MONASH University

Anti-inflammatory therapies for bronchopulmonary dysplasia

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This thesis is dedicated to my parents,

Chris and Fil,

To my brother,

Phillip,

And to my grandparents,

Phillip and Voula

This would not have been possible without your support.

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Summary

Bronchopulmonary dysplasia (BPD) is a potentially fatal chronic lung disease that represents the principal cause of morbidity in infants born before 28 weeks of gestation. Survivors of BPD suffer higher-than-normal rates of chronic respiratory illness and adverse neurological outcomes, including cerebral palsy. There is no therapy for BPD.

Clinically, BPD is defined as the requirement for at least 28 days of respiratory support. The pathogenesis of BPD is understood incompletely, but antenatal inflammation and mechanical ventilation are common antecedents. Thus, BPD likely arises from antenatal- and ventilator-induced inflammation in preterm infants. Prevention or attenuation of lung inflammation is a realistic therapeutic prospect for BPD.

Postnatal steroids are effective in alleviating respiratory distress and reducing lung inflammation in preterm infants, but there is hesitation in their use due to undefined dosing regimes and, at high doses, adverse neurological outcomes. Human amnion epithelial cells (hAECs) show promise in mitigating the pathological processes associated with BPD development. Human AECs are easily obtained from placentae, which are usually discarded at birth.

Long-term mechanical ventilation of preterm lambs causes lung injury and alterations in lung development consistent with the pathology of BPD. The only preclinical intensive care research unit in the southern hemisphere was recently established for long-term care of preterm lambs, which allows us to manage lambs for a period of weeks, in a way that mimics human neonatal care. We utilised this research facility to perform preclinical studies investigating the efficacy of postnatal steroids or hAECs to treat and prevent BPD.

In Chapter 3, we exposed fetal sheep to *in utero* inflammation and 48 hours later delivered the lambs preterm. Lambs were managed for one week in the preclinical research facility and received postnatal dexamethasone as a low-tapered dose over the first week of life. We found that postnatal dexamethasone did not alter ventilation requirements in preterm lambs that were exposed to antenatal inflammation. Tapered low-dose postnatal dexamethasone thinned lung parenchyma (indicative of rapid maturation of the lungs) and reduced

inflammation in the lungs of preterm lambs exposed to antenatal inflammation. These data support low-dose postnatal dexamethasone administration during the perinatal period.

Before the application of hAECs in preterm lambs we interrogated their viability and function in response to changing temperatures. In Chapter 4, we cultured hAECs at 33 °C, 37 °C and 39 °C and saw no change in rates of cellular apoptosis at 24, 48 and 72 hours. However, we noted that time in culture increased the proportion of cells undergoing apoptosis, irrespective of culture temperature. There were otherwise no observed functional changes in hAECs across culture temperatures. We determined that hAECs are viable across a range of temperatures.

In Chapter 5, we administered hAECs to preterm lambs following exposure to antenatal inflammation. We found that hAEC-treated preterm lambs did not have reduced ventilation requirements over the first week of life. However, hAECs improved lung structure and reduced lung injury in preterm lambs albeit upregulated pro- and anti-inflammatory mediators of the pulmonary immune system in preterm lambs following exposure to antenatal inflammation.

In conclusion, these studies better elucidate the use of low-dose postnatal dexamethasone and hAECs in preterm infants at risk of developing BPD. Our data suggests tapered low-dose dexamethasone reduces lung inflammation and alters lung parenchyma, whilst hAECs improve lung morphology and have pro-reparative potential in BPD-like lung pathology.

Declaration

This declaration is to be included in a standard thesis. Students should reproduce this section in their thesis verbatim.

In accordance with Monash University Doctor of Philosophy regulations I declare the following:

I declare that the material in this thesis has not 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.

I declare that this thesis contains original papers that have been submitted or are in preparation for being submitted to peer reviewed journals. The ideas, development and writing up of original papers was my responsibility, working within the Perinatal Inflammation Research Group under the supervision of A/Prof. Tim J.M. Moss and the Perinatal Intensive Care Research Unit under the supervision of Prof. J. Pillow. A description of the nature of co-author contributions is outlined before each chapter.

Signature 

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Publications

Publications

Papagianis, P. C., Pillow, J. J., Moss, T. J. (2018) Bronchopulmonary dysplasia: pathophysiology and potential anti-inflammatory therapies. Paediatric Respiratory Reviews. Accepted July 2018

Conference presentations

Papagianis, P.C., Pillow, J.J., Noble, P.B. & Moss, T.J. (2018) Cells from the placenta: can we give back to the preterm infants? Endocrine and Reproductive Biology Society.

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Limwan, A., Widiasmoko, L., **Papagianis, P. C.,** Burgner, D. & Moss, T. J. (2018) The effects of pre- and postnatal inflammation on atherosclerosis in ApoE knockout mice. Fetal and Neonatal Physiological Society.

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Papagianis, P.C. Noble P.B., Ahmadi-Noorbakhsh, S., Dahl, M. J., Albertine, K. H. & Moss, T.J. & Pillow, J.J. (2017) The effect of postnatal steroids on lung development and inflammation in preterm lambs exposed to antenatal inflammation. Endocrine and Reproductive Biology Society.

Papagianis, P. C., Ahmadi-Noorbakhsh, S., Noble, P. B., Pillow, J.J. & Moss, T. J. (2017) Modulating antenatal inflammation and alveolarisation in preterm lambs with postnatal dexamethasone and human amnion epithelial cells. Perinatal Society of Australia and New Zealand.

Papagianis, P.C., McDonald, C., Pillow, J.J. & Moss, T.J. (2017) Viability and function of human amnion epithelial cells is not altered by temperature. The Fetal and Neonotal Workshop of Australia and New Zealand 31th Annual Meeting

Papagianis, P. C., Pillow, J.J., Ahmadi-Noorbakhsh, S., Noble, P. B. & Moss, T. J. (2017) Human amnion epithelial cells alter lung development in 7-day-old preterm lambs exposed to inflammation before birth. Australian Physiological Society

Papagianis, P.C. Noble P.B., Ahmadi-Noorbakhsh, S., Dahl, M. J., Albertine, K. H. & Moss, T.J. & Pillow, J.J. (2017) The effect of postnatal steroids on lung development and inflammation in preterm lambs exposed to antenatal inflammation. Monash Health Research Week

Chan, K., **Papagianis, P.C.,** Nitsos, I., Zahra, V., LaRosa, D., McDonald, C. A., Miller, S., Alahmari, D. & Polglase, G. R. &. (2017) Isolating the effects of the initiation of ventilation on the preterm lamb brain. Australian Society of Medical Research.

Papagianis, P.C., Noble P.B., Ahmadi-Noorbakhsh, S., Moss, T.J. & Pillow, J.J. (2016) The effect of postnatal steroids on the lung structure of ventilated preterm lambs exposed to

chorioamnionitis. The Fetal and Neonotal Workshop of Australia and New Zealand 30th Annual Meeting

Papagianis, P.C., McDonald, & Moss, T.J. (2016) Incubation temperature does not alter viability or function of human amnion epithelial cells in culture. Monash Health Research Week

Papagianis, P.C., Nguyen, V., Polglase, G. & Moss, T.J. (2015) Optimising continuous positive airway pressure for preterm infants. The Fetal and Neonotal Workshop of Australia and New Zealand 29th Annual Meeting.

Papagianis, P.C., Nguyen, V., Polglase, G. & Moss, T.J. (2015) Optimising continuous positive airway pressure for preterm infants. Monash Health Research Week

Invited talks

Papagianis, P. C., Pillow, J. J. & Moss, T. J. (2017) Modulating pulmonary inflammation using dexamethasone or amnion epithelial cells in preterm lambs exposed to antenatal inflammation. NHMRC Preterm Infants Centre of Research Excellence Seminars

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Papagianis, P. C., Pillow, J. J. & Moss, T. J. (2015) Human amnion epithelial cells as a therapy for bronchopulmonary dysplasia. NHMRC Preterm Infants Centre of Research Excellence Annual Meeting

Thesis including published works declaration

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 1 original paper accepted in a peer review journal and 1 submitted publication. The core theme of the thesis is anti-inflammatory therapies for bronchopulmonary dysplasia.

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 under the supervision of Tim Moss and Graeme Polglase and within the Department of School of Health Sciences under the supervision of Jane Pillow.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

Thesis Chapter	**Publication Title**	**Status** *(published, in press, accepted or returned for revision, submitted)*	**Nature and % of student contribution**
Chapter 1	*Bronchopulmonary dysplasia: pathophysiology and potential anti-inflammatory therapies*	*In Press*	*90 %. Concept and writing first draft*
Chapter 3	*The effect of postnatal steroids on lung development and inflammation in preterm lambs exposed to antenatal inflammation*	*Manuscript form*	*70 %. Performed all experiments and analyses and wrote original manuscript drafts*
Chapter 4	*Viability and function of human amnion epithelial cells*	*Submitted/*	*70 %. Performed all lab work and wrote original*

	is not altered by temperature	*manuscript form*	*manuscript drafts*
Chapter 5	*The effect of human amnion epithelial cells on lung development and inflammation in preterm lambs exposed to antenatal inflammation*	*Manuscript form*	*90 %. Performed all experiments and analyses and wrote original manuscript drafts.*

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

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Paris Clarice Papagianis,

PhD Candidate,

3rd 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.

Main Supervisor signature:

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Timothy Moss

3rd August, 2018

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Abbreviations

ABG	Arterial blood gas
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
B-CPAP	Bubble continuous positive airway pressure
BAL	Bronchoalveolar lavage
BE	Base excess
BP	Blood pressure
BPD	Bronchopulmonary dysplasia
cDNA	Complementary deoxyribonucleic acid
E. Coli	*Escherichia coli*
FBS	Fetal bovine serum
FiO_2	Fraction of inspired oxygen
GA	Gestational age
hAEC	Human amnion epithelial cells
Hb	Haemoglobin
HBSS	Hank's balanced salt solution
HCO_3	Bicarbonate
HHF	Humidified high flow
HR	Heart rate
IA	Intra-amniotic
ICU	Intensive care unit
ID	Inner diameter
IgG	Immunoglobulin

IL	Interleukin
IV	Intravenous
IVH	Intraventricular haemorrhage
LPS	Lipopolysaccharide
MAP	Mean airway pressure
mmHg	Millimeters of mercury
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
MV	Mechanical ventilation
n	Nasal
NCS	Newborn calf serum
NICU	Neonatal intensive care unit
NLR	Nod-like receptor
OD	Outer diameter
OI	Oxygen index
$PaCO_2$	Partial pressure of carbon dioxide
PaO_2	Partial pressure of oxygen
PBS	Phosphate buffered saline
PEEP	Positive end expiratory pressure
pF	Arterial oxygen partial pressure to fractional inspired oxygen ratio
pH	Potential hydrogen
PIP	Positive inspiratory pressure
RDS	Respiratory distress syndrome
RNA	Ribonucleic acid
RNase	Ribonuclease
ROI	Region of interest
rpm	Revolutions per minute

RR	Respiratory rate
SaO_2	Arterial oxygen saturation
SD	Standard deviation
SI	Sustained inflation
SP	Surfactant protein
VILI	Ventilation-induced lung injury
V_T	Tidal volume
w/v	Weight per volume
x g	Times gravity

Declaration for Thesis Chapter 1: Bronchopulmonary dysplasia: pathophysiology and potential anti-inflammatory therapies

Declaration by candidate

Nature of the contribution	Extent of contribution
Conceived the idea and drafted manuscript	90 %

The following authors contributed to the work. If the co-authors are students at Monash University their contribution in percentage is stated:

Nature of the contribution		Extent of contribution
J. Jane Pillow	Conceived the idea and drafted manuscript	N/A
Tim J. Moss	Conceived the idea and drafted manuscript	N/A

Introduction

Globally, 15 million infants are born preterm each year, representing more than 1 in 10 live births.[1] Extremely preterm infants (born before 28 weeks' gestational age (GA)) are the most vulnerable. The most common complication they experience is the chronic lung disease, bronchopulmonary dysplasia (BPD).[2] BPD is the only complication of preterm birth for which the incidence is increasing at the same time that all other co-morbidities are decreasing[2].

There is no cure for BPD. The use of antenatal corticosteroids and exogenous surfactant administration has improved the acute respiratory complications of preterm birth dramatically, but these treatments do not prevent BPD.[3, 4] The early application of non-invasive respiratory support is an alternative strategy aimed at preventing BPD but inconsistently reducing its incidence.[5-7]

Although BPD is clearly a respiratory disease of prematurity, BPD may not simply be a consequence of lung immaturity. The immature immune system of preterm neonates coupled with the immune modulating effects of factors that commonly accompany preterm birth (e.g. antenatal corticosteroids, intrauterine infection/inflammation and postnatal sepsis),[8] may be responsible for aberrant regulation of lung inflammation. Thus, targeting the chronic lung inflammation that underlies BPD with the use of new anti-inflammatory therapies offers the prospect of preventative and reparative treatment for infants with BPD.

Bronchopulmonary dysplasia

BPD was first described by Northway in the 1960s as a form of chronic lung disease in moderately preterm infants, characterised predominately by fibrosis.[9] Northway attributed the injurious effects of high oxygen levels and airway pressures used in mechanical ventilation as the main causes of the disease[9]. However, changes in respiratory management strategies for preterm infants has transformed BPD into a disease characterised by more subtle lung abnormalities, including alveolar hypoplasia (fewer and larger simplified alveoli),[10, 11] dysmorphic pulmonary vasculature and chronic pulmonary inflammation.[12-14] The lungs of infants with severe BPD exhibit a lack of septation within the developing airspace, resulting in reduced surface area for

gas exchange in the lungs, hyperconstrictive vasculature that further compromises gas exchange, and impaired surfactant synthesis,[15] favouring atelectasis. The National Institute of Child Health and Human Development defines this "New BPD" as the requirement for at least 28 days of supplemental oxygen, with the severity of BPD indicated by the level of respiratory support required at 36 weeks postmenstrual age.[16]

The aetiology of BPD

Prenatal inflammation

Inflammation within the uterus during pregnancy is a common antecedent of preterm birth that manifests as chorioamnionitis; inflammation of the chorion and amnion. Chorioamnionitis is commonly classified as clinical or histological: clinical chorioamnionitis is diagnosed prior to labour, when women present with symptoms including fever, a tender uterus and preterm rupture of membranes (PROM) with purulent liquor; histological chorioamnionitis is an asymptomatic inflammation of the membranes.[17] Histological chorioamnionitis is more common than clinical chorioamnionitis but may be undiagnosed because post-partum histological examination of the placenta is required.[17] Histological chorioamnionitis complicates between 30–70 % of preterm births with PROM and spontaneous labour, with incidence inversely related to GA. Thus, rates of histological chorioamnionitis exceed 70 % for infants at highest risk of BPD (23 weeks GA).[18]

Prenatal inflammation alters fetal lung development, with consequences that may be detrimental or beneficial for preterm newborns. Histological chorioamnionitis reduces the risk of respiratory distress syndrome (RDS),[18-20] likely due to elevated surfactant production in the lungs.[21-23] However, despite lower risk of RDS, infants exposed to chorioamnionitis may require longer term respiratory support and have higher rates of BPD and persistent pulmonary hypertension of the newborn (PPHN).[24] Although increases[20, 25] or decreases[18,26] in the incidence of BPD are reported after exposure to chorioamnionitis, the relationship is complicated by low-birth-weight and postnatal events, such as sepsis. The avoidance of prolonged mechanical ventilation in low-birth-weight newborns exposed to chorioamnionitis is associated with decreased incidence of BPD, compared to newborns exposed to chorioamnionitis who received prolonged ventilation.[27] It is unclear whether a prenatal (chorioamnionitis) or postnatal (ventilator-

induced) origin of lung inflammation is the greater contributor to the pathogenesis of BPD.

Fetal sheep exposed to inflammation induced by intra-amniotic (IA) injection of lipopolysaccharide (LPS) have lung abnormalities like those observed in infants who die of BPD: alveolar hypoplasia[21, 28] and decreased septation,[21, 29] impaired surfactant secretion (despite increased surfactant protein),[21] and impaired pulmonary vascular development and function.[30] The similarities in the lungs of fetal lambs exposed to inflammation and the pathological features of BPD support a prenatal origin of BPD. Although preterm infants exposed to chorioamnionitis have less RDS, experimental intrauterine inflammation does not reduce the postnatal respiratory support required by preterm baboons[31] and lambs.[32]

Respiratory Support

Mechanical ventilation initiates an influx of neutrophils and macrophages into the alveoli.[33] These cells produce cytokines,[33] which can disrupt lung development and may be used as biomarkers in serum and tracheal aspirates to identify infants at risk of BPD.[12-14] Infants who develop BPD have persistently elevated pro-inflammatory cytokines (IL-1β, IL-6 and IL-8) in tracheal aspirates and blood, compared to infants who recover from initial RDS.[34] Preterm infants may become increasingly dependent on respiratory support, which exacerbates pulmonary inflammation, inducing the production of reactive oxygen species (ROS).[35] ROS promote inflammation and epithelial cell death in the lungs via the cleavage, and thus activation, of caspase-1 (Figure 1).[36]

In preterm lambs, 2 hours of mechanical ventilation initiates inflammation within the lungs resulting in similar upregulation of IL-1β, IL-6 and IL-8, the same biomarkers as infants that are ventilated or were exposed to chorioamnionitis.[37, 38] Longer ventilation of preterm lambs (3-4 weeks) increases neutrophil and macrophage infiltration into the lungs and causes non-uniform inflation patterns and abnormal lung vascular development, similar to that observed in infants who die from BPD.[39]

Long-term pulmonary consequences of BPD

The long-term sequelae of new BPD are described by very few studies. Autopsies reveal its major pathological features: abnormal alveolar architecture (alveolar hypoplasia) and impaired pulmonary vascular development, where vessels are distant from airspaces.[10, 11] Pulmonary gas exchange is impaired in 2-year-old infants with BPD compared to non-BPD controls; however, alveolar volume was normal in both cohorts, suggesting a lower alveolar surface area, consistent with an alveolar hypoplasia lung phenotype.[40] Persistent abnormalities in lung parenchyma have long-term functional consequences: 7-to-8-year-olds who had BPD and received surfactant during the perinatal period had lower forced expiratory volume and higher airway resistance, compared to age- and sex- matched controls without BPD, indicative of increased work of breathing.[41]

Steroidal approaches to prevent pulmonary inflammation

Antenatal corticosteroids

Antenatal corticosteroids administered to women at risk of preterm delivery accelerate fetal lung maturation to prevent RDS but do not prevent BPD.[3] Antenatal corticosteroids cause remodeling of the lung parenchyma, which improves gas exchange but results ultimately in fewer, larger alveoli,[42] like the lungs of fetal sheep exposed to prenatal inflammation.[10, 21] Antenatal corticosteroids alter immune activity by suppressing lymphocytes but increasing neutrophils in preterm infants,[43] and altering immune cell function,[44] which may underlie an increased risk of early-onset sepsis.[3]

Antenatal corticosteroid treatment can suppress lung inflammation (induced by IA injection of LPS) in fetal sheep but the reduction of LPS-induced inflammation is transient.[45] Thus, the timing of antenatal corticosteroid administration in humans may influence the lung inflammation that accompanies chorioamnionitis. The optimal timing, dose, and frequency of administration of glucocorticoids to women at risk of preterm birth are unknown. While chorioamnionitis is not a contraindication to the use of antenatal corticosteroids, their interaction likely affects lung development differently to either in isolation;[21, 46] the impact of this interaction on rates of BPD is not known.

Few studies investigate any effect of antenatal glucocorticoids on postnatal ventilator requirements and lung inflammation. In adult animals, pretreatment with corticosteroids reduces ventilation-induced lung injury.[47, 48] Preterm lambs ventilated following exposure to antenatal glucocorticoids have less lung injury and inflammation in comparison to ventilated preterm lambs exposed to antenatal saline.[49] The maturational and anti-inflammatory effects of antenatal glucocorticoids in the preterm lungs appear to be maintained for the initial ventilation period, consistent with lower RDS incidence. However, meta-analyses show that antenatal corticosteroids do not reduce BPD.[3]

Postnatal glucocorticoids

Postnatal glucocorticoid use peaked in the late 1990s[50] after observations of improved extubation rates in a small trial.[51] Later meta-analyses revealed reduced BPD incidence in preterm infants who received postnatal glucocorticoids, but increased incidence of cerebral palsy and death.[52-55] Thus, postnatal glucocorticoids are now used reluctantly in infants with intractable BPD: fewer than 10 % of preterm infants receive them.[2]

Ventilator management of preterm infants has evolved since the 1990s, aiming for respiratory management with lower airway pressures and inspired oxygen concentrations, and avoidance of prolonged periods of intubation.[5, 56] The practice of more 'gentle' ventilation coupled with significantly less postnatal steroid exposure may result in infants receiving longer periods of respiratory support than may have been used prior to adoption of these changes in practice. It is unclear whether the increasing BPD incidence is attributable to longer periods of respiratory support and/or the decrease in steroid use. Typically, infants who receive postnatal steroids are those who require prolonged periods of invasive ventilation, indicating a stronger predisposition for BPD development, and complicating any assessment of the impact of postnatal steroids on BPD incidence.

Early studies of postnatal high-dose dexamethasone therapy (~1 mg/kg/day over 42 d)[51] focused on immediate respiratory outcomes, at the expense of neurological follow-up. Optimal postnatal steroid dosing regimens are undefined, contributing to reluctance for their clinical use. Early administration of lower dexamethasone doses may be safe and effective in infants at high-risk of developing BPD.[55]

Despite the key role of inflammation in the pathogenesis of BPD, few studies of postnatal steroids include inflammation as an outcome. Small studies describe a reduction in neutrophils[57, 58] and IL-1 concentration in bronchoalveolar lavage (BAL) of ventilated preterm infants receiving dexamethasone.[57, 59] Similarly, IL-1β expression is lower in the lungs of preterm lambs receiving a single dexamethasone dose (0.5 mg/kg) immediately before initiation of ventilation compared to placebo controls.[49] No animal studies assess the ability of postnatal dexamethasone to treat established lung inflammation and injury.

Neurological impairment is associated with high dose postnatal steroids. The risk of cerebral palsy increases by 40 % for every 1 mg/kg increase in dexamethasone dose.[60] The most premature infants are not the worst affected (despite presumably more immature organ systems); treatment after 33 weeks' postmenstrual age is associated with greatest neurological deficit at follow-up.[60] Adverse clinical neurological outcomes associated with dexamethasone are consistent with animal studies.[61-63] Other complications of high-dose postnatal steroid use include stunted growth[64], intraventricular haemorrhage (IVH),[55, 65] gastrointestinal bleeding,[54] sepsis and hypertension.[53-55, 66] Combined use of dexamethasone and indomethacin (for closure of the ductus arteriosus) increases the likelihood of gastrointestinal perforation three-fold.[64]

The DART trial aimed to investigate the ability of a low-dose 10-day tapered course of postnatal dexamethasone (0.89 mg/kg cumulative over 10 days) to prevent BPD in preterm infants born before 28 weeks GA.[67] The trial was terminated because of low (10 %) recruitment,[67] but infants who received dexamethasone spent less time intubated on mechanical ventilation; although this did not reach statistical significance. Major disability and cerebral palsy were not different between dexamethasone-treated and placebo-treated preterm infants at 2 years follow-up.[68]

Non-steroidal approaches to prevent pulmonary inflammation

Inflammation associated with BPD involves the activation or over-expression of a number of inflammatory cytokines and pro-inflammatory mediators (Figure 1) providing opportunity for multiple therapeutic targets to prevent lung inflammation in newborn

infants. Inhibition of cell signaling that exacerbates inflammation or inhibition of specific pro-inflammatory cytokines in the lungs may prevent BPD progression.

Suppressing inflammation at various sites: Pentoxifylline, NLRP3 inhibition, IL-1Ra and Adenosine Monophosphate Proteins

Pentoxifylline

Pentoxifylline is a synthetic theobromine derivative, structurally similar to caffeine.[69] Pentoxifylline is a an immunological agent sometimes used in septic shock due to its ability to lower blood viscosity and improve tissue perfusion.[69] Pentoxifylline acts by inhibiting erythrocyte phosphodiesterase, which increases expression of the anti-inflammatory protein adenosine monophosphate protein kinase (AMPK), suppressing neutrophils and pro-inflammatory cytokines[69, 70] and likely preventing chronic inflammation.

Pentoxifylline is well tolerated by neonates, in whom it is predominately used during sepsis. Pentoxifylline infusion (5 mg/kg/hour for 6 hours) over six days lowers plasma IL-6 and TNF in preterm infants with sepsis, when compared to placebo.[71] Infants receiving pentoxifylline had less hypotension and overall improved clinical course,[71] highlighting its immuno- and vasculo-modulatory effects. However, comparison of pentoxifylline and dexamethasone in low-birth-weight infants with oxygen requirements >30 % at 72 hours of age revealed neither treatment impacted BPD incidence.[72]

It is unclear if pentoxifylline is beneficial for BPD prevention. Current studies are limited by small sample size and poor design (e.g. no blinding is apparent in any of the studies). Hypotension, arrhythmia[69] and more rarely, IVH, are noted in adult trials using pentoxifylline.[73, 74] It is unclear whether pentoxifylline may have particular side effects in infants with BPD. Preclinical studies using pentoxifylline for BPD are rare, and animal studies are warranted to ensure effective translation of pentoxifylline into larger randomised controlled trials (RCTs). One RCT is currently recruiting preterm infants to receive either pentoxifylline or placebo for preventing sepsis and necrotising enterocolitis (NEC), with BPD as a secondary outcome (ACTRN: 12616000405415). No RCTs using pentoxifylline assess BPD as a primary outcome.

In newborn rats exposed to hyperoxia, pentoxifylline administration increased survival and expression of lung vasculogenic markers compared to normoxic controls.[75] However, hyperoxia produces a classic fibrotic BPD phenotype, not contemporary

BPD. Adult rats subjected to intratracheal hydrochloric acid (HCl) have lung inflammation and develop acute RDS.[76] Prophylactic, but not rescue, pentoxifylline reduces HCl-induced lung inflammation and normalises alveolar architecture,[76] indicating the timing of pentoxifylline may be important when considering its application in preterm infants.

NLRP3 inflammasome

The NLRP3 inflammasome is part of the innate immune system, responsible for sensing pathogens and initiating inflammation.[77] The NLRP3 inflammasome is activated by numerous stimuli,[78] forming a complex with other molecules, including procaspase-1.[77, 79] Procaspase-1 is activated upon formation of the NLRP3 complex and induces IL-1β maturation.[77, 79] Expression of IL-1β is tightly regulated by activation of the NLRP3 complex.

NLRP3 is implicated in adult ventilator-induced lung injury[80] and is upregulated in BAL after 5 hours of ventilation.[80] There are no data suggesting activation of NLRP3 in neonatal ventilation, however NLRP3 is part of the innate immune system and should be present at birth.

The NLRP3 inflammasome can be activated by ROS (through injurious ventilation), or TLR activation (through LPS; Figure 1).[81] Ventilation using low or high tidal volumes both stimulate NLRP3 and IL-1β expression in mice lungs.[80] Overexpression of NLRP3 interrupts alveolar formation and leads to abnormal lung morphogenesis of mice.[82] NLRP3-deficient mice are protected from ventilator-induced lung injury and have low IL-1β in their lungs.[80, 83]

NLRP3 activity is altered in the presence of glucocorticoids and LPS, which may compromise NLPR3 blockade for BPD prevention in preterm infants. Cultured human and mouse macrophages pre-treated with LPS have elevated NLRP3 and IL-1β following exposure to dexamethasone, despite glucocorticoids being anti-inflammatory (dexamethasone alone blocks IL-1β).[81] The use of postnatal steroids may be less effective in reducing NLRP3-induced inflammation in preterm infants exposed to both chorioamnionitis and ventilation. Targeting inflammatory inhibition downstream of NLRP3 may be more appropriate in these infants.

Other avenues for NLRP3 suppression include administration of the antidiabetic drug glibenclamide and IL-1 inhibitors. In ventilated adult mice receiving glibenclamide,

compared to placebo, NLRP3 and IL-1β in the lungs was reduced.[80] Glibenclamide is used antenatally in mothers with gestational diabetes[84] and in neonates with permanent neonatal diabetes mellitus,[85] but not in neonatal lung inflammation.

IL-1 receptor antagonist

IL-1 plays a crucial role in inflammation.[86] IL-1α and IL-1β enhance their own upregulation and recruit other pro-inflammatory cytokines, including IL-6 and IL-8, to aggravate inflammation.[86, 87] Upregulation of IL-1 is apparent in tracheal aspirates of preterm infants who were exposed to chorioamnionitis[88] or who have BPD.[34] Elevated IL-1 in tracheal aspirates between days 1-3 of life may better predict BPD than GA alone for infants born <27 weeks GA.[83]

Imbalance between IL-1 and its endogenous IL-1 receptor antagonist (Ra) may be involved in the pathogenesis of BPD. Preterm infants <30 weeks GA have elevated IL-1 and lower IL-1Ra,[89, 90] an imbalance that can persist for the first month of life.[89] However, levels of IL-1 and IL-1Ra are both higher than non-BPD controls,[90] indicating an inability to inhibit IL-1β by IL-1Ra in preterm infants at risk of BPD. Increases in IL-1:IL-1Ra, favouring inflammation, may contribute to prolonged pulmonary inflammation and BPD development. Elevated IL-1 in tracheal aspirates preceded increased macrophage activity between 7-10 days of life in preterm infants who develop BPD, indicating a hyperactive immune system.

The ratio of IL-1:IL-1Ra increases exponentially in tracheal aspirates of baboons delivered at 70 % of full gestation and ventilated for 2, 6 or 14 days,[83] suggesting an ongoing inflammatory response. Synthetic IL-1Ra prevents BPD-like lung pathology in mice and rats by reducing pulmonary inflammation and normalising alveolar development.[91,92] Synthetic IL-1Ra reduces lung inflammation and suppresses IL-1β in fetal lambs exposed to LPS. [93]

IL-1Ra has a well-established safety profile for therapeutic use in adults,[94] but 100-fold levels of IL-1Ra to IL-1 are required for functional inhibition of IL-1.[94] Synthetic IL-1Ra is used in neonatal-onset multisystem inflammatory disease to control relapsing inflammation.[95, 96] However, clinical use for BPD prevention is not reported. There are no guidelines for IL-1Ra use in the neonate and only one IL-1Ra (Anakinra) has shown to be safe in patients less than 2 years old.[97, 98] Other FDA-approved IL-1 inhibitors have not been tested in neonates.[99,100]

Adenosine monophosphate proteins

AMPs are produced by macrophages, neutrophils and epithelial cells in response to inflammation, ROS or infection, and suppress the release of inflammatory mediators.[101] Excess ROS activates AMP to AMPK, which suppress NLRP3.[102] AMPs are elevated in tracheal aspirates of newborn ventilated infants with pulmonary infections compared to ventilated infants without infection[103] but it is unclear if these AMPs are active.

Intratracheal LPS administration in mice increases lung endothelial cell permeability and white cell infiltration, in parallel with AMPK inhibition.[104] Pretreatment, but not rescue treatment, of wild-type mice with an AMPK activator reduces LPS-induced inflammation and injury.[104] Infants exposed to prenatal inflammation have similar lung morphology to that observed in mice exposed to intratracheal LPS; thus these infants may have reduced AMPK activity.

AMPK activity is enhanced by resveratrol in obese patients (still in clinical trials),[105] consistent with inhibition of inflammation in LPS-exposed macrophages from mice *in vitro.*[106] The use of resveratrol has not been investigated in lung inflammation, but stimulation of innate AMPK may inhibit inflammation and prevent BPD lung pathology in preterm infants.

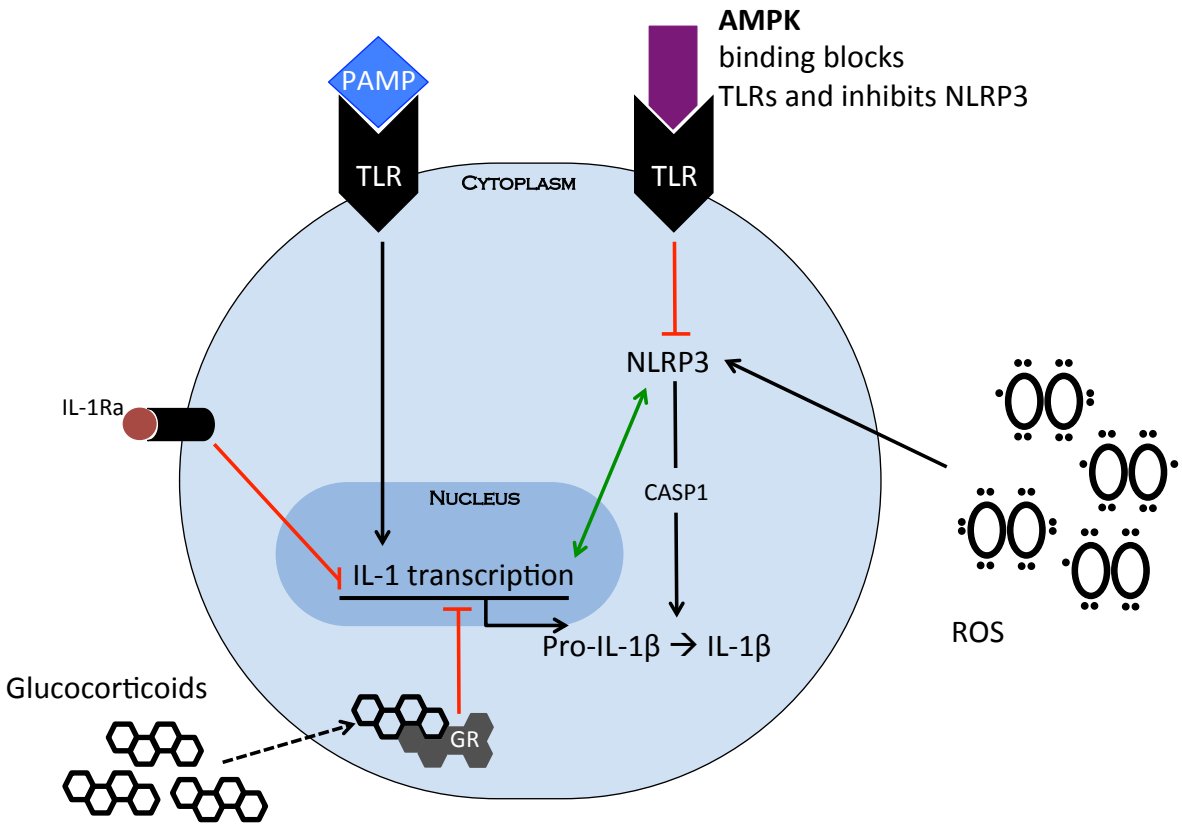


Figure 1. Interplay of factors that may contribute to chronic lung inflammation and the development of BPD in preterm infants. NLRP3 and pathogen-associated molecular patterns (PAMP) both increase transcription of potent pro-inflammatory cytokine IL-1. Imbalances in IL-1 and IL-1 antagonists (i.e. IL-1 receptor antagonist: IL-1Ra) may predispose infants to BPD. IL-1 transcription can be prevented by IL-1Ra. IL-1 production positively feeds back to increase NLRP3 activity (green arrow). Reactive oxygen species (ROS) induced by ventilation also increase NLRP3 activity, inducing caspase-1 (CASP1), and thus IL-1 transcription. Glucocorticoids bind to glucocorticoid receptors (GR) in the cytoplasm and interfere with the transcription of IL-1 in the nucleus. Adenosine monophosphate-activated protein kinase (AMPK) binding to toll-like receptors (TLR) inhibits NLRP3 (red line).

Cell therapies for prevention of pulmonary inflammation

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) with multi-lineage differentiability are usually derived from bone marrow. MSCs home to sites of injury and possess immunomodulatory functions.[107] In culture, MSCs can differentiate into alveolar epithelium.[107, 108] *In vivo,* MSCs engraft in the lung and produce surfactant,[108] but engraftment rates are low,[109, 110] suggesting MSCs work via paracrine effects. Prophylactic administration of MSCs mitigate lung injury in mice but are ineffective in repairing established lung injury.[110]

Meta-analysis of RCTs in adult diseases indicates MSCs are safe;[111] they do not increase rates of infection, death or malignancy,[111] despite previous concerns about tumourogenicity.[112] Transient fever was noted in trials using MSCs[111] but it is unclear whether this is the consequence of an immune reaction to MSCs.

Clinical studies of MSCs in preterm infants are limited. One trial counted MSCs in BAL and another administered MSCs before BPD diagnosis. The presence of endogenous MSCs within BAL of preterm infants was associated with increased risk of developing BPD.[113] However, the source of MSCs in BAL was unclear, and may be a result of injured lung epithelium. A phase I dose-escalation trial examined administration of MSCs intratracheally to preterm infants at risk of developing BPD (23-29 weeks GA) who required continuous ventilator support.[114] Pro-inflammatory cytokines in BAL were lower at day 7 compared to day 3 after MSC transplantation, highlighting the paracrine effects of the cells. Thirty-three percent of preterm infants enrolled developed BPD. This trial targeted infants before BPD developed, who may or may not have had established lung inflammation. Six patients in the trial developed serious adverse events up to 84 days after MSC transplantation, including pneumothorax, NEC and IVH (< grade 3). The majority of adverse events occurred in infants receiving the highest MSC dose (20 million cells/kg), but there were 6 infants in the high dose MSC group and 3 in the lower MSC dose group, which may have skewed the results.

The origin or handling of MSCs may influence their therapeutic potential. The yield of cord-blood-derived MSCs is low and MSCs need to be expanded for adequate cell numbers for delivery to a patient, but culturing induces ageing.[115] Cultured, MSCs have weaker

immunomodulatory properties *in vitro* compared to primary MSCs,[115] potentially compromising the therapeutic effects of MSCs. Additionally, MSC expansion often requires growth factors (containing animal products),[114] or plating onto a glycoprotein-rich fibronectin matrix.[116] The impact of media, growth factors or matrices used with MSCs, and whether these alter MSC function, must be considered when proposing MSC transplantation in preterm infants.

Human amnion epithelial cells

The amniotic membrane is the innermost placental membrane surrounding the fetus,[117] made up of a single layer of cuboidal columnar epithelial cells.[117] The amnion predominately provides the developing fetus with protection, but also produces growth factors, cytokines, prostaglandins and erythropoietin.[118] The amniotic epithelium is formed before gastrulation, and thus is pluripotent even at term gestation.[117]

Amniotic membranes were first used over a century ago as biological dressings for skin wounds.[119] This practice continues, highlighting the safety of these cells as a therapeutic for human disease.[120] Unlike MSCs, isolation of human amnion epithelial cells (hAECs) from a single placenta yields enough cells for administration to multiple patients. Primary hAECs may be used immediately after isolation, obviating concerns about cell manipulation.

Human AECs do not express telomerase,[121] and have low tumourigenicity.[122] Rejection does not occur when hAECs are administered to humans[123] or other animals,[121, 124] likely due to low HLA class II expression.[124 125] Thus, hAECs can be applied without concerns of tumorigenesis or rejection.

Human AECs reduce lung collagen and fibrosis in mice,[126, 127] up to 14 days after bleomycin insult.[127] Hyperoxia-exposed mice treated with hAECs have increased expression of vascular endothelial growth factor receptor and angiogenin1 in their lungs and this correlates with normalized pulmonary vasculature.[128] Human AEC administration normalises alveolar structure in hyperoxia-exposed mice,[128] LPS-exposed fetal sheep[129] and in fetal and preterm sheep following injurious ventilation.[130, 131] Thus, hAECs prevent BPD-like lung pathology, independent of the model, and this reduction in BPD-like lung pathology is consistently accompanied by reduced lung inflammation.

The immunomodulatory effects of hAECs are demonstrated by their ability to downregulate pro-inflammatory cytokines, IL-6,[130, 132-134] IL-1α and IL-1β,[133] and upregulate anti-inflammatory cytokine IL-10.[131] However, hAECs increase total immune cell numbers in the

lungs of LPS-exposed fetal sheep and mechanically ventilated preterm lambs[129, 131] demonstrating the ability of hAECs' to augment inflammatory cell recruitment but seemingly without proinflammatory effects. IL-10 has a role in activating M2 pro-reparative macrophages and opposing differentiation of pro-inflammatory M1 macrophages[135] and is upregulated following hAEC administration.[131, 134] Indeed, lung and liver macrophage activity shifts from a predominately M1 to M2 phenotype after hAEC administration in bleomycin-exposed mice.[136, 137] Similar to MSCs, hAECs modulate the immune system and prevent BPD-like lung pathology, likely via paracrine effects.

The safety of hAECs has been explored in an unpublished phase I trial of hAECs in infants with intractable BPD (ACTRN: 12614000174684). Subsequent trials are required to determine the appropriate dose of hAECs and when to administer hAECs in preterm infants with BPD.

Final comments and future research

BPD remains a major cause of morbidity and mortality in preterm infants. Historically, therapies like postnatal steroids have been used without appropriate preclinical data regarding safety and long-term outcomes. Pharmacological inhibitors of inflammation, such as IL-1 inhibitors, pentoxifylline and AMP activators, are promising therapies for pulmonary inflammation but are in preclinical stages. Cellular therapies, arguably, have more preclinical evidence surrounding their use for BPD and are approaching RCTs. However, it is unlikely that a one-drug-fix-all approach to BPD will eventuate. Likely, the most beneficial outcomes for infants developing, or who have developed, BPD will be achieved with combined therapies. If an infant is unresponsive to a therapy (e.g. steroids or hAECs) within several days an alternative therapy should be considered. Future studies will need to consider interactions between therapies in preterm infants if tailoring therapies for neonates with BPD is necessary.

Educational aims

The reader will appreciate:

- The incidence of BPD is increasing, despite advancements in clinical care of preterm infants.
- Maturational agents to improve lung architecture, including antenatal glucocorticoids and surfactant therapy, have not improved BPD incidence.
- Anti-inflammatory therapies may be beneficial over maturational agents in preventing BPD incidence.
- Glucocorticoids reduce BPD incidence but have poor neurological outcomes. Glucocorticoid use in the newborn requires significant optimisation.
- Cellular therapies likely modulate lung inflammation associated with BPD, without adverse outcomes.

Future research directions

BPD is a complex disease involving aberrant regulation of lung inflammation. The use of preclinical, translational studies to identify or optimise pre-existing therapies, such as postnatal steroids or IL-1Ra, need to be conducted and compared to less conventional cellular therapies. Future studies should aim to encompass the inflammatory, structural and vascular complications of BPD for the best outcomes in preterm infants.

Summary and project aims

Bronchopulmonary dysplasia is a potentially fatal chronic lung disease that represents the principal cause of morbidity in infants born before 28 weeks of gestation. Survivors of BPD suffer higher-than-normal rates of chronic respiratory illness and adverse neurological outcomes. There is no therapy for BPD.

We chose to investigate the use of postnatal steroids or hAECs following exposure to antenatal inflammation, as antenatal inflammation accounts for up to 70% of preterm deliveries worldwide.

Long-term mechanical ventilation of preterm lambs causes lung injury and alterations in lung development consistent with the pathology of BPD. The preclinical intensive care research unit (PICRU) was recently established for long-term care of preterm lambs, which allows us to manage lambs for a period of weeks, in a way that mimics human neonatal care.

Overall Aim

To investigate the therapeutic effects of tapered low-dose postnatal dexamethasone or postnatal hAECs in preterm lambs that were exposed to antenatal inflammation.

Overall Hypothesis

Postnatal anti-inflammatory therapies, dexamethasone and hAECs, will reduce lung inflammation and injury in preterm lambs following antenatal inflammation.

General Methodology

Human amnion epithelial cell isolation

The Monash Human Ethics Committee, Monash University, approved all procedures for human amnion epithelial cell (hAEC) isolation (ref #: MUHREC-CF13/2144-2013001109). Placentae were obtained from women undergoing term caesarean sections. All women provided written informed consent. Exclusion criteria included: preterm birth, preeclampsia, intrauterine growth restriction (IUGR), clinical chorioamnionitis, active labor or preexisting maternal disease including diabetes. The isolation of term hAECs from placentas was described previously [138]. The amnion was manually peeled from the adjacent coriodecidua and placed into a sterile container with DMEM/F12 media (Gibco, Invitrogen, Australia) supplemented with 1 % penicillin. The amnion underwent 3-5 washes in Hanks Balanced Salt Solution (HBBS; Invitrogen, Vic, Australia) to remove blood. Any remaining blood clots were cut away with sterile scissors. Human AECs were isolated by two, 1-hour digests in 0.05 % trypsin-EDTA (Gibco, Invitrogen, Australia) at 37 °C for 60 minutes, with gentle agitation in a water bath. The supernatant was collected and the Trypsin was inactivated using 5 mL of newborn calf serum (NCS). The supernatant was centrifuged at 1800 rcf for 10 minutes at 21 °C and the pellet of cells was resuspended in DMEM supplemented with 10 % fetal bovine serum (FBS; Gibco, Invitrogen, Vic, Australia). This cell suspension was drained through a 70 µm filter before cell count and viability assay by trypan blue exclusion.

Cryopreservation and thawing of hAECs

For cryopreservation, hAECs were frozen at a cell density of $5x10^6$ cells/mL in FBS supplemented with 5 % dimethyl sulfoxide (DMSO; Sigma Aldrich), stored in cryovials and frozen in freezing containers (MrFrosty, Thermo Fisher Scientific) at -80 °C overnight. Cells were transferred to liquid nitrogen until required.

To thaw, cryovials containing hAECs were taken directly out of liquid nitrogen, and placed into a 37 °C water-bath until thawed. Human AECs were transferred into a falcon tube containing DMEM/F12 supplemented with 10 % FBS to prevent DMSO toxicity. This cell suspension was centrifuged at 500 rcf for 5 minutes at 4 °C and the supernatant discarded.

Preparation of hAECs injections

Cells were resuspended in saline and counted and assessed for viability, first using trypan blue exclusion and then with automated cell counter, for consistency (Bio-Rad TC20 Automated Cell Counter, NSW, Australia). From cell counts, doses of $30x10^6$ cells in 20 mL of saline were made by extracting or adding the appropriate volume of saline to the cells. Cells were strained through a 200 µm filter (Pluristrainer, PluriSelect, Cell Systems Biology, Australia) into a 20mL syringe to ensure the selection of epithelial cells for injection. The syringe was sealed with a cap at the open end and immediately taken to the neonatal bedside. Cells were administered intravenously (IV) at 1 mL/minute. At completion the intravenous line was flushed with 3 mL of saline.

Animals

The University of Western Australia Animal Ethics Committee approved all animal experimentation (RA 3/100/1301 and RA 3/100/1454). Pregnant ewes of known mating date were housed in pens at the University of Western Australia's Large Animal Facility at least one week prior to onset of experiments. Ewes were fed twice daily and had unlimited access to water. They were maintained at room temperature (18-20 °C), with a 12-hour light/dark cycle.

Animal experiments

Pregnant ewes received intramuscular (IM) medroxyprogesterone (150 mg; Pfizer, Australia) 7 days prior to planned caesarean section to avoid subsequent betamethasone-induced preterm labour. Ewes received 2 IM injections of betamethasone (5.7mg/dose; Celestone, Merck Sharp & Dohme Pty Ltd, NSW, Australia) at 48 and 24 hours prior to delivery, equivalent to 50 % of clinical dosing. Pregnant ewes were randomly assigned to receive an intra-amniotic (IA) injection of either LPS (4 mg, 2 mg/mL; *Escherichia coli* 055:B5; Sigma) or equivalent volume of saline, guided by ultrasound, at 126 days' gestational age (GA; term ~150 d).

Ewes were fasted for 24 hours before delivery; access to water was not limited. Ewes were pre-medicated with buprenorphine (0.01 mg/kg, 300 mcg/mL; Indivior, Pty, Ltd, NSW, Australia) and acepromazine (0.02 mg/kg, 2mg/mL; Ceva Animal Health Australia, NSW, Australia) 1 h prior to IV induction of anesthesia (15 mg/kg sodium thiopental; Troy

Laboratories, NSW, Australia). Inhalational isoflurane (2-3 %; Henry Schein, Qld, Australia) was initiated after endotracheal intubation, and delivered using positive pressure ventilation (General Electric Healthcare Datex-Ohmeda A-AUF, Carestation Anesthesia Machine, Chicago, Illinois, US).

Caesarean Delivery

The ewe was placed in supine position in preparation for aseptic surgery and the abdomen was shorn and cleaned with chlorhexidine gluconate (0.5 % w/v in 70 % ethanol; Johnson & Johnson, Australia), 3 washes of betadine surgical scrub (7.5 % w/v povidine-iodine; Fauldings, Australia) and application of betadine antiseptic solution (10 % w/v povidine-iodine; Fauldings, Australia).

Surgical drapes, towels, gowns and instruments were sterilized prior to surgery using autoclave. Fetal catheters were sterilized by exposure to ethylene oxide. Surgeons scrubbed their fingers, hands and arms with chlorhexidine gluconate and wore hair nets, masks, sterile surgical gowns and sterile latex gloves.

Ligonocaine (20 mg/mL, Troy Laboratories, NSW, Australia) was injected subcutaneously prior to making a midline incision through the skin of the ewes' abdomen down to the linea alba. The peritoneum was then incised, exposing the abdominal cavity so the uterus could be accessed. The fetal head and neck were located within the uterus through palpitation. The uterus was incised and the fetal head and neck were exteriorised for catheterization of fetal blood vessels

An incision was made on the right side of the fetal neck for the placement of two polyurethane catheters (Argyle™ ID 0.86 mm, OD 1.52 mm; Medtronic, NSW, Australia) filled with heparinised saline into the carotid artery and jugular vein, for blood gas sampling and drug administration, respectively. The incision at the fetal neck was sutured closed. The fetus was intubated with a 3.5 mm endotracheal tube (Portex®, Smiths Medical, Qld, Australia) and excess lung liquid was drained before administration of surfactant (3 mL, 80 mg/mL, poractant alfa; Chiesi Farmaceutici, S.p.A., Italy) via a feeding tube passed down the tracheal tube.

The umbilical cord was clamped and cut and the lamb was weighed, dried and placed prone, suspended in a veterinary sling in a neonatal baby warmer. The lamb received a sustained inflation at 30 cmH_2O for 30 seconds before the initiation of ventilation (see *respiratory management*), consistent with previous ventilation studies in preterm lambs. A sustained

inflation is required to recruit the lungs of preterm lambs, who otherwise have difficulty weaning.[139] Body temperature was maintained between 38 °C and 39 °C. Cord blood was collected for fetal blood gas measurements, along with plasma and full blood counts. Heparinised maternal blood was retained for lamb transfusion, if needed. The ewe was killed immediately after the lamb was delivered (150 mg/kg pentobarbitone; Valobarb, Jurox, Rutherford, Australia).

Postnatal interventions

We randomly administered all postnatal interventions to preterm lambs.

Saline vehicle in preterm lambs

Separate groups of animals received antenatal IA LPS and postnatal saline (LPS/Sal) or antenatal and postnatal saline (Sal/Sal) at equivalent volumes (Table 1).

Postnatal treatment interventions

Postnatal anti-inflammatory interventions in this thesis were either IV dexamethasone or IV hAECs, which were analysed as separate studies.

Dexamethasone treatment of preterm lambs, following exposure to antenatal LPS

Dexamethasone was only administered to preterm lambs that were exposed to antenatal LPS (LPS/Dex). A tapered low-dose of dexamethasone (dexamethasone sodium phosphate; Alphapharm Pty Ltd, NSW, Australia) was administered IV over 7 days (0.15 mg/kg/day for 3 days, 0.1 mg/kg/day for 2 days and 0.05 mg/kg/day for 2 days: total 0.75 mg/kg), beginning immediately after delivery. Dexamethasone doses were modeled from the tapered low-dose DART randomised controlled trial [67].

Human AEC treatment of preterm lambs, following exposure to antenatal LPS

Prophylactic IV hAECs were administered as a single bolus to preterm lambs exposed to antenatal LPS (LPS/hAECs) within 2 hours of birth. hAECs were suspended in 20 mL of saline and delivered at a rate of 1 mL per minute. Our decision to deliver hAECs IV is based on reports showing no difference in IV or intratracheal (IT) administration of hAECs in sheep[129] and takes into consideration IV administration of hAECs to preterm infants in a phase I safety trail.[140]

Table 1. Experimental groups and interventions

Treatments	Antenatal Intervention	Postnatal Intervention	Number
Sal/Sal	Saline	Saline	10
LPS/Sal	Lipopolysaccharide	Saline	10
LPS/Dex	Lipopolysaccharide	Dexamethasone	9
LPS/hAECs	Lipopolysaccharide	Human amnion epithelial cells	7

Sal, Saline; LPS, lipopolysaccharide; Dex, dexamethasone; hAECs, human amnion epithelial cells

Respiratory Management

Ventilated lambs were managed in accordance with best clinical practice. Over the 7-day experimental period, lambs received graded de-escalation of respiratory support aimed at early weaning from mechanical ventilation (Babylog VN500 Ventilator, Dräger Medical, Lübeck, Germany) onto non-invasive forms of respiratory support. The general pattern involved: mechanical ventilation, then endotracheal bubble continuous positive airway pressure (B-CPAP) by 3-4 hours after birth, then extubation onto nasally delivered heated humidified high flow (HHF; Fisher Paykel Healthcre, Auckland, NZ) or CPAP via nasal cannulae and eventual unassisted breathing of room air. We commenced ventilation with volume guarantee (5-7 mL/kg), a fraction of inspired oxygen (FiO_2) of 0.3, peak inspiratory pressure (PIP) of 30 cmH_2O, positive end expiratory pressure (PEEP) of 9 cmH_2O and ventilator rate of 50 breaths per minute (breaths/min). Ventilatory settings; PEEP, FiO_2, inspiratory time and rate, ventilation rate and mean airway pressure (MAP) were adjusted with each type of respiratory support (where possible) to target oxygen saturation (SpO_2) of 90-95 % at the lowest achievable FiO_2. Amplitude during non-invasive nCPAP and nHHF was adjusted to maintain $PaCO_2$ of 45-55 mmHg. FiO_2 and MAP were adjusted to maintain SpO_2 of 90-95 %.

Lambs were reintubated if they experienced more than one of the following: 1) ventilatory failure ($PaCO_2$ > 80 mmHg on 2 consecutive arterial gases > 30 min apart, unresponsive to altered nasal amplitude and frequency settings); 2) severe metabolic acidosis; 3) > 4 apneas

requiring resuscitation within an hour, or 4) persistent SpO_2 < 80 % despite FiO_2 > 0.8 and increased mean airway pressure to a maximum 12 cmH_2O.

General postnatal management

Lambs received daily IV antibiotics twice daily (piperacillin/tazobactam 100 mg/kg, 100 mg/mL, Sandoz Pty Ltd, Australia and gentamicin 6 mg/kg, 100 mg/mL Troy Laboratories, Australia). Caffeine (WAMF, Perth, WA, Australia) was administered IV as a loading dose (20 mg/kg) then daily as required (5mg/kg over ~10 minutes), in accordance with routine neonatal ICU protocol.

Arterial blood samples from the carotid arterial catheter were obtained every 30 minutes for the first hour of life, every hour for the next 4 hours of life, and 4-6 hours thereafter. If the lamb was considered to be stable and on minimal respiratory support, blood gas samples were taken 6-12 hourly. Arterial blood gas samples were used to aid clinical decisions regarding ventilation, fluid management and general lamb well-being.

General observations were taken every hour for the duration of experiments. Figure 2 shows an observation sheet outlining the parameters recorded each hour.

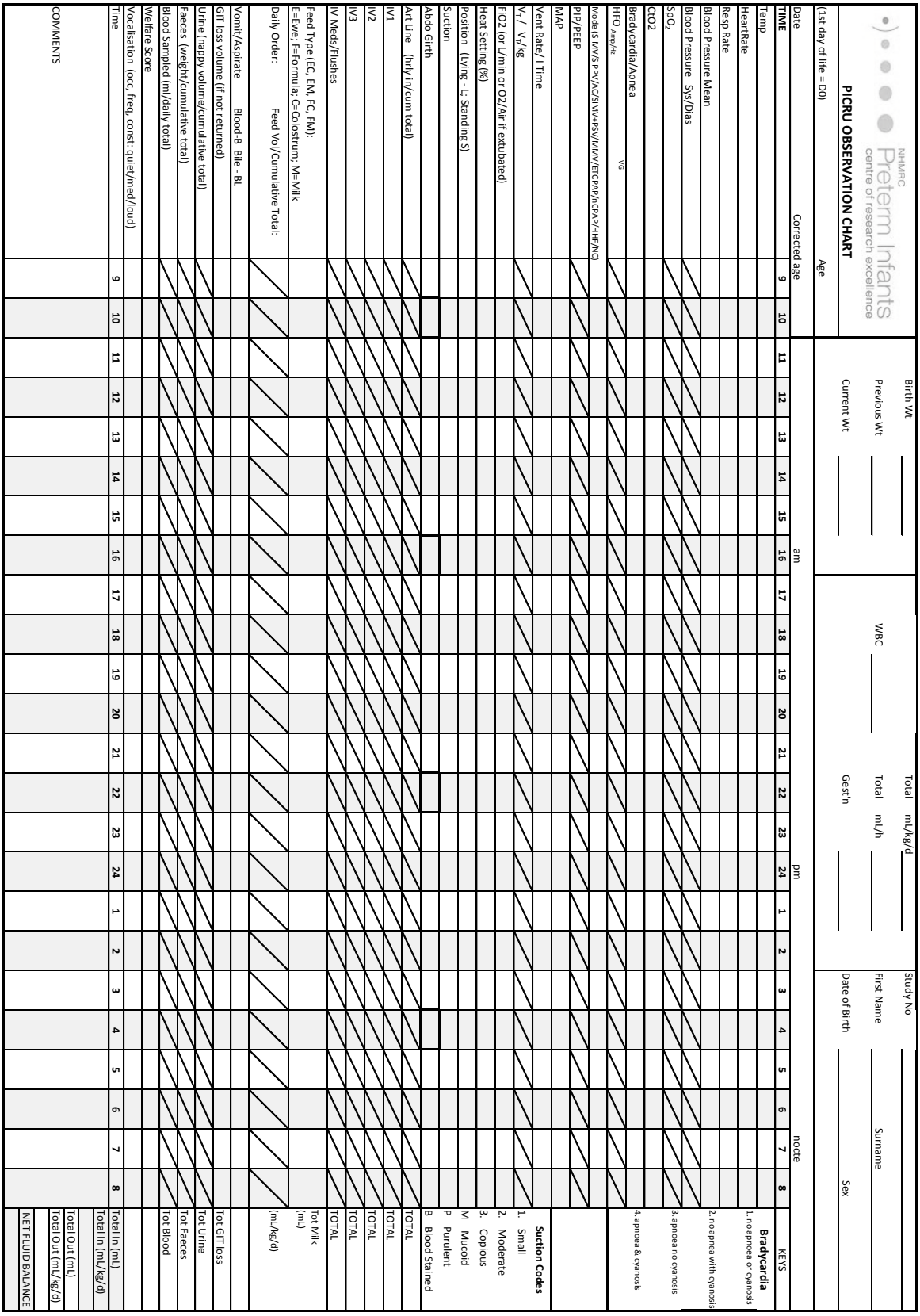
Lambs received increasing aliquots of enteral feeds (colostrum from ewe) commencing at 2 hours after birth, given at 2-hour intervals for 24 hours, followed by 50:50 feeds (ewe colostrum:lamb formula; Maxcare®, Qld, Australia). With time, lambs were encouraged to suckle and feed from a syringe or bottle. Feeds increased by 1 mL every 6 hours for the first 72 hours, then by 1 mL every 4 hours thereafter.

Cerebral tissue oximetry (Fore-sight absolute tissue oximeter, CAS Medical Systems Inc., CT, USA) was recorded hourly. Urine and stool outputs were recorded over the 7 days of postnatal life.

All observations were recorded by individuals blinded to the treatment interventions of preterm lambs. Hourly observations were entered and managed using REDCap electronic data capture tools (hosted at The University of Western Australia, Perth, Australia). REDCap (Research Electronic Data Capture) is a secure, web-based application designed to support data capture for research studies, providing: 1) an intuitive interface for validated data entry; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures

for importing data from external sources (REDCap, version 7.6.10 © Vanderbily University, Nashville, Tennessee).

Figure 2. Perinatal intensive care research unit (PICRU) hourly observation sheet



Post Mortem

On day 7 of life, ~20 mL of blood was collected in a heparinised sterile syringe just before completion of experiments and centrifuged at 4100 rpm for 15 minutes at 4 ºC for collection of plasma. Lambs were then killed with an overdose of sodium pentobarbitone (150 mg/kg; Jurox Pty Ltd, NSW, Australia).

The left lung was inflation-fixed at 30 cmH_2O with 10 % formaldehyde (FA) for morphometric and histological analysis. Static compliance was calculated as the volume of fixative infused at 30 cmH_2O divided by the birth weight. The right middle lobe of the lung was lavaged five times with 10 mL of PBS per lavage (i.e. total 50 mL). The supernatant from the bronchoalveolar lavage fluid (BALF) was discarded and BALF cell pellet was resuspended and stored at -80 ºC until required. Subpleural segments of the right lower lobe of the lung were collected and snap frozen in liquid nitrogen and stored at -80 °C until required for molecular analysis. Small segments from the midline of the liver were snap frozen in liquid nitrogen and stored at -80 ºC until required for subsequent molecular analyses.

Histological Analyses

The left lung was cut into 5-mm slices and three random sections of fixed left lung were embedded in paraffin. Embedded lung tissue was cut at 5 µm and transferred into a 40 ºC water bath before mounting onto glass slides (Superfrost plus+, Menzel Glaser, Saabruckner, Germany) and baked in a 60 ºC oven for one hour. Five regions of interest (ROI) per section, were used for histological analyses. A total of 15 ROIs were analysed per animal. We avoided any large vessels or airspaces when selecting ROIs for analyses.

Hart's elastin stain

Hart's resorcin-fuschin elastin stain was used to identify tissue and airspace fractions, elastin fibres present on secondary septa of alveoli, and total elastin content. Paraffin-embedded sections of lung tissue were de-waxed with xylene (2 x 5 minutes), rehydrated with absolute ethanol (3 x 3 minutes), rinsed in tap water (3 x 30 seconds), immersed in 0.25 % potassium permanganate (5 minutes) and rinsed in distilled water (2 x 30 seconds). Tissue was briefly submerged in 5 % oxalic acid to dissolve potassium permanganate and then rinsed in tap water. Sections were stained with Hart's resorcin-fuschin stain for elastin, incubated for 6

hours, rinsed in tap water (3 x 30 seconds) and counterstained with 0.25 % tartrazine in saturated picric acid (3 minutes). Sections were dehydrated with xylene (2 x 5 minutes) and mounted in disyrene plasticiser xylene (DPX; Brittish Drug House Chemicals, United Kingdom). Hart's elastin stain shows elastic fibres in black and tissue in yellow.

Quantification of Hart's elastin stain

Five random ROIs per section of fixed lung were captured at X20 magnification and used to quantify tissue and airspace fraction and septal crest density. A linear point counting grid was overlaid onto each ROI. Points placed 10 pixels apart, with a total 676 points per ROI. Points that overlaid tissue, air and septal crests were identified using a partly automated ImageJ plugin (Copyright © 2015, Keith Schulze, Monash Micro Imaging, Monash University; NIH Image). The plugin automatically detects points that fall on tissue in green and points that fall on airspace in grey, based on yellow and white colour, respectively. Points falling on septal crests are manually counted.

The points falling on lung tissue or airspace were divided by the total points (676) in the ROI, so as to express the areal fraction of tissue or airspace in that ROI. Septal crest areal fraction was determined by number of points falling on septal crests divided by total points falling on tissue. The mean tissue, airspace and septal crest areal fractions were calculated for each animal.

Total elastin deposition was calculated with a separate automated ImageJ plugin (Copyright © 2015, Keith Schulze, Monash Micro Imaging, Monash University) which created two images, one isolating total tissue from background, and one separating elastin from tissue and background, based on the black colour of elastin after staining with Harts resorcin-fuschin. We selected a threshold to identify black-stained elastin by initially trialing the ImageJ plugin on 15 ROI from each treatment group. Once satisfied with the ability of the macro to detect elastin content separately to tissue content all ROIs were run at the set threshold in one analysis. The area of total elastin was divided by the total area of tissue to measure the proportion of elastin deposition in the lungs.

Haematoxylin and eosin stain

Haematoxylin and eosin (H&E) staining was used to asses lung injury. Paraffin-embedded sections of lung tissue were de-waxed with xylene (2 x 5 minutes), rehydrated with absolute ethanol (3 x 3 minutes) and rinsed in tap water (3 x 30 seconds). Sections were stained with Meyer's haematoxylin for 3 minutes and washed in running tap water for 3 minutes. Sections

were dipped into acid ethanol 2-3 times and washed in running tap water for 3 minutes before being dipped briefly into ammonia water until colour changed from a strong purple to blue. Sections were washed with running tap water (3 x 30 seconds) and counterstained with eosin (10 dips) before being dehydrated with xylene (2 x 5 minutes) and mounted in DPX. Haematoxylin stains tissue in pink, nuclei in purple/blue colour and muscle fibres a deep red.

Quantification of H&E stain

Lung injury scoring was described previously.[38] Briefly, ROIs were scored between 0 and 4, were 0 is none and 4 is severe, for wall thickness and epithelial sloughing. A rough guide for scoring wall thickness can be seen in Figure 3. For epithelial sloughing we scored, per ROI: no events = 0, <5 events as 0.5, 5-10 events as 1-2, 10-20 events as 2.5-3 and >20 events as 3.5-4.

Picrosirus red stain

Lung sections were stained with picrosirus red for detection of type I and III collagen fibers. Paraffin-embedded sections were de-waxed with a series of xylene and ethanol washes, as per H&E staining protocol. The nuclei of sections were stained with Weigert's haematoxylin for 8 minutes then washed in running tap water for 10 minutes and placed in 0.2 % Molybdophosphoric acid for 2 minutes. Sections were stained with picrosirus red for one hour and washed in acidified water (0.5 % acetic acid in dH_2O; 2 x 2 minutes). Sections were dehydrated with a series of ethanol and xylene washes, as per H&E staining protocol, and mounted in DPX. Picrosirus red stains collagen red and tissue yellow.

Analyses of picrosirus red stain

The birefringence of collagen was visualized using a Lecia Abrio polarizing microscope (512 X 512 CCF black and white camera controlled by CRI Abrio software). Images were analysed using the image-processing package Fiji. Total area of collagen within each image was calculated and normalised to the area of tissue using an automated ImageJ plugin (Copyright © 2015, Keith Schulze, Monash Micro Imaging, Monash University; NIH Image).

Immunohistochemistry

In general, sections underwent dewaxing and rehydration steps, as per H&E protocol. Antigen retrieval with citrate buffer (pH 6) was used to recover antigen immunoreactivity followed by 3 x 5 minute washes with PBS. Endogenous peroxidases were blocked with 3% hydrogen peroxide (H_2O_2) diluted in distilled H_2O (dH_2O), followed by an additional 3 washes

in PBS. Non-specific protein binding was blocked for 30 minutes using serum raised in the same species as the secondary antibody (e.g. normal rabbit serum (NRS) or normal goat serum (NGS)) in conjunction with bovine serum albumin (BSA). The primary antibody was immediately applied and incubated overnight at 4^{o}C. Specific staining of the primary antibody was confirmed through the omission of the primary antibody.

On the second day, the primary antibody was removed with 3 x 5-minute washes with PBS. The secondary antibody was applied and slides were incubated for 60 minutes at room temperature before undergoing another 3 x 5-minute washes with PBS. Slides were incubated with an ABC kit for 30-45 minutes at room temperature and washed for 3 x 5 minutes with PBS. DAB (1 DAB tablet dissolved in 10 mL of dH_2O and 3 µL of 30% H_2O_2) was applied to each section for 1–5 minutes, depending on the antibody, and stopped once brown staining was visible under a microscope. Sections were counterstained with haematoxylin, rehydrated and cover-slipped as per H&E protocol.

Quantification of immunohistochemistry

All sections were scanned using ImageScope (Aperio Technologies, California, USA). Sections of tissue stained for CD45, CD163, Ki67 and SP-C were analysed using ImageJ (NIH Image). Ki67-positive cells are represented as areal density of tissue by counting total cells and positive cells. The number of CD45+, CD163+ and SP-C+ cells were counted and averaged for the number of ROIs per animal. Positive stained is expressed as the average number of cells per animal.

Sections stained for α smooth muscle actin (αSMA) were analysed using ImagePro Plus (version 9.2, Build 6156, 2012-2015, Media Cybernetic, Silver Spring, MD). For each ROI the area of tissue stained for αSMA was expressed as a percentage of the total area of tissue in that ROI. All sections were analysed on sections of lung which excluded major airways or blood vessels. The % αSMA = area of total stain/by total area of tissue x 100.

Table 2. Antibodies, dilutions indications and alterations to immunohistochemistry in paraffin-embedded lamb lung sections

Antibody	Cell detected	Primary antibody [dilution]	Secondary antibody [dilution]	Alterations
CD45	Leukocytes	Mouse anti-CD45, Bio Rad [1:100]	Goat anti-mouse, biotinylates IgG, Vector Laboratories [1:500]	None
CD163	Macrophages	Mouse anti-CD163, Acris Antibodies [1:2000]	Goat anti-mouse, biotinylates IgG, Vector Laboratories [1:200]	None
Ki67	Proliferating cells in late G1-, S-, M- and G2-phases of the cell cycle	Rabbit anti-human, ThermoFisher Scientific [1:1000]	Goat anti-rabbit, biotinylates IgG, Vector Laboratories [1:200]	None
α smooth muscle actin	Myofibroblasts	Mouse anti-human, Dako [1:800]	Goat anti-mouse, biotinylates IgG, Vector Laboratories [1:400]	Antigen retrieval: 10mmol/L Tris, 1 mmol/L EDTA, pH9 Block: 10%BSA, 0.1%Triton Primary: incubated in humid chamber (30min)
SP-C	Type II alveolar epithelial cells	Rabbit anti-SP-C [1:2000]	Goat anti-rabbit, biotinylates IgG, Vector Laboratories [1:200]	No antigen retrieval

IgG = immunoglobulin G; Tris = trisaminomethane; EDTA = ethylenediaminetetraacetic acid; SP = surfactant protein

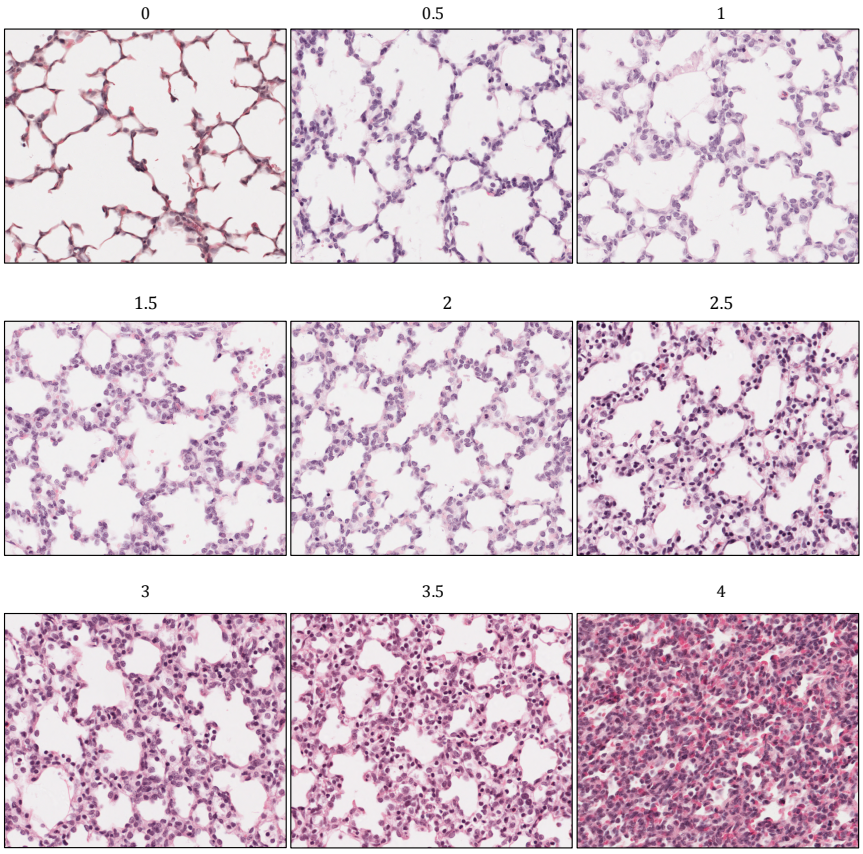


Figure 3. Representative images used as a guide when scoring lung wall thickness. Lung sections are stained with haematoxylin and eosin. The number on top of each image represents an injury score.

Molecular Analyses

RNA extraction

Snap frozen pieces of lung and liver were weighed and homogenised (Ultra Turraz T-25; Janke & Kunkel, IKA-Laboritechnik, Germany) with 1 % β-mercaptoethanol RLT Buffer (RNeasy Kit, Qiagen, USA). Tissue lysate was centrifuged for 10 minutes at 4200 x g and the supernatant was carefully transferred into a new tube. Ethanol (70 %) was added to the supernatant and shaken vigorously and immediately applied to a RNeasy column for centrifugation for 5 minutes at 4200 x g; flow through was discarded. DNase treatment was carried out in the same column to avoid any DNA contamination in the RNA sample. RW1 buffer was applied to the column before centrifugation for 3 minutes at 4200 x g; flow through was discarded. DNase mix was applied to the column and incubated for 15 minutes at room temperature before addition of another volume of RW1 buffer and incubation for a further 1 minute at room temperature. The samples were then centrifuged for 5 minutes at 4200 x g; flow through was discarded. Two washes with RPE buffer followed and flow through was discarded. The sample was then eluted twice in RNase-free water with centrifugation for 3 minutes at 4200 x g. The flow through then contained RNA. RNA concentration was determined using spectrophotometry (NanoPhotometer® N50; Germany). Integrity of RNA was determined by gel electrophoresis and RNA was stored at -80 ºC.

For gel electrophoresis, 2 µL of RNase-free water and 2 µL of RNA sample were combined and heated at 65 ºC for 5 minutes to denature RNA. Loading buffer (2 µL; Bromophenol Blue, Xylene cyanol FF, ThermoFisher) was added to each sample before it was loaded into wells of a 1 % agarose gel (0.5 g agarose; Agarose, Scientifix, France). Gel electrophoresis was performed for 30-45 minutes at 90 V, or until bands were sufficiently separated. The gel was visualised and photographed using an ultraviolet transilluminator (Molecular Imager ChemiDox XRS System, USA). Samples were considered sufficient if they showed 28S and 18S ribosomal RNA bands and sometimes a fainter 5S band, with no smearing or other bands present.

Complimentary DNA synthesis

Complimentary DNA (cDNA) was transcribed from RNA using the Superscript III reverse transcription kit (Invitrogen, Australia) with provided reagents. RNA was diluted with RNase free water, 1 µL of random primers and 1 µL of dNTPs (single unites of DNA; 10 mM) and

heated for 5 minutes at 65ºC to denature RNA. Reverse transcription was completed by addition of 4 µL of 5 x First-Strand buffer, 1 µL of dithiothreitol (DDT; a reducing reagent), 1 µL of RNaseOUT (recombinant RNase inhibitor) and 1 µL of Superscript III Reverse Transcriptase to each sample. The combined mixture was incubated at room temperature for 5 minutes, then at 50 ºC for 60 minutes, then was heat-activated at 70 ºC for 15 minutes and at 4 ºC until stored at -20 ºC.

Fluidigm® Taqman® RT-PCR gene expression assay

TaqMan probes were obtained from ThermoFisher or if unavailable were designed with help from the ThermoFisher technical team using NCBI BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast); TaqMan probes IL-8 and SP-D were designed. A table of the probes used in this thesis are outlined in Table 3.

TaqMan RT-PCR assays are outlined elsewhere.[141] Initially, a quality control (QC) report was completed in order to determine the integrity of cDNA. During the QC, samples were considered to be clean (no gDNA contamination) if Ct values were above 30. The samples then underwent a pre-amplification phase, where 3.75 µL of Sample Pre Mix (Life Technologies TaqMan® PreAmp Master Mix and Pooled Taqman assays) was combined with 1.25 µL of each cDNA sample. All samples were pre-amplified over 14 cycles. Following pre-amplification, samples were diluted 1:5 by the addition of 20 µL Tris EDTA buffer pH 8.0 to the final 5 µL volume for a total volume of 25 µL.

For completion of the full Fluidigm® TaqMan® gene expression assay, each target gene was run in triplicate cDNA samples on an ABI Prism 7900HT Real-Time PCR System (PE Applied Biosystems). PCR parameters were: 50 ºC for 2 minutes, 95 ºC for 10 minutes, 40 cycles of 95 ºC for 15 seconds and 60 ºC for 1 minute. Two RT-negative samples were prepared by exclusion of Superscript III Reverse Transctiptase during the formation of cDNA. This RT-negative sample was included to test for genomic DNA contamination within the samples.

Fluidigm® Taqman® gene expression analysis

Ct values were generated for each cDNA sample and provided by the MHTP Medical Genomics Facility. We analysed Ct values using qBase+ software, version 3.1.[142] We selected reference gene ribosomal (r)18S using qBase+ geNorm algorithm for assessing stability of reference genes and optimal number of reference genes.[143] The expression of all genes are expressed as calibrated normalized relative quantity (CNRQ; i.e. normalized to r18S and expressed relative to Sal/Sal) where Sal/Sal values are normalized to ~0.00.

Table 3. Probe selection for Taqman® Assays

Gene	Assay ID
r18S	Oa4906333_g1
IL-1α	Oa04658682_m1
IL-1β	Oa04656322_m1
IL-4	Oa04927178_s1
IL-6	Oa04656315_m1
IL-8	CUSTOM
IL-10	Oa03212724_m1
TGF-β1	Oa04259484_m1
TNF	Oa04656867_g1
Surfactant protein A1	Oa04657758_m1
Surfactant protein B	Oa04750908_g1
Surfactant protein C	Oa04656841_m1
Surfactant protein D	CUSTOM
VEGF-A	Oa04653812_m1
VEGFR1	Oa04694159_m1
Angiopoeitin-1	Oa04757067_m1
EGR1	Oa03237885_m1
CYR61	Oa04673852_g1
CTGF	Oa04659069_g1
PECAM	Oa04677168_m1
MMP9	Oa03215996_g1
MMP12	Oa04744924_g1
CCL2	Oa04677078_m1
SAA	Oa04924154_s1
Hepcidin	Oa04656982_m1

IL = interleukin; TGF = transforming growth factor; VEGF = vascular endothelial growth factor; EGR = early growth response; CYR = cysteine-rich; CTGF = connective tissue growth factor; PECAM = platelet endothelial cell adhesion molecule; MMP = matrix metallopeptidase; CCL = chemokine ligand; SAA = serum amyloid A.

Statistical Analyses

Statistical analyses differed between chapters. For chapters 3 and 5, complete data sets were obtained utilising sequential regression modelling to impute missing physiological data points (<5 %). All serial physiological data were analysed using a 2-way repeated measures (RM) ANCOVA (SPSS, IBM). Influential covariates were identified using Pearson's correlation (SPSS, IBM). Covariates varied between tests, but consistently significantly correlated with the dependent variable. The covariates used are outlined in the results of each experimental chapter. Post-hoc comparisons were made using the Holm-Sidak method.

Umbilical cord blood gas samples were analysed separately to serial ABGs after birth, using Kruskal-Walis with Dunn's multiple comparison post hoc test (GraphPad Prism v.7).

All histological and molecular data are expressed as mean ± SD. Histological data were compared between groups by Kruskal-Wallis and Dunn's multiple comparisons post-hoc test. Molecular data are expressed as mean ± SD, relative to the Sal/Sal group and analysed using qBase+ software, version 3.1.

For chapter 4, apoptotic data were analysed using 2-way ANOVA with Holm-Sidak's multiple comparison test. Wound healing assay and phagocytosis assay were analysed using one-way ANOVA with Tukey's post hoc test.

There is slight variability in animal number between different types of analyses (i.e. physiological versus histological) due to incomplete data collection in some treatment groups.

Declaration for Thesis Chapter 3: The effect of postnatal steroids on lung development and inflammation in preterm lambs exposed to antenatal inflammation

Declaration by candidate

Nature of the contribution	Extent of contribution
Performed experiments, collected data, analysed data and drafted manuscript	70 %

The following authors contributed to the work. If the co-authors are students at Monash University their contribution in percentage is stated:

Nature of the contribution		Extent of contribution
Peter B. Noble	Conceived the idea, obtained funding for the work and drafted manuscript	N/A
Andy W. Gill	Conceived the idea, obtained funding for the work and drafted manuscript	N/A
Siavash Ahmadi-Noorbakhsh	Conducted experiments	N/A
Tim J. Moss	Contributed to data analyses, interpretation and and drafted manuscript	N/A
J. Jane Pillow	Conceived the idea and obtained funding for the work. Participated in design and performed the experiments and drafted manuscript	N/A

Chapter Three

Introduction

Bronchopulmonary dysplasia (BPD) is a chronic inflammatory lung disease, most common in preterm infants born before 28 weeks gestational age (GA).[2] Clinically, BPD is defined as the persistent requirement for respiratory support beyond 28 days of life.[144]

The pathogenesis of BPD is not completely understood, but antenatal inflammation and mechanical ventilation (MV) are common antecedents. Antenatal inflammation, manifest as chorioamnionitis, is present in more than 70 % of infants who subsequently develop BPD.[18] Antenatal inflammation reduces the risk of neonatal RDS but the likelihood of developing BPD increases when antenatal inflammation is coupled with a secondary inflammatory insult, such as MV.[18] Prevention or attenuation of lung inflammation in response to chorioamnionitis and mechanical ventilation is considered a realistic therapeutic approach for BPD.[145]

Postnatal dexamethasone for ventilator-dependent preterm infants facilitates extubation and reduces lung inflammation,[55] potentially reducing the likelihood of BPD. However, the use of postnatal dexamethasone is controversial. Initial studies using postnatal high-dose dexamethasone therapy focussed on immediate respiratory outcomes at the expense of neurological outcomes. Later, long-term follow-up studies showed high-dose dexamethasone (~0.5 mg/kg/day or ~8 mg/kg over 42 days) is associated with adverse neurological outcomes and increased incidence of cerebral palsy.[51, 53, 146] Now, high-dose postnatal dexamethasone is not recommended for preventing or treating BPD,[147] causing ambiguity regarding optimal steroid dosing regimens. Thus, there is hesitation around postnatal dexamethasone use in the preterm population.

The DART trial conducted by Doyle and colleagues[67] aimed to administer a low-tapered dose of postnatal dexamethasone in low-birth-weight, ventilator-dependent preterm infants in the first week of life, hoping to reduce BPD incidence. Unfortunately the DART trial was statistically underpowered because of a 10 % recruitment rate. Infants receiving dexamethasone in the DART trial tended to spend less time intubated on MV but, because of low statistical power, this finding is not definitive.

Traditional animal experiments modelling BPD utilise hyperoxia or injurious ventilation strategies, which result in lung fibrosis dissimilar to the pathology of contemporary BPD. The lungs of infants who have died from contemporary BPD are characterised by simplified lung architecture, with larger airspaces, thinner lung tissue and a lack of septation (alveolar hypoplasia).[10, 15] We utilise preterm lambs to investigate the multifactorial effects of prenatal inflammation, intensive care protocols and respiratory support in BPD development. We exposed fetal sheep to inflammation and then treated a subgroup of preterm lambs with a low-tapered dose of dexamethasone. We hypothesised dexamethasone would reduce ventilator requirements and prevent inflammation and BPD-like lung pathology, following antenatal inflammation.

Methods

The University of Western Australia Animal Ethics Committee approved all animal experimentation. Studies were conducted in accordance with RA 3/100/1301.

Antenatal interventions

Pregnant ewes received intramuscular (IM) injections of medroxyprogesterone (150 mg, Pfizer, Australia) 7 days prior to planned induction of labour, to avoid subsequent betamethasone-induced preterm labour. Ewes received two IM injections of betamethasone (5.7 mg/dose; Celestone, Merck Sharp & Dohme Pty Ltd, Australia) at 48 h and 24 h prior to planned Caesarean section delivery. Pregnant ewes were randomly assigned to receive ultrasound guided intra-amniotic (IA) lipopolysaccharide (4 mg, 2 mg/mL LPS; *Escherichia coli,* 055:B5; Sigma-Aldrich, NSW, Australia; n=10) or IA saline (n=10), at 126 days gestational age (GA; term ~150 d). This dose of LPS results in a well characterised fetal response, which peaks 48 h after injection.[148]

Preterm delivery

Pregnant ewes at 128 d GA were premedicated with IM buprenorphine (0.01 mg/kg, 300 mcg/mL, Indivor, Pty Ltd, Australia) 1 h prior to anaesthetic induction (IV, 15 mg/kg sodium thiopental; Troy Laboratories, NSW, Australia) and inhalational anaesthesia (2-3 % Isoflurane; Bomac Animal Health, Australia). The fetal head and neck were exposed via maternal laparotomy and hysterotomy. The right carotid artery and right jugular vein of the fetus were catheterised for later blood gas sampling and drug administration, respectively.

The fetus was intubated and excess lung liquid was drained passively before administration of surfactant (3 mL, 80 mg/mL, proactant alfa, Chiesi Farmaceutici S.pA., Italy) through the tracheal tube. The umbilical cord was clamped and cut. The lamb was weighed, dried and placed in ventral recumbency in a sling on a neonatal baby warmer. Cord blood was collected for a baseline blood gas measurement. The lamb received a sustained inflation (30 cmH_2O for 30 s) before initiation of ventilation. Body temperature was maintained between 38 ºC and 39 ºC. The ewe was killed immediately after the lamb was delivered (150 mg/kg pentobarbitone; Valobarb, Jurox, Rutherford, Australia).

Postnatal interventions

Lambs exposed to IA LPS randomly received tapered twice-daily intravenous (IV) dexamethasone (LPS/Dex; n=9) or equivalent volumes of saline (LPS/Sal; n=10), beginning immediately after birth. The postnatal dexamethasone dose (0.15 mg/kg/day for 3 days, 0.1 mg/kg/day for 2 days and 0.05 mg/kg/day for 2 days) was modified from the DART trial.[67] Lambs exposed to IA saline received IV saline (Sal/Sal; n=9) as a control.

Respiratory support

Lambs received graded respiratory support and ventilation (Evita® Infiity® V500 Ventilator, Dräger) in accordance with best clinical practice, beginning with mechanical ventilation (MV), then bubble continuous positive airway pressure (B-CPAP), extubation onto heated humidified high flow (HHF) via nasal (n) cannulae and eventual unassisted breathing of room air. MV was initiated with volume guarantee (5-7 mL/kg), a fraction of inspired oxygen (FiO_2) of 0.3, peak inspiratory pressure (PIP) of 30 cmH_2O, positive end-expiratory pressure (PEEP) of 9 cmH_2O and ventilator rate of 50 breaths/min. Ventilator adjustments were determined by clinical examination and arterial blood gas measurements. Ventilator settings were adjusted (where possible) to target oxygen saturation (SpO_2) of 90-95 % at the lowest achievable FiO_2. We attempted rapid weaning from MV, allowing permissive hypercapnia. Amplitude during nCPAP and nHHF was adjusted to maintain $PaCO_2$ in the range of 45-55 mmHg. Lambs remained on nCPAP or nHHF if they displayed sufficient respiratory drive, had minimal oxygen requirements and acceptable arterial blood gas levels. Lambs were reintubated if they experienced one or more of the following: 1) ventilatory failure ($PaCO_2$ > 80 mmHg on 2 consecutive occasions > 30 min apart and unresponsive to altered nasal pressure amplitude and frequency settings); 2) severe metabolic acidosis; 3) > 4 apnoeas requiring resuscitation within an hour, or 4) persistent SpO_2 < 80 %.

PaO_2 to FiO_2 ratio (pF) and oxygen index (OI = [(MAP x FiO_2 x 100)] / [(PaO_2 x 1.36)) were

calculated daily to assess pulmonary gas exchange.

General postnatal management

Lambs received IV antibiotics twice-daily (piperacillin/tazobactam 100 mg/kg,100 mg/mL; Sandoz Pty Ltd, Australia and gentamicin 6 mg/kg, 100 mg/mL Troy Laboratories, Australia). Caffeine (WAMF, Australia) was administered as an IV loading dose (20 mg/kg) then daily as required (5 mg/kg) in accordance with routine neonatal intensive care protocol.

Arterial blood gases (ABGs) were measured every 30 minutes for the first hour of life, every hour for the next 4 hours and 4-6 hourly thereafter. ABGs were taken 6-12 hourly if lambs were considered stable and on minimal respiratory support.

Lambs received increasing aliquots of enteral feeds (colostrum from ewe) commencing at 2 h, given at 2 h intervals for 24 h followed by 50:50 (ewe colostrum:lamb formula), then lamb milk replacement formula only from 48 h of life. Feeds were increased 1 mL/6 h for the first 72 h and 1 mL/4 h thereafter.

General observations were made every hour for the duration of experiments. Observations included, but were not limited to: body temperature, heart rate (HR), arterial blood pressure (BP), respiratory rate (RR), SaO_2, mean airway pressure (MAP), FiO_2, feed volumes and urine and faeces output.

Post mortem measurements and tissue collection

Lambs were killed with an overdose of IV sodium pentobarbitone (150 mg/kg; Jurox Pty Ltd, Australia) on day 7. The left lung was inflation-fixed with 10 % formaldehyde for morphometric[29] and histological analyses. Three random sections of fixed left lung, and five regions of interest (ROI) per section (total 15 ROIs/animal) were used for all morphometric, histological and immunohistochemical analyses. All images were taken at x40 magnification, unless otherwise specified. Subpleural segments of the right lower lobe of the lung and the midline of the liver were snap frozen in liquid nitrogen and stored at -80 °C until required for molecular analysis. All analyses were performed by P.C. Papagianis who was blinded to treatments.

Morphometric analyses

Lung sections were stained with Harts resorcin-fuschin[149] for identification of tissue, airspace, septal crests and elastin. A point grid detecting tissue and airspace was placed over each

ROI using a semi-automated ImageJ (NIH image, Bethasda, Maryland, USA) plugin (Copyright © 2015, Keith Schulze, Monash Micro Imaging, Monash University). Total points falling on tissue or airspace were divided by total overlaid points (676) for each ROI, expressing the areal fraction of tissue or airspace in the lungs. Septal crests were counted by P. C. Papagianis (Septal crest areal fraction = [points on septal crests] / [points on tissue]).

Total elastin was calculated with an automated ImageJ plugin (Copyright © 2015, Keith Schulze, Monash Micro Imaging, Monash University), which calculated area of elastin or tissue based on the colour black or yellow, respectively. The proportion of elastin was calculated by dividing the area of elastin by area of tissue.

Collagen was visualised with a picrosirus red (PSR) stain.[150] The birefringence of collagen was visualised using a Leica Abrio polarising microscope (512 X 512 CCD black and white camera by CRI Abrio software). The total area of collagen within each ROI was normalised to area of tissue using an automated ImageJ plugin (Copyright © 2015, Keith Schulze, Monash Micro Imaging, Monash University; NIH Image).

Immunohistochemistry

Leukocytes (CD45)[151], macrophages (CD163)[152], proliferating cells (Ki67)[153], myofibroblasts (α-smooth muscle (SM) actin)[153] and type II alveolar epithelial cells (pro-surfactant protein (SP)-C)[154] were visualised with immunohistochemistry. All sections were scanned using ImageScope (Aperio Technologies, California, USA) and analysed by P.C. Papagianis using ImageJ. Cells positive for Ki67 and PSR are presented as areal density of tissue. α-SM actin staining density was analysed using ImagePro Plus software (v. 9.2, Build 6156, 2012-2015 Media Cybernetics© Inc.) and is outlined elsewhere.[153] CD45+, CD163+ and SP-C analyses are presented as the average number of positive cells across 15 ROIs per animal.

Molecular analyses

Total RNA was isolated from lung and liver samples (RNeasy Kit, Qiagen) and reverse-transcribed into cDNA (SuperScript III reverse transcriptase, Invitrogen). Genes of interest (Table 1) were measured using TaqMan® probes.

TaqMan® RT-PCR assays, detailed elsewhere,[141] were used to measure gene expression. Briefly, each target gene was assayed in triplicate cDNA samples on an ABI Prism 7900HT Real-Time PCR System (PE Applied Biosystems). Ct values were analysed using qBase+ software, version 3.1.[142] We selected reference gene ribosomal (r)18S using the qBase+ geNorm algorithm for assessing stability of reference genes and optimal number of reference

genes.[143] The expression of all genes is expressed as calibrated normalised relative quantity (CNRQ; i.e. normalised to r18S and expressed relative to the Sal/Sal group), where 18S Sal/Sal is normalised to ~0.00.

Statistical Analyses

Statistical analyses were undertaken using SPSS (v. 24, IBM). Complete data sets were obtained utilising sequential regression modelling to impute missing physiological data points (<5 %). Serial physiological data were analysed using two-way repeated measures ANCOVA (SPSS, IBM). Influential covariates were identified using Pearson's correlation (SPSS, IBM). Post-hoc comparisons were made using the Holm-Sidak method. Histological data were analysed using Kruskal-Wallace with Dunn's multiple comparisons post-hoc test (GraphPad Prism version 7 for Mac OS X). Data are expressed as mean (SD).

Table 4. Probe selection for TaqMan® Assays

Gene	Assay ID
r18S	Oa4906333_g1
IL-1α	Oa04658682_m1
IL-1β	Oa04656322_m1
IL-4	Oa04927178_s1
IL-6	Oa04656315_m1
IL-8	CUSTOM
IL-10	Oa03212724_m1
TGF-β1	Oa04259484_m1
TNF	Oa04656867_g1
Surfactant protein A1	Oa04657758_m1
Surfactant protein B	Oa04750908_g1
Surfactant protein C	Oa04656841_m1
Surfactant protein D	CUSTOM
VEGF-A	Oa04653812_m1
VEGFR1	Oa04694159_m1
Angiopoeitin-1	Oa04757067_m1
EGR1	Oa03237885_m1
CYR61	Oa04673852_g1
CTGF	Oa04659069_g1
PECAM	Oa04677168_m1
MMP9	Oa03215996_g1
MMP12	Oa04744924_g1
CCL2	Oa04677078_m1
SAA	Oa04924154_s1
Hepcidin	Oa04656982_m1

IL = interleukin; TGF = transforming growth factor; VEGF = vascular endothelial growth factor; EGR = early growth response; CYR = cysteine-rich; CTGF = connective tissue growth factor; PECAM = platelet endothelial cell adhesion molecule; MMP = matrix metallopeptidase; CCL = chemokine ligand; SAA = serum amyloid A.

Results

Birth weights were not different between lambs (Table 5).

Umbilical cord pH, $PaCO_2$, PaO_2, HCO_3 or base excess (BE) measurements were not different between groups (P>0.05).

Table 5. Characteristics of lambs

Variable	Experimental group		
	Sal/Sal	LPS/Sal	LPS/Dex
GA (d)	129.7 ± 1.4	128.9 ± 0.9	129.2 ± 1.2
N (% male)	10 (44.4)	10 (50)	8 (77.8)
Birth body weight (kg)	2.89 ± 0.14	3.29 ± 0.13	3.19 ± 0.16
PM body weight (kg)	2.78 ± 0.13	3.02 ± 0.09	2.94 ± 0.16
Left lung weight/PM body weight (g/kg)	11.5 ± 1.2	17.9 ± 1.9*	15.7 ± 0.9
Static lung compliance ((mL/kg)/cmH_2O)	0.97 ± 0.87	0.76 ± 0.05	1.01 ± 0.07#
Liver weight/PM body weight (g/kg)	33.5 ± 2.3	33.6 ± 2.0	33.4 ± 1.9
Thymus weight/PM body weight (g/kg)	1.96 ± 0.37	2.27 ± 0.22	2.11 ± 0.22
Spleen weight/PM body weight (g/kg)	1.90 ± 0.32	2.38 ± 0.18	2.94 ± 0.21*
Adrenal weight/PM body weight (g/kg)	0.22 ± 0.02	0.22 ± 0.02	0.15 ± 0.01#

Data are mean ± SD. PM = post mortem; GA = gestational age. *P<0.05 significantly different to Sal/Sal and #significantly different to LPS/Sal

Respiratory support and gas exchange

The duration of MV correlated with birth-weight (r=0.488; P<0.001) and year of delivery (r=0.272; P<0.001). There was no difference in the proportion of time spent on MV between groups when controlled for birth-weight and year of delivery (P=0.57; Table 7). The proportion of time spent on MV (Table 6), ET-CPAP, nB-CPAP, nHHF or no respiratory support for each animal is represented in Figure 5.

Rates of extubation were highest in Sal/Sal lambs (100 % extubated on day 6), followed by LPS/Dex lambs (55.6 % extubated on day 6) and lastly in LPS/Sal lambs (51.9 % extubated on day 6). Time intubated correlated with birth-weight (r=0.344; P<0.001) and year of delivery (r=0.530; P<0.001). There was no difference in time intubated between groups when controlled for birth-weight and year of delivery (P=0.82; Table 6).

MAP correlated with hours of MV (r=0.678; P<0.001) and time intubated (r=0.656; P<0.001). Overall, MAP was highest in LPS/Sal lambs, but was only significantly higher when compared to LPS/Dex lambs (mean difference: 1.9, 95 % CI [0.6, 3.3]; P=0.002; Figure 4;Table 7). MAP did not change between treatment groups on days 1-7 of life (P=0.84).

Arterial pH, pCO_2 and pO_2 did not change between groups, or with time (Figure 4). There was a small increase in HCO_3 and BE (F(5, 133) = 7.4 and F(5, 133) = 8.6, respectively; P<0.001) with time. FiO_2 requirements increased slightly over the study duration, irrespective of treatment (F(5, 149) = 2.8; P=0.02; Table 6). There was no difference in average FiO2 delivery to animals (Table 6). The pF and OI values were not different between groups (P>0.05), with the exception of increased pF in Sal/Sal lambs compared to LPS/Dex lambs (F(10, 125) = 4.8; P=0.02), and decreased OI (better oxygenation) in Sal/Sal lambs compared to LPS/Sal lambs (F(25, 125) = 6.1; P=0.04; Figure 6), on day 5 of life.

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Table 6. Physiological parameters over the first 6 days of life in preterm lambs

Variable	Day	Experimental group		
		Sal/Sal	**LPS/Sal**	**LPS/Dex**
Proportion of time on mechanical ventilation (%)	1	19	43.98	35.65
	2	11.11	57.41	22.22
	3	10.19	51.81	24.54
	4	26.85	36.57	31.48
	5	29.17	33.80	41.20
	6	0	41.67	40.74
Hours/day intubated	1	8.1 ± 6.5	17.2 ± 8.9	18.2 ± 7.1
	2	3.7 ± 8.2	15.3 ± 11.3	8.1 ± 10.8
	3	4.1 ± 8.0	12.6 ± 11.4	7.1 ± 8.8
	4	7.3 ± 11.0	13.4 ± 12.5	9.1 ± 11.4
	5	5.3 ± 8.7	13.1 ± 12.2	10.3 ± 12.3
	6	0.0 ± 0.0	8.4 ± 11.0	10.7 ± 12.7
FiO_2	1	0.23 ± 0.00	0.22 ± 0.01	0.22 ± 0.02
	2	0.21 ± 0.01	0.23 ± 0.02	0.23 ± 0.03
	3	0.23 ± 0.02	0.23 ± 0.02	0.23 ± 0.02
	4	0.23 ± 0.02	0.27 ± 0.09	0.24 ± 0.03
	5	0.24 ± 0.04	0.30 ± 0.16	0.27 ± 0.07
	6	0.24 ± 0.04	0.26 ± 0.07	0.25 ± 0.04
BP (mmHg)	1	61 ± 6	62 ± 4	65 ± 6
	2	50 ± 7	57 ± 5	65 ± 5$^{\#*}$
	3	62 ± 8	60 ± 6	67 ± 5
	4	62 ± 7	63 ± 5	69 ± 3
	5	64 ± 6	63 ± 7	69 ± 5
	6	62 ± 6	67 ± 8	69 ± 5
HR (beats/min)	1	221 ± 17	212 ± 22	206 ± 14
	2	201 ± 16	191 ± 27	186 ± 63
	3	201 ± 28	190 ± 26	210 ± 13
	4	215 ± 30	186 ± 26	221 ± 31$^{\#}$
	5	213 ± 24	193 ± 32	221 ± 18
	6	222 ± 22	208 ± 29	225 ± 34
RR (breaths/min)	1	54 ± 6	55 ± 8	56 ± 7
	2	58 ± 6	49 ± 11	60 ± 11$^{\#}$
	3	54 ± 8	50 ± 6	55 ± 11
	4	54 ± 9	53 ± 6	61 ± 13
	5	52 ± 11	56 ± 8	62 ± 15
	6	61 ± 12	55 ± 12	61 ± 11

Data are mean ± SD. *P<0.05 compared to Sal/Sal; $^{\#}$P<0.05 compared to LPS/Sal. FiO_2: fraction of inspired oxygen; BP: blood pressure; HR: heart rate; RR: respiratory rate.

Table 7. **Mechanical ventilation, time intubated and mean airway pressure in preterm lambs.**

		Mean difference [95 % CI]	P-value
MV (%)			
Sal/Sal	LPS/Sal	-2.45 [-7.00, 2.10]	0.479
	LPS/Dex	0.49 [-4.65, 3.66]	0.989
Time intubated (h)			
Sal/Sal	LPS/Sal	-2.92 [-7.52, 1.89]	0.337
	LPS/Dex	-5.14 [-6.05, 2.36]	0.644
MAP (mmHg)			
Sal/Sal	LPS/Sal	-1.21 [-2.67, 0.25]	0.132
	LPS/Dex	0.73 [-0.68, 2.15]	0.513

Mean difference between Sal/Sal and LPS/Sal lambs and between Sal/Sal and LPS/Dex lambs [95 % confidence intervals]. Year of delivery was used as a covariate in all analyses, with the addition of birth-weight for MV and time intubated analysis and MV for MAP analyses. MV: mechanical ventilation; MAP: mean airway pressure. MAP is taken from ventilator and set continuous positive airway pressure.

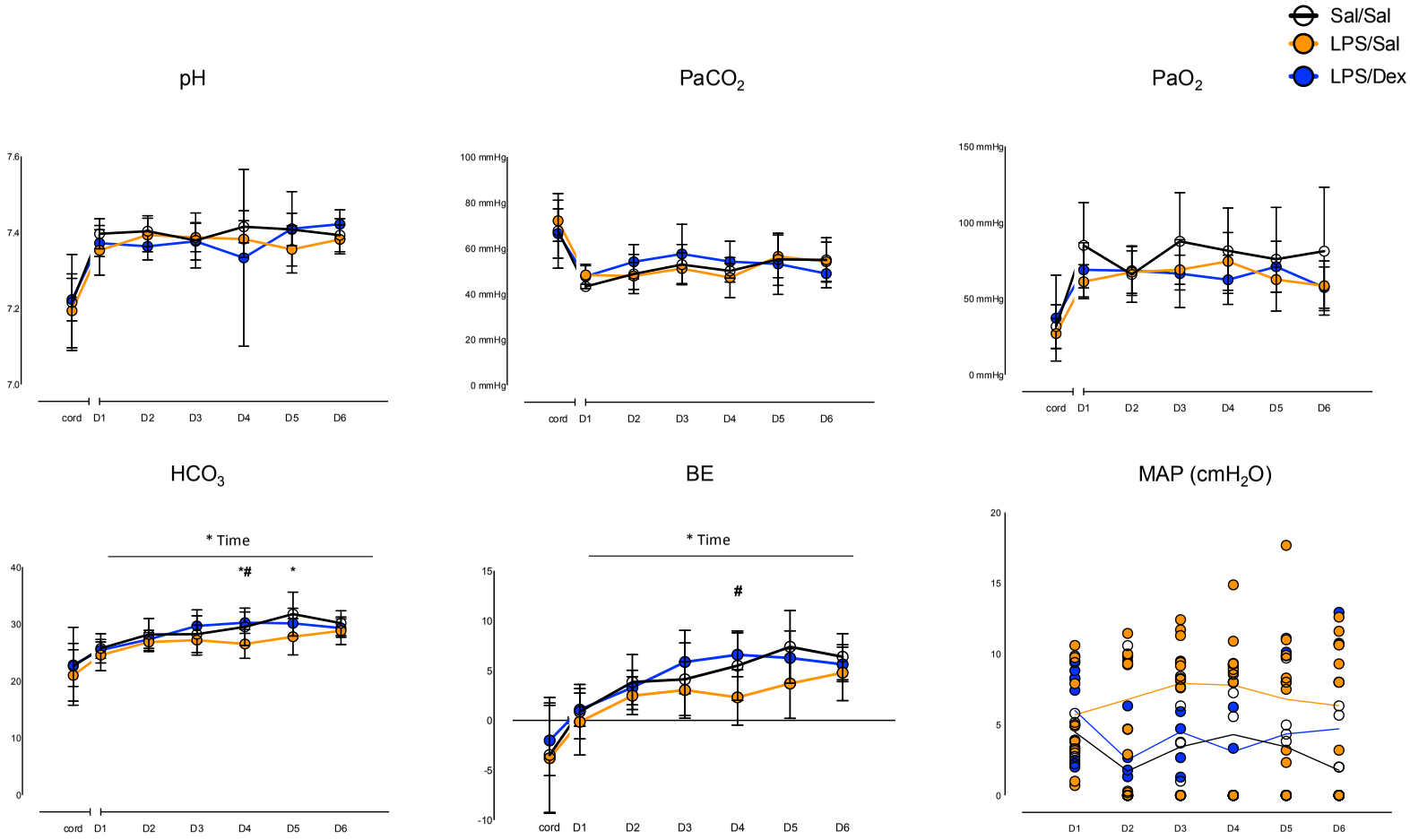


Figure 4. pH, $PaCO_2$, PaO_2, HCO3, BE and mean airway pressure (MAP) of Sal/Sal lambs (open circles), LPS/Sal lambs (orange circles) or LPS/Dex lambs (blue circles). Cord blood gas values were analysed separately to days 1-6 (D1-D6). *Signifies P<0.05 between Sal/Sal and LPS/Sal. #Signifies P<0.05 between LPS/Sal and LPS/Dex. pH, $PaCO_2$, PaO_2, HCO3 and BE data are mean ± SD. MAP data points are individual animals with mean connected by a line. MAP measurements are recorded from mechanical ventilation or continuous positive airway pressure. MAP was calculated as 0 cmH_2O in extubated lambs.

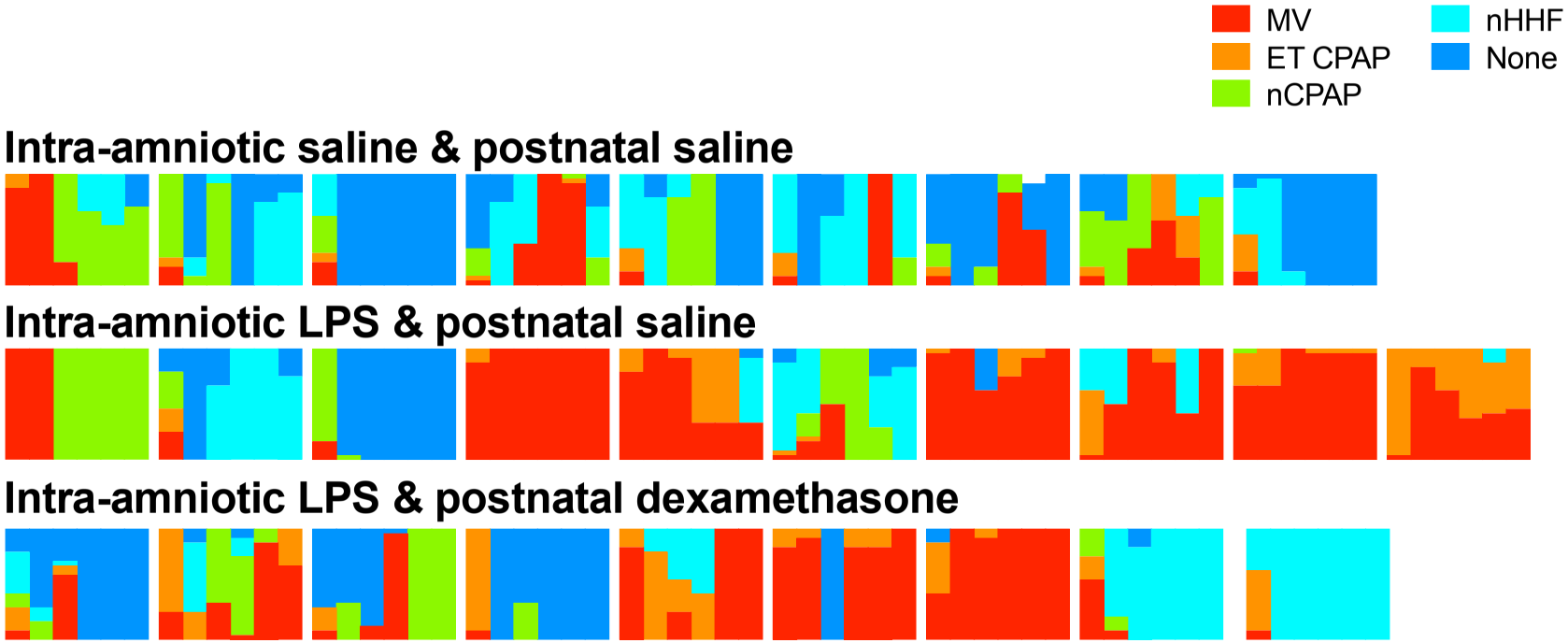


Figure 5. Representative graphs of ventilator requirements in preterm lambs. Types of respiratory support are graded with intubated variations, mechanical ventilation (MV) and endotracheal continuous positive airway pressure (ET CPAP), in red and orange and non-intubated variations, nasal (n)CPAP, nasal humidified high flow (nHHF) and no support (none), in green, light blue and blue, respectively. Lambs within Sal/Sal, LPS/Sal and LPS/Dex groups are represented in left-to-right, in order of delivery. The first 6 days of respiratory support are presented as columns, form left-to-right, as the proportion of time spent on any mode of respiratory support on that day.

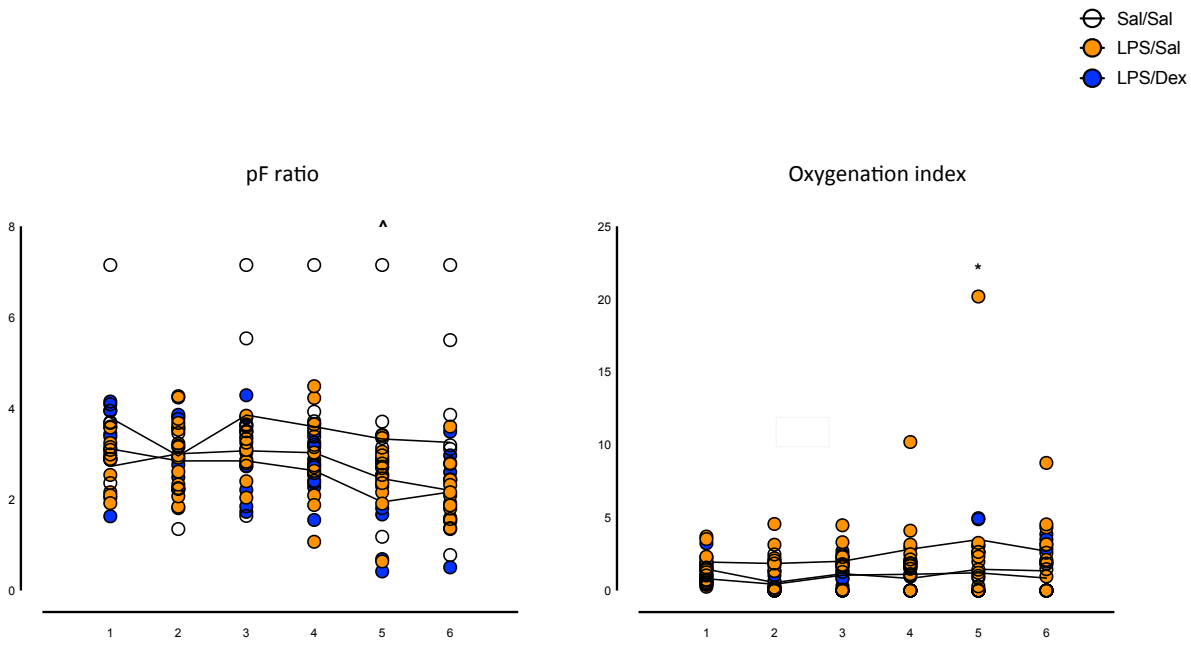


Figure 6. pF ratio and oxygenation index (OI) of Sal/Sal lambs (open circles), LPS/Sal lambs (orange circles) or LPS/Dex lambs (blue circles). Days 1-6 of life are presented on the x-axis. Data points are individual animals with mean connected by a line. *Signifies P<0.05 between Sal/Sal and LPS/Sal. ^Signifies P<0.05 between Sal/Sal and LPS/Dex.

Physiology

Physiological measures are provided in Table 6. Mean BP was generally higher in LPS/Dex lambs compared to both Sal/Sal (P=0.03) and LPS/Sal lambs (P=0.02), but this was only significantly higher on day 2. HR was higher in LPS/Dex compared to LPS/Sal (P=0.02) lambs on day 4. RR was higher in LPS/Dex compared to LPS/Sal lambs (P=0.03) on day 2.

Body and organ weights

The body weights and organ weights of lambs at 7 days of age were not different between treatment groups (Table 5). There were more males in the LPS/Dex group than the Sal/Sal and LPS/Sal groups.

Lamb characteristics are outlined in Table 5. At post mortem, LPS/Dex lambs had increased static lung compliance compared to LPD/Sal lambs. Spleen weight was increased in LPS/Dex compared to Sal/Sal lambs (P=0.03), but similar to LPS/Sal lambs (H(2) = 6.7; P=0.26; mean ranks: 8.7 for Sal/Sal, 12.4 for LPS/Sal and 18.4 for LPS/Dex). Adrenal-to-body weight ratio was decreased in LPS/Dex compared LPS/Sal lambs (P=0.02), but not compared to Sal/Sal lambs (H(2) = 7.8; P=0.15; mean ranks: 15.7 for Sal/Sal, 18.0 for LPS/Sal and 8.1 for LPS/hAECs).

Lung morphometry

Lung tissue thickness varied, with some portions of the lungs displaying atelectasis and others showing simplistic and dysmorphic lung architecture on day 7 of life, regardless of treatment (Figure 7). LPS/Dex lambs had less tissue in their lungs (46.5 ± 4.8 %) compared to LPS/Sal lambs (55.8 ± 8.5 %; P=0.046), but not compared to Sal/Sal lambs (47.6 ± 7.5 %; H(2) = 7.6, P>0.99; mean ranks: 10.8 for Sal/Sal, 18.7 for LPS/Sal and 9.6 for LPS/Dex; Figure 7). LPS/Sal lambs tended to have lower airspace areal fraction in their lungs compared to Sal/Sal lambs (H(2) = 7.6, P=0.07; mean ranks: 16.2 for Sal/Sal, 8.3 for LPS/Sal and 17.4 for LPS/Dex; Figure 7). Septal crest areal fraction was not different between groups (Sal/Sal 1.8 ± 0.8 %; LPS/Sal 1.4 ± 1.2 % and LPS/Dex 2.6 ± 1.3 %; P>0.05; Figure 7).

The areal density of elastin deposition in the lungs was not different between groups (Sal/Sal 9.1 ± 1.3 %; LPS/Sal 9.2 ± 3.0 % and LPS/Dex 10.8 ± 2.7 %; P>0.05). Similarly, the areal density of tissue collagen was not different between groups (Sal/Sal 30.8 ± 5.1 %; LPS/Sal 33.1 ± 8.3 % and LPS/Dex 31.4 ± 6.2 %; P>0.05).

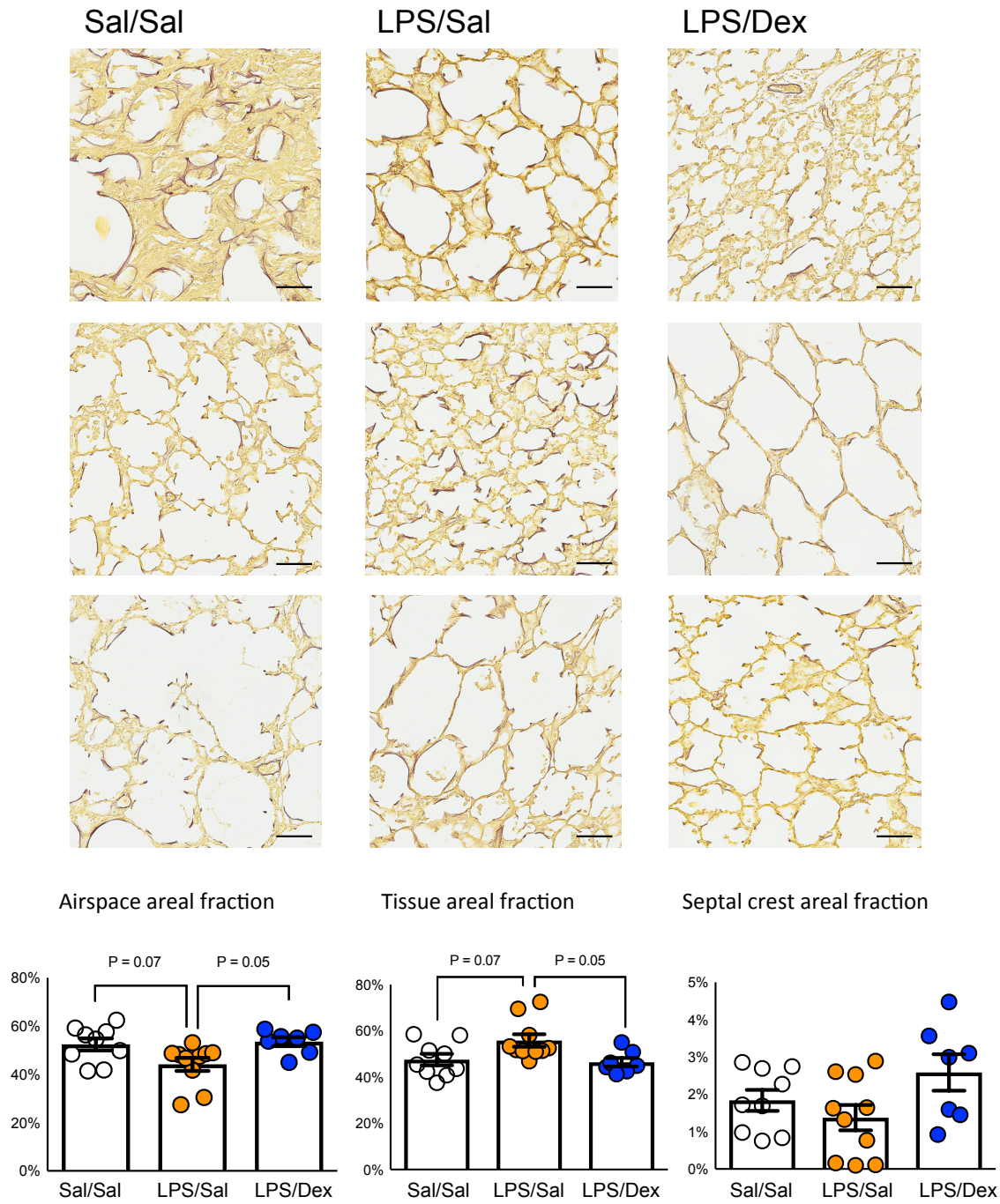


Figure 7. Representative images depicting variability in the lung parenchyma of preterm lambs within the same treatment group. Quantification of airspace, tissue and septal crest areal fractions in the lungs of preterm lambs. Sal/Sal preterm lambs were exposed pre- and postnatally to saline, LPS/Sal preterm lambs were exposed prenatally to LPS and postnatally to saline and LPS/Dex preterm lambs were exposed prenatally to LPS and postnatally to low-dose dexamethasone (Dex). All images are taken at 20X magnification. Scale bar = 50 µm.

Lung inflammation and cell characteristics

The number of CD45+ cells in the lungs was lower in LPS/Dex lambs (52.3 ± 15.6 %) compared to Sal/Sal lambs (93.1 ± 37.4 %; P=0.03) and LPS-only lambs (121 ± 41.1 %; P=0.001; H(2) = 13.02; mean ranks: 16.3 for Sal/Sal lambs, 20.6 for LPS/Sal lambs and 6.25 for LPS/Dex lambs; Figure 8). The number of CD163+ cells in the lungs of LPS/Dex lambs (29.0 ± 11.7 %) was lower than in the lungs of LPS/Sal lambs (LPS/Sal 55.9 ± 28.4 %; P=0.04), but not different to Sal/Sal lambs (36.8 ± 24.6 %; P>0.99) on day 7 of life (H(2) = 6.3; mean ranks: 14.1 for Sal/Sal, 21.0 for LPS/Sal and 11.2 for LPS/Dex; Figure 8).

The areal density of Ki67+ cells in the tissue and airspaces was lower in Sal/Sal (7.8 ± 3.0 %) and LPS/Dex (6.7 ± 7.9 %; P=0.036) lambs compared to LPS/Sal lambs (19.5 ± 11.7 %; P=0.011), but not different between Sal/Sal and LPS/Dex lambs (H(2) = 10.3, P>0.99; mean ranks: 10.7 for Sal/Sal, 19.5 for LPS/Sal and 8.6 for LPS/Dex; Figure 9). Ki67+ cells in the airspaces of the lungs of Sal/Sal lambs (1.4 ± 0.9 %) were not different to LPS/Sal (8.4 ± 8.0 %; P=0.075) or LPS/Dex (1.2 ± 0.7 %; P>0.99) lambs. There were more Ki67+ cells in the airspaces of LPS/Sal (8.4 ± 8.4 %) compared to LPS/Dex lambs (1.2 ± 0.7 %; H(2) = 8.3, P=0.024; Figure 9). The percentage of Ki67+ cells within lung tissue was not different between groups (Sal/Sal 6.4 ± 2.4 %; LPS/Sal 10.5 ± 5.9 % and LPS/Dex 5.5 ± 2.5 %; P>0.05).

The areal density of α-SMA expression was not different in the lungs of preterm lambs (Sal/Sal 38.2 ± 10.9 %; LPS/Sal 28.0 ± 21.26 % and LPS/Dex 39.3 ± 8.7 %; P>0.05).

The number of pro-SP-C cells in the lungs of preterm lambs was not different (Sal/Sal 23.6 ± 12.7 cells/ROI; LPS/Sal 19.1 ± 10.6 cells/ROI and LPS/Dex 18.1 ± 9.1 cells/ROI; P>0.99).

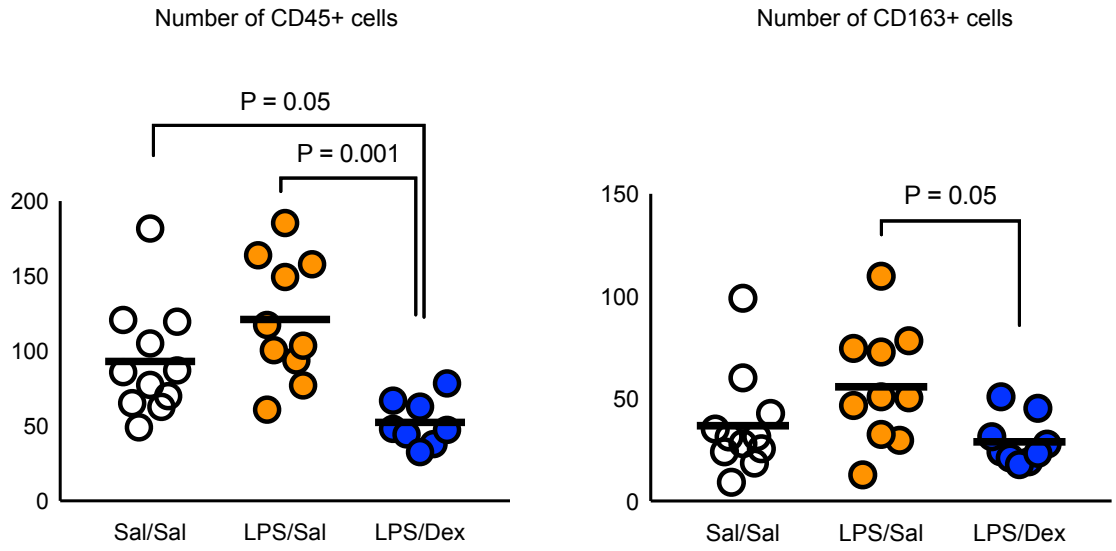


Figure 8. The number of CD45+ and CD163+ cells in the lung tissue of Sal/Sal, LPS/Sal and LPS/Dex preterm lambs on day 7 of life. Line represents mean.

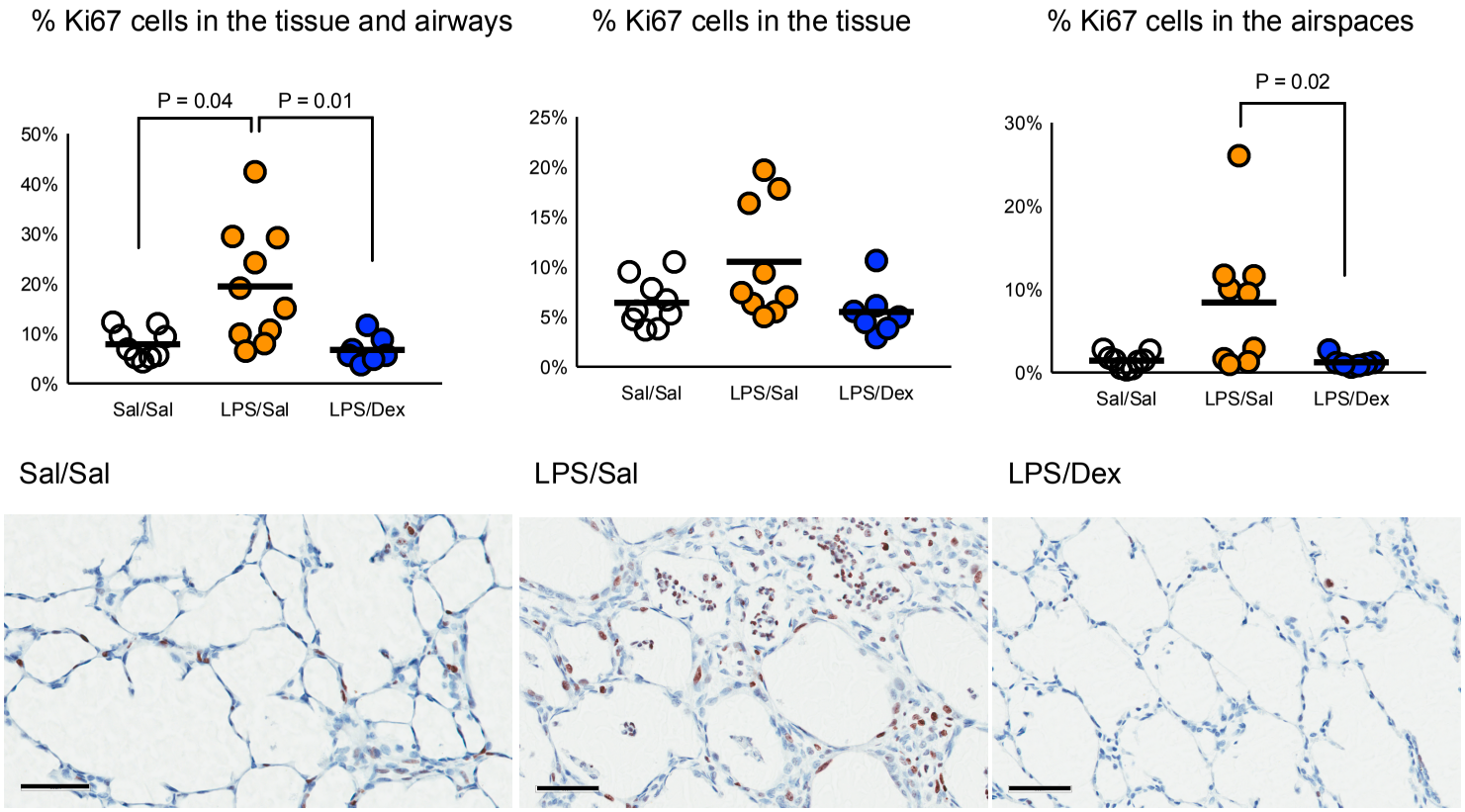


Figure 9. Proliferating cells within the tissue and airspace of the lungs, within the tissue only, or within the airspaces only of preterm lambs on day 7 of life. Representative images of the lungs of Sal/Sal, LPS/Sal and LPS/Dex treated preterm lambs. Clumps of proliferating cells were often noted in the airspaces of animals exposed to antenatal LPS. Line represents mean. Scale bar = 50 μm.

Gene expression in the lungs

LPS/Dex animals had similar mRNA expression of *IL-1α IL-1β, IL-6* and *IL-8* to Sal/Sal lambs (Figure 10). *IL-1α IL-1β, IL-6* and *IL-8* were upregulated in LPS/Sal lambs compared to Sal/Sal lambs ($P<0.05$), but not in comparison to LPS/Dex lambs. Other inflammatory, vascular and surfactant protein genes were not differentially expressed between groups (Table 8).

Gene expression in the liver

TGB-β was upregulated in the livers of LPS/Dex lambs compared to Sal/Sal ($P=0.03$) and LPS/Sal ($P=0.01$) lambs. PECAM mRNA expression was upregulated in the livers of LPS/Dex lambs in comparison to LPS/Sal lambs ($P=0.002$) and tended to be elevated in comparison to Sal/Sal ($P=0.07$). Other inflammatory genes were not differentially expressed between groups (**Table 9**).

Table 8. Fold change mRNA expression of genes in the lungs of preterm lambs

Gene of interest	Experimental group		
	Sal/Sal	LPS/Sal	LPS/Dex
IL-1α	-0.15 ± 0.08	0.25 ± 0.10*	-0.06 ± 0.12
IL-1β	-0.13 ± 0.08	0.26 ± 0.12*	-0.16 ± 0.17
IL-4	0.02 ± 0.12	0.09 ± 0.15	-0.11 ± 0.19
IL-6	-0.13 ± 0.09	0.17 ± 0.12*	-0.09 ± 0.08
IL-8	-0.26 ± 0.12	0.33 ± 0.20*	-0.16 ± 0.17
IL-10	-0.14 ± 0.10	0.15 ± 0.12	-0.00 ± 0.12
TGF-β	-0.03 ± 0.05	0.10 ± 0.03	-0.22 ± 0.11
TNF	-0.07 ± 0.07	0.21 ± 0.09	-0.12 ± 0.16
MMP9	-0.00 ± 0.09	0.13 ± 0.07	-0.08 ± 0.13
MMP12	0.10 ± 0.07	0.07 ± 0.12	0.10 ± 0.12
CCL2	-0.06 ± 0.09	0.19 ± 0.09	-0.10 ± 0.10
PECAM	-0.01 ± 0.05	0.06 ± 0.04	0.00 ± 0.11
VEGF-A	-0.04 ± 0.05	0.08 ± 0.04	0.00 ± 0.11
VEGF-R1	-0.00 ± 0.16	0.05 ± 0.06	0.00 ± 0.08
Angiopoietin-1	0.03 ± 0.12	0.09 ± 0.06	0.02 ± 0.07
SP-A	-0.16 ± 0.15	0.04 ± 0.12	-0.04 ± 0.15
SP-B	-0.12 ± 0.23	0.08 ± 0.09	0.01 ± 0.13
SP-C	0.08 ± 0.09	0.02 ± 0.06	0.05 ± 0.08
SP-D	-0.16 ± 0.20	0.12 ± 0.11	0.02 ± 0.11
EGR1	0.01 ± 0.14	0.13 ± 0.07	-0.05 ± 0.06
CTGF	-0.00 ± 0.12	0.04 ± 0.06	-0.04 ± 0.13
CYR61	-0.01 ± 0.18	0.08 ± 0.06	0.04 ± 0.0.06

Data are mean ± SD relative to 18S and as a fold change from Sal/Sal group. *Significantly different from Sal/Sal

Figure 10. IL-1α, IL-1β, IL-6 and IL-8 messenger RNA expression in the lungs of Sal/Sal lambs, LPS/Sal lambs or LPS/Dex lambs. *Signifies P<0.05 from Sal/Sal. Data are mean ± SD in duplicates. Sal/Sal group is normalised to approximately 0.00 using qBase+ software. LPS/Sal and LPS/Dex groups are expressed as a fold change from Sal/Sal.

Table 9. Messenger RNA expression of genes in the liver of preterm lambs

Gene of interest	Experimental group		
	Sal/Sal	LPS/Sal	LPS/Dex
IL-1α	-0.04 ± 0.08	0.14 ± 0.13	0.05 ± 0.06
IL-1β	-0.05 ± 0.06	0.13 ± 0.10	0.08 ± 0.07
IL-4	-0.06 ± 0.15	0.10 ± 0.22	-0.22 ± 0.24
IL-8	-0.11 ± 0.12	0.33 ± 0.19	-0.06 ± 0.08
IL-10	-0.10 ± 0.11	0.34 ± 0.16	-0.01 ± 0.14
TNF	-0.12 ± 0.08	0.24 ± 0.12	0.06 ± 0.085
TGF-β	0.01 ± 0.05	-0.03 ± 0.05	0.22 ± 0.05*#
PECAM	0.05 ± 0.04	-0.05 ± 0.05	0.16 ± 0.03#
Hepcidin	0.06 ± 0.08	0.03 ± 0.05	0.05 ± 0.07
SAA	0.09 ± 0.10	0.11 ± 0.11	0.10 ± 0.13

Data are mean ± SD relative to r18S and as a fold change from Sal/Sal group. * Indicates significantly different from Sal/Sal and #significantly different from LPS/Sal.

Discussion

Tapered low-dose postnatal dexamethasone supressed lung inflammation and altered lung structure without reducing the proportion of time spent on MV, in preterm lambs exposed to antenatal inflammation. To our knowledge, no other studies investigate the effects of postnatal dexamethasone therapy on altered lung inflammation and architecture induced by antenatal inflammation and postnatal ventilation, in the setting of contemporary neonatal care.

High-dose postnatal dexamethasone facilitates extubation[51] but is associated with neurological deficit in humans.[155] Cumulative high-dose dexamethasone is thought to increase lung maturity and improve respiratory outcomes in preterm infants,[156] aiding earlier extubation. The impact of tapered low-dose postnatal dexamethasone on ventilator requirements is unclear. The DART trial administered tapered low-dose dexamethasone to ventilator-dependent preterm infants, which facilitated extubation without impacting the rates of BPD or death in the first week of life, compared to placebo.[67] However, the DART trial was statistically underpowered due to low recruitment.[67] The administration of tapered low-dose postnatal dexamethasone in our study did not result in decreased time intubated, suggesting tapered low-dose postnatal dexamethasone following antenatal inflammation does not reduce respiratory requirements in preterm lambs.

There are no clinical data on ventilation requirements when chorioamnionitis and postnatal dexamethasone are combined, despite exposure to chorioamnionitis increasing the likelihood of moderate and severe BPD.[18] Overall, the administration of dexamethasone in the presence of antenatal LPS did not alter ventilator requirements in preterm lambs during the first week of life.

Single or multiple injection(s) of IA LPS correlate with decreasing ventilation pressures in the first hour of life in preterm lambs, attributable to lung maturation.[21] Despite many lambs being extubated from MV for at least some time in our 7-day studies, we did not observe LPS-induced lung maturation in LPS/Sal lambs, suggesting that factors other than chorioamnionitis-induced lung maturation contribute to weaning from MV. Maternal betamethasone has similar maturational effects on the lungs as IA LPS in fetal sheep.[157] Preterm lambs exposed to maternal betamethasone receive lower ventilation pressures in

the first hour of life, compared to saline-exposed controls.[157] Lung maturation induced by maternal betamethasone, rather than LPS or postnatal dexamethasone, may have facilitated weaning of preterm lambs from MV across all treatment groups in our study. Administration of either antenatal betamethasone[3, 42] or IA LPS[158] appears to improve lung outcomes, but there may be an interaction when exposed to both antenatal betamethasone and IA LPS.

Exposure to antenatal betamethasone before IA LPS reduces immediate ventilation requirements and lung inflammation,[158] but reduction of LPS-induced lung inflammation is transient in fetal sheep.[45, 159, 160] We controlled for any effect of antenatal betamethasone on postnatal ventilation requirements and lung inflammation by exposing all lambs to antenatal corticosteroids. Antenatal betamethasone is routinely administered to women at risk of preterm delivery to aid in lung maturation and relieve immediate respiratory distress, and in this way our study mimics clinical practice.[3] Additionally, exposure to antenatal betamethasone was essential to the survival of control (Sal/Sal) preterm lambs. We are unaware of interactions between antenatal corticosteroid and postnatal corticosteroid therapies for weaning infants from MV or reducing lung inflammation.

Tapered low-dose dexamethasone may be less effective at aiding weaning from MV in the presence of chorioamnionitis, despite reduced lung injury and inflammation. Chorioamnionitis results in pulmonary inflammation, and pulmonary inflammation is associated with higher ventilator requirements in neonates.[34] Thus, prenatal inflammation may be one explanation for reduced weaning in preterm lambs receiving postnatal dexamethasone who were exposed to both antenatal inflammation and postnatal ventilation. The only indicator of reduced respiratory support was increased static lung compliance and decreased MAP delivery to LPS/Dex lambs compared to LPS/Sal lambs, but reduced MAP should be interpreted cautiously as LPS/Sal lambs had increasing FiO_2 with increasing study duration. FiO_2 requirements were highest on day 5 in individual LPS/Sal lambs indicative of atelectasis of the lungs.

LPS exposure before birth reliably thins lung parenchyma, resulting in alveolar hypoplasia and arrested septation in the lungs of fetal sheep,[42, 46] consistent with antenatal corticosteroids in fetal sheep.[49] However, we investigated the impact of antenatal and postnatal inflammation in preterm lambs, not fetal sheep. We show that IA LPS caused lung inflammation and altered lung development; LPS/Sal lambs had thickened tissue, less airspace, more inflammatory cells and more proliferative cells in their lungs at one week of age. This, antenatal LPS and postnatal ventilation appear to exacerbate lung inflammation

and injury in LPS/Sal lambs on day 7 of life. Postnatal dexamethasone prevented lung inflammation and injury, and lambs had similar lung morphology to Sal/Sal lambs. LPS/Dex lambs had thinner lung tissue, increased airspace and reduced inflammatory and proliferative cells within the lungs compared to LPS/Sal lambs at one week of age.

IL-1α IL-1β, IL-6 and *IL-8* expression was increased in the lungs of LPS/Sal lambs compared to Sal/Sal lambs on day 7 of life, suggesting increased lung injury. Elevated inflammation in the lungs of LPS/Sal lambs coincided with elevated Ki67 staining. Clumps of proliferating cells in the airways likely represent inflammatory cells capable of releasing growth factors and cytokines that stimulate cell proliferation and increase lung wall thickness.[153] Thus, increased proportion of proliferating cells in LPS-exposed lambs may contribute to increased wall thickness and increased inflammation in the lungs.

LPS/Dex lambs had intermediate vascular and surfactant protein gene expression in their lungs, compared to Sal/Sal and LPS/Sal lambs, and the expression of pro-inflammatory cytokines tended to be lower in LPS/Dex compared to LPS/Sal lambs. A lack of significant difference in lung cytokine expression between LPS/Sal and LPS/Dex lambs may be due to a limited effect of tapered low-dose postnatal dexamethasone after established LPS-induced lung inflammation. The tapered doses of dexamethasone used initially may differentially impact inflammatory gene expression on the first days of life compared to at day 7. Even though the number of inflammatory cells noted histologically in the lungs were reduced by dexamethasone, the transcription of these inflammatory cytokines may be temporarily different to inflammatory cell number at day 7 of life. The administration of antenatal corticosteroids 7 days prior to IA LPS suppresses lung inflammation in fetal sheep, but suppression is transient.[158, 161] Until now, sheep studies have only used prophylactic high-dose antenatal and postnatal corticosteroids to prevent lung inflammation, dissimilar to clinical practice[42, 46, 49, 162-164] and to our dexamethasone dosing. Lower doses of dexamethasone used in our study may increase variability in cytokine expression in dexamethasone-treated preterm lambs.

Elevated liver *TGF-β* and PECAM was coupled with increased spleen weights in LPS/Dex lambs, indicative of a systemic inflammatory response, despite an anti-inflammatory pulmonary response. *TGF-β* and PECAM stimulate and aid in the passage of immune cells, exacerbating liver inflammation[165] and causing splenomegaly.[166] Spleen, liver and adrenal weights are all reduced in preterm and term lambs exposed to singular and repeated injections of antenatal betamethasone,[167] suggesting antenatal corticosteroids reduce

growth, despite inducing organ maturation.[168] Exposure to antenatal betamethasone and LPS elevates expression of acute pro-inflammatory genes in fetal sheep livers,[168] consistent with our postnatal observations. Antenatal betamethasone may mature immune cell function, which could explain elevated expression of pro-inflammatory genes in the liver following exposure in fetal sheep[168] and preterm lambs in our study. Our observation of increased spleen weight in LPS/Dex lambs at day 7 of life are contrary to reports that antenatal and postnatal corticosteroids retard growth,[169] but may reflect corticosteroid-induced organ maturation and resultant enhanced systemic inflammatory response.

Limitations

Preterm lambs in our study were treated pragmatically leading to large variability within our datasets, which likely contributed to lack of statistical significance. The lung architecture of chronically ventilated (3-4 weeks) preterm lambs also display non-uniform inflation patterns and alveolar hypoplasia,[39] similar to that observed in our histological analyses.

Our decision to undertake a pragmatic study design meant we could not compare some variables. In particular, ventilation parameters such as PIP, PEEP and tidal volume could not be compared between groups, as many lambs were extubated within the first 72 hours. Comparing ventilation parameters between groups would have biased the outcomes towards the sickest animals (who remained ventilated at day 7). To overcome this we chose to only analyse MAP, as we could assign a value of 0 cmH_2O to extubated animals.

The proportion of males in the LPS/Dex group was higher than in the other groups, which may have prevented revealing statistically significant findings. Male preterm lambs and preterm infants have worse clinical courses than females,[170-173] and the response to steroids is superior in females compared to males.[174, 175] Clinically relevant study designs with increased animal numbers are required to account for additional variability or unpredictable nature of experiments for future short term postnatal studies.

Conclusion

We show that tapered low-dose postnatal dexamethasone following antenatal inflammation has no impact on ventilation requirements throughout the first week of life, but reduces lung inflammation and injury in preterm lambs. The efficacy and safety of tapered low-dose dexamethasone has not been appropriately studied in preterm infants with BPD. Thus,

additional studies should investigate low-dose dexamethasone in naïve animals, examining both short and long term lung and neurological outcomes.

Declaration for Thesis Chapter 4: Viability and function of human amnion epithelial cells is not altered by temperature

Declaration by candidate

Nature of the contribution	Extent of contribution
Performed experiments, collected data, analysed data and drafted manuscript	90 %

The following authors contributed to the work. If the co-authors are students at Monash University their contribution in percentage is stated:

Nature of the contribution		Extent of contribution
Courtney A. McDonald	Contributed to the design of experiments and drafting of the manuscript	N/A
J. Jane Pillow	Conceived the idea and contributed to drafting of the manuscript	N/A
Rebecca Lim	Contributed to tissue collection and drafting of the manuscript.	N/A
Tim J. Moss	Conceived the idea and obtained funding for the work. Participated in design and performed the experiments and drafted manuscript	N/A

Chapter Four

Introduction

Human amnion epithelial cells (hAECs) are potent anti-inflammatory cells with regenerative potential and low immunogenicity, enabling their clinical application in corneal surgery and as skin grafts for burns victims.[124, 176-180] Their low human leukocyte antigen (HLA) class II and telomerase expression allows for hAEC use with little or no immunosuppressive requirements or concerns of rejection or tumorigenesis.[124] Human AECs are isolated from placentas, normally discarded at birth. Thus, ethical and technical issues that complicate use of other cell types (such as embryonic stem cells and bone marrow stromal cells) do not apply to hAECs.

Human AECs are an attractive therapy for inflammatory-based diseases. They suppress lymphocyte infiltration,[176, 177] likely damping a pro-inflammatory immune response. In culture, hAECs inhibit T and B cell activity following LPS stimulation.[176] The anti-inflammatory properties of hAECs are preserved *in vivo*: macrophage infiltration decreases in mice subjected to fibrotic lung and liver injury after treatment with hAECs, and the ratio of M1:M2 macrophages is reversed, favouring the pro-reparative M2 macrophage phenotype.[136, 181] These anti-inflammatory effects likely underlie the beneficial effects of hAECs in large animal models of perinatal lung and brain injury,[129, 130, 182] in which hAECs reduce interleukin (IL) -1 and -6 expression.[129, 130] The efficacy of hAECs have been investigated in mice [183] and sheep,[184] which are normothermic at 36 ºC and 39 ºC respectively, different to the normothermic environment (37 ºC) of the cells' native human host.

The effect of different body temperatures on hAEC viability and function is unknown, and is an important consideration for interpretation of research findings prior to clinical application in neonates. Therapeutic hypothermia is recommended for term newborns whose births are complicated by hypoxic ischemic encephalopathy (HIE), involving reduction of core body temperature to ~33 °C.[185] Additionally, preterm infants are at risk of hypothermia due to high body surface area to weight ratio, allowing for rapid heat loss (< 36 ºC).[186] Preterm and term neonates born in Australia and the US experience rates of sepsis between 1 and 9 per 1000 live births, of which the predominant symptom is fever (>38 °C).[187] For implementation of hAECs in human newborns, the cells must be viable and functional across such temperatures.

Fluctuations in temperature impact cell mechanics and arrests the G_1 and M stages of the cell cycle in mouse leukemic cells (L5178Y) and HeLa cell lines, respectively.[188, 189] L5178Y cell viability is optimal when cultured at 37 °C but culture temperatures of 28 °C and 40 °C result in significant cell death.[189] Cellular death at culture temperatures outside 37 ºC is consistent with other mammalian cell lines,[190-192] and is noted in some stem cell colonies.[193, 194] Culturing mesenchymal stromal cells (MSCs) at 38 °C, 48 °C or 58 °C for up to 150 seconds has detrimental outcomes on cell viability for up to 3 days following temperature insult.[193] MSCs cultured at 58 °C for 45 – 150 seconds die.[193] We are unaware of studies examining the viability of hAECs across a range of clinically relevant temperatures. Thus, we aimed to investigate the effect of temperature on the viability and function of hAECs cultured at 33 °C, 37 °C or 39 °C. We hypothesised that hAECs will maintain viability and function when cultured at 33 °C, 37 °C or 39 °C.

Methods

Isolation and cryopreservation of hAECs

All experiments were performed with approval from the Monash University Human Ethics Committee. Placentae were obtained from women undergoing elective term caesarean section. All women provided written informed consent. Exclusion criteria included preterm birth, preeclampsia, intrauterine growth restriction (IUGR), clinical chorioamnionitis, active labour or preexisting maternal disease, including diabetes. The isolation of term hAECs from placentas was described preciously.[138] Briefly, the amnion was stripped from adjacent choriodecidua and rinsed in Hanks Balanced Salt Solution (HBSS; Invitrogen, San Diego, CA). Human AECs were isolated by two 1-hour digests in 0.05 % Trypsin (Invitrogen, San Diego, CA), and collected by centrifugation. Live-cell counts and viability were determined by trypan blue exclusion. For cryopreservation, hAECs were frozen at a cell density of 5×10^6 cells/mL in fetal bovine serum (FBS; Gibco, Life Technologies) + 5 % dimethyl sulfoxide (DMSO; Sigma Aldrich), stored in cryovials and cryopreserved in freezing containers (MrFrosty, Thermo Fisher Scientific) at -80 °C overnight. Cells were transferred to liquid nitrogen until required. To thaw, hAECs were taken directly out of liquid nitrogen, into a 37 °C water-bath until thawed. hAECs were washed to remove DMSO, and cell counts and viability were determined.

Flow cytometry

hAECs were seeded at 5 x 10^5 cells/well on a 6-well plate and placed into separate incubators set at temperatures of 33 °C, 37 °C or 39 °C. Cells were cultured in standard DMEM/F12 media (Gibco, Life Technologies) supplemented with 10% FBS and 1 % antibiotics (Penicillin-Streptomycin, Gibco, Life Technologies), in 5 % CO_2 in room air. Cultured hAECs (5 x 10^5 cells) at 33 °C, 37 °C and 39 °C were then harvested at 24, 48 and 72 hours and stained with Annexin V and 7AAD (PE Annexin V Apoptosis Kit I, BD Biosciences, USA) for 15 minutes at room temperature (RT), to assess apoptotic activity. Unstained hAECs were used for controls. Cells positive for Annexin V were considered to be in early apoptosis. Cells positive for 7AAD were considered to be in late apoptosis. Cells were washed with FACS buffer (1 % FBS in PBS) and were centrifuged (Heraeus Megafuge, Thermo Fisher Scientific) at 400 rcf for 5 minutes at 4 °C. Data were acquired with BD FACS-Canto II flow cytometer. Conditioned hAEC medium was collected at 72 hours and stored at -80 °C for future use in phagocytosis assays.

Wound-healing assay

The wound-healing properties of hAECs were assessed with a scratch assay. The hAECs were seeded in DMEM + 10 % FBS + 1 % antibiotics at 5 x 10^5 cells/well in a 6-well plate and incubated at 37 °C until confluent (7-12 days). A cross was scratched in the middle of the well with a 1,000µl pipette tip at 100 % confluency and hAECs were incubated at temperatures 33 °C, 37 °C or 39 °C for 3 days. Images were taken at the corner of the cross at 0 and 72 hours, using a phase contrast microscope (Axiovert 25, Ziess, Germany) so that the exact position could be replicated. Recovery of the scratch area was analysed using ImageJ.[195]

Phagocytosis assay

The day before phagocytosis assays, immortalised mouse macrophages (iMACs; generously provided by A/Prof Ashley Mansell, Hudson Institute of Medical Research) were plated in DMEM + 10 % FBS + 1 % antibiotics, at a density of 5 x 10^5 cells/well in a 96-well plate. The next day, media were removed from wells and replaced with 100 µl of FITC-marked fluorescent beads (2 x 10^4 beads/µl; diameter: 1 µm; Sigma-Aldrich, Fluka, USA) in DMEM + 10 % FCS and 100µl of hAEC-conditioned media (from cultures incubated at 33 °C, 37 °C or 39 °C for 72 hours). As negative controls, iMACs were cultured in 200 µl standard DMEM media, with and without 7AAD. As positive controls, iMACs were cultured in 100 µl standard DMEM media and 100 µl fluorescent beads in DMEM + 10 % FCS, with and without 7AAD.

All treated iMACs were incubated for 3 hours at 37 °C to allow phagocytosis of beads. After incubation, media was removed and iMACs were washed with PBS. Trypsin 0.05 % (100 µl) was used to detach iMACs from the plate; they were resuspended in PBS and centrifuged at 1400 rpm for 10 minutes at RT, and then resuspended in FACS buffer for analysis of positive FITC staining by flow cytometry. 7AAD was added to the relevant controls immediately before flow cytometry data acquisition was performed and analysed using a BD FACS-Canto II flow cytometer. Uptake was expressed as a percentage of fluorescent cells, corresponding to the percentage of iMACs with phagocytosed beads.

Statistics

Apoptotic data were analysed using 2-way ANOVA with Holm-Sidak's multiple comparison test. Wound healing assays and phagocytosis assays were compared using a one-way ANOVA with Tukey's post hoc test. Negative control samples were not included in statistical analysis of phagocytosis data.

Results

Apoptotic activity of hAEC at 33 °C, 37 °C and 39 °C

The percentage of cells positive for Annexin V increased at 48 hours and 72 hours, compared to 24-hour cultures, regardless of culture temperature ($p=0.003$ and $p=0.001$, respectively). Human AECs positive for Annexin V (single positive) or 7AAD (single positive) were not statistically different between temperatures at 24 hours, 48 hours or 72 hours (Figure. 11).

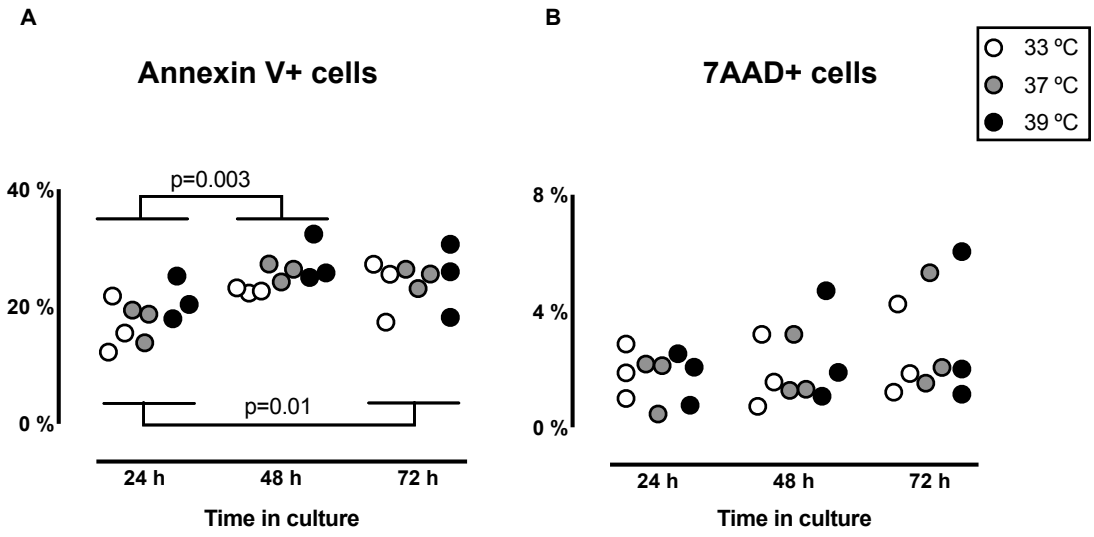
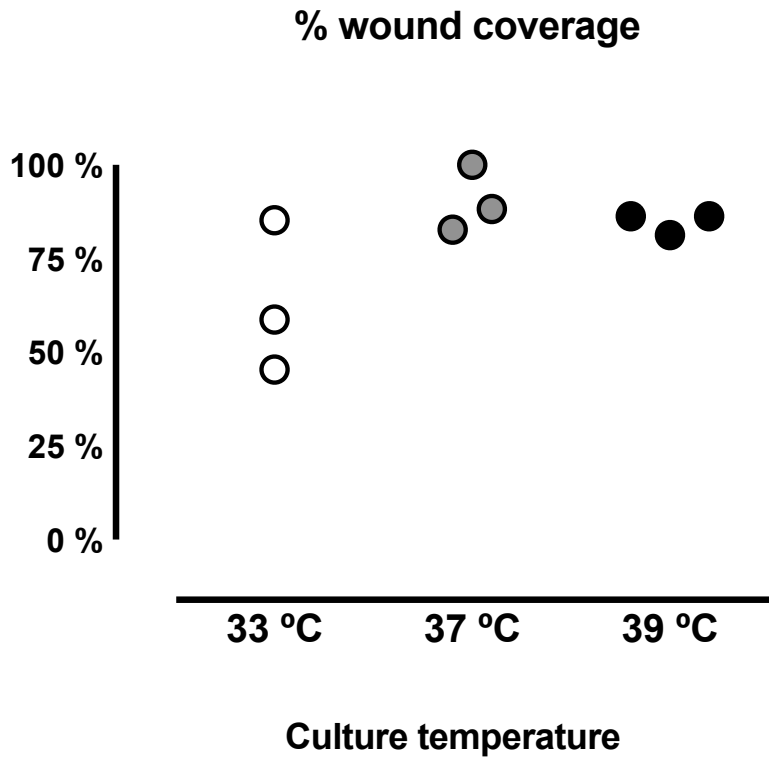


Figure 11. hAECs apoptotic activity is influenced by time, not culture temperatures of 33 °C, 37 °C or 39 °C. (A) The proportion of cells positive for Annexin V significantly increased with time (*p<0.05), irrespective of culture temperature (n=9, performed in triplicate). (B) hAECs positive for 7AAD did not change with culture temperature or time (n=9, performed in triplicate). Open circles are representative of hAECs cultured at 33 ºC, grey circles are hAECs cultured at 37 ºC and black circles are hAECs cultured at 39 ºC.

Effect of temperature on wound healing capacity of hAECs

Wound healing was not different between hAECs cultured at 33 °C, 37 °C and 39 °C (Figure 12).

A


% wound coverage
100 %
75 %
50 %
25 %
0 %
33 ºC
37 ºC
39 ºC
Culture temperature

B

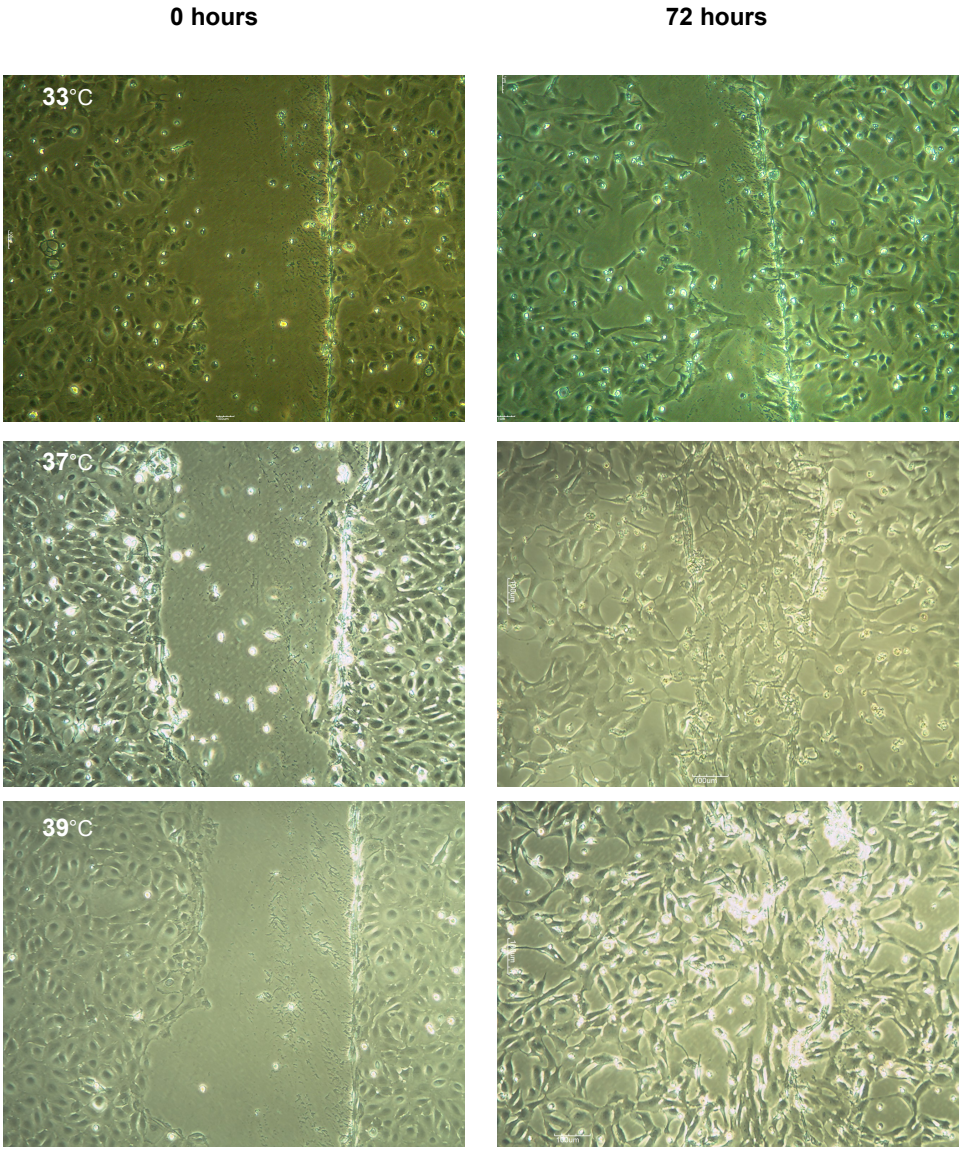


Figure 12. hAECs wound healing capacity is the same in 33 °C, 37 °C or 39 °C cultures. (A) Percentage change in the wound area recovered by hAECs exposed to culture temperatures 33 °C, 37 °C and 39 °C. (B) Representative images of cells cultured at 33 °C, 37 °C and 39 °C, at 0 and 72 hours (n=9, performed in triplicate). Open circles are representative of hAECs cultured at 33 ºC, grey circles are hAECs cultured at 37 ºC and black circles are hAECs cultured at 39 ºC.

Phagocytic activity of iMACs incubated in hAEC-conditioned media

Phagocytic activity of fluorescent beads was evident in all iMAC cultures incubated at 37 °C (Figure. 13). Viability was not different between control iMACs incubated with (90.4 % ± 0.3 %) or without beads (94.5 % ± 0.08 %).

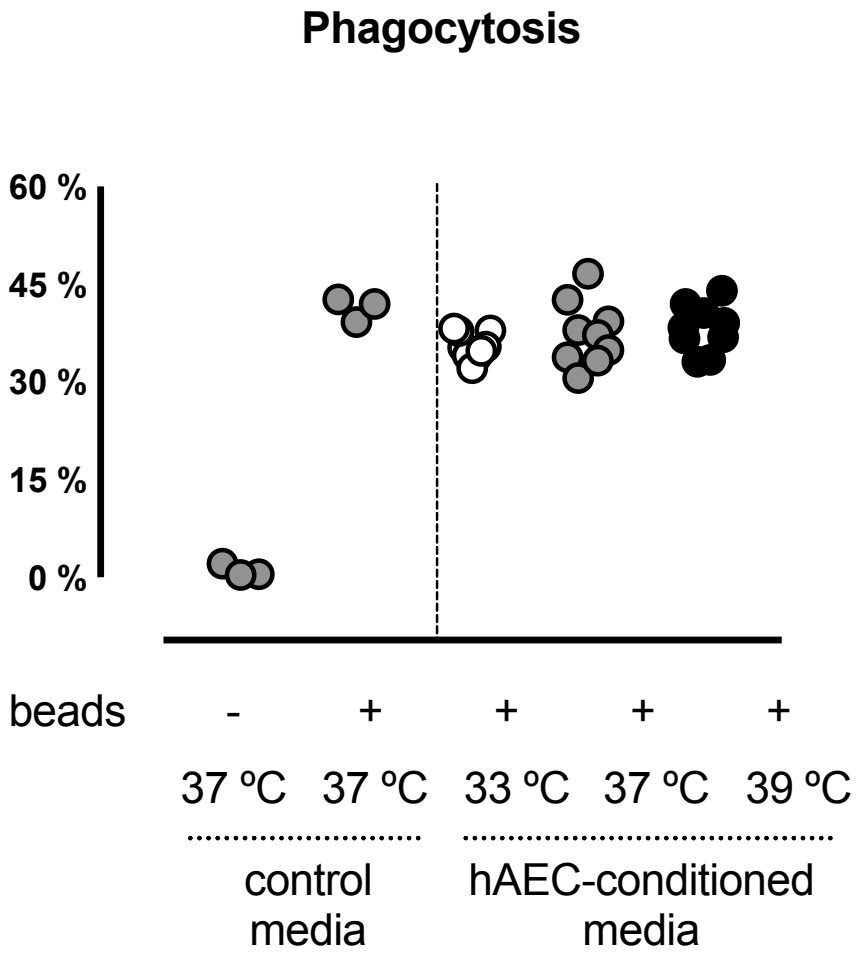


Figure 13. hAEC conditioned media at 33 °C, 37 °C or 39 °C does not influence mouse macrophage phagocytic activity. Immortalized mouse macrophages (iMACs) were incubated with standard media or with hAEC conditioned media from temperatures 33 °C, 37 °C and 39 °C (n=9, performed in triplicate). Percentage represents proportion of iMACs that have phagocytosed FITC-marked fluorescent beads. Open circles are representative of hAECs cultured at 33 ºC, grey circles are hAECs cultured at 37 ºC and black circles are hAECs cultured at 39 ºC.

Discussion

Viability and function of hAECs are not affected by temperatures ranging from 33 ºC – 39 ºC, indicating that efficacy of hAECs is unlikely compromised across a realistic range of temperatures for human neonates. Furthermore, factors secreted by hAECs at 33 °C, 37 °C and 39 °C do not alter innate macrophage phagocytic activity.

Human AECs are a potential therapeutic in prenatal inflammation and HIE and are currently used in adult corneal transplants and burns patients. Scientific investigation of these scenarios often involves the use of animals with different core body temperatures: mice (36 °C), rats (37 °C) and sheep (39 °C). Additionally, HIE is treated with therapeutic hypothermia, involving cooling the newborn to 33 °C[185] and fever associated with sepsis is common in preterm and term newborns.[187] Our data show that hAECs retain viability and function at temperatures that may be encountered clinically.

The rate of apoptosis in cultured hAECs was unchanged at 33 °C, 37 °C and 39 °C, over 72 hours. However, significantly more cells entered early apoptosis over 72 hours of culture at all temperatures, compared to 24 hours. Similarly, apoptosis increases with increasing temperatures (38 °C – 58 °C) in MSCs, and this persists for 3 days of culture.[193] MSCs have recovered cell number by day 7 of culture. The decrease in viability within the first 3 days in our study may have been transient, however we did not analyse cell death after day 3. The proliferation of live hAECs after more than 3 days in culture may improve the observed cell viability proportions, in a fashion similar to that observed in MSCs.

Human AECs achieved up to 90 % repopulation of wound areas when incubated at 33 °C, 37 °C and 39 °C, demonstrating efficient cell migration at these temperatures. The apparent slower repopulation of wound area in 33 ºC cultures compared to 37 ºC and 39 ºC cultures was not significant. Incubation temperatures between 27 °C and 40 °C increase the migration index of adult guinea pig skin cells.[196] However, this study was published over 40 years ago: migration was calculated subjectively using a numbered scoring system developed by the authors without statistical analyses.[196] We believe our data are the first to examine the effect of temperature on cell migration and wound healing capacity *in vitro*.

Inflammation is key to the development of perinatal lung and brain injury. Infants at greatest risk of perinatal lung and brain injury are those born preterm,[2, 197] who are likely to have been exposed to inflammation before birth.[18] Postnatal interventions following preterm birth, such as mechanical ventilation, can initiate or exacerbate lung inflammation,[198, 199] and cause a cascade of events that induce brain injury.[200] Administration of hAECs reduces inflammation in the lungs and brains of fetal and neonatal preterm lambs.[129-131, 182] The findings from our study suggest that these beneficial effects in sheep, with body temperatures of ~39 ºC, would be reproducible at temperatures ranging from 33 ºC – 39 ºC, and therefore in human neonates. Indeed, anti-inflammatory effects of hAECs are observed in mice, a species with a body temperature close to that of humans.[126, 127, 133, 136]

One known mechanism by which hAECs influence inflammation in mice is by the modulation of macrophage function.[136, 181] We observed consistent phagocytic activity of macrophages incubated with conditioned media from hAECs incubated at different temperatures.

Limitations

We did not measure cell viability during wound healing assays, which may have been important considering the extended amount of time hAECs spent in culture (first reaching confluency before the wound healing assays). Viability measurements may have answered questions regarding initially high apoptosis rates by day 3 of culture, but would have compromised wound healing assays. All hAECs undergoing wound healing assays were initially cultured at 37 °C to ensure that hAECs reached 100 % confluence at the same time, before being scratched and incubated at 33 °C, 37 °C or 39 °C. Consequently, we do not know if incubation at temperatures other than 37 °C prior to scratching cells would influence cell migration. Lastly, we did not investigate factors hAECs were secreting when cultured at 33 °C, 37 °C and 39 °C, and whether these were different. The specific factors secreted by hAECs may be important, depending on the intended clinical application of hAECs.

Conclusion

Viability and function of hAECs is not altered *in vitro* by temperatures ranging from 33 ºC – 39 °C. Thus, hAECs are likely to have therapeutic efficacy in patients across a realistic range of body temperatures.

Acknowledgements

We thank Professor Euan Wallace's research group for assistance in tissue collection. We also thank Nikeh Shariatian for assistance in isolating hAECs and running tissue culture experiments. We thank Associate Professor Ashley Mansell for providing mouse macrophages.

Declarations

Ethics approval

All experiments were approved by the Monash University Human Research Ethics Committee (2013001109).

Competing interests

We have no competing interests to report.

Funding

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Declaration for Thesis Chapter 5: The effect of human amnion epithelial cells on lung development and inflammation in preterm lambs exposed to antenatal inflammation

Declaration by candidate

Nature of the contribution	Extent of contribution
Performed experiments, collected data, analysed data, contributed to experimental design and drafted manuscript	70 %

The following authors contributed to the work. If the co-authors are students at Monash University their contribution in percentage is stated:

Nature of the contribution		Extent of contribution
Siavash Ahmadi-Noorbakhsh	Conducted experiments	N/A
Peter B. Noble	Conducted experiments	N/A
Rebecca Lim	Helped conceive the idea, obtain funding for the work, contributed to data interpretation and drafting manuscript	N/A
Euan Wallace	Helped conceive the idea, obtain funding for the work, contributed to data interpretation and drafting manuscript	N/A
J. Jane Pillow*	Obtained funding for the work, helped to conceive the idea, participated in design and performed the experiments and drafted manuscript	N/A
Tim J. Moss*	Conceived the idea and obtained funding for the work. Designed and performed experiments, assisted with analysis, interpretation and presentation of data and drafted manuscript	N/A

*Joint senior author

Chapter Five

Introduction

Antenatal inflammation often precedes preterm birth and may increase the likelihood of bronchopulmonary dysplasia (BPD).[201] Preterm infants often require respiratory support, which contributes to BPD development. The combination of chorioamnionitis and postnatal ventilation increases the risk of BPD.[18] Both chorioamnionitis and mechanical ventilation exacerbate lung inflammation in preterm infants, which is pivotal to the pathogenesis of BPD. Airway inflammation and elevated levels of interleukin (IL)-1, IL-6 and IL-8 are observed in tracheal aspirates of preterm infants who were exposed to chorioamnionitis or received ventilation after birth.[88, 202, 203] Prevention or attenuation of lung inflammation in response to chorioamnionitis and mechanical ventilation may provide a realistic therapy for BPD.[145]

Human amnion epithelial cells (hAECs) are a potential anti-inflammatory therapy for BPD. Stem cell-like hAECs can differentiate along any cell lineage, including into lung epithelial cells *in vitro*[138] and *in vivo.*[134] Human AEC treatment reduces lung inflammation in fetal sheep exposed to intra-amniotic lipopolysaccharide (LPS)[129] or ventilated *in utero*[130] and in preterm lambs ventilated for 2 hours[131] or 2 days[204]. No one has examined the effects of hAECs in the presence of pre- and post-natal inflammatory insults.

Infants who died from BPD have simplified lung architecture with apparent inhibition of alveolarisation.[10, 15] The airspaces are large and secondary septae are not sufficiently formed.[15] Similar lung morphology is seen in preterm lambs after exposure to antenatal inflammation[21] or periods of ventilation.[153]

We hypothesised that administration of hAECs to ventilated preterm lambs, exposed to antenatal intrauterine inflammation, would decreased the duration of ventilation, reduce markers of lung injury and inflammation and, consequently, reduce abnormalities in lung development.

Methods

Human AEC isolation

All procedures were approved by Monash University Human Ethics Committee (ref #: MUHREC-CF13/2144-2013001109). Placentae were obtained from women undergoing elective term caesarean section. All women provided written informed consent. Exclusion criteria included preterm birth, intrauterine growth restriction, clinical chorioamnionitis or pre-existing maternal disease, including diabetes. The isolation of hAECs from placentas was described previously.[138] Briefly, the amnion was stripped from adjacent choriodecidua and rinsed in Hanks Balanced Salt Solution (HBSS; Invitrogen, San Diego, CA). Human AECs were isolated by two, 1-hour digests in 0.05 % Trypsin (Invitrogen, San Diego, CA), and collected by centrifugation. Live-cell counts and viability were determined by trypan blue exclusion (>85 % viability was required) at isolation (before cryopreservation) and prior to administration to preterm lambs (immediately after thawing). Human AECs from at least 2 donors were thawed, counted and resuspended in sterile saline ($30x10^6$ hAECs in 20 mL) for delivery to preterm lambs at 1 mL/minute.

Animals

The University of Western Australia Animal Ethics Committee approved all animal experimentation (RA 3/100/1454).

Antenatal interventions

Pregnant ewes received intramuscular (IM) medroxyprogesterone (150 mg; Pfizer, Australia) 7 days prior to planned induction of labour, to avoid subsequent betamethasone-induced preterm labour. Ewes received two IM injections of betamethasone (5.7 mg/dose; Celestone, Merck Sharp & Dohme Pty Ltd, Australia) at 48 h and 24 h prior to planned Caesarean section delivery. Pregnant ewes randomly received ultrasound guided intra-amniotic (IA) LPS (4 mg; 2 mg/mL; *Escherichia coli* 055:B5; Sigma-Aldrich, NSW, Australia; n=10) or IA saline (n=10), at 126 days' gestational age (GA; term ~150d). This dose of LPS results in a well characterised fetal inflammatory response that peaks 48 h after injection.[148]

Preterm delivery

Pregnant ewes at 128 d GA were premedicated with IM buprenorphine (0.01 mg/kg, 300 mcg/mL, Indivor, Pty Ltd, Australia) 1 h prior to IV induction of anaesthesia (15 mg/kg sodium

thiopental; Troy Laboratories, Australia). Inhalational anaesthesia (2-3 % Isoflurane; Bomac Animal Health, Australia) was maintained via an endotracheal tube using a ventilator. The fetal head and neck were exposed through maternal laparotomy and hysterotomy. The right carotid artery and right jugular vein of the fetus were catheterised for blood gas sampling and drug administration, respectively. The fetus was intubated and excess lung liquid was passively drained before administration of surfactant (3 mL, 80 mg/mL, poractant alfa, Chiesi Farmaceutici S.pA., Italy) via the endotracheal tube. The umbilical cord was clamped and cut. The lamb was weighed, dried and placed in ventral recumbency, suspended in a sling, on a neonatal baby warmer. Cord blood was collected for blood gas measurement, full blood counts and collections of plasma. The lamb received a sustained inflation (30 cmH_2O for 30 s) before initiation of ventilation. Body temperature was maintained between 38 ºC and 39 ºC. The ewe was humanely killed immediately after the lamb was delivered (150 mg/kg pentobarbitone; Valobarb, Jurox, Australia).

Postnatal interventions

Human AECs were randomly administered to preterm lambs exposed to IA LPS (LPS/hAEC; $30x10^6$ cells IV; n=7), beginning immediately after delivery. Remaining lambs (delivered after either IA saline or LPS) received postnatal saline (control). Numbers of subjects are shown in Table 10.

Table 10. Experimental groups and interventions

Treatment	Antenatal	Postnatal	Number per group
Sal/Sal	Saline	Saline	10
LPS/Sal	LPS	Saline	10
LPS/hAEC	LPS	hAECs	7

LPS, lipopolysaccharide; hAECs, human amnion epithelial cells

Respiratory support

Lambs received graded respiratory support and ventilation (Evita® Infiity® V500 Ventilator, Dräger) in accordance with best clinical practice, beginning with mechanical ventilation (MV), then bubble continuous positive airway pressure (B-CPAP), extubation onto heated humidified high flow (HHF) via nasal (n) cannulae and eventual unassisted breathing of room air. MV was initiated with volume guarantee (5-7 mL/kg), a fraction of inspired oxygen (FiO_2) of 0.3, peak inspiratory pressure (PIP) of 30 cmH_2O, positive end-expiratory pressure (PEEP) of 9 cmH_2O and ventilator rate of 50 breaths/min. Ventilator adjustments were determined by clinical examination and arterial blood gas measurements. Ventilator settings were adjusted (where possible) to target oxygen saturation (SpO_2) of 90-95 % at the lowest achievable FiO_2. We attempted rapid weaning from MV, allowing permissive hypercapnia. Amplitude during nCPAP and nHHF was adjusted to maintain $PaCO_2$ in the range of 45-55 mmHg. Lambs remained on nCPAP or nHHF if they displayed sufficient respiratory drive had minimal oxygen requirements and acceptable arterial blood gas levels. Lambs were reintubated if they experienced one or more of the following: 1) ventilatory failure ($PaCO_2$ > 80 mmHg on 2 consecutive occasions > 30 min apart and unresponsive to altered nasal pressure amplitude and frequency settings); 2) severe metabolic acidosis; 3) > 4 apneas requiring resuscitation within an hour, or 4) persistent SpO_2 < 80 %.

Pulmonary gas exchange was assessed using PaO_2 to FiO_2 (pF) ratio and oxygenation index (OI = [(MAP x FiO_2 x 100)] / [(PaO_2 x 1.36)].

General postnatal management

Lambs received IV antibiotics twice-daily (piperacillin/tazobactam 100 mg/kg, 100 mg/mL;

Sandoz Pty Ltd, Australia and gentamicin 6 mg/kg, 100 mg/mL Troy Laboratories, Austrlia). Caffeine (WAMF, WA) was administered as an IV loading dose (20 mg/kg) then daily as required (5 mg/kg) in accordance with routine neonatal intensive care unit protocol.

Arterial blood gases (ABG) were measured every 30 minutes for the first hour of life, every hour for the next 4 hours of life and 4-6 hourly thereafter. ABGs were taken 6-12 hourly if lambs were considered stable, on minimal respiratory support.

Lambs received increasing aliquots of enteral feeds (colostrum from ewe) commencing at 2 h, given at 2 h intervals for 24 h followed by 50:50 (ewe colostrum:lamb formula), then lamb milk replacement formula from 48 h of life. Feeds were increased 1 mL/6 h for the first 72 h and 1 mL/4 hours thereafter.

General observations were made every hour for the duration of experiments. Observations included, but were not limited to: body temperature, heart rate (HR), blood pressure (BP), respiratory rate (RR), SaO_2, mean airway pressure (MAP), FiO_2, feed volumes and urine and faeces output.

Post mortem measurements and tissue collection

On day 7 of life, lambs were killed with an overdose of sodium pentobarbitone (150 mg/kg; Jurox Pty Ltd, Australia). The left lung was inflation-fixed at 30 cmH_2O with 10 % formaldehyde[29] for morphometric and histological analyses. Static compliance was calculated as the volume of fixative infused at 30 cmH_2O divided by the birth-weight. Three random sections of fixed left lung and 5 regions of interest (ROI) per section were used for all histological and immunohistochemical analyses. A total of 15 ROIs at x40 magnification (unless otherwise stated) were analysed per animal. Small subpleural segments of the right lower lobe of the lung and the midline of the liver were frozen in liquid nitrogen for molecular analysis. All analyses were carried out by P.C. Papagianis who was blinded to treatment interventions.

Morphometric analyses

Lung sections were stained with Harts resorcin-fuschin[149] for elastin and identification of tissue, airspace and septal crests. A semi-automated ImageJ (NIH image, Bethasda, Maryland, USA) plugin (Copyright © 2015, Keith Schulze, Monash Micro Imaging, Monash University) was used to overlay a point grid on each ROI, which detected tissue and airspace. Total points falling on tissue or airspace were divided by total overlaid points (676)

for each ROI, so as to express the areal fractions of tissue or airspace in the lungs. Septal crests were counted by P. C. Papagianis (Septal crest areal fraction = [points on septal crests] / [points on tissue]).

Total elastin was calculated with an automated ImageJ plugin (Copyright © 2015, Keith Schulze, Monash Micro Imaging, Monash University). The area of elastin was divided by the total area of tissue to measure the proportion of elastin deposition in the lungs.

Haematoxylin and eosin (H&E)-stained sections were used for scoring epithelial sloughing (a sign of mechanical disruption of the lung epithelium)[38,205] using a scale between 0 and 4 per ROI: no events = 0, < 5 events = 0.5, 5 - 10 events = 1 - 2, 10 - 20 events = 2.5 - 3 and > 20 events = 3.5 - 4.

Collagen was visualised with picrosirus red (PSR) stain.[150] The birefringence of collagen was visualised using a Leica Abrio polarising microscope (512 X 512 CCD black and white camera by CRI Abrio software). The area of collagen within each ROI was normalised to area of tissue using an automated ImageJ plugin (Copyright © 2015, Keith Schulze, Monash Micro Imaging, Monash University; NIH Image).

Immunohistochemistry

Immunohistochemistry was used to identify leukocytes (CD45)[151], macrophages (CD163)[152], proliferating cells (Ki67)[153], myofibroblasts (α-smooth muscle (SM) actin)[153] and type II epithelial cells (pro-surfactant protein (SP)-C)[154], as detailed elsewhere. All sections were scanned using ImageScope (Aperio Technologies, USA). Positive cells were manually counted using ImageJ. Cells positive for Ki67 and PSR are presented as areal density of tissue. α-SM actin staining density was analysed using ImagePro Plus software (c. 9.2, Build 6156, 2012-2015 Media Cybernetic© Inc.) and is outlined elsewhere.[153] CD45+, CD163+ and SP-C analyses are presented as the average number of positive cells across 15 ROIs per animal.

Molecular analyses

Total RNA was isolated from lung and liver samples (RNeasy Kit, Qiagen) and reverse-transcribed into cDNA (SuperScript III reverse transcriptase, Invitrogen). Genes of interest (Table 11) were measured using TaqMan® probes.

TaqMan® RT-PCR assays, outlined elsewhere,[141] were used to measure gene expression. Briefly, expression of each target gene was measured in triplicate cDNA samples on an ABI

Prism 7900HT Real-Time PCR System (PE Applied Biosystems). We analysed Ct values using qBase+ software, version 3.1.[142] We selected reference gene ribosomal (r)18S using the qBase+ geNorm algorithm for assessing stability of reference genes and optimal number of reference genes.[143] The expression of all genes is expressed as calibrated normalised relative quantity (CNRQ; i.e. normalised to r18S and expressed relative to the Sal/Sal group), where 18S Sal/Sal is normalised to ~0.00.

Table 11. Probe selection for TaqMan® Assays

Gene	Assay ID
r18S	Oa4906333_g1
IL-1α	Oa04658682_m1
IL-1β	Oa04656322_m1
IL-4	Oa04927178_s1
IL-6	Oa04656315_m1
IL-8	CUSTOM
IL-10	Oa03212724_m1
TGF-β1	Oa04259484_m1
TNF	Oa04656867_g1
Surfactant protein A1	Oa04657758_m1
Surfactant protein B	Oa04750908_g1
Surfactant protein C	Oa04656841_m1
Surfactant protein D	CUSTOM
VEGF-A	Oa04653812_m1
VEGFR1	Oa04694159_m1
Angiopoeitin-1	Oa04757067_m1
EGR1	Oa03237885_m1
CYR61	Oa04673852_g1
CTGF	Oa04659069_g1
PECAM	Oa04677168_m1
MMP9	Oa03215996_g1
MMP12	Oa04744924_g1
CCL2	Oa04677078_m1
SAA	Oa04924154_s1
Hepcidin	Oa04656982_m1

IL = interleukin; TGF = transforming growth factor; VEGF = vascular endothelial growth factor; EGR = early growth response; CYR = cysteine-rich; CTGF = connective tissue growth factor; PECAM = platelet endothelial cell adhesion molecule; MMP = matrix metallopeptidase; CCL = chemokine ligand; SAA = serum amyloid A.

Statistical Analyses

We identified < 5 % of physiological data points were missing and utilised sequential regression modelling to impute values (SPSS v. 24, IBM). Serial physiological data were analysed using two-way repeated measures ANCOVA (SPSS, IBM). Pearson's correlations were used to identify covariates (SPSS, IBM). Year of delivery was used as a covariate in all physiological analyses. Additional covariates are stated where included. Cord blood gas measurements were analysed separately to serial postnatal ABGs. Post-hoc comparisons were made using the Holm-Sidak method. Histological data are expressed as mean ± SD and were analysed using Kruskal-Wallis with Dunn's multiple comparisons post-hoc test (GraphPad Prism version 7 for Mac OS X). TaqMan® data are expressed as mean ± SD, relative to the Sal/Sal group.

Results

Characteristics of lambs

Lamb characteristics are outlined in Table 12. Body weights at birth and postnatal day 7 were similar between groups. Lung-to-body-weight ratio was heavier in LPS/Sal and LPS/hAEC lambs compared to Sal/Sal lambs (P=0.03 and P=0.04, respectively; H(2) = 8.2; mean ranks: 6.9 for Sal/Sal, 15.7 for LPS/Sal and 16.1 for LPS/hAECs). Liver, thymus, spleen and adrenal weights, relative to body weight, were not different between groups (P>0.05). Static lung compliance was lowest in LPS/hAEC lambs compared to Sal/Sal lambs (H(2) = 12.42; P=0.001) but not different to LPS/Sal lambs (P=0.09; mean ranks: 19.3 for Sal/Sal, 13.8 for LPS/Sal and 5.7 for LPS/hAECs).

Umbilical cord pH, $PaCO_2$, PaO_2, HCO_3 and base excess (BE) measurements were not different between groups (P>0.05).

Table 12. Lamb characteristics

Variable	Experimental group		
	Sal/Sal	LPS/Sal	LPS/hAEC
GA (d)	129.7 ± 1.4	128.9 ± 0.9	126.6 ± 0.79
N (% male)	10 (44.4)	10 (50.0)	7 (42.9)
Birth body weight (kg)	2.89 ± 0.14	3.29 ± 0.13	3.12 ± 0.8
PM body weight (kg)	2.78 ± 0.13	3.02 ± 0.09	2.90 ± 0.05
Left lung weight/PM body weight (g/kg)	11.5 ± 1.2	17.9 ± 1.9*	17.0 ± 1.8*
Static lung compliance ((ml/kg)/cmH_2O)	0.97 ± 0.87	0.76 ± 0.05	0.53 ± 0.06*
Liver weight/PM body weight (g/kg)	33.4 ± 2.3	33.6 ± 2.0	35.7 ± 1.0
Thymus weight/PM body weight (g/kg)	1.96 ± 0.37	2.27 ± 0.22	2.96 ± 0.43
Spleen weight/PM body weight (g/kg)	1.90 ± 0.32	2.38 ± 0.18	2.67 ± 0.26
Adrenal weight/PM body weight (g/kg)	0.28 ± 0.02	0.22 ± 0.02	0.22 ± 0.02

Data are mean ± SD. PM = post mortem; GA = gestational age. *P<0.05 significantly different to Sal/Sal.

Types of respiratory support and gas exchange

Year of delivery and birth-weight correlated with duration of MV (r=0.602, P<0.01 and r=0.398, P<0.001, respectively). When corrected for year of delivery and birth-weight LPS/hAEC lambs spent an increased proportion of time on MV compared to Sal/Sal lambs ($F(2, 135) = 3.0$; P=0.05; **Table 14**). The proportion of time spent on MV was not different between other treatment groups or between groups for any given day (P>0.05; Table 13).

Total hours of intubation were not different between groups (**Table 14**). The proportion of time spent on MV, ET-CPAP, nB-CPAP, nHHF or no respiratory support for each animal is outlined in Figure 15.

Year of delivery and proportion of time spent on MV strongly correlated with MAP (r=0.497; P<0.001 and r=0.728, P<0.05, respectively) and when considered as covariates we saw no differences between MAP of Sal/Sal, LPS/Sal and LPS/hAEC lambs (P>0.05; Figure 16).

Over the course of the study, all animals had increasing $PaCO_2$, HCO_3 and BE (P<0.05; Figure 14). During the course of experiments pH did not change (P>005; Figure 14). PaO_2 was higher in Sal/Sal lambs (85.1 ± 29.0 mmHg) compared to LPS/Sal (61.2 ± 11.1 mmHg; P=0.05) and LPS/hAEC (57.1 ± 12.8 mmHg; P=0.030) lambs on day 1, but there were no differences in PaO_2 between days 2-7 (P>0.05; Figure 14). SaO_2 was not different between groups (P>0.05; Figure 3). FiO_2 did not change in Sal/Sal or LPS/hAEC lambs over the course of the study (P>0.05). LPS/Sal lambs required increasing FiO_2 over the 7-day experiments ($F(5, 136) = 2.5$; P=0.03), but there was no difference in FiO_2 requirements between groups (P=0.13; Table 13). The pF ratio and OI values were not different between groups (P>0.05), with the exception of decreased OI (better oxygenation) in Sal/Sal lambs on day 6 compared to LPS/hAEC lambs (P=0.02; Figure 16).

Physiology

Physiological measures are shown in Table 13. Mean BP was not different between treatment groups. The HR of lambs was not different between groups, with the exception of day 4 when LPS/Sal lambs had higher HR than Sal/Sal lambs (P=0.04). Respiratory rate was not different between groups, with the exception of a higher RR in Sal/Sal lambs compared to LPS/hAEC lambs on day 6 (P=0.03).

Table 13. Physiological variables in preterm lambs over the first 6 days of life.

Variable	Day	Experimental group		
		Sal/Sal	**LPS/Sal**	**LPS/hAECs**
Proportion of time on mechanical ventilation (%)	1	19.91	43.98	75.69
	2	11.11	57.41	89.90
	3	10.19	51.81	79.86
	4	26.85	36.57	75
	5	29.17	33.80	94.44
	6	0	41.67	83.33
FiO_2	1	0.23 ± 0.00	0.22 ± 0.01	0.22 ± 0.01
	2	0.21 ± 0.01	0.23 ± 0.02	0.22 ± 0.01
	3	0.23 ± 0.02	0.23 ± 0.02	0.23 ± 0.02
	4	0.23 ± 0.02	0.27 ± 0.09	0.23 ± 0.03
	5	0.24 ± 0.04	0.30 ± 0.16	0.24 ± 0.03
	6	0.24 ± 0.04	0.26 ± 0.07	0.27 ± 0.04
Mean BP (mmHg)	1	61 ± 2	62 ± 1	60 ± 3
	2	50 ± 2	58 ± 2	59 ± 1
	3	63 ± 3	60 ± 2	62 ± 1
	4	62 ± 2	63 ± 2	62 ± 2
	5	64 ± 2	63 ± 2	62 ± 4
	6	62 ± 2	68 ± 3	62 ± 3
HR (beats/min)	1	221 ± 17	212 ± 22.09	195 ± 17
	2	200 ± 16	191 ± 26.94	195 ± 31
	3	201 ± 28	190 ± 26.44	186 ± 31
	4	215 ± 30	186 ± 26*	197 ± 28
	5	213 ± 24	193 ± 32	202 ± 23
	6	222 ± 22	208 ± 29	211 ± 17
RR (breaths/min)	1	54 ± 2	55 ± 2	51 ± 2
	2	58 ± 2	49 ± 3	50 ± 4
	3	54 ± 3	50 ± 2	47 ± 4
	4	54 ± 3	53 ± 2	49 ± 3
	5	52 ± 4	56 ± 3	53 ± 4
	6	61 ± 4	55 ± 4	49 ± 3*

Data are mean ± SD. *P<0.05 compared to Sal/Sal. FiO_2: fraction of inspired oxygen; BP: blood pressure; HR: heart rate; RR: respiratory rate.

Table 14. Mechanical ventilation, time intubated and mean airway pressure in preterm lambs.

		Mean difference [95 % CI]	P-value
MV (%)			
Sal/Sal	LPS/Sal	-2.63 [-6.92, 1.67]	0.367
	LPS/hAEC	-6.33 [-12.64, -0.03]	0.049
Time intubated (h)			
Sal/Sal	LPS/Sal	-2.64 [-6.78, 1.51]	0.333
	LPS/hAEC	-5.14 [-11.27, 1.00]	0.129
MAP (mmHg)			
Sal/Sal	LPS/Sal	-1.19 [-2.55, 1.95]	0.145
	LPS/hAEC	-0.30 [-2.66, 0.27]	0.984

Mean difference between Sal/Sal and LPS/Sal or LPS/hAEC lambs and [95 % confidence intervals]. Year of delivery was used as a covariate in all analyses, with additional covariates of birth-weight for MV analysis and MV for MAP analyses. MV: mechanical ventilation; MAP: mean airway pressure. MAP is taken from ventilator and set continuous positive airway pressure.

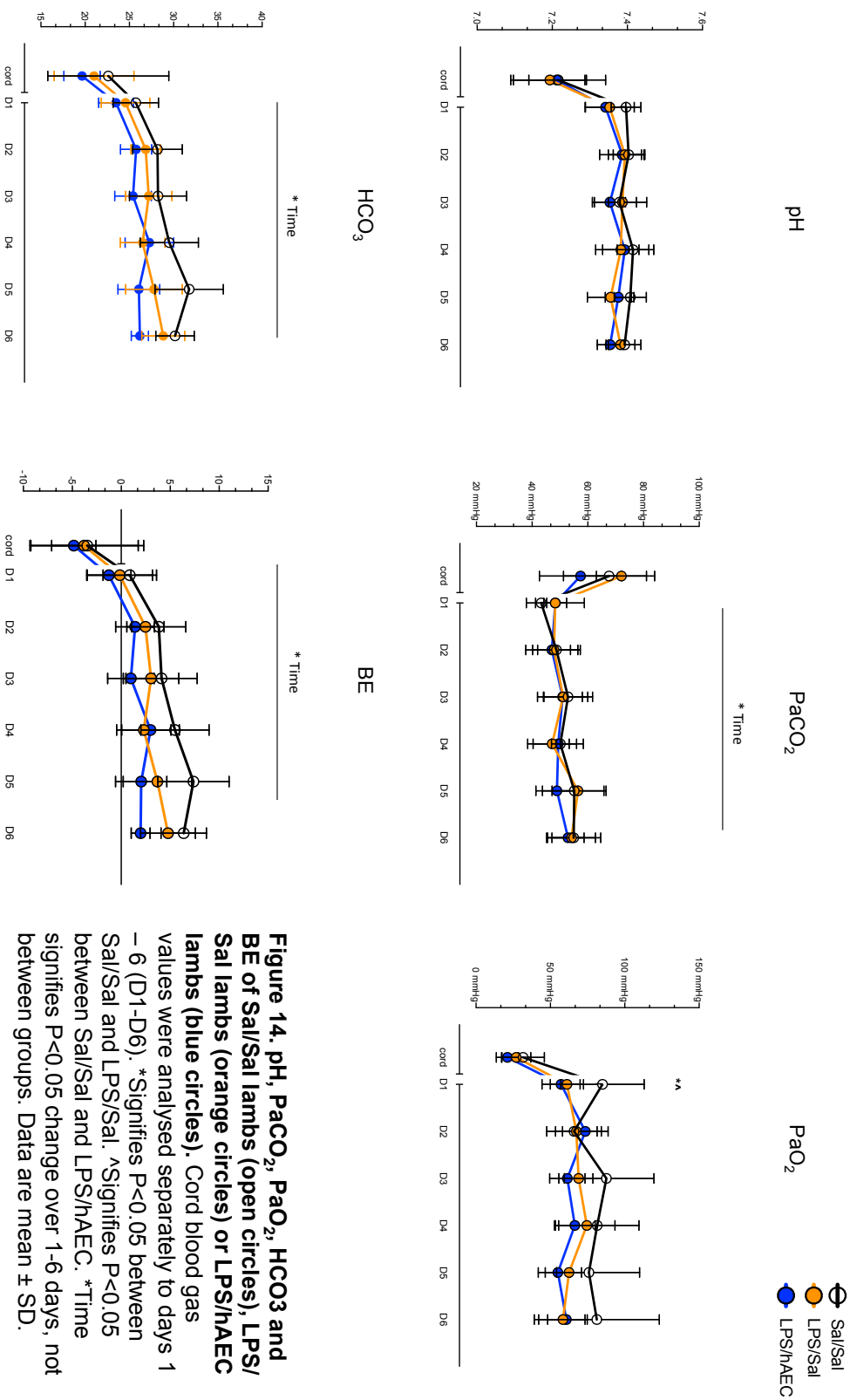

Figure 14. pH, $PaCO_2$, PaO_2, HCO3 and BE of Sal/Sal lambs (open circles), LPS/Sal lambs (orange circles) or LPS/hAEC lambs (blue circles). Cord blood gas values were analysed separately to days 1 – 6 (D1-D6). *Signifies P<0.05 between Sal/Sal and LPS/Sal. ^Signifies P<0.05 between Sal/Sal and LPS/hAEC. *Time signifies P<0.05 change over 1-6 days, not between groups. Data are mean ± SD.

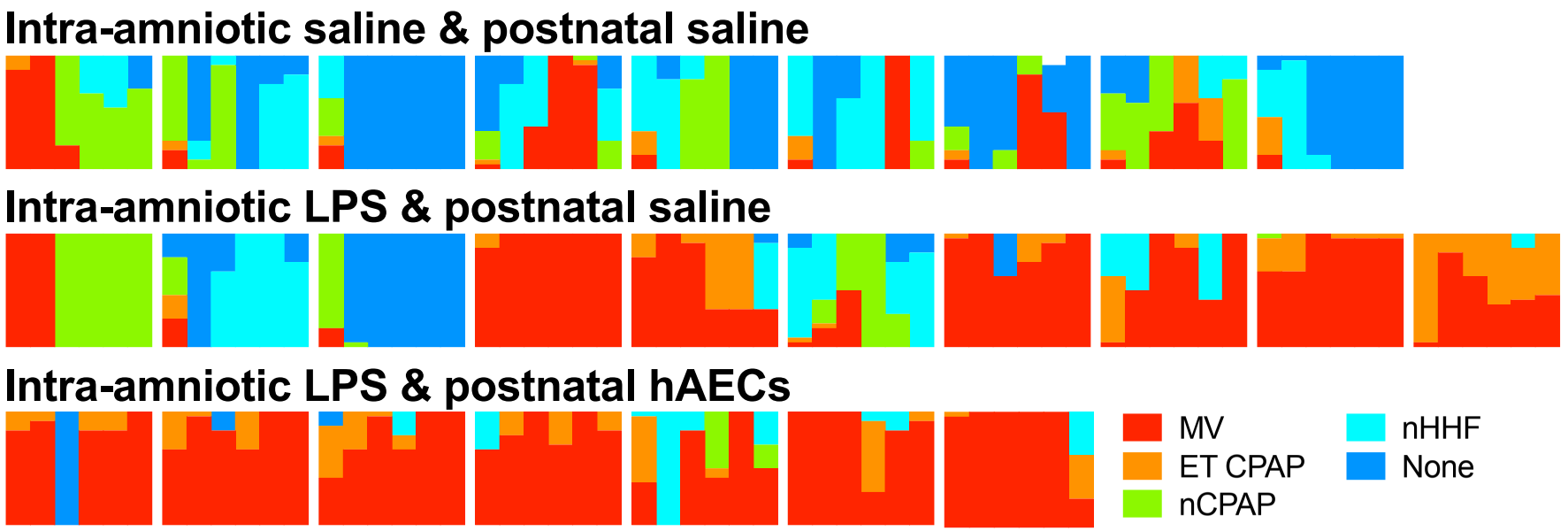

Figure 15. Representative graphs of ventilator requirements in preterm lambs. Types of respiratory support are graded with intubated variations, mechanical ventilation (MV) and endotracheal continuous positive airway pressure (ET CPAP), in red and orange and non-intubated variations, nasal (n)CPAP, nasal humidified high flow (nHHF) and no support (none), in green, light blue and blue, respectively. Lambs within Sal/Sal, LPS/Sal and LPS/hAEC groups are represented in left-to-right, in order of delivery. The first 6 days of respiratory support are presented as columns, form left-to-right, as the proportion of time spent on any mode of respiratory support on that day.

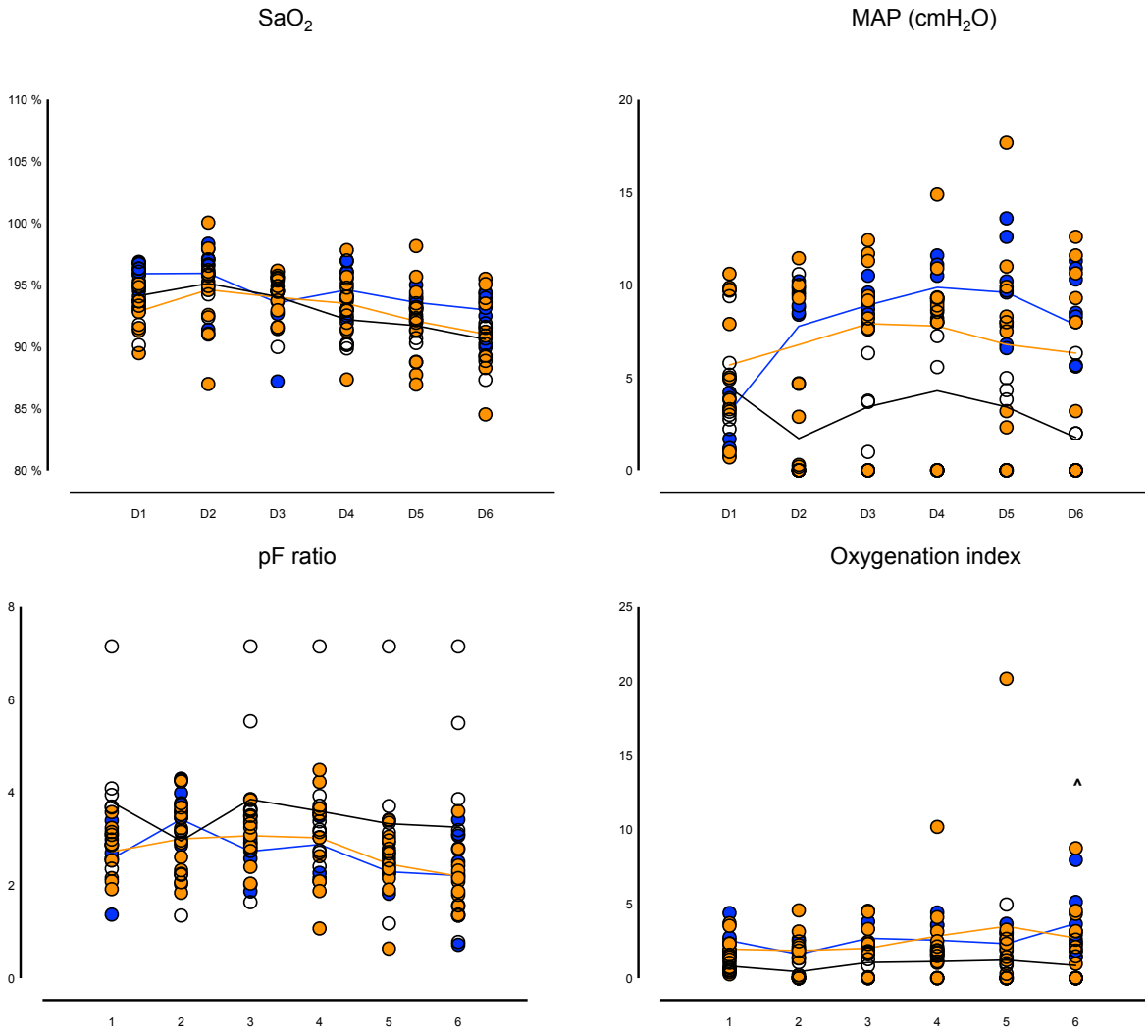

Figure 16. SaO_2, mean airway pressure (MAP) pF ratio and oxygenation index (OI) of Sal/Sal lambs (open circles), LPS/Sal lambs (orange circles) or LPS/hAEC lambs (blue circles). Days 1-6 of life are presented on the x-axis. MAP is taken from mechanical ventilation and continuous positive airway pressure. MAP was calculated as 0 cmH_2O in extubated lambs. Data points are individual animals connected by mean line. ^Signifies P<0.05 between Sal/Sal and LPS/hAEC lambs.

Lung morphology

There was large variability in lung architecture within treatment groups (Figure 17). The proportion of tissue in the lungs was not different between LPS/hAEC (50.3 ± 2.4 %), Sal/Sal (47.6 ± 2.5 %) or LPS/Sal (55.9 ± 2.7 %; P>0.05) lambs, although LPS/Sal lambs tended to have increased tissue areal fraction compared to Sal/Sal lambs (H(2) = 5.5; P=0.06; mean ranks: 9.8 for Sal/Sal, 17.8 for LPS/Sal and 12.1 for LPS/hAECs; Figure 17).

Septal crest areal fraction was not different between Sal/Sal and LPS/Sal lambs (1.8 ± 0.3 vs. 1.4 ± 0.3 %; P>0.99). There was a tendency for increased septal crest areal fraction in LPS/hAECs lambs (3.1 ± 0.7 %) compared to LPS/Sal lambs (H(2) = 4.9, P=0.08; mean ranks: 13.4 for Sal/Sal, 10.1 for LPS/Sal and 18.4 for LPS/hAEC; Figure 17).

The areal density of elastin within lung tissue was not different between groups (Sal/Sal: 9.1 ± 0.4 %; LPS/Sal: 9.2 ± 1.1 % and LPS/hAECs: 9.6 ± 0.6 %; P>0.99). The areal density of collagen within lung tissue was not different between groups (Sal/Sal: 30.8 ± 1.7 %; LPS/Sal: 33.1 ± 2.6 % and LPS/hAECs: 32.7 ± 2.6 %; P>0.05). Epithelial sloughing was lower in LPS/hAEC lambs compared to LPS/Sal (P=0.02) and Sal/Sal groups (H(2) = 10.1, P=0.01; mean ranks: 17.8 for Sal/Sal, 16.8 for LPS/Sal and 6.0 for LPS/hAEC; Figure 18). Epithelