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Human Umbilical Cord Blood Therapy Protects Cerebral White Matter from Systemic LPS Exposure in Preterm Fetal Sheep

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Keywords

Brain injury · Cerebral palsy · Fetal sheep · Infection · Inflammation · Neonatal neuroinflammation · Stem cells · White matter · White matter damage

Abstract

Background: 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. ***Methods:*** Chronically instrumented fetal sheep at 0.65 gestation were administered lipopolysaccharide (LPS, 150 ng, 055:B5) intravenously over 3 consecutive days, followed by 100 million human UCB mononuclear cells 6 h 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. ***Results:*** 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. ***Conclusions:*** UCB cell treatment following inflammation reduces preterm white matter brain injury, likely mediated via anti-inflammatory actions.

Introduction

Preterm birth is the most significant contributor to neonatal morbidity and mortality [1]. Infants who survive preterm birth, particularly those born very preterm

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(<32 weeks) or extremely preterm (<28 weeks), are at high risk for neurodevelopmental deficits [2]. In utero inflammation of the fetal membranes and/or placenta, termed chorioamnionitis, is the principal underlying cause of preterm birth [3]. Chorioamnionitis affects up to 70% of extremely preterm births [4], with fetal clinical condition influenced by gestational age and whether the infection is clinical or subclinical [5]. 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 [4]. 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 [4]. However, subclinical chorioamnionitis is associated with adverse short- and long-term outcomes including subtle but clinically important brain injury [6]. 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 [7].

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 [8]. 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 [9, 10]. 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 [11]. This includes disorganised white matter reflected in altered fibre density and complexity compared to those infants not exposed to chorioamnionitis [11]. These subtle changes to white matter microstructure contribute to life-long learning and behavioural deficits [12]. In turn, it is well described that white matter injury underlies most cases of CP [12].

In modern cohorts of preterm infants, white matter pathology most commonly takes the form of non-cystic diffuse periventricular leukomalacia [13], wherein cerebral injury is microscopic and isolated to the central white matter with evidence of glial scarring, but without large cystic formations [12]. 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 [14]. Indeed, there are a number of clinical trials for the examination of UCB in infants and children with CP [15], 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 [16]. 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 [17].

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 [18, 19]. However, to date the majority of studies have used early intervention UCB therapy in experimental models of perinatal hypoxic-ischaemic brain injury at term [20], 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 [6]. 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 [18, 19].

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 [21] 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.

Materials and Methods

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.

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 approx. 147 days). Under isoflurane (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 hindlimbs 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 after surgery, and then for the next 3 days with 0.1 mg/kg oxytetracycline (Engemycin; MSA Animal Health, Wellington, New Zealand) and 500 mg 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 day 4.

At 95 days of gestation, fetuses were randomised to one of 3 groups: (i) control: intravenous 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 experimental days 1, 2, and 3, plus 100×10^6 human UCB stem cells via the jugular vein on experimental day 3, 6 h after the final LPS administration (see online suppl. Fig. 1; see www.karger.com/doi/10.1159/000490943 for all online suppl. material). LPS and/or cell treatment was administered over a 5-min period, and the catheter was cleared with a subsequent 2-mL saline flush over 2 min. Blood samples (1 mL) were collected via the fetal femoral artery catheter prior to each saline, LPS, or cell administration, 3, 6, and 12 h 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 18-gauge 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 5-mm sections and frozen at –80 °C in optimal cutting temperature compound (Tissue-Tek OCT; Sakura, USA). The brain was coronally sectioned at 10 μm with both fixed and frozen tissues mounted on SuperFrost Plus (Thermo Fisher Scientific, Scoresby, Australia) glass slides.

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 1,000 *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 bicarbonate, and EDTA in MilliQ water). The lysis was stopped with excess media (16.5% fetal bovine serum in DMEM:F12; Gibco). Cells were manually counted, and viability was assessed using trypan blue exclusion dye (Gibco). Cells were cryopreserved in dimethyl sulphoxide (10% DMSO, Sigma, in fetal bovine serum) and stored in liquid nitrogen.

