BASIC SCIENCE ARTICLE

Umbilical cord blood versus mesenchymal stem cells for inflammation-induced preterm brain injury in fetal sheep

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BACKGROUND: Chorioamnionitis and fetal inflammation are principal causes of neuropathology detected after birth, particularly in very preterm infants. Preclinical studies show that umbilical cord blood (UCB) cells are neuroprotective, 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 show strong anti-inflammatory benefits. We aimed to compare the neuroprotective benefits of UCB versus MSCs in a large animal model of inflammation-induced preterm brain injury. We hypothesized that MSCs would afford greater neuroprotection.
METHODS: Chronically instrumented fetal sheep at 0.65 gestation received intravenous lipopolysaccharide (150 ng; 055:B5, $n = 8$) over 3 consecutive days; or saline for controls ($n = 8$). Cell-treated animals received 10^8 UCB mononuclear cells ($n = 7$) or 10^7 umbilical cord MSCs ($n = 8$), intravenously, 6 h after the final lipopolysaccharide dose. Seven days later, cerebrospinal fluid and brain tissue was collected for analysis.
RESULTS: Lipopolysaccharide induced neuroinflammation and apoptosis, and reduced the number of mature oligodendrocytes. MSCs reduced astrogliosis, but UCB did not have the same effect. UCB significantly decreased cerebral apoptosis and protected mature myelinating oligodendrocytes, but MSCs did not.
CONCLUSION: UCB appears to better protect white matter development in the preterm brain in response to inflammation-induced brain injury in fetal sheep.

INTRODUCTION

Preterm birth is strongly associated with brain injury and life-long disability.[1] Chorioamnionitis affects up to 70% of infants born preterm[2] 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.[3] Accordingly, chorioamnionitis is associated with developmental deficits at school age.[1] 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.[4] UCB therapy is being trialed currently in term neonatal encephalopathy and established neurological disorders including cerebral palsy.[4] UCB is easily accessible, has low immunogenicity, and has proven safety and neuroprotective efficacy.[5] Preclinical models of preterm white matter injury show that UCB suppresses inflammation and normalizes oligodendrocyte development.[6,7] 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[8,9] leaving patients to rely on allogeneic UCB, which present temporal and human leukocyte antigen (HLA)-matching barriers.[6]

Mesenchymal stem cells (MSCs) also have neuroprotective properties and are readily isolated from healthy umbilical cord tissue.[10,11] Currently, >450 clinical trials are using MSCs for various conditions (https://clinicaltrials.gov/), including one for term infants with neonatal encephalopathy. MSCs secrete cytokines and soluble factors in response to inflammation and regulate immune processes and tissue repair.[7] Engraftment of MSCs within tissues is not necessary.[12] MSCs are easily sourced, do not require HLA matching, have an established safety profile, and tolerate long-term cryopreservation without compromising function.[13,14]

Mild and diffuse white matter brain injury, consistent with the neuropathology identified in modern cohorts of infants with chorioamnionitis and cerebral palsy,[3] can be simulated in fetal sheep by intravenous 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 hypothesize that MSCs would reduce neuroinflammation and protect white matter development to a greater extent than UCB.

METHODS

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 cesarean sections.

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Umbilical cord blood versus mesenchymal stem cells for…
MCB Paton et al.

166

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

Umbilical cord blood
UCB mononuclear cells were isolated as previously described.[15] Briefly, blood was centrifuged, the buffy coat collected, and cells counted using trypan blue exclusion (Gibco), before cryopreservation in dimethyl sulfoxide (DMSO, 10% (Sigma) in fetal bovine serum (FBS)), following clinical practice standards,[16] and storage in liquid nitrogen.

For intravenous administration, cells were thawed and washed with phosphate-buffered saline (PBS) to remove DMSO, and then cells were counted using trypan blue exclusion. Cells were fluorescently labeled with carboxyfluorescein succinimidyl ester (CFSE, Thermo Fisher, Australia, following the manufacturer's instructions) before administration. Each animal received 10^8 mononuclear cells (pooled from three donors) in 2 ml PBS.

Mesenchymal stem cells
Umbilical cord tissue was processed immediately for MSC isolation as previously described.[17] Briefly, a 1–2 cm section of the cord was minced manually (>3 min), media were added (16.5% FBS in DMEM:F12; Gibco), and incubated (37 °C, 21% oxygen, 5% CO_2). Media were changed every 3 days (d) and passaged at 80% confluency. Cells were cryopreserved after passage 2 (10% DMSO in FBS). Cells were rapidly thawed 10 days prior to use and plated. On administration day, passage 3 MSCs were harvested, counted, and fluorescently labeled with CFSE, and an aliquot (3 million cells) was retained for karyotyping (G-banding, Cytogenetics Laboratory, Victoria, Australia). Each animal received intravenous 10^7 MSCs (2 donors) in 2 ml PBS.

Flow cytometry analysis
MSCs and UCBs were incubated with primary antibodies for flow cytometry analysis following the manufacturer's instructions. Cells were assessed using BD FACSCantoII and analyzed with the 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).

Animal Surgery and LPS administration
Animal experiments were undertaken with approval from Monash Medical Centre Animal Ethics Committee and conducted in accordance with National Health and Medical Research Council of Australia guidelines.

Pregnant Border Leicester-Merino ewes with a single fetus underwent sterile surgery at 91.1 ± 0.2 days (mean ± SD) gestation (term is ~150 days) as previously described.[15] Briefly, ewes were placed under general anesthesia and catheters inserted into the fetal jugular vein, femoral artery, and amniotic cavity. The ewe recovered for 4 days prior to experimental intervention.

