

Cell therapies present novel and potentially efficacious neuroprotective treatments for preterm infants. UCB therapy in humans is safe, and has shown efficacy in improving developmental outcomes in established CP [38]. However, UCB collection volumes may be very low in infants born preterm, and cellular composition in preterm birth and

chorioamnionitis may be altered [87]. Therefore we compared UCB with MSCs, which have potential as an off-the-shelf allogeneic alternative.

5.5.3 Mechanisms of cell action and interpretation

UCB contains a mixed cell population, as shown by our identification of stem and progenitor cell populations of EPCs, Tregs and monocytes, plus a minute proportion of MSCs (<0.1%). We have previously shown that individual populations of EPCs, Tregs and monocytes show differential neuroprotective benefits when administered following hypoxia-ischemia in neonatal rats [127]. EPCs support angiogenesis and blood brain barrier structure and indirectly modulate peripheral immune responses, while T-regulatory cells and monocytes are immunosuppressive and modify inflammatory cell infiltration into the brain[127]. The heterogeneity of UCB cells suggests that their neuroprotective benefits may be due to effects on immunomodulatory, angiogenic, and apoptotic pathways [128]. It is also important to highlight that some of the effects of UCB could be in part due to the presence of non-stem cell populations, such as immune cells, and this is an important consideration.

UCB treatment reduced pro-inflammatory IL-1β in CSF 10 days post-LPS, a critical finding given that this cytokine is upregulated in human chorioamnionitis and implicated in white matter injury [83, 129]. IL-1β is principally released from microglia in response to insult [130], but interestingly, UCB did not decrease inflammatory microglial cell aggregates in this study. Following insult, activated microglia mediate both damage and repair responses within the immature brain [127], and our findings suggest that the complete dampening of neuroinflammation as seen with MSCs does not translate to white matter protection.

Unlike UCB, MSCs are quite a homogenous cell population that mediate neuroprotective effects via direct immuno-modulation and secretion of anti-inflammatory cytokines, and indirectly via cell recruitment to injury sites and release of trophic factors [67, 131]. Cell engraftment in the brain is not necessary for neuroprotection, as confirmed by our observations. In the present study, the most compelling effect of MSC therapy was a

considerable dampening of brain inflammation, often below basal levels. MSCs significantly reduced gene expression for *Foxp3*, well-known for its role in programming regulatory T cells [132]. While other inflammatory genes were not significantly different, we observed a broad down-regulation of inflammatory mediators with MSC treatment. It might be considered that downregulation of classical inflammatory genes is beneficial and perhaps reparative for the brain following insult, but a careful inflammatory cell balance is essential for healthy brain development [127]. For example, Foxp3 is necessary for normal astrocytic development [133]. Therefore, reducing an aberrant pro-inflammatory response within the brain is likely to better support brain development than a profound and global anti-inflammatory dampening. Consistent with the link between Foxp3 and astrocytes, we also observed reduced astrocyte density with MSC therapy, together with microglial cell coverage below control levels. MSC treatment also decreased white matter expression of *IGF-1*, which is essential for normal oligodendrocyte maturation and is produced by multiple cell types within the CNS, particularly the glial cells [134]. Indeed, *IGF-1* is proposed as a rescue therapy for hypomyelination [135], and is protective for oligodendrocytes following LPS exposure [136]. In the current study downregulation of *IGF-1* might, at least partially, account for the lack of protective benefit of MSCs on mature oligodendrocyte development. Alongside the anti-inflammatory effects, MSCs protected blood brain barrier structure with increased tight junction protein expression, and decreased *MPO* (neutrophil and vascular compromise marker) [137], which in turn is reflected in neuropathology findings, with no red blood cell extravasation into white matter in MSC-treated fetuses.

5.5.4 Clinical implications, limitations and future directions

While the actions of UCB cells and MSCs appear to be different, the pronounced dampening of inflammation and growth factor expression by MSCs is somewhat concerning. Inflammation and cytokine release helps regulate the immune response, astrocytic development and activation, oligodendrocyte maturation and blood brain barrier maintenance [138]. Potentially the dose of MSCs administered in this study may be too high, equivalent to ~6 million/kg. The total number of cells administered in the UCB group (100 million) was greater than for MSCs alone (10 million), but the number

of MSCs in the UCB cell mix was likely very low (<70,000). UCB cells are generally small, and large doses of 25-50 million cells/kg can safely be administered to neonates without adverse effects [139]. In contrast MSCs require expansion by tissue culture and therefore are a much larger cell (average 25μm), limiting the number that can be administered in a single dose without risk of embolism [140]. These are important considerations as preclinical results move towards clinical application.

In the present study, cells were administered 6h after the third LPS dose in an attempt to mimic a clinical application of cell therapy after birth and upon diagnosis of chorioamnionitis. Clinically, cell administration could occur once the infant was stable, in the hours after birth. Administration at different timepoints, and postnatally after delivery, are vital next steps. In previous work we showed that treating with UCB cells early after insult is more efficacious for the preterm brain than delaying administration until day 5 [141-143].

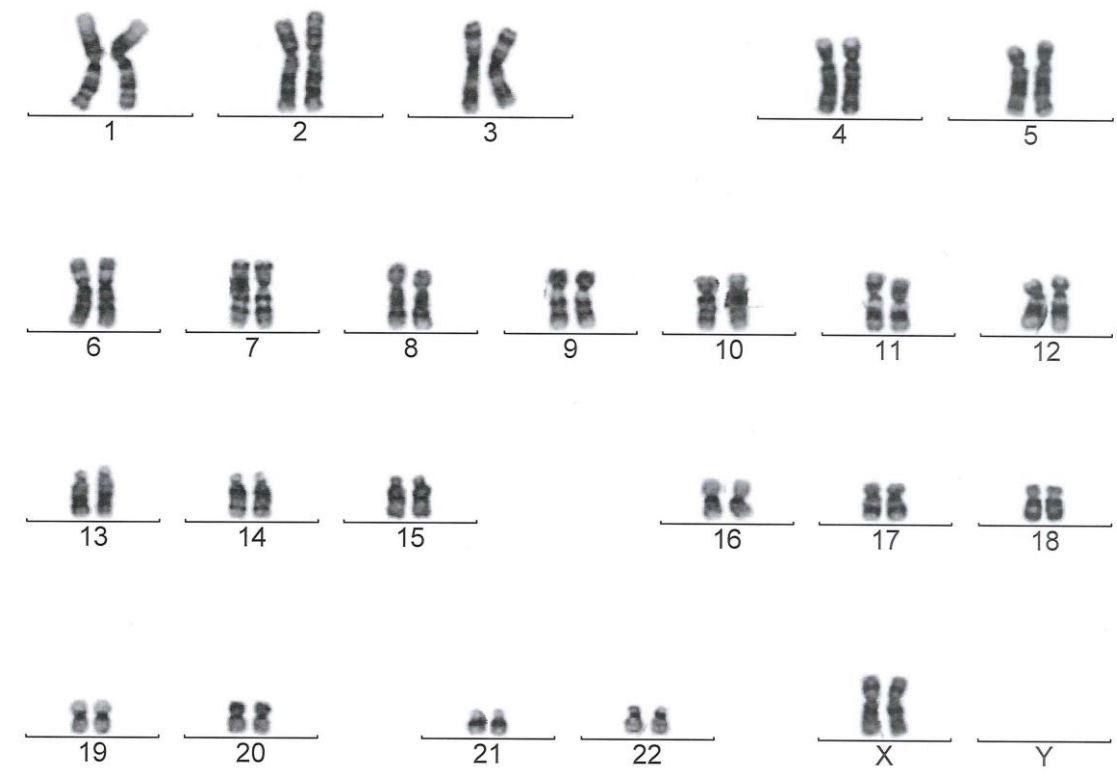
5.5.5 Conclusion

In response to LPS-induced preterm brain injury, administration of MSCs had a profound and global effect on dampening brain inflammation, which in turn may have detrimental effects on brain repair and development. MSCs did not improve survival of critical oligodendrocytes and did not prevent apoptosis-mediated cell death. In contrast, UCB was neuroprotective against cell death and normalised the number of mature myelinating oligodendrocytes, but did not display the same anti-inflammatory effects as MSCs. Our results indicate that UCB is a comprehensive therapy for protecting white matter brain development, likely contributed by the mixed cell population in UCB, and their differential actions.