On the day of cell infusion (day 3, 6 h 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 100×10^6 mononuclear cells resuspended in 2 mL PBS.

Cells were administered directly to the fetus via slow infusion into the jugular vein catheter over 5 min, ensuring that the line was cleared with a subsequent flush of 2 mL saline over 2 min. 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.

CSF Cytokine Analysis

CSF, collected post mortem on day 10, was analysed for pro-inflammatory cytokine IL-1β using recombinant cytokines via ELISA, as previously reported [22].

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 subcortical white matter (SCWM) and periventricular white matter (PVWM).

3,3′-Diaminobenzidine Immunohistochemistry

Cellular apoptosis was quantified in the brain via immunostaining on fixed tissue with activated caspase-3 (1:1,000; R&D Systems, Minneapolis, MN, 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 myeloperoxidase (MPO, 1:500, Dako). Total numbers of oligodendrocytes were visualised by staining for oligodendrocyte transcription factor 2 (Olig-2, 1:1,000; Millipore, Billerica, MA, USA), while

Table 1. Arterial blood gas, heart rate, and blood pressure variability

	Baseline D1				D2		D3		D4		D10
	pre-LPS	3 h	6 h	12 h	pre-LPS	12 h	pre-LPS	12 h	pre-LPS	12 h	
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±0.07	0.98±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, mm Hg											
Control	26.60±1.51	23.55±1.01	28.44±2.89	24.18±1.03	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, mm Hg											
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											
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±033	−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											
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 percent change (Δ) from daily baseline (each time point taken from the mean of 3 × 1-min epochs, averaged per time point). Data expressed as means ± SEM. D, day; pO_2, arterial partial pressure of oxygen; pCO_2, arterial partial pressure of carbon dioxide. Two-way repeated measures ANOVA, * $p \leq 0.05$.

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Table 2. Post-mortem body weight and organ:body weight ratios

	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	2.49±0.00[b]	2.04±0.00[a,b]	>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[a,b]	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). Data expressed as means ± SEM; *p* value is ANOVA *p* value summary. One-way ANOVA, different superscript letters indicate $p \leq 0.05$.

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 min to block endogenous peroxidases. The sections were then rinsed and blocked with animal serum (caspase-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 min. 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, Burlingame, USA; 1:200 GFAP: biotinylated rabbit anti-mouse IgG antibody; Wako Pure Chemical Industries; 1:200 Olig-2 and 1:500 MBP: goat anti-mouse IgG) was then incubated on the sections for 1 h. Lectin immunohistochemistry did not require a secondary label. Staining was visualised with 3,3′-diaminobenzidine (Pierce Biotechnology, Rockford, IL, USA) as per the manufacturers' instructions. The slides were then cleared and coverslipped 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, MD, USA) at 400× magnification, with densitometry performed using threshold coverage measurements, that reads percentage coverage of staining as grey levels in contrast to background staining.

Fluorescence Immunohistochemistry

For O4 and O1 (oligodendrocyte marker-4, i.e. marker of premyelinating, immature, and mature oligodendrocytes, and oligodendrocyte marker-1, i.e. marker for myelinating immature/mature oligodendrocytes [23]) fluorescence immunohistochemistry, frozen brain tissues were dried on slides for 20 min, incubated in detergent solution (PBS-Tween 20), and then quenched for endogenous peroxidases using hydrogen peroxide. Sections were then blocked (Dako Protein Block), incubated with O4 or O1 primary antibody for 72 h at 4 °C (mouse monoclonal IgM, 1:200; O4 and O1 antibodies generated in house by J.M.D.; hybridoma cell lines gifted from Dr. Ben Emery, OHSU, Portland, OR, USA), followed by anti-mouse Alexa 594 secondary antibody (1:1,000; goat anti-mouse Alexa 594; Invitrogen, Mount Waverley, Australia) for 1 h. The slides were rinsed in water, dried, then mounted in medium for analysis. Images were captured at 200× magnification.