At 95 days gestation (experimental d1) fetuses were assigned to: (i) control: saline; (ii) LPS: intravenous 150 ng LPS (055:B5 from *Escherichia coli*, courtesy of Dr. Phillip Bird, University of Queensland); (iii) LPS + UCB: intravenous 150 ng LPS and 10^8 UCB mononuclear cells; or LPS + MSCs: intravenous 150 ng LPS and 10^7 cord tissue MSCs. LPS/saline administration occurred daily from experimental days 1 to 3. UCB/MSC administration occurred on day 3, 6 h after the final dose of LPS.

On experimental day 10 animals were humanely killed via pentobarbitone overdose (Lethabarb, Virbac, Australia). Fetal cerebrospinal fluid (CSF) was collected using an 18 g needle inserted between the first two cervical vertebrae and then centrifuged. The brain was removed and weighed. CSF concentrations of interleukin-1β (IL-1β) were later measured by enzyme-linked immunosorbent assay (ELISA).[3,18] The right hemisphere of the brain was immersion-fixed in 4% paraformaldehyde. After paraffin embedding, the brain was coronally sectioned at 10 μm and mounted on SuperFrost Plus (Thermo Fisher Scientific, Australia) slides. The left side was snap frozen for PCR.

Neuropathological assessment
Cerebral white matter was evaluated at the level of the lateral ventricles, with full exposure of the periventricular and subcortical white matter (SCWM). 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 (1:1000, R&D Systems) and glial fibrillary acidic protein (GFAP, 1:800, Sigma), respectively. Brain macrophages and microglia were visualized using peroxidase-labeled lectin (1:200, Sigma). Mature myelinating oligodendrocytes were stained with myelin basic protein (MBP, 1:500, Millipore). Immunostaining was revealed using 3,3′-diaminobenzidine (Pierce Biotechnology).

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

Quantitative real-time PCR
Messenger RNA (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 synthesized from mRNA (SuperScript III Reverse Transcriptase; Thermo Fisher, Australia), and custom Taqman gene primers (Applied Biosystems: *interleukin (IL)-1β, IL-6, IL-18, forkhead box p3 (Foxp3), IL-4, nuclear factor kβ subunit-1 (NFkβ1), toll-like receptor-4 (TLR-4), tight junction protein (TJP)-2, TJP-3, occludin (OCLN), myeloperoxidase gene (MPO), tumor protein gene 53 (TP53)*, and *insulin-like growth factor-1 (IGF-1)*) were used for quantitative real-time 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 normalized relative quantities of each gene were calculated and exported for analysis. Gene expression is expressed as fold-change relative to calculated Ct values normalized to geometric mean of housekeeping genes.

Statistics
Analysis was performed using GraphPad Prism 6 (GraphPad software). Immunohistochemistry and molecular gene analysis were analyzed using a one-way analysis of variance (ANOVA) with post hoc individual *t* test or Tukey's post hoc. For the *z*-scores of neuroinflammation, outcomes from GFAP and lectin were pooled and then analyzed using a two-way ANOVA with Tukey's post hoc. Data are presented as mean ± SEM, and the statistical significance was set at $p < 0.05$.

RESULTS
In total, 31 fetuses were studied: control, $n = 8$; LPS, $n = 8$; LPS + UCB, $n = 7$; and LPS + MSCs, $n = 8$. We have previously presented some physiological and histological outcomes from these subjects.[15] Fetal characteristics were not different between groups (Table 1).

Umbilical cord blood versus mesenchymal stem cells for…
MCB Paton et al.

167

Table 1. Cohort characteristics and white matter neuropathology

Variable	Control	LPS	LPS+UCB	LPS+MSCs
Animals, n	8	8	7	8
Gestational age at surgery (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 number (n), 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 the number of animals per group with abnormality
MSC mesenchymal stem cell, *UCB* umbilical cord blood, *LPS* lipopolysaccharide

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 hematopoietic (CD34+/45low) and 14.2 ± 10.9% were T-regulatory cells (CD25+/Foxp3, CD4+). Isolated MSCs met the standard MSC criteria[19] and karyotyping (Sup Fig. S1).

Neuropathology and neuroinflammation

Control animals did not show any white matter abnormalities (Table 1). Cystic white matter damage, 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. 1). MSC fetuses did not display white matter lesions, inflammatory aggregates, or hemorrhage.

CSF concentrations of IL-1β were elevated in LPS animals (control: 11.1 ± 3.7 versus LPS: 28.1 ± 7.2 ng/ml, $p = 0.06$, $n = 6$–8 per group). IL-1β concentrations in CSF were lower in LPS + UCB (7.23 ± 1.24 ng/ml, $p = 0.03$) and LPS + MSC (12.41 ± 1.63 ng/ml, $p = 0.06$) animals, compared to the LPS group.

Astrocyte activation (GFAP coverage, Fig. 2g) was higher in LPS animals than in 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+; Fig. 2g) in SCWM was lower in MSC animals than in 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 in controls (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).

Cell death and white matter integrity

Activated caspase-3 in the SCWM was higher in LPS fetuses than in control (Fig. 3a, e, $p = 0.04$) and UCB animals ($p = 0.048$), but not in 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 (MBP-positive cells) in SCWM was lower in LPS animals than in controls (Fig. 3c, e, $p = 0.02$). Oligodendrocyte number was higher in UCB animals compared to LPS ($p = 0.04$), but not in MSC animals. Mature oligodendrocyte cell counts in PVWM were higher in UCB animals compared to LPS (Fig. 3d, $p = 0.004$), but not in MSC animals.