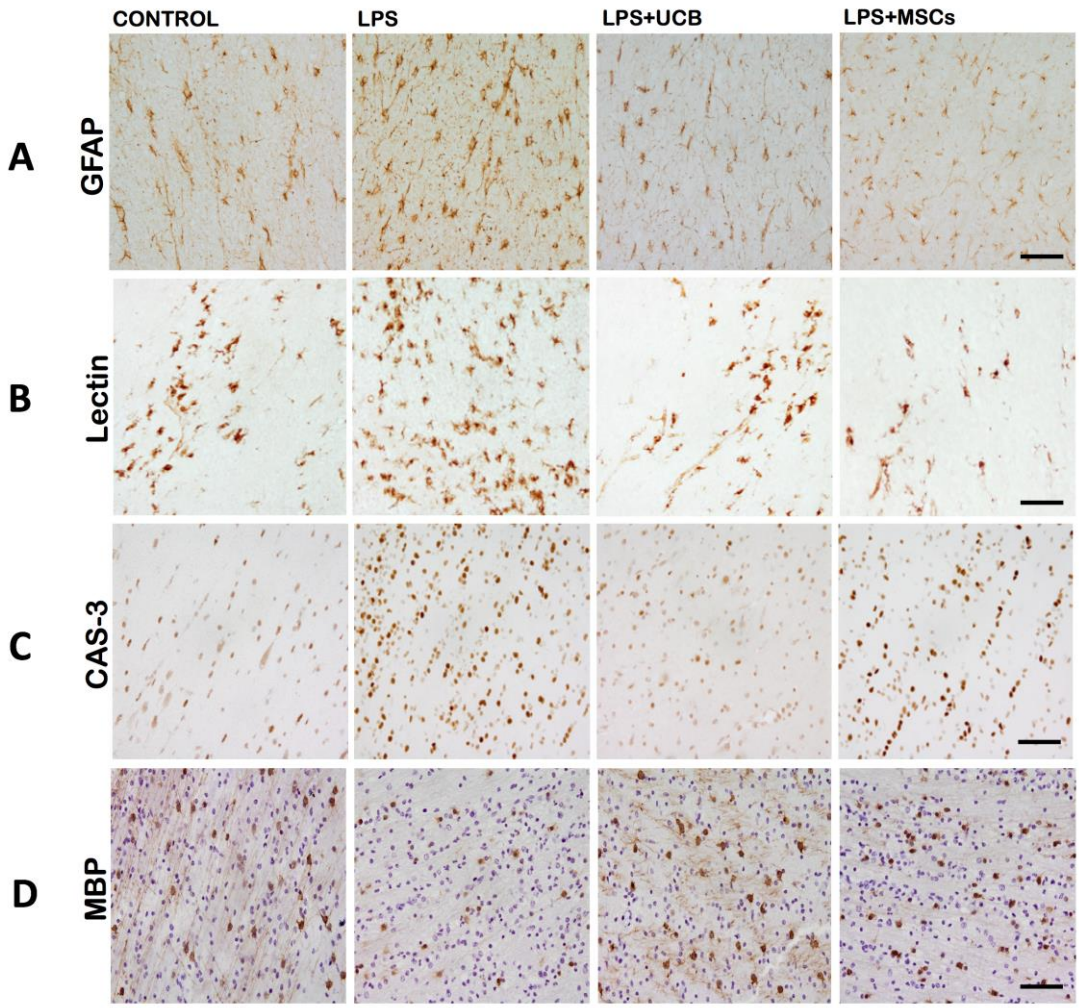
5.6 Acknowledgements

The authors would like to thank Jamie Mihelakis, Dalibor Stanojkovic and Arielle Kogan for their technical assistance. This project was made possible with financial support from the Cerebral Palsy Alliance, Inner Wheel Australia, the National Health and Medical Research Council, as well as an Australian Research Council Future Fellowship to SM, NHMRC and Cerebral Palsy Alliance Early Career Research Fellowship to CM, and Kahli Sargent Research Studentship and Australian Government Research Training Program Scholarship to MP.

5.7 Supplementary figures



Supplementary Figure 5.1: Representative image of G-banding karyotype for cord tissue MSC donors. Regular female XX karyotype with 23 pairs of chromosomes from passage-3 MSCs.



Supplementary Figure 5.2: Representative images of neuroinflammation, cell death and mature white matter cells. (A) glial fibrillary astrocytic protein, (B) lectin, (C) activated caspase-3 and (D) myelin basic protein staining in the subcortical white matter. Scale bar is 10μm.

Chapter 6

General Discussion

Nearly 43% of all cases of cerebral palsy (CP) can be attributed to preterm birth [144]. Chorioamnionitis and fetal inflammation affect up to 70% of all preterm births, particularly those with premature rupture of placental membranes [112]. Chorioamnionitis increases the risk of neurological complications, learning deficits and CP. Along with this comes a substantial economic impact, with the annual cost, including the burden of lifelong disability and premature death, estimated at $115,000 (AUD) per person in 2007 [145]. The rate of CP in Australia has been declining for the last decade due to advances in medical care, particularly with compelling results supporting the use of maternal magnesium sulphate to protect the preterm brain [146]. However, there is still no treatment that can be applied soon after birth that targets and protects the brain when an infant is born preterm following perinatal inflammation and chorioamnionitis.

There has been significant interest in developing stem cell therapies to address this unmet need. Umbilical cord blood (UCB) is a heterogenous mix of immune privileged and easily accessible stem and progenitor cells, with proven neuroprotective and immunomodulatory effects in adults and children for a range of conditions [87]. However, many questions regarding the use of cell therapies remain unanswered; we do not know which cell therapy may be most useful for preterm infants following perinatal inflammation and, with respect to the use of UCB, whether whole mononuclear cells (MNCs) are superior to single cell therapy, for example mesenchymal stem cells (MSCs). This question was investigated in this series of studies. In addition, factors such as treatment dosing, route and timing of administration have yet to be thoroughly investigated. There is, thus, a need for the development and standardisation of efficacious protocols for potential cell therapies based on preclinical scientific evidence.

CP describes a spectrum of motor and postural deficits and is associated with extensive comorbidities and multiple major organ involvement [147]. There is increasing interest in the application of stem cell therapies for patients with established CP. While stem cells are not the only therapy being examined for treating CP, they are favoured due to their multiple potential therapeutic targets and the ability to respond to environmental cues compared to classical pharmaceutical drugs [148]. Therefore, when administered following organ damage and inflammation, stem cells are purported to home to sites of

injury, such as the brain or peripheral organs, and contribute to both immunomodulation and recruitment of other cells to sites of injury to initiate repair [87, 149]. Stem cells can come from a variety of sources, however, placental-derived cells are particularly favourable for clinical studies as they are virtually free of ethical issues associated with their collection, and are considered an immune-privileged cell type [150, 151]. As they are derived from fetal tissues, placental cells require less patient human leukocyte antigen (HLA)-matching and some cells, including MSCs, may not require matching at all [84, 152].

In Australia alone, there are over 300,000 live births every year, and almost all placentas are discarded [153]. Accordingly, placentas are an excellent potential source of stem cells that can be collected and stored for later use. Within the placenta and the umbilical cord are a variety of stem cell populations that can be harvested and that could be of therapeutic use, including MSCs derived from umbilical cord tissue, MNCs and endothelial progenitor cells (EPCs) derived from UCB, and human amnion epithelial cells (hAECs) from the membranes [154]. This thesis investigated the use of different stem cell therapies derived from UCB and umbilical cord tissue specifically for brain injury associated with preterm brain inflammation. I have compared the effects of UCB therapy versus a potential off-the-shelf cell treatment, cord tissue-derived MSCs, in a fetal ovine model of preterm brain inflammation, induced via fetal lipopolysaccharide (LPS) administration.