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 100× 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 400× magnification. MPO analysis was conducted via selection of 6 fields of view per region containing blood vessels. All analyses were conducted with the assessor blinded to the group.

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

Data were presented as means ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6 Software (GraphPad Software, San Diego, CA, USA). Physiological recordings were analysed using a 2-way repeated measures ANOVA with Tukey's post hoc analysis. Parametric histological and cytokine data were analysed using a 1-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 $p \leq 0.05$.

Results

Fetal Arterial Blood Gas and Physiological Parameters

There were no significant differences between control, LPS or LPS + UCB arterial blood gas parameters

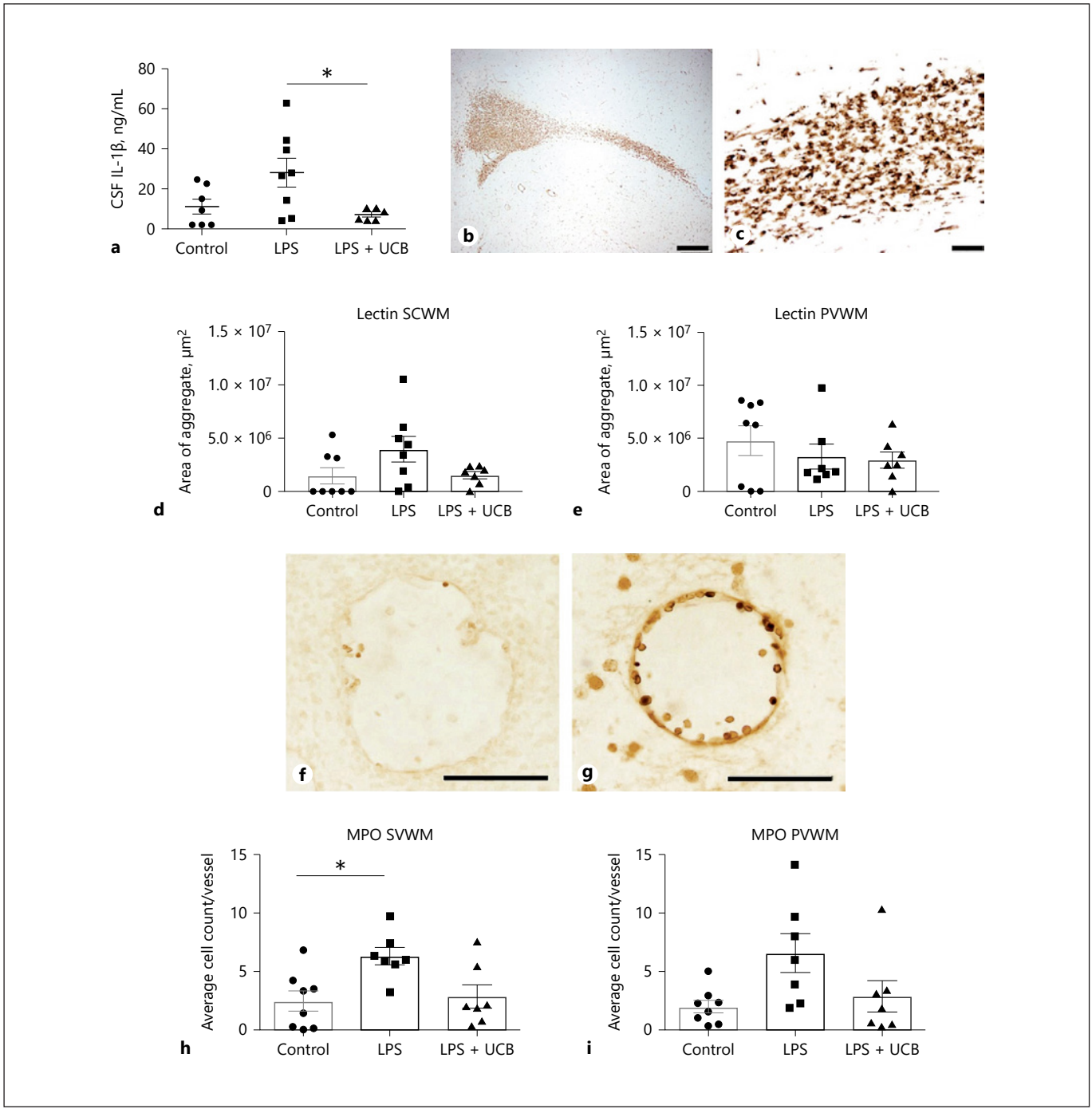

Fig. 1. Neuroinflammation within white matter. **a** IL-1β concentration (ng/mL) in CSF at post-mortem examination. **b–e** Immunohistochemical staining showing lectin accumulation in the subcortical white matter (SCWM) and periventricular white matter (PVWM). **b** Scale bar 500 µm. **c** Scale bar 50 µm. Quantification of lectin staining in SCWM (**d**) and PVWM (**e**) in control, LPS and LPS + UCB groups. **f–i** Myeloperoxidase (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 50 µm. Quantification of MPO expression within blood vessels of SCWM (**h**) and PVWM (**i**) regions in control, LPS and LPS + UCB brains. Data expressed as means ± SEM, $n = 6–8$, 1-way ANOVA, * $p \leq 0.05$.

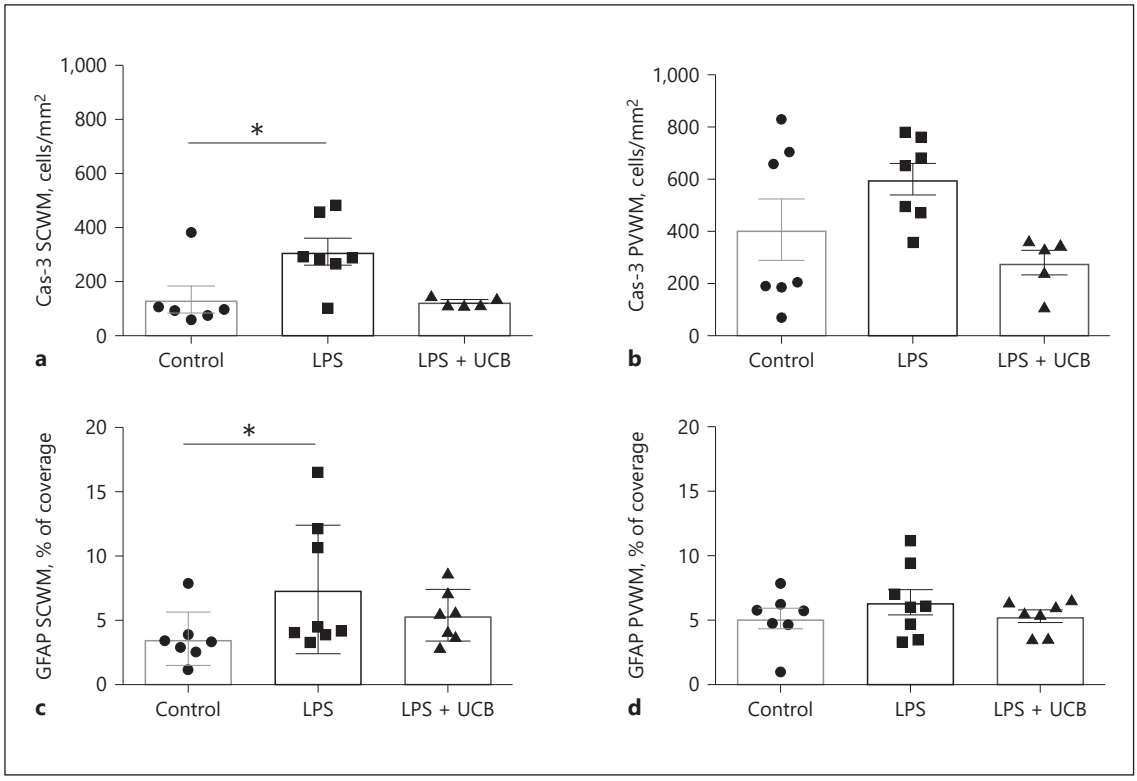

Fig. 2. White matter cell death and astrogliosis. Expression of caspase-3 (Cas-3) in the subcortical white matter (SCWM) (**a**) and periventricular white matter (PVWM) (**b**). Expression of glial astrocytic protein (GFAP) in the SCWM (**c**) and PVWM (**d**) (% coverage of staining). Data expressed as means ± SEM, n = 5–8, 1-way ANOVA, * $p \leq 0.05$.

including pH, lactate, pO_2 or pCO_2 across the experimental period, including the immediate period following LPS (Table 1). Fetal heart rate and blood pressure throughout the experiment in all groups remained unchanged (Table 1).

Post-Mortem Body and Organ Weight Results

Body weight at the post mortem time (approx. 105 days) was not different between groups (Table 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 in 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 2).

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 100× magnification (Fig. 1b). The aggregates viewed at 400× magnification were principally comprised of microglia with an activated/amoeboid morphology (Fig. 1c) [24]. 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

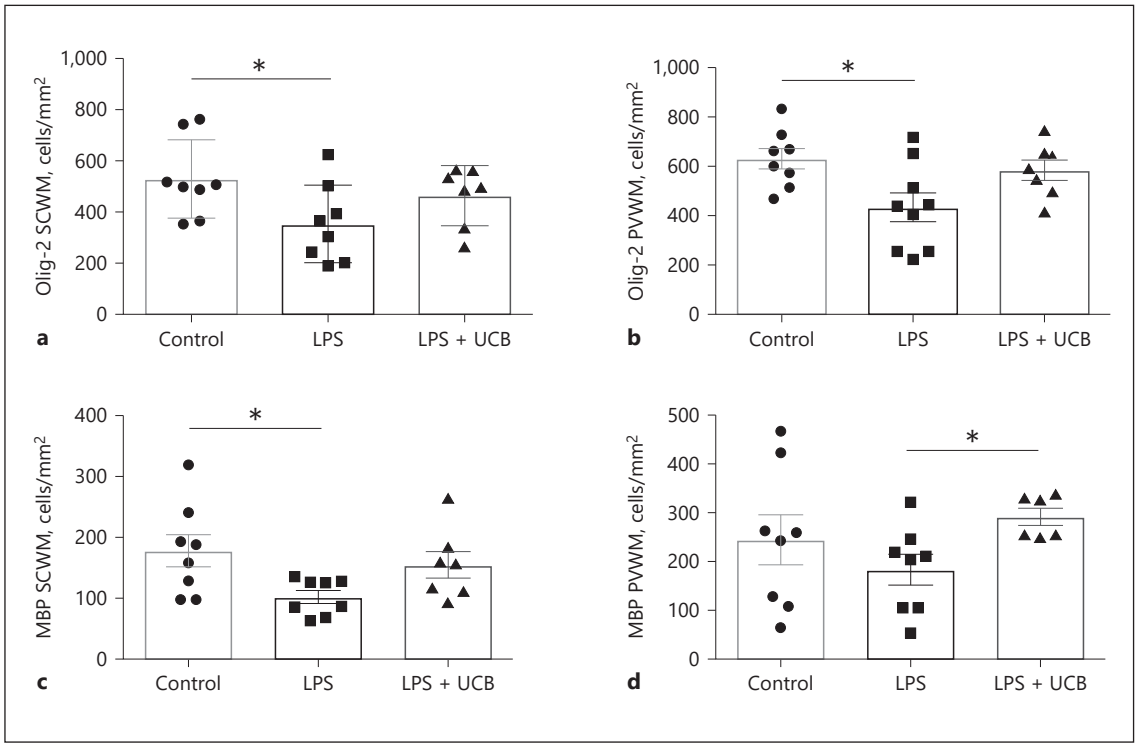

Fig. 3. White matter integrity. Expression of oligodendrocyte transcription factor (Olig-2-positive cells) in the subcortical white matter (SCWM) (**a**) and periventricular white matter (PVWM) (**b**). Myelin basic protein (MBP) expression in the SCWM (**c**) and PVWM (**d**). Data expressed as means ± SEM, n = 6–8, 1-way ANOVA, * $p \leq 0.05$.

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.

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 that in 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 (Fig. 2d). Astrocyte morphology did not vary between groups.

Cerebral White Matter Integrity

Olig-2 counts were significantly reduced in SCWM and PVWM 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 approximately 105 days (Fig. 4a, d). How-

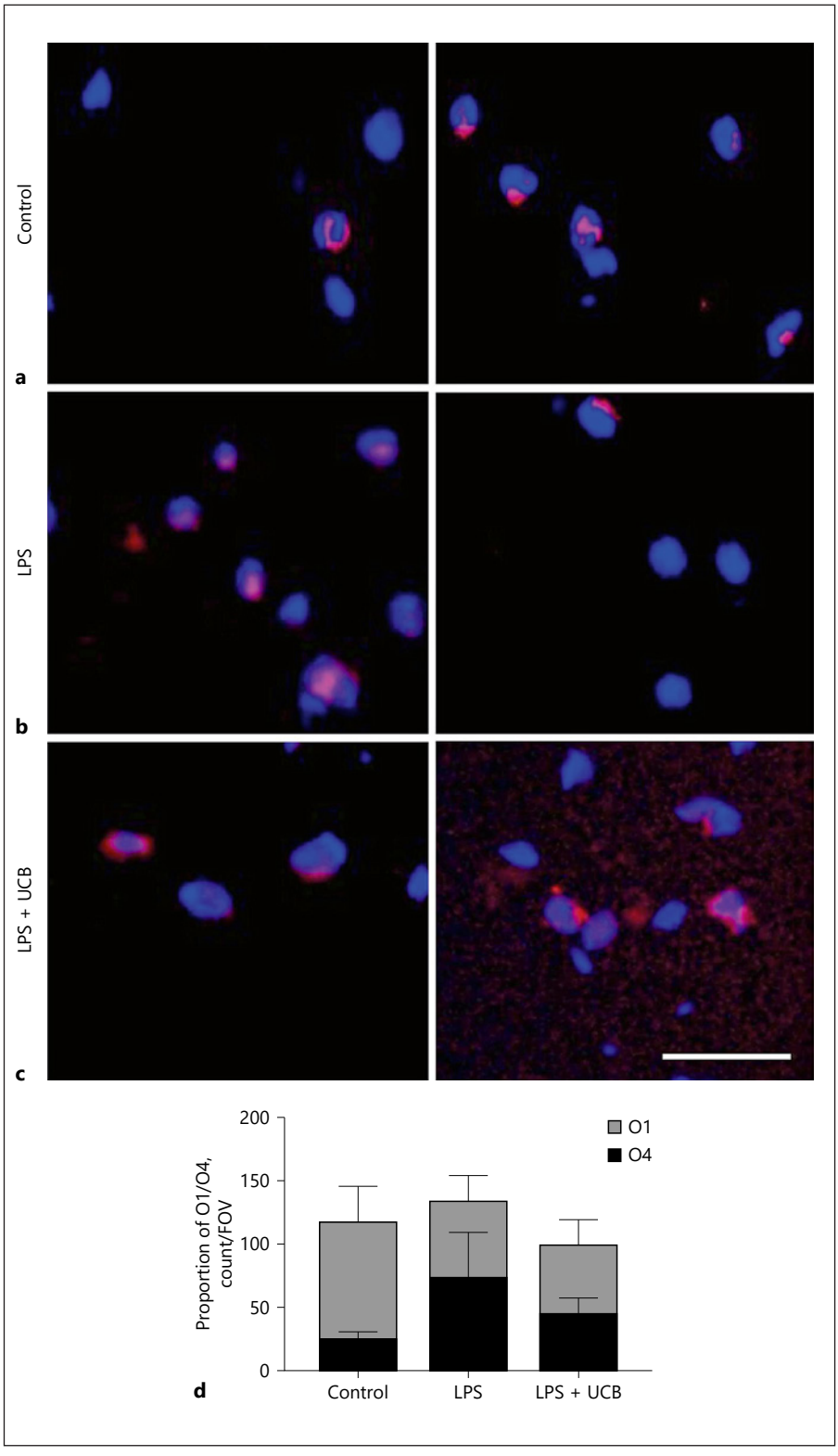

Fig. 4. O4 and O1 fluorescence immunohistochemical analysis. Representative images of O4 and O1 immunohistochemistry (red) with Hoescht nuclear stain (blue) in control (**a**), LPS (**b**) and LPS + UCB (**c**) animals. Scale bar 200 μm. **d** Counts for O1 and O4 cell expression in subcortical white matter. Data expressed as means ± SEM, $n = 3$, 1-way ANOVA, * $p \leq 0.05$. FOV, field of view.

ever, 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).

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 [2]. 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 [25]. 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 [4, 11].

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 [14]. 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 [8, 15, 20]. The cell types present in UCB include mesenchymal stromal cells, haematopoietic stem cells, endothelial progenitor cells, T regulatory cells, and monocyte-derived suppressor cells [17], 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 [26, 27]. 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 [28], and microglial activation and aggregation, and release of pro-inflammatory cytokines are fundamental mechanisms that contribute to cell degeneration and interruption of brain development [28, 29]. 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 CSF 10 days after the first LPS administration. That IL-1β was not profoundly increased likely reflects the 7-day period between final LPS administration and tissue collection, together with the well-characterised tolerance which can develop over repeated LPS exposure [30]. 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 [31]. 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 [32]. Neutrophil localisation around the cerebral blood vessels contributes to inflammatory neonatal brain injury [31], 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 [33], and increases in IL-1β in placental tissues are correlated with poor neurological outcome and CP [34]. Activated microglial aggregates within the brain are associated with

oligodendrocyte death via inflammatory cytokine secretion and oxidative stress [35, 36]. The results from this study indicate that the anti-inflammatory actions of UCB cells are mediating neuroprotective benefit, as shown previously in hypoxia-ischaemia-mediated preterm and term perinatal brain injury [18, 19].

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 [4]. While subtle, this subclinical fetal inflammation contributes to diffuse white matter injury [11], which is now the most commonly reported neuropathology associated with CP [12]. 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 was within the normal range for fetal sheep at this preterm gestational age [21]. LPS administration did result in increased spleen and liver weights compared to control animals, indicative of an immune-modulated response to LPS exposure [37], 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 the local release of cytokines [38, 39]. 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 mesenchymal stromal cells are neuroprotective for the fetus in response to LPS exposure in pregnant mice, principally via actions at the placenta [40]. Combined, these results suggest that UCB/mesenchymal stromal 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 (approx. 30% fewer cells than controls), and specifically, a significant decrease in the number of mature MBP-positive oligodendrocytes in the SCWM (approx. 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 [23]. This maturational progress can be examined by investigating the proportion of O4- and O1-positive oligodendrocytes [41], 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 [23]), 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 (Fig. 1–4). This could be attributed to the well-characterised selective vulnerability of white matter regions and cells as gestation progresses [42]. 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 [13]. 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/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 the number of oligodendrocytes. These were all modified by

administration of UCB cells. Critically, this experiment provides 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 with poor neurological outcome [43]. 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 reduce 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-ischaemic brain injury [18, 19]. 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 [44]. Further studies are warranted to better understand the potential role of constituent cells, and to better understand whether one cell type is better than a mixed population of whole UCB for treatment of this condition.

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.

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Disclosure 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.

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