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

Mechanisms of cell action

Inflammatory genes. mRNA levels for IL-1β, IL-6, IL-18, Foxp3, IL-4, NFkβ1, and TLR-4 were not different between control and LPS animals (Fig. 4a–c, e–h, respectively). TGF-β1 mRNA levels tend to be higher in LPS animals than in controls (3.72-fold increase, Fig. 4d).

mRNA levels for inflammatory proteins were not different between LPS and UCB animals.

mRNA levels for Foxp3 were lower in MSC fetuses than in controls ($p = 0.03$, Fig. 4e) and LPS ($p = 0.04$) fetuses. mRNA levels for IL-1β, IL-6, IL-18, TGF-β1, IL-4 ($p = 0.06$), NFKβ1, and TLR-4 tend to be lower in MSC animals than in LPS animals.

Blood–brain barrier and regulatory growth genes. Brain mRNA levels for TJP-2, TJP-3, OCLN, MPO, TP53, and IGF-1 were similar for control and LPS animals (Fig. 5a–f). UCB and MSC administration increased the expression of TJP-3 mRNA levels in UCB ($p = 0.02$) and MSC animals than in 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 in controls.

DISCUSSION

Principal findings

This study is the first to compare the neuroprotective efficacy of UCB cells versus MSCs for inflammation-induced 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 multiple 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 <0.1% of the total mononuclear cells within cord blood
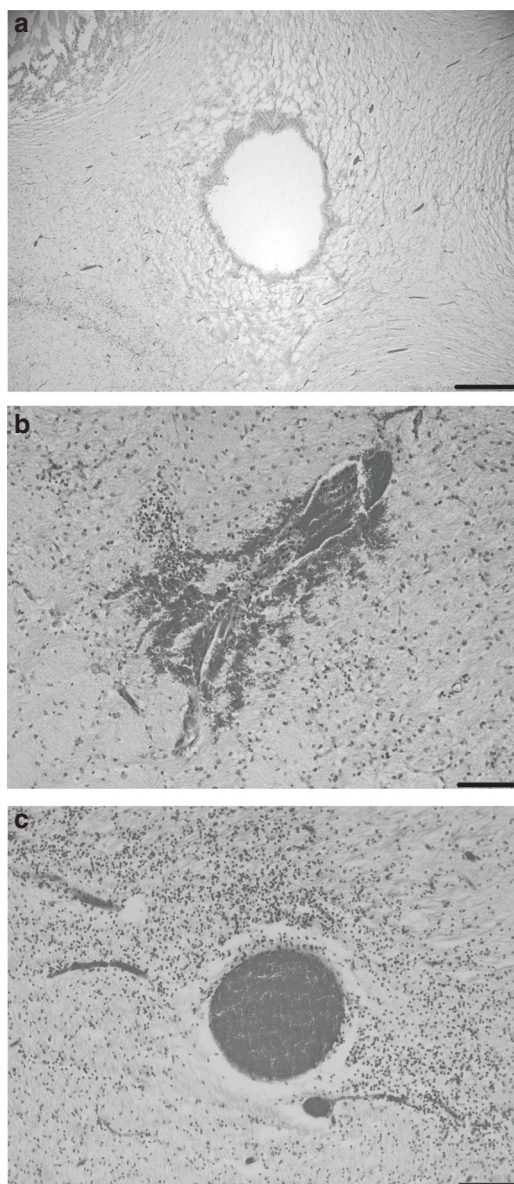
Umbilical cord blood versus mesenchymal stem cells for...
MCB Paton et al.

168

Fig. 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 500 μm, and **b**, **c** is 100 μm

samples used for this study, and UCB efficacy is likely attributable to the remaining cell populations.

Clinical importance
Chorioamnionitis and preterm birth are causal factors for neurological deficits and subsequent life-long disability, learning, and behavior dysfunctions.[20] Globally, preterm birth rates are high and may be increasing,[21,22] and neurological dysfunctions are increased at all levels of prematurity.[23] 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.[15] These subtle changes in white matter can lead to devastating neurological outcomes, with diffuse white matter injury being the most common neuropathology underlying cerebral palsy.[24] 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 and motor outcomes in established cerebral palsy.[5] However, UCB collection volumes may be very low in infants born preterm, and cellular composition in preterm birth and chorioamnionitis may be altered.[7] Therefore, we compared UCB with MSCs, where MSCs have potential as an off-the-shelf allogeneic alternative.

Mechanisms of cell action and interpretation
In our study, we compared the efficacy of UCB, which is a heterogeneous population of cells that contains many different stem and progenitor cells, to an enriched MSC treatment. We have previously shown that individual populations found within UCB (endothelial progenitor cells (EPCs), T-regulatory cells (T-regs), and monocytes) show differential neuroprotective benefits when administered following hypoxia–ischemia in neonatal rats.[25] Of particular relevance to this study, we found that EPCs were the most potent cell type within UCB to suppress apoptotic cell death. Therefore, the enhanced anti-apoptotic effect we observed in this study with UCB treatment compared to MSC treatment may indeed be due to the presence of EPCs. This finding requires further investigation. In addition, EPCs support angiogenesis and blood–brain barrier structure and indirectly modulate peripheral immune responses, while T-regs and monocytes are immunosuppressive and modify inflammatory cell infiltration into the brain.[26] The heterogeneity of UCB stem and progenitor cells suggests that their neuroprotective benefits may be due to effects on immunomodulatory, angiogenic, and apoptotic pathways.[4]

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.[27] IL-1β is principally released from microglia in response to insult,[28] 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,[28] and our findings suggest that the dampening of neuroinflammation as seen with MSCs does not translate to white matter protection. LPS administration is also known to trigger systemic inflammation.[29] We performed serial analysis of plasma cytokines (IL-1β and IL-6) for this study, but unfortunately all cytokines tested were below detectable levels using our sheep-specific assays (ELISA), even in response to LPS administration.