6.1 Clinical relevance

6.1.1.1 Clinical relevance of the ovine model of preterm brain inflammation

For this thesis, a model of preterm brain inflammation was optimised in fetal sheep. Preliminary studies demonstrated that a dosage of 150 ng administered once a day for three consecutive days, directly (i.v.) to the fetus, during a critical period of brain development was optimal to induce diffuse brain inflammation and white matter injury that resembles the neuropathology observed in clinical cases of chorioamnionitis [89]. This dose was chosen based on an extensive review of the literature and modified from a published LPS regime previously used in sheep [88]. We came to use a lower dose than

that used in other published studies, and we administered the LPS systemically (i.v.) to the fetus, rather than intra-amniotically as used in many other recent studies. Our rationale for this LPS administration regimen was to induce a standardised fetal inflammatory response, with reproducible injury to the brain versus other typical target organs such as the lung [155-158]. I optimised the dose of LPS in the early stages of this study by titrating the dose from a relatively high starting dose, in stepwise progression towards a lower concentration that produced mild and diffuse white matter injury. The LPS used in my studies was synthesised and verified for potency at the University of Queensland (from *E.Coli* 055:B5; courtesy of Dr Phillip Bird). Therefore studies using this formulation may not be directly comparable to that used in other studies where LPS had been purchased commercially [9, 75]. A commercial source of LPS was not available for us to use in these studies at the commencement of my project due to changes in Australia's import regulations.

We aimed to induce brain injury at 0.65 ovine gestation (~91 days) which is comparable to human fetal brain development at approximately 28 weeks (Figure 6.1). In humans, this very preterm gestation is an important stage where oligodendrocytes are maturing but not yet producing functional myelin within the white matter [108]. The period of greatest risk for periventricular white matter injury is from 24-32 weeks of gestation, which corresponds to the period in human brain development prior to the onset of myelination. Thus, pregnancy compromise and preterm birth over this period have a very high risk of white matter damage and subsequent development of CP [108, 159]. The LPS model we developed reflects a sub-clinical inflammatory insult, the most commonly observed clinical presentation of chorioamnionitis, wherein only subsequent histopathological assessment of the placenta can reveal inflammation [160]. Thus, in our experimental paradigm, no physiological or blood gas changes were detected in the fetus throughout the period of study. On histopathological assessment of the brain, we did, however, observe mild and diffuse white matter brain injury and inflammation following administration of LPS, reflecting what occurs in the majority of human patients born preterm following *in utero* inflammation [80, 159].

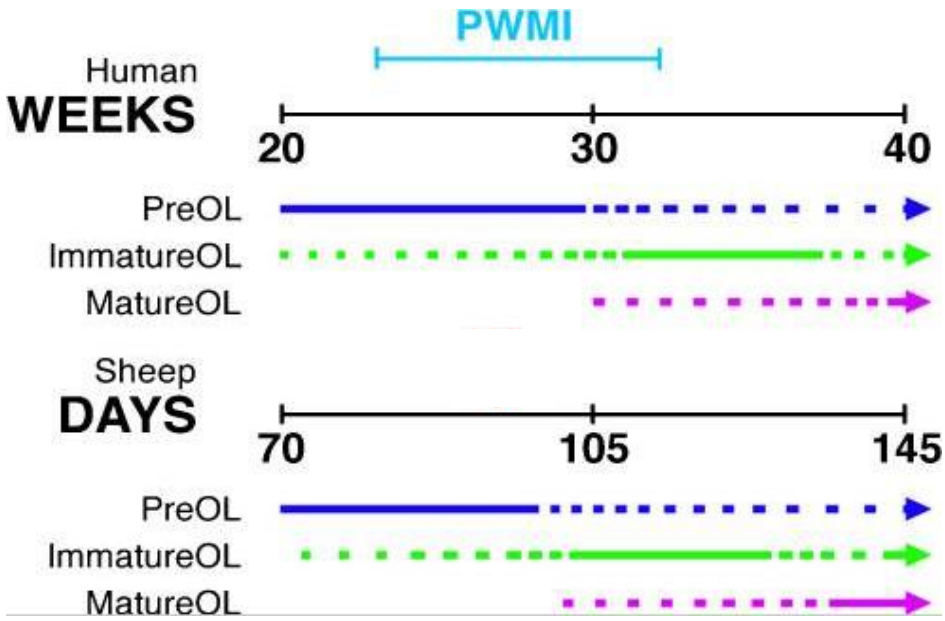


Figure 6.1: Comparison of gestational white matter development in human versus sheep, adapted from Back, et al. 2006 [161].

6.1.1.2 Clinical relevance of stem cell dosing and administration timing

In this thesis we used stem cell doses of either 100 million UCB MNCs or 10 million MSCs administered to fetal sheep, equating to around 77 x 10^6 and 7.7 x 10^6 cells per kilogram, respectively, based on an estimated fetal weight of ~1.3kg at 98 days gestation. This fetal weight at 98 days gestation was derived from n=35 post-mortem records from our lab over the past several years. Cells were intravenously administered to the fetus at 6 hours after the final LPS dose. These cell doses are considered to be in the moderate-to-high dose range when compared with those used in current clinical trials, which typically use 10^6 MSCs per kilogram, or repeated doses of up to $2x10^7$ UCB cells per kilogram [37, 48]. Each cell dose was chosen based on current literature, information regarding the heterogenous progenitor and stem cell mix in UCB [83], previous clinical studies that have shown safe and neuroprotective UCB doses [25, 139], as well as taking the difference in cell size into consideration [25, 139, 140]; the size of MSCs after expansion is large (~25μm) compared to UCB cells. Administering MSCs at higher

doses, equivalent to UCB, increases the risk of embolism [140]. Therefore doses of MSCs must be lower than UCB.

We strategically chose a cell administration timepoint that was clinically relevant, reflecting the time it might take to deliver and stabilise a very preterm infant, allowing feasible delivery of cells. Autologous UCB mononuclear cell administration has been shown to take on average 6.5 hours from time of collection to reinfusion in newborns following hypoxia-ischemia [139]. Administering cells 6 hours after the final LPS dose, is the proposed ideal time for stem cell therapies, where there is a *window of opportunity* for repair and immunomodulation (commonly between 6-12 hours) [10]. Early administration at this gestational timepoint is critical, as pre-myelinating oligodendrocytes are still the principal cell type within the brain's white matter and potential long term-damage of progenitors can be addressed and avoided, rather than delaying cell therapy which must then treat established brain injury (Figure 6.2). For other models of brain injury in sheep, early intervention timepoints (12-24 hours) has been trialled with hAECs following *in utero* inflammation and UCB after hypoxia ischemia, with neuroprotective effects [8, 86]. Other ovine studies have also demonstrated that delaying cell administration for 5 days after preterm brain injury, compared to 12 hours after insult, is associated with poorer neurological outcomes [28].

Here, we assessed efficacy of our selected therapies at seven days after cell administration. It is common in rodent models of brain injury to assess the effects of cell therapies on myelination days or weeks after insult [162, 163]. However, in ovine models, assessing the efficacy of cell therapies 1-4 days after administration [9, 12, 86] is common, with limited examination at weeks after administration [13]. This later post mortem timepoint was essential so that we could analyse longer term reparative mechanisms following stem cell treatment and observe whether white matter cell recovery had resulted. Shorter term experiments are relevant to investigate the acute effects of cells and any short-term reparative properties they might possess. Acutely, cell therapies have been shown to reduce neuroinflammation and cell death [12, 86]. However, for the experiments utilised in this thesis, we wanted to understand longer-term reparative properties of the cells and, particularly, their effects on the white matter and

their ability to protect oligodendrocyte progenitor populations. This reflects a *proof of concept* model, where we focus on the effects of cells directly on the brain following LPS exposure, without the involvement of other confounders such as delivery and ventilation of the lamb. This allowed a useful comparison of the potential differences between UCB and MSC therapies, where changes in white matter cell populations and brain structures could be investigated.

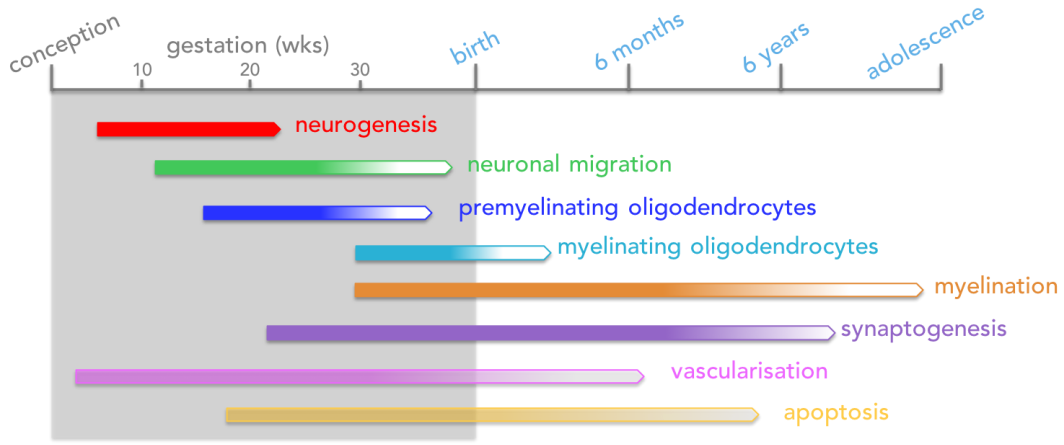


Figure 6.2: Dominant neurodevelopmental processes across gestation, at delivery, and through to adolescence (Figure courtesy of Margie Castillo-Melendez).

6.1.1.3 Clinical relevance of human stem cell therapies in an ovine model

This work is the first to demonstrate the use of human UCB cells and MSCs applied to an ovine model of preterm brain inflammation. UCB or MSC-derived stem cells used in this thesis were sourced from healthy human, term pregnancies. Although we have previously shown that the use of ovine UCB and MSCs in an ovine model of preterm hypoxic ischemic brain injury are efficacious [86, 164], using human as opposed to ovine cells allows for more rapid clinical translation and increased the potential translational impact of results from this study by using cells from the same origin as those that would be considered for human trials. Additionally, the cells sourced from non-self donors is clinically relevant as autologous administration of stem cells for preterm babies would not always be feasible. Previous studies have postulated that if preterm births were not

complicated by other co-morbidies such as chorioamnionitis, an adequate volume and cell number could be sourced for autologous transplantation [40, 165]. However, routine collection of UCB from very small and preterm placentas is difficult, with potentially low collection volumes and compromise in cellular integrity with the presence of chorioamnionitis [87]. Term UCB samples were used in these studies, which reflects the best cell source of cells for use in clinical trials due to collection feasibility. This thesis also investigated the use of MSCs as a potential source of cells for an *off the shelf* therapy. Whilst preterm cord tissue may be a viable source of MSCs, there is a need for isolation and lengthy expansion to obtain clinical doses – this is not the case for UCB cells. This time consuming and costly procedure would mean that autologous MSCs could not be administered during an optimal therapeutic window within hours of life. However allogeneic *off-the-shelf* therapy using MSCs is quite feasible as an early intervention therapy as they do not require HLA-matching and validation of cell integrity, and efficacy validation and storage can be completed in advance from a universal donor.

6.2 Main thesis findings

While EPCs could not be adequately expanded and characterised under the multiple conditions investigated (*Chapter 3*), UCB MNCs and MSCs sourced from umbilical cord tissue were collected and MSCs were successfully expanded. These cells were isolated, easily stored and expanded to produce relevant clinical doses. Both cell types have a proven safety profile and are shown to be efficacious in human trials for other indications [39, 48], and both UCB and MSC are now under investigation as potential therapies for established CP [26, 37]. However, early intervention, during the critical period of brain plasticity after birth (the critical *window of opportunity*), of cell therapies has not been investigated. Therefore, this thesis aimed to investigate the potential of early administration of cells (6 hours after the final LPS dose) in the context of neuroprotection, as opposed to neuroregeneration. We already know that these cells hold potential as a therapy for brain injury and inflammation, and that placental cell therapies have many other benefits over the use of drugs, and other stem cell sources, such as those from adult tissues [41]. However, these treatments have not previously been assessed in the setting

of fetal inflammation and white matter brain injury, nor have different cell types been directly compared for efficacy within one experimental paradigm.

In *Chapters 4* and *5* we showed that UCB stem cell therapy administered following inflammatory brain injury, induced by LPS, selectively reduced markers of neuroinflammation and normalised the developmental profile of oligodendrocytes. These observations confirm findings in rats exposed to hypoxia-ischaemia, wherein results showed that UCB cells mediated neuroprotective benefits via actions on Akt signalling, release of neurotrophic factors and cytokines, and UCB promoted survival of oligodendrocyte mediators which contributed to white matter cell survival [166]. In the current study, UCB administered following LPS significantly reduced IL-1β within the CSF and was associated with significantly less white matter cell death, compared to LPS alone. Other markers of neuroinflammation (lectin+ and GFAP) were not significantly altered by UCB treatment. UCB animals did, however, show increased expression of total oligodendrocytes (Olig-2), and a significant increase in mature myelinating oligodendrocytes (MBP+) compared to LPS animals. In the LPS+UCB fetuses, gene expression of inflammatory markers was not different within white matter brain tissue at seven days after the final LPS dose, when compared to all other groups, relative to control. However, there was an altered expression of blood-brain barrier proteins compared to control. The overall effect of UCB in this model therefore was mainly potentiated by anti-apoptotic actions which prevented myelinating oligodendrocyte cell loss. This work contributes to supportive literature using UCB therapy in rats and sheep for other conditions, where treatment with UCB for stroke and birth asphyxia significantly reduced neuronal deficits and improves inflammatory markers in the brain [86, 167].

Treating preterm fetal sheep with MSCs following exposure to LPS, improved general brain morphology, with no animals displaying any signs of white matter lesions, red blood cell infiltration or inflammatory aggregates, unlike LPS animals. MSC therapy demonstrated a strong effect on modulating neuroinflammation, with significantly less astrocytic coverage and macrophage/ microglia cells present in the white matter. This finding is consistent with current literature examining the neuroprotective benefits of MSCs in fetal sheep exposed to acute hypoxia-ischamia, where MSCs were shown to

dampen neuroinflammation and subsequently mediate repair [164]. However, in our experiments, MSC therapy did not reduce overall cell death, compared to LPS alone animals, nor did it recover the loss of mature myelinating oligodendrocytes, which is in contrast to results observed following UCB therapy. MSC administration was associated with altered white matter gene expression; this included both anti-inflammatory and pro-inflammatory markers, a number of blood brain barrier proteins and important regulatory white matter development genes. It has been reported in term-equivalent neonatal rats exposed to hypoxic-ischaemic stroke that MSCs can protect the oligodendrocyte lineage cells, aiding progenitor migration, restoring total oligodendrocyte cell number and preventing the loss of mature myelinating cells, particularly when studied within the first few days of injury [168, 169]. Here, we have demonstrated different results. MSC treatment following LPS induced a global dampening of inflammation, often below control levels, and consistent downregulation of expression in gene markers of both anti- and pro-inflammatory cytokines. This response may interfere with critical reparative processes that in turn protect oligodendrocytes. It may be a matter of optimising the time of MSC administration or reducing the cell dose to get optimal immunomodulation.

We could, therefore, conclude from *Chapter 5* that, while MSCs effectively reduced neuroinflammation, this did not translate into a protective benefit for the white matter of the brain, thus resulting in a lack of comprehensive neuroprotection. This effect was in contrast to the results of UCB therapy which proved more neuroprotective, resulting in significantly less cell death and inhibiting the loss of critical white matter cell populations. It is important to note, that UCB is a heterogenous source of cells unlike MSCs, that include non-stem cells, progenitors and immune cells. This may in part explain the differences in function. This work is novel, with UCB and MSCs never having been directly compared for any neurological injury or previously tested in the setting of preterm brain inflammation as an early intervention in an ovine model. Results from this thesis will help guide clinical practice in support of the application of cell therapies in the treatment of chorioamnionitis.

6.3 Study limitations

6.3.1.1 LPS-induced brain inflammation

We administered LPS in a relatively low dose, directly to the fetus, in an attempt to mimic mild and diffuse white matter injury, however this group did not demonstrate consistent brain inflammation or neuropathology. Some animals (n=2 from LPS cohort) were found to have small cystic lesions in the white matter, with the majority of LPS animals showing diffuse and mild signs of neuroinflammation and injury. Cohort variability impacted on outcomes of interest and overall analysis, with histology to identify large inflammatory infiltrates or brain bleeds not being fully appropriate end points for these studies. Instead, immunohistochemical analysis of specific inflammatory cells proved more informative. However, our model does reflect the human condition, where nearly 30% of all patients with CP will have a normal MRI at around 1.5 years of age with no profound apparent structural deficits [128]. Therefore, subclinical chorioamnionitis and premature delivery may not overtly cause initial detectable injury, however CP may still be diagnosed in later life. Longer-term functional studies using this model are, therefore, warranted.