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.[25,30] Cell engraftment in the brain is not necessary for effects on the brain, as confirmed by our observations. In the present study, the most compelling effect of MSC therapy was a profound dampening of brain inflammation. MSCs significantly reduced gene expression for *Foxp3*, well known for its role in programming Tregs.[31] 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 down-regulation of classical inflammatory genes is beneficial and perhaps reparative for the brain following insult, but a careful inflammatory cell balance is also essential for healthy brain development.[28] For example, Foxp3 is necessary for normal astrocytic development,[32] and while LPS alone did not increase Foxp3 expression, MSC treatment induced a strong down-regulation of this gene. Consistent with the link between Foxp3 and astrocytes, we also observed reduced astrocyte density with MSC therapy, similarly reflected in a reduction in microglial cell coverage below that seen in LPS and UCB-treated animals. Therefore, a global dampening of inflammatory mediators with
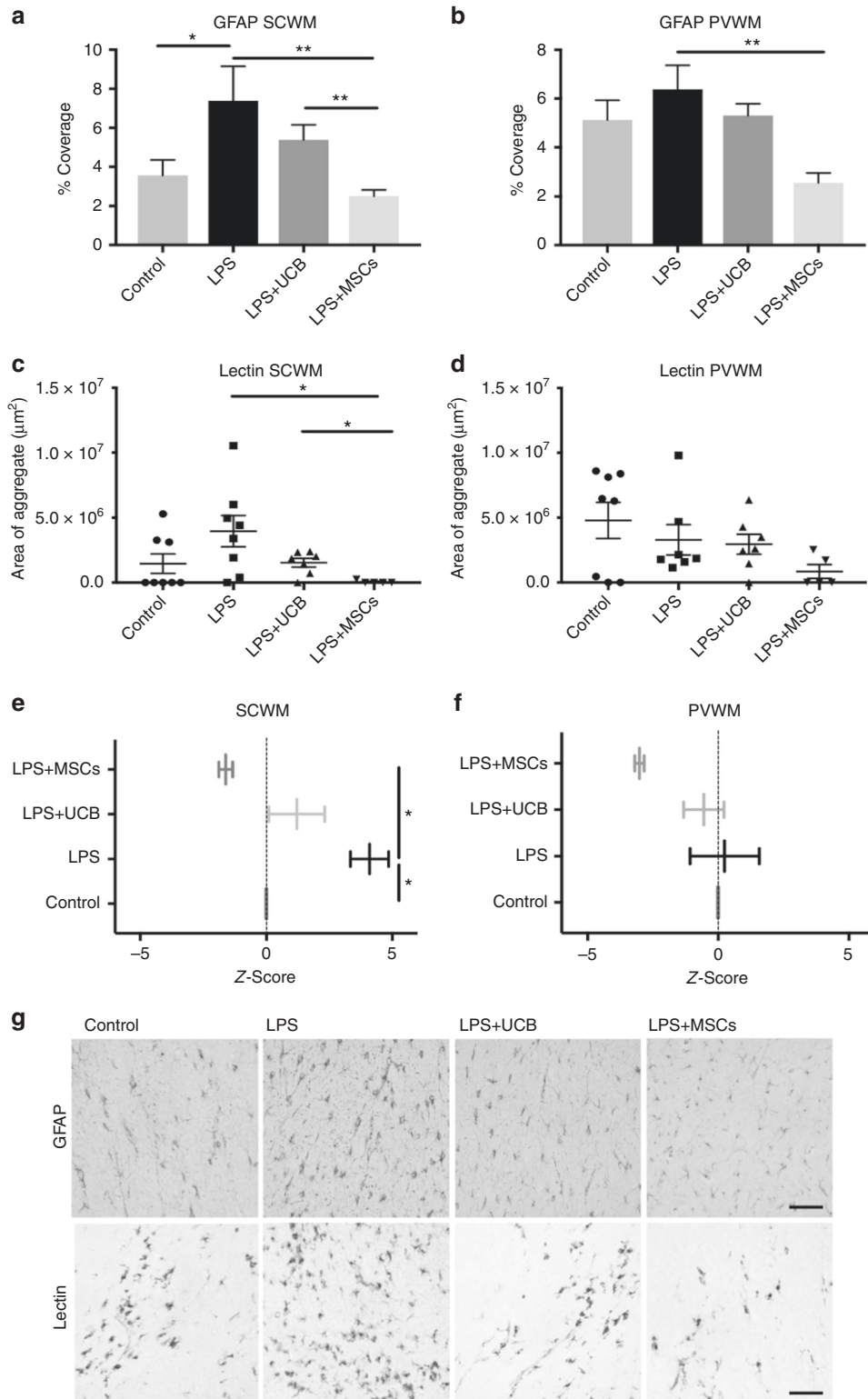
Umbilical cord blood versus mesenchymal stem cells for...
MCB Paton et al.

169

Fig. 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. **g** Representative images of glial fibrillary astrocytic protein and lectin in the subcortical white matter. Scale bar is 10 μm. **a–d** One-way analysis of variance (ANOVA), with post hoc t tests (mean ± SEM). **e**, **f** Two-way ANOVA with Tukey's post hoc (mean ± min/max), $n = 6–8$

MSC administration (as seen in the directional change of the bars in Fig. 4) may interrupt normal brain development and it may be such that reducing an aberrant pro-inflammatory cerebral response with cell treatment is preferable. MSC treatment

decreased white matter expression of *IGF-1*, which is essential for normal oligodendrocyte maturation produced by multiple cell types within the CNS, particularly the glial cells.[33] Indeed, *IGF-1* is proposed as a rescue therapy for hypomyelination,[34] and is