Results from *Chapters 4 and 5* display large variations, as reflected with large error bars, particularly on histological outcomes. Although preliminary power calculations suggested that sample sizes of 7-8 animals would be sufficient, due to unexpected cohort variability, it may have been appropriate to increase the sample size to establish more robust and less variable cohorts. Cohort variation meant that while some animals in the LPS group displayed identifiable brain injury compared to controls, overall this effect remained statistically non-significant for some end-points. This was a result that pertained even after optimisation of LPS dosing to attempt to mimic the most common clinical presentation of white matter injury following chorioamnionitis. It is important to note that, during development and validation of our model, a higher dose of 200ng of the LPS used in this study (data not presented), delivered to the fetus daily for three days, resulted in such severe brain tissue damage that it meant that fixation was not possible as tissue integrity was too low. This occurrence was likely due to a combination of the very preterm gestation of the brain and its low lipid (myelin) content compared to that in more mature brains, together with the adverse effects of LPS [170]. The extensive tissue degradation

resulting from this higher LPS dose was, therefore, a more extensive injury than we wished to use in the current study, and the use of this dose of LPS was not pursued. However, future experiments may look at refining the dosing concentration and the regimen to induce more consistent and somewhat more severe injury that may be better detected using classical immunohistological methods of assessing brain inflammation.

6.3.1.2 Experimental duration

For experiments used in this thesis, there was 7 days between the last LPS dose and post-mortem. This duration is long in comparison to other studies [9, 12] (discussed previously), and resulted in no direct changes in the fetus in response to LPS exposure in many outcomes investigated. These included cytokine arrays of the CSF and plasma, molecular analysis and major brain histological outcomes of consistent lesions and severe inflammation. Therefore, if the injury did manifest after LPS, as previously shown in other studies [88, 171, 172], injury may have resolved by the time that post-mortem was undertaken in these studies. Thus analysis, with a further cohort of animals at an earlier timepoint, may have provided insight as to whether an initial response had occurred, and if the cells had any early effects on these changes, or whether the brain could resolve injury on its own during this preterm period of highly plastic neuronal processes (Figure 6.2). Whilst we could not confirm early brain injury for this thesis, current studies are underway that demonstrate preliminary evidence of inflammatory infiltrates and cortical disorganisation at 6 hours after the final dose of LPS, when cells would be administered.

Current literature supports that, in this study, we were administering cells at an appropriate time to protect white matter and prevent long-term oligodendrocyte damage and cell death [28, 83]. As previously stated, stem cell therapies are being trialled for established CP, however plasticity of the brain is reduced in childhood and adolescence and we were therefore interested in an early intervention, in the hope of targeting and protecting the pre-myelinating oligodendrocytes (Figure 6.1 and 6.2). However, the mechanism of action and the time course of effects from each cell therapy need to be investigated to better understand optimal therapeutic timing. These additional experiments are also critical to definitively understand our observations that MSCs had

persistent effects on the brain which UCB administration did not demonstrate, yet UCB overall appeared to be a better potential neuroprotective therapy for the developing cerebral white matter.

6.3.1.3 Very preterm gestation and experimental feasibility in sheep

Another limitation of these studies is the very preterm gestation of fetal sheep at the time of the initial surgeries and experiments. LPS was administered to the fetus at 91 days gestation, reflecting a critical stage of brain development where, if damage were to occur, we would expect to see significant alterations in white matter cell populations that inevitably impact on maturation (Figure 6.1). Oligodendrocyte progenitors are a particularly vulnerable cell type, and are the predominant cell within the white matter at around 28 weeks of human gestation (equivalent to ~90 days in the sheep) [159]. As a result, infants born preterm from 24-32 weeks have a high risk of white matter injury and subsequent development of CP. This was, therefore, the most relevant gestation to target to implement a cell therapy for preterm brain injury. Selecting this gestation was novel, with limited studies analysing the effects of an intervention on the brain in an LPS model at this very preterm gestation. Such experiments in sheep are normally conducted around 109-117 days gestation [8, 12]. Brain development during this time is undergoing significant neuronal migration and myelination (Figure 6.2). Therefore, studies using the gestation chosen by us are critical to study grey matter infarcts, periventricular white matter injury and periventricular leukomalacia (PVL). Because of the current shifting paradigm where preterm babies are being delivered earlier, with no abnormal brain MRI showing PVL, but notable neurodevelopmental and cognitive impairments [89, 128], this is the type of injury that studies in this thesis were designed to model, capturing a critical period of oligodendrocyte vulnerability.

Whilst the study design allowed for us to assess the efficacy of cell therapies on vulnerable early pre-oligodendrocytes in inflammatory brain injury, this stage of gestation meant that we were restricted in what catheter instrumentation could be performed and the length of time we could maintain the delicate fetuses. For example,

the catheters were as small as possible so that adequate blood sampling could be collected, unfortunately the catheter's narrow bore resulted in a loss of patency over time and this resulted in the inability to collect adequate samples over the experimental period while the fetus was mobile and growing.

This extremely preterm gestation also meant that delivery and maintenance of the fetus would be complicated and very challenging due to their relatively underdeveloped and immature lungs. As previously mentioned, this would have introduced multiple additional variables due to the major interventions necessary to maintain such neonates *ex utero*. Preterm birth and delivery itself is a known cause of systemic and cerebral inflammation [173, 174]. Additionally, studies involving the delivery and maintenance of preterm lambs are only considered currently feasible after ~126 days gestation [31, 156].

6.4 Future directions

6.4.1.1 EPCs for preterm brain injury

Examination of the neuroprotective benefits of EPCs for the preterm brain were limited by our ability to expand EPCs adequately. However just this year, two alternative methods have been developed and published for EPC expansion which may yield sufficient cells for these proposed studies to take place in the future [175, 176]. We still believe that it is very important to obtain EPCs and examine their effects on preterm brain inflammation, as these cells have proven efficacy in repairing blood vessel networks and supporting repair after injury [67, 177]. As our published review (*Chapter 1*) states, we initially wanted to compare UCB with a targeted cellular therapy using individual cell types; in particular the administration of MSCs and/or EPCs at specific times when inflammation could be targeted, and cerebral blood vessels could be protected. The efficacy of a combined MSC and EPC treatment also remains to be investigated as a potential therapeutic option given the results of differential effects of each cell type described in this thesis.

6.4.1.2 Longer-term neonatal ovine studies

This study was designed as *proof of concept*, isolating brain inflammation from other confounding injury associated with ventilation and the transition after birth by using an *in utero* ovine model of chorioamnionitis. In doing so, our results provide insight into the ability of the stem cell therapies to directly protect the brain following the effects of fetal inflammation. We were able to study histological neuroinflammation and apoptosis, as well as white matter gene expression, to gain an understanding of how stem cell therapies prevented injury. To progress these studies further, postnatal intervention in a clinically relevant model of preterm delivery following *in utero* inflammation should be considered. Exposure to LPS antenatally, followed by preterm caesarean delivery, neonatal intensive care with ventilation, and then cell treatment at 6 hours after delivery would be an excellent way to mimic more completely the clinical scenario, where inflammation may have occurred some time before delivery and ongoing damage accumulates before an opportunity for therapeutic intervention would be possible. This would be a technically challenging series of experiments to undertake, but is potentially feasible as such experiments are now being attempted in Western Australia with some success, with delivery of preterm sheep at ~126 days gestation and maintenance for 7 days [178]. Whilst this is a highly advanced, intensive experimental setup, it would have the advantage of determining whether the injury to the brain is more profound after delivery and ventilation, and whether our cells of interest not only prevent brain injury but also have positive effects on other major organs such as the premature lungs. A protective and anti-inflammatory effect of stem cell administration has previously been reported in other models in which the effects of mechanical ventilation following hAEC administration in an LPS model has been studied [31, 156].

Studies of long-term postnatal outcomes could contribute to elucidating if cell therapies have persistent effects on behaviour and neurodevelopment. Such studies, potentially carried out after LPS exposure, delivery and maintenance of animals (over a number of weeks), could uncover functional and behavioural deficits that closely reflect human diagnosis of CP at a later stage of development; replicating the subtle and diffuse white matter injury that is not apparent through MRI at birth, but evolves into deficits later on

in life. Similar studies are already underway in our lab, testing the administration of UCB cells after fetal growth restriction. These studies characterise behavioural deficits of animals four weeks after delivery following fetal growth restriction, and then compare these outcomes to animals treated with early intervention cell therapies.

6.4.1.3 Repeated cell-dosing trials of UCB and MSCs

Human trials using stem cell therapies have supported the use of repeated cell doses [179, 180]. As suggested in the published review [41], MSCs could be administered in multiple doses, or in conjunction with other cells at critical stages of injury to protect against neuroinflammation, breakdown of the blood brain barrier, and help support repair processes. Multiple doses of UCB should also be assessed in our preterm LPS model to see if there are additive effects. Using this model that we have developed, cells could be administered 6 hours after the last LPS dose as well as an additional dose at 18 and/or 24 hours. This timing would reflect interventions at critical times including a period of late primary inflammation, breakdown of the blood brain barrier, and a final period where cells could induce neurorepair and support oligodendrocytes [94, 181]. Previous studies have shown additive effects of multiple dosing regimes of hAECs in sheep [12, 179]. In human trials, repeated autologous UCB infusions in babies around 2 months of age has been proven safe [180]. It is postulated that preterm infants born less than 28 weeks may require repeated cell dosing to cover periods of pre-myelinating oligodendrocyte vulnerability, until at least 32 weeks of gestation [87] (Figure 6.1). Delayed dosing would also be necessary to understand if treating at a later time point as opposed to 6 hours, has equal or equivalent benefit. If so, this may increase the feasibility of cell therapies as an intervention following delivery, increasing the time of the potential therapeutic window.

Delayed cell dosing studies are also highly relevant as chorioamnionitis can be associated with weeks to months of fetal exposure to *in utero* inflammation [182]. Chorioamnionitis and inflammation of the placenta often goes undetected, referred to as subclinical, with no maternal or fetal symptoms of infection [183]. If infection is detected during pregnancy or soon after delivery, the likelihood that the fetus has been exposed to longer-term inflammation and infection is high [184, 185]. Therefore, delaying the

administration of cells in this model and elucidating their reparative and anti-inflammatory effects after established brain injury but while the brain is still in a period of neuroplasticity, is essential. It also becomes even more relevant, knowing that, upon delivery of the neonate, inflammation and brain injury may become exacerbated further by ventilation [174].

6.5 Review of thesis aims and hypotheses

6.5.1.1 Global thesis aim and outcomes

The overall aim of this thesis was to investigate different cell therapies for the preterm brain exposed to *in utero* inflammation and injury.

We demonstrated in *Chapter 3* that we could successfully collect and characterise both UCB and MSCs. EPCs were difficult to expand to adequate cell numbers for preclinical studies. Therefore, they were excluded from further use.

Chapter 4 showed that UCB was effective in reducing inflammatory brain injury in an ovine LPS model, and also protected against the loss of oligodendrocytes.

In *Chapter 5*, we demonstrated that, while MSC had some long-lasting effects on immune regulation, gene expression and astrocytic white matter coverage, this therapy was not able to protect against white matter cell damage and loss. Our results supported that UCB was superior to MSC therapy in this LPS model of preterm brain injury, protecting developing white matter.

6.6 Conclusion

Both UCB and MSCs are well-characterised stem cell therapies that have been proposed for use in the treatment of multiple conditions, including neurological compromise. Our studies show, for the first time using a clinically relevant large animal model, that these

two cell types have a potential to reduce LPS-induced white matter injury. This thesis shows that both cell treatments have specific and different effects in reducing aspects of brain inflammation in a large animal model of LPS-induced white matter injury. However, UCB is far more efficacious than MSCs for preterm brain injury, with their ability to reduce white matter cell death and oligodendrocyte injury. UCB is therefore a potential comprehensive therapy in this scenario, providing significant neuroprotection when contrasted with MSC therapy. Future studies will need to address whether higher doses or multiple dosing provides additive beneficial effects, and if a later timepoint of administration, which may increase feasibility of administration, still results in neuroprotection.

Chapter 7

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Chapter 8

Appendix

Appendix Table 8.1: Patient eligibility criteria

Criteria	**Comment**
Nil known maternal complications	No Type 1/ type 2 or gestational diabetes, preeclampsia, fetal growth restriction/ intrauterine growth restriction
Nil maternal prescribed drugs	No hormones, insulin or other prescribed medications
Nil illicit drug use/smoking	No history of illicit drug use, and no smoking use over gestation
Placental membranes intact at caesarean section	No sign of premature rupture of membranes
Elective caesarean section delivery	Non-emergency delivery, with adequate time to consent patient
Term gestation	Pregnancy carried to at least 37 weeks
Normal maternal blood pathology	No infections or blood disorders e.g. Hepatitis, HIV

Appendix Document 8.1: Patient Tissue Donation Consent Form

Participant Information Sheet and Consent Form
Perinatal research

Project title	**The Ritchie Centre Human Tissue Bank**
HREC No	**01067B**
Project Sponsor	**Monash Health**
Location	**Monash Health and Jessie McPherson Private Hospital**
Coordinating Principal Investigator	**Associate Professor Caroline Gargett** **The Ritchie Centre, Hudson Institute of Medical Research**

SHF 6057

Principle Investigators: Perinatal research

- **Professor Euan M. Wallace, Carl Wood Professor of Obstetrics and Gynaecology, Monash University; Head Department of Obstetrics and Gynaecology, Monash University; Director, The Ritchie Centre, Hudson Institute of Medical Research; Director Obstetric Services, Monash Health.**
- **Professor Graham Jenkin, Deputy Director, The Ritchie Centre, Hudson Institute of Medical Research; Professor, Department of Obstetrics and Gynaecology, Monash University.**
- **Dr Rebecca Lim, Research Fellow, The Ritchie Centre, Hudson Institute of Medical Research Lecturer, Department of Obstetrics and Gynaecology, Monash University.**

Those with direct involvement: Perinatal research

- **Ms Joanne C. Mockler, Research Midwife Consultant. Manager, Perinatal (Obstetric) Clinical Trials, The Department of Obstetrics and Gynaecology, Monash University and Monash Health.**
- **Ms Anne Clare, Research Nurse. Research Nurse Coordinator. The Ritchie Centre, Hudson Institute of Medical Research.**

1 Introduction

You are being invited to contribute to the *Ritchie Centre* Human Tissue Bank because you are pregnant. We would like to ask mothers who do not have any problems in their pregnancies and those who have experienced complications, to participate. We are approaching women who are planning a vaginal birth as well as those who are having a caesarean section.

This participant information sheet tells you about the *Ritchie Centre* Human Tissue Bank. Knowing what is involved will help you to decide if you would like to take part in *the Ritchie Centre* Human Tissue Bank or not.

Please read this participant information sheet carefully. We encourage you to ask us questions if there is anything that you do not understand or if there is something that you would like to know more about. Before deciding whether or not to take part, you might like to talk about it with a relative, friend or the doctors who are caring for you.

Participation in the *Ritchie Centre* Human Tissue Bank is voluntary. If you do not want to take part, you do not have to. You will receive the best possible care whether or not you decide to take part.

If you decide that you would like to take part in the *Ritchie Centre* Human Tissue Bank, you will be asked to sign the consent section. By signing it you are telling us that you:
- **Understand what you have read**
- **Consent to take part in the *Ritchie Centre* Human Tissue Bank**
- **Consent to the research that is described**
- **Consent to the use of your tissue and personal/health information as described.**

You will be given a copy of the signed consent form to keep.

2 What is the purpose of *the Ritchie Centre* Human Tissue Bank?

The Ritchie Centre, the Hudson Institute of Medical Research and the Department of Obstetrics and Gynaecology, Monash University has an established perinatal research program for the study of both healthy and complicated pregnancies, fetal growth and development, as well as the ongoing health of the newborn. Our aim is to further the understanding of pregnancy, the different conditions that can affect pregnant women and babies and to ultimately develop better treatments for these.

For many of our research studies, there is a need for pregnant women to donate tissue samples to them. It is for this reason that we have established *the Ritchie Centre* Human Tissue Bank. The purpose of the tissue bank is to collect a variety of tissue samples from pregnant women such as; maternal blood and, after the baby has been born, placenta, for our scientists to use in their research projects, now and in the future.

3 Who has reviewed the *Ritchie Centre* Human Tissue Bank?

All research in Australia involving humans is reviewed by an independent group of people called a Human Research Ethics Committee (HREC). The ethical aspects of the *Ritchie Centre* Human Tissue Bank have been reviewed and approved by the HREC of Monash Health.

The *Ritchie Centre* Human Tissue Bank will operate in accordance with the National Statement on Ethical Conduct in Human Research (updated May 2014). This statement has been developed to protect the interests of people who agree to participate in human research.

4 What does participation in the *Ritchie Centre* Human Tissue Bank involve?

- Your baby will not be touched for this research project or placed at any risk of harm.

Because we study a range of different conditions which can affect pregnant mothers and babies, the tissue needed for each of individual research projects we supply, can and does, vary. Therefore, your contribution to *the Ritchie Centre* Human Tissue Bank project may involve you being asked to consider donating one or more of the following tissues:

- *During your pregnancy or before your labour begins or you have your caesarean section*:-

- *Donating a sample of your urine.*
- Donating 20 mLs (or less) of maternal blood.

As far as possible, we will try to collect the maternal blood sample at the same time as blood is being collected for your clinical care or an intravenous 'bung' is being sited. If this is not possible, we will ask if we may collect blood from you for the purposes of this research project only.

- *After your baby has been born*:-
- Allowing us to collect the placental inner membranous sac (amnion).
- Allowing us to collect a small piece (no more than 2cm x 2cm) of the placental tissue (placental lobes).
- Allowing us to collect the umbilical cord.
- Allowing us to collect blood samples from the umbilical cord (both artery and vein).

For the majority of women, once the baby is born, the placenta is simply thrown away. Allowing us to keep, and make use of, your placenta would make a valuable contribution to the research work that is being undertaken in our department.

- *If you are having a caesarean section*:-
- Donating adipose tissue (fat) - approximately 1cm x 1cm
- Donating myometrium (uterus/womb muscle) - approximately 1cm x 1cm

- Donating some amniotic fluid –approximately 20mLs

➢ <u>During your pregnancy or after you have your baby</u>:-
- Allowing us to brush the inside of your cheek so that we can collect cells from the cheek lining.

In addition:

- For some of our research studies, we would like to collect cells from inside of your cheek and/or the placental tissue (placenta lobes), so that we can identify and study genetic material i.e. DNA, from within these cells. We are especially interested in looking at DNA because we know that small differences in some peoples' genes can affect pregnancy outcome. By looking at some of the genes from women who have had complications in their pregnancy and comparing them to those from women who have had healthy pregnancies without any problems, we hope to be able to better understand and in future develop methods to predict and even prevent, poor outcomes. Please note: a) we will not be looking for, or testing for, any genetic problems in you or your baby and b) not all the tissues we collect will be examined in this way, when we are collecting cheek cells and/or placental tissue (placental lobes) for genetic analysis, we will specifically tell you at the time of counseling and consenting.

- Allowing us to collect relevant information from your medical records with regard to your pregnancy, labour and birth outcome.

- Allowing us to use the results (only) from the research projects that will use your tissue, in the design of other research projects in the future.

❖ The donation of your tissue(s) to the *Ritchie Centre* Human Tissue Bank, does <u>not</u> involve: changes to your clinical care, any additional investigations or tests, you staying in hospital any longer or completing any paperwork other than signing this consent form. You will not be asked to return to the hospital for any appointments related to the donation of your tissue(s) to the *Ritchie Centre* Human Tissue Bank.

❖ Please note: the tissue samples collected for the *Ritchie Centre* Human Tissue Bank will not be used to diagnose any conditions in you or your baby or to guide your clinical care. We do not clone from your tissue or, where placental stem cells have been collected, generate ongoing cell lines.

❖ Please note: the results from the research projects that will subsequently use your tissue will **not** provide any information that will be of use to you, your baby or your family members now or in the future.

5 Do I have to take part in the *Ritchie Centre* Human Tissue Bank?

You do not have to donate tissue to the *Ritchie Centre* Human Tissue Bank to receive treatment at this hospital. Your participation in the *Ritchie Centre* Human Tissue Bank or in any research project is entirely voluntary. This means that if you do not wish to take part, you do not have to. If you decide to take part and later change your mind, you are free to withdraw at any stage.

Your decision whether to take part or not to take part, or to take part and then withdraw, will not affect your routine treatment, your relationship with those treating you or your relationship with Monash Health or Jessie McPherson Private Hospital.

6 What are the possible benefits of taking part?

There will be no clear benefit to you from your participation in the *Ritchie Centre* Human Tissue Bank. However, we hope that the results generated from the research projects that are using your tissue, will benefit pregnant mothers and their babies in the future.

8 What are the possible risks and disadvantages of taking part?

- There are no risks to your baby if you donate tissue to the *Ritchie Centre* Human Tissue Bank. When we collect the tissue you donate, there will be no need for us to touch your baby.
- Having a blood sample taken may cause you some discomfort or bruising. Sometimes, the blood vessel may swell, or blood may clot in the blood vessel, or the spot from which tissue is taken could become inflamed. Rarely, there could be a minor infection or bleeding. If this happens, it can be easily treated.
- There are no risks to you or your baby for the collection of any placental tissue samples. All of the placental samples and umbilical cord blood samples are collected after you have given birth and the placenta has separated from you and your baby.
- There are no risks to you for the collection subcutaneous (tummy wall) fat or myometrium (uterus/womb muscle). These samples are taken after the placenta has been delivered just prior to repair of the uterus/womb. There is no discomfort associated with the collection because the samples are taken under the same regional anaesthetic (spinal or epidural) as the caesarean section itself.
- There are no risks to you or your baby during the collection of amniotic fluid.
- There are no risks to you in the collection of buccal (cheek) swabs.

If you do become upset or distressed as a result of your participation in the *Ritchie Centre* Human Tissue Bank, the researcher is able to arrange for counselling or other appropriate support. Any counselling or support will be provided by staff who are not members of the research team. In addition, you may prefer to suspend or end your participation in the research if distress occurs.

As with any project, there may be additional risks that the researchers do not expect or do not know about.

7 What will happen to my tissue samples?

Any tissues donated by you to our perinatal research projects are stored in *the Ritchie Centre* Human Tissue Bank. Before the scientists can gain access to and use the tissue samples, they must seek approval from the Ethics Committees of Monash University and/or Monash Health in order to be able to conduct their research project. Tissue samples are only released from *the Ritchie Centre* Human Tissue Bank on condition that the Ethics Committees are satisfied that the proposed research meets guidelines of the *National Statement on Ethical Conduct in Human Research* (2007) produced by the National Health and Medical Research Council of Australia. This statement has been developed to protect the interests of all the people who agree to participate in human research studies.

- ❖ All donated tissue will be de-identified after collection. This means that any information which could identify you, such as: your name, address, date of birth and hospital record number will be removed before your tissue sample goes into the laboratory for the scientists to use.

The *Ritchie Centre* has a recognised national and international reputation for its research. For this reason, it may sometimes be necessary for our scientists to share their tissue samples and/or results, with other professionals employed outside *The Ritchie Centre*, who have specific expertise or special interest in our work. Such collaboration can take place locally at Monash University and Monash Health, or other academic institutions within Australia and sometimes overseas.

All the researchers from outside Monash University and Monash Health who collaborate with us, are also required to seek and obtain Human Research Ethics Committee (HREC) approval from their own local institution before we can share our de-identified tissue samples with them.

8 What will happen to information about me?

By signing the consent form, you consent to: the collection and use of your tissue sample(s) and any relevant information about you, that is required for the ethics committee approved laboratory research project(s) using your tissue.

Any information collected about you will only be disclosed to the researchers who are using your tissue, with your permission, except as required by law. The information will always be disclosed to them in a de-identified form, that is without your: name, initials, date of birth, address, telephone number or hospital record number being attached to it.

Any information obtained in connection with this research project that can identify you will remain confidential. The information will be stored in a locked filing cabinet and password protected database, accessible only by those named in this participant information sheet and consent form.

After the research studies that use your tissue have been completed, the information will be securely stored for 7 years by the principal investigator, as currently recommended by the National Health and Medical Research Council (NHMRC) and Monash Health HREC. After this time, all the information will be disposed of in a secure and confidential manner.

In accordance with relevant Australian and Victorian privacy and other relevant laws, you have the right to request access to the information collected and stored by the research team about you. You also have the right to request that any information with which you disagree be corrected. Please contact the research team member named at the end of this document if you would like to access your information.

Your health records and any information obtained during the research project are subject to inspection (for the purpose of verifying the procedures and the data) by the relevant authorities and authorised representatives of the Sponsor, Monash Health, Monash University, the Hudson Institute of Medical Research or as required by law. By signing the consent form, you authorise release of, or access to, this confidential information to the relevant study personnel and regulatory authorities as noted above.

- Information about your participation in the *Ritchie Centre* Human Tissue Bank may be recorded in your health records.
- It is anticipated that the results from the research projects that will use your tissue will be published and/or presented in a variety of forums. In any publication and/or presentation, information will be provided in such a way that you cannot be identified, except with your permission.

9 Other relevant information about the *Ritchie Centre* Human Tissue Bank.

- The *Ritchie Centre* Human Tissue Bank has been established for many years. It is oversighted by Associate Professor Caroline Gargett, *The Ritchie Centre*, the Hudson Institute of Medical Research. Funding for the *Ritchie Centre* Human Tissue Bank is derived from: departmental funding, research grants and private donations.

Reimbursement: there are no costs to you associated with participating in the *Ritchie Centre* Human Tissue Bank.

- Your tissue will not be sold, nor will *The Richie Centre* knowingly transfer your samples to anyone who has expressed intent to sell the samples.
- No member of the research team will receive a personal financial benefit from your involvement in the *Ritchie Centre* Human Tissue Bank.

By donating tissue to the *Ritchie Centre* Human Tissue Bank, you understand and agree that your tissue sample (or data generated from its analysis) may lead to discoveries that are directly or indirectly of commercial value to Monash University, if, for example, the project assists the *Ritchie Centre*, the Hudson Institute of Medical Research

and Monash University, to obtain an approval for a new treatment. You or your family will not be able to benefit financially from this.

Please note: that once tissue sample(s) have been collected for the research project(s) that the *Ritchie Centre* Human Tissue Bank supplies, they will not be retrievable by you, or anyone else, in the future.

10 Can I have other treatments when I donate tissue to the *Ritchie Centre* Human Tissue Bank?

It would be appreciated if you could inform the study staff about any treatments or medications you may be taking, including over-the-counter medications, vitamins or herbal remedies, acupuncture or other alternative treatments.

11 What if I withdraw from the *Ritchie Centre* Human Tissue Bank project?

If you decide to withdraw from the *Ritchie Centre* Human Tissue Bank project, please notify a member of the research team before you withdraw. A member of the research team will inform you if there are any special requirements linked to withdrawing.

12 Results of the research using tissue from the *Ritchie Centre* Human Tissue Bank

The results of research that have used your tissue are not likely to be available in the foreseeable future. This is because research can take a long time and must use tissue samples from many people before results are known. However, information on all the research being undertaken at *the Ritchie Centre*, the Hudson Institute of Medical Research is available on the web at:

http://hudson.org.au/research-centres/the-ritchie-centre/

13 Complaints and compensation

In the unlikely event that you suffer any injuries or complications as a result of participating in the *Ritchie Centre* Human Tissue Bank, you should contact the study team as soon as possible and you will be assisted with arranging appropriate medical treatment. If you are eligible for Medicare, you can receive any medical treatment required to treat the injury or complication, free of charge, as a public patient in any Australian public hospital.

If you have any complaints about any aspect of the *Ritchie Centre* Human Tissue Bank, the way it is being conducted or any questions about being a research participant in general, then you may contact:

Local HREC Office contact

Name	Mrs Deborah Dell
Position	Manager, Human Research Ethics Committees
Telephone	+61 3 9594 4605
Email	deborah.dell@monashhealth.org

14 Further information and who to contact

If you would like any further information concerning the *Ritchie Centre* Human Tissue Bank, please contact:

Name	Ms Anne Clare
Position	Research Nurse
Telephone	0415 909 082
Email	anne.clare@hudson.org.au

HUDSON INSTITUTE OF MEDICAL RESEARCH

MonashWomen's Monash Health Maternity Services

MONASH University

Consent Form
Perinatal research

Title	**The Ritchie Centre Human Tissue Bank**
HREC Number	**01067B**
Project Sponsor	**Monash Health**
Coordinating Principal Investigator	**Associate Professor Caroline Gargett**
Principle Investigators- Perinatal research	**Professor Euan M. Wallace, Professor Graham Jenkin and Dr Rebecca Lim**
Location	**Monash Health and Jessie McPherson Private Hospital**

SHF 60657

Declaration by Participant

- I have read the Participant Information Sheet or someone has read it to me in a language that I understand.
- I understand the purposes, procedures and risks of the *Ritchie Centre* Human Tissue Bank.
- I have had an opportunity to ask questions and I am satisfied with the answers I have received.
- I freely agree to participate in the *Ritchie Centre* Human Tissue Bank as described and understand that I am free to withdraw at any time during the project without affecting my future health care.
- I understand that I will be given a signed copy of this document to keep.
- I consent to the collection & examination of the following tissue(s): participant *please tick and initial as appropriate:*

Placenta (all)		Maternal buccal (cheek) swab		Maternal urine sample	
Placental tissue (lobe) 2cm x 2cm		Maternal blood sample(s)		Other(s) state:	
Placental amniotic sac		Amniotic fluid		I consent to analysis of DNA-Epigenetic markers	
Umbilical cord		Adipose tissue (fat) 1cmx1cm		After any placental tissue donation, I would like to keep my placenta, please return it to me	
Umbilical cord blood sample(s)		Myometrium (uterus/ womb muscle) 1cm x 1cm		After any placental tissue donation, I do not want to keep my placenta, please throw it away	

- Name of participant (please PRINT):

Signature: Date: Time:

- Name of witness to participant's signature (please PRINT):

Signature: Date: Time:

* Witness is not to be the investigator, a member of the study team or their delegate. In the event that an interpreter is used, the interpreter may not act as a witness to the consent process. Witness must be 18 years or older.

Declaration by Senior Researcher*

I have given a verbal explanation of the *Ritchie Centre* Human Tissue Bank, its procedures and risks and I believe that the participant has understood that explanation.

- Name of senior researcher (please PRINT):

Signature: Date: Time:*

*A senior member of the research team must provide the explanation of, and information concerning, the *Ritchie Centre* Human Tissue Bank. Note: All parties signing the consent section must date their own signature.

Form for Withdrawal of Participation
Perinatal research

Title	**The Ritchie Centre Human Tissue Bank**
HREC Number	**01067B**
Project Sponsor	**Monash Health**
Coordinating Principal Investigator	**Associate Professor Caroline Gargett**
Principle Investigators- Perinatal research	**Professor Euan M. Wallace, Professor Graham Jenkin and Dr Rebecca Lim**
ation	**Monash Health and Jessie McPherson Private Hospital**

laration by Participant

sh to withdraw from participation in the *Ritchie Centre* Human Tissue Bank and understand that such withdrawal not affect my routine treatment, my relationship with those treating me or my relationship with Monash Health.

- **Name of participant (please PRINT):**

Signature: **Date:** **Time:**

In the event that the participant's decision to withdraw is communicated verbally, the Study Doctor/Senior Researcher will need to provide a description of the circumstances below.

Declaration by Senior Researcher*

I have given a verbal explanation of the implications of withdrawal from the *Ritchie Centre* Human Tissue Bank and I believe that the participant has understood that explanation.

- **Name of senior researcher (please PRINT):**

Signature: **Date:** **Time:**

*A senior member of the research team must provide the explanation of and information concerning withdrawal from the *Ritchie Centre* Human Tissue Bank.
Note: All parties signing the consent section must date their own signature.