Umbilical cord blood versus mesenchymal stem cells for…
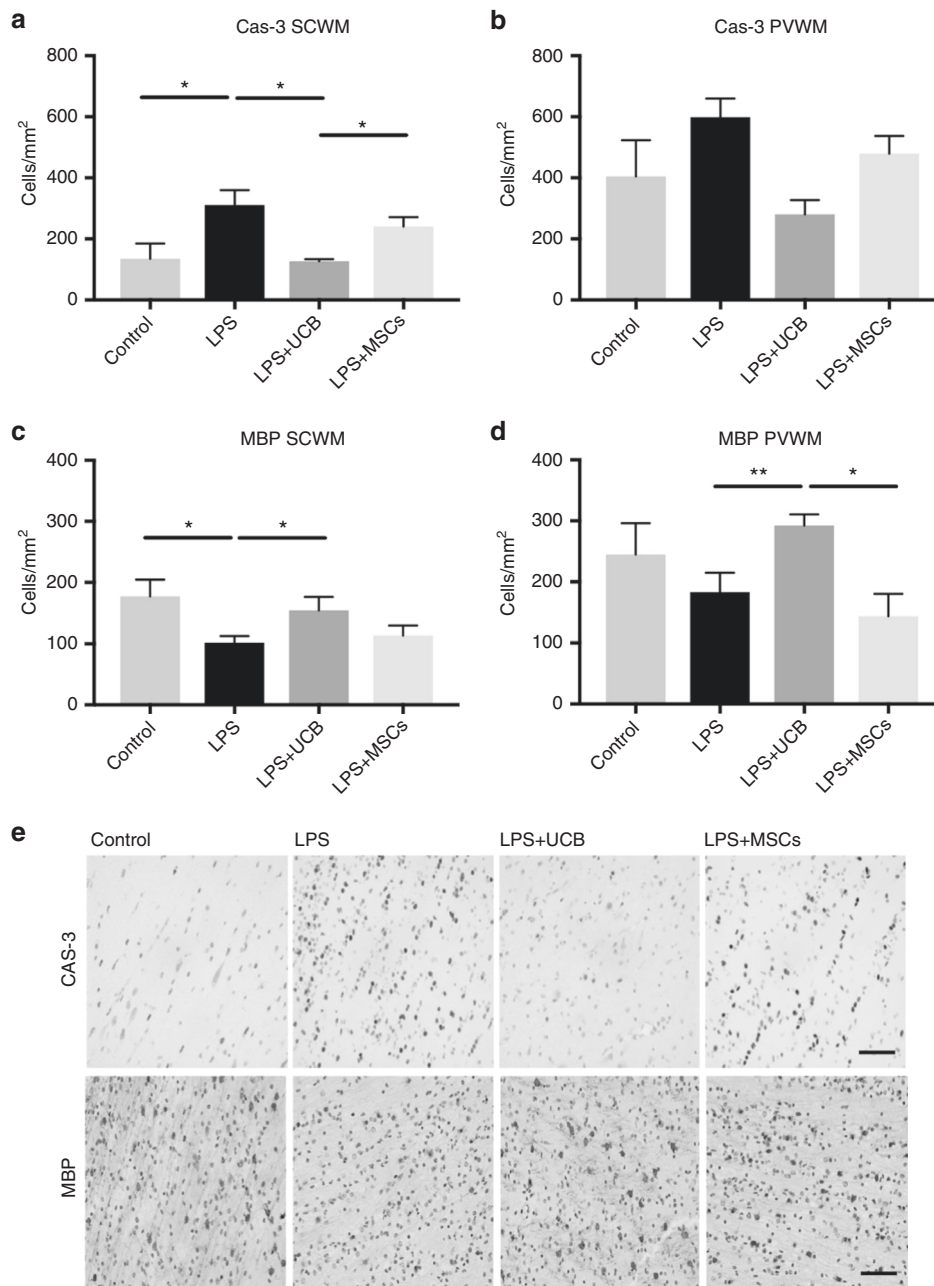MCB Paton et al.

170

Fig. 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. **e** Representative images of activated caspase-3 and myelin basic protein in the subcortical white matter. Scale bar is 10 μm. **a–d** One-way analysis of variance (ANOVA), with post hoc t tests, $n = 6$–8

protective for oligodendrocytes.[35] In the current study down-regulation of *IGF-1* might, at least partially, account for the lack of protective benefit of MSCs on mature oligodendrocyte development. Where MSC therapy significantly reduced white matter IGF-1 below control levels, UCB did not, which sits well with the known role of IGF-1 in mediated programmed cell death and promoting oligodendrocyte survival and maturation,[34] and offers insight into the possible mechanisms by which UCB but not MSCs protect oligodendrocytes. 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),[36] which in turn is reflected in neuropathology findings, with no red blood cell extravasation into white matter in MSC-treated fetuses.

Clinical implications, limitations, and future directions
The dampening of inflammation and growth factor expression by MSCs may be a concern, further investigation into this finding is required. Potentially, the dose of MSCs administered in this study may be too high, equivalent to ~6 million cells/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 very low (<0.1%). UCB cells are generally small, and large doses of 25–50 million cells/kg can safely be administered to neonates without adverse effects.[37] In contrast, MSCs require expansion by tissue culture and are a much larger cell (average 25 μm), limiting the concentration that can be administered in a single dose without risk of embolism.[38] We have previously shown in preterm

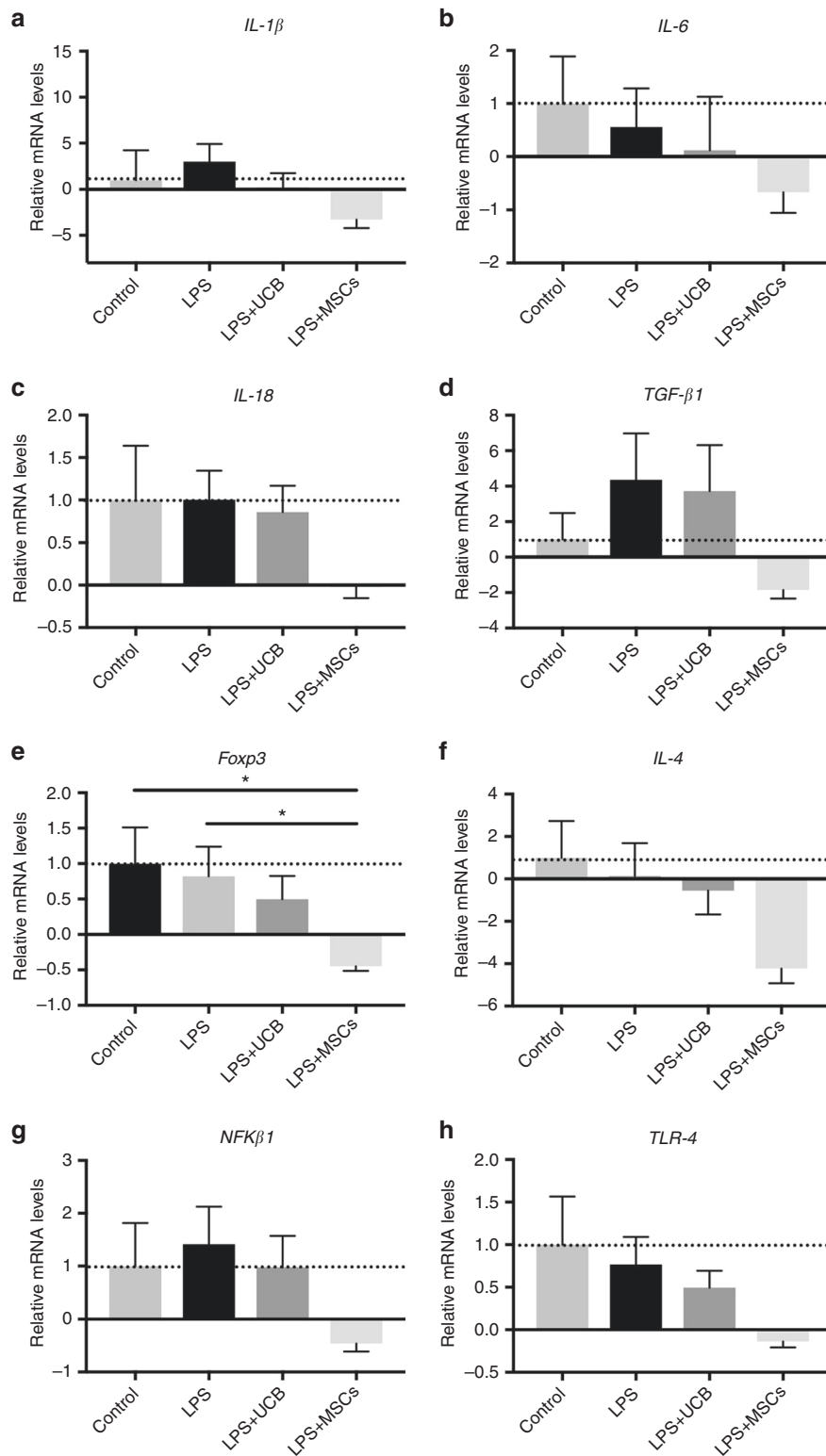Umbilical cord blood versus mesenchymal stem cells for...
MCB Paton et al.

171

Fig. 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 normalized relative quantities from three housekeeping genes. One-way analysis of variance (ANOVA) (mean ± SEM), $n = 7$–8

hypoxic–ischemic brain injury that a similar dose of MSCs, as used in our current study, could effectively protect the brain.[39] In addition, multiple doses of up to 3.5 million MSCs per kg body weight have recently been shown to be safe in children aged 3–12 years.[40] However, doses need to be considered for infant cohorts. Accordingly, the cell doses selected in the current study were based on preclinical and clinical doses in published literature, and the differences in cell size. These are important
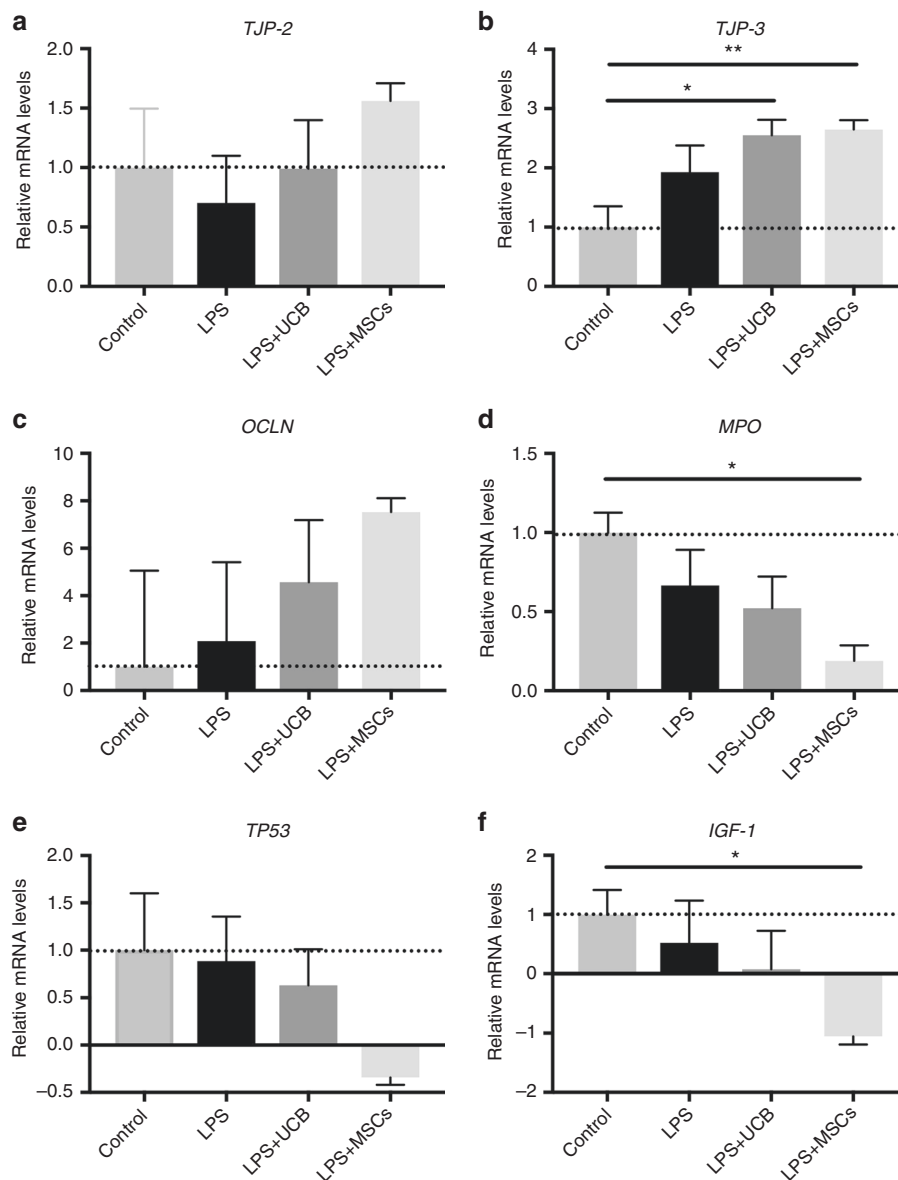
Umbilical cord blood versus mesenchymal stem cells for...
MCB Paton et al.

172

Fig. 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**) *tumor protein 53*, and (**f**) *insulin growth factor-1 gene* expression shown as relative messenger RNA (mRNA) change from controls, normalized from the calibrated normalized relative quantities from three housekeeping genes. One-way analysis of variance (ANOVA) (mean ± SEM), $n = 7$–8

considerations as preclinical results move towards clinical application.

In the present study, cells were administered 6 h after the third LPS-inflammatory stimuli in an attempt to mimic clinical application of cell therapy after preterm birth and upon diagnosis of chorioamnionitis or known exposure to inflammation. 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 the 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.[8]

A limitation of this study is that we used only one marker of oligodendrocytes to examine the effects of cell therapy on myelin-producing cells (MBP + mature oligodendrocytes). We observed a restoration in the number of myelinating oligodendrocytes following administration of UCB, but not with MSC therapy in response to LPS. In earlier work, we showed that the total number

of oligodendrocyte lineage cells (olig2 + cells) was significantly decreased following LPS, coincident with an increase in caspase-3-mediated apoptotic cell death.[15] Taken together, these data suggest that UCB cells, but not MSCs, may interrupt the process of programmed cell death of oligodendrocytes after an inflammatory insult. These data require validation and investigation of further cell types in the oligodendrocyte lineage, but are very promising for the preterm brain with white matter injury.

CONCLUSION
In response to LPS-induced preterm brain injury, administration of MSCs had a global effect on dampening brain inflammation, which in turn may have detrimental effects on brain repair and normal 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 normalized the number of mature myelinating oligodendrocytes,

Umbilical cord blood versus mesenchymal stem cells for...
MCB Paton et al.

173

but did not display the same anti-inflammatory effects as MSCs. Our results indicate that UCB is a more comprehensive therapy for protecting white matter brain development, likely contributed by the mixed cell population in UCB, and their differential actions.

ADDITIONAL INFORMATION
The online version of this article (https://doi.org/10.1038/s41390-019-0366-z) contains supplementary material, which is available to authorized users.

Competing interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

REFERENCES
1. Pascal, A. et al. Neurodevelopmental outcome in very preterm and very-low-birthweight infants born over the past decade: a meta-analytic review. *Dev. Med. Child Neurol.* **60**, 342–355 (2018).
2. Tita, A. T. N. & Andrews, W. W. Diagnosis and management of clinical chorioamnionitis. *Clin. Perinatol.* **37**, 339–354 (2010).
3. Anblagan, D. et al. Association between preterm brain injury aand exposure to chorioamnionitis during fetal life. *Scientific Rep.* **6** (2016).
4. McDonald, C. A. et al. in *Umbilical Cord Blood Banking for Clinical Application and Regenerative Medicine* (2017).
5. Novak, I. et al. *Concise review: stem cells interventions for people with cerebral palsy: systematic review with meta-analysis. Stem Cells Transl. Med.* **5**, 1014–1025 (2016).
6. Paton, M. C. B. et al. Perinatal brain injury as a consequence of preterm birth and intrauterine inflammation: designing targeted stem cell therapies. *Front. Neurosci.* **11** (2017).
7. Li, J. et al. Could cord blood cell therapy reduce preterm brain injury?. *Front. Neurol.* **5**, 200 (2014).
8. Li, J. et al. Preterm white matter brain injury is prevented by early administration of umbilical cord blood cells. *Exp. Neurol.* **283**, 179–187 (2016).
9. Li, J. et al. Term vs. preterm cord blood cells for the prevention of preterm brain injury. *Pediatr. Res.* (2017).
10. Zheng, G. et al. Mesenchymal stromal cells affect disease outcomes via macrophage polarization. *Stem Cells Int.* **2015**, 989473 (2015).
11. Hass, R. et al. Different populations and sources of human mesenchymal stem cells (MSC): a comparison of adult and neonatal tissue-derived MSC. *Cell Commun. Signal.* **9**, 12 (2011).
12. Zhao, Q., Ren, H. & Han, Z. Mesenchymal stem cells: immunomodulatory capability and clinical potential in immune diseases. *J. Cell. Immunother.* **2**, 3–20 (2016).
13. Berglund, A. K. et al. Immunoprivileged no more: measuring the immunogenicity of allogeneic adult mesenchymal stem cells. *Stem Cell Res. Ther.* **8**, 288 (2017).
14. Kean, T. J. et al. MSCs: delivery routes and engraftment, cell-targeting strategies, and immune modulation. *Stem Cells Int.* **2013**, 732742 (2013).
15. Paton, M. C. B. et al. Human umbilical cord blood therapy protects cerebral white matter from systemic LPS exposure in preterm fetal sheep. *Dev. Neurosci.* **40**, 258–270 (2018).
16. Page, K. M. et al. Optimizing donor selection for public cord blood banking: influence of maternal, infant, and collection characteristics on cord blood unit quality. *Transfusion* **54**, 340–352 (2014).
17. Li, L. et al. Human umbilical cord mesenchymal stem cells reduce systemic inflammation and attenuate LPS-induced acute lung injury in rats. *J. Inflamm.* **9**, 33 (2012).
18. Khwaja, O. & Volpe, J. J. Pathogenesis of cerebral white matter injury of prematurity. *Arch. Dis. Childhood Fetal Neonatal Ed.* **93**, 2 (2008).
19. Melville, J. M. et al. Human amnion epithelial cells modulate the inflammatory response to ventilation in preterm lambs. *PLoS ONE* **12**, e0173572 (2017).
20. Galinsky, R. et al. The consequences of chorioamnionitis: preterm birth and effects on development. *J. Pregnancy* **2013**, 412831 (2013).
21. Wu, Y. W. & Colford, J. M. Chorioamnionitis as a risk factor for cerebral palsy. *JAMA* **284**, 1417–1424 (2000).
22. Oh, K. J. et al. Twenty-four percent of patients with clinical chorioamnionitis in preterm gestations have no evidence of either culture-proven intraamniotic infection or intraamniotic inflammation. *Am. J. Obstet. Gynecol.* **216**, 6 (2017).
23. March of Dimes et al. in *Born too Soon: The Global Action Report on Preterm Birth* (eds. Howson, C. P., Kinney, M. & Lawn, J. E.) (National Academy Press, 2012). Geneva: World Health Organization.
24. Cheong, J. L. et al. Association between moderate and late preterm birth and neurodevelopment and social-emotional development at age 2 years. *JAMA Pediatr.* **171**, e164805 (2017).
25. McDonald, C. A. et al. Effects of umbilical cord blood cells, and subtypes, to reduce neuroinflammation following perinatal hypoxic–ischemic brain injury. *J. Neuroinflamm.* **15**, 47 (2018).
26. Castillo-Melendez, M. et al. Stem cell therapy to protect and repair the developing brain: an overview of mechanisms of action of cord blood and amnion epithelial derived cells. *Front. Neurosci.* **7**, 194 (2013).
27. Yoon, B. H. et al. Amniotic fluid inflammatory cytokines (interleukin-6, interleukin-1β, and tumor necrosis factor-α), neonatal brain white matter lesions, and cerebral palsy. *Am. J. Obstet. Gynecol.* **177**, 19–26 (1997).
28. Czeh, M., Gressens, P. & Kaindl, A. M. The yin and yang of microglia. *Dev. Neurosci.* **33**, 199–209 (2011).
29. Kallapur, S. G. et al. IL-1 mediates pulmonary and systemic inflammatory responses to chorioamnionitis induced by lipopolysaccharide. *Am. J. Respir. Crit. Care Med.* **179**, 955–961 (2009).
30. Shi, Y. et al. How mesenchymal stem cells interact with tissue immune responses. *Trends Immunol.* **33**, 136–143 (2012).
31. Kyurkchiev, D. et al. Secretion of immunoregulatory cytokines by mesenchymal stem cells. *World J. Stem Cells* **6**, 552–570 (2014).
32. Hannenhalli, S. & Kaestner, K. H. The evolution of Fox genes and their role in development and disease. *Nat. Rev. Genet.* **10**, 233–240 (2009).
33. Suh, H. et al. Insulin-like growth factor 1 and 2 (IGF1, IGF-2) expression in human microglia: differential regulation by inflammatory mediators. *J. Neuroinflamm.* **10**, 37 (2013).
34. Mason, J. L. et al. Insulin-like growth factor-1 inhibits matute oligodendrocyte apoptosis during primary demyelination. *J. Neurosci.* **20**, 5703–5708 (2000).
35. Yao, D. et al. Insulin-like growth factor 1 treatment reduces demyelination and up-regulates gene expression of myelin-related proteins in experimental autoimmune exephalomyelitis. *Proc. Natl Acad. Sci. USA* **92**, 6190–6194 (1995).
36. Ullen, A. et al. Myeloperoxidase-derived oxidants induce blood–brain barrier dysfunction in vitro and in vivo. *PLoS ONE* **8**, e64034 (2013).
37. Cotten, C. M. et al. Feasibility of autologous cord blood cells for infants with hypoxic–ischemic encephalopathy. *J. Pediatr.* **164**, 973–979 e1 (2014).
38. Janowski, M. et al. Cell size and velocity of injection are major determinants of the safety of intracarotid stem cell transplantation. *J. Cereb. Blood Flow Metab.* **33**, 921–927 (2013).
39. Li, J. et al. Preterm umbilical cord blood derived mesenchymal stem/stromal cells protect preterm white matter brain development against hypoxia–ischemia. *Exp. Neurol.* **308**, 120–131 (2018).
40. Huang, L. et al. A randomized, placebo-controlled trial of human umbilical cord blood mesenchymal stem cell infusion for children with cerebral palsy. *Cell Transplant.* **27**, 325–334 (2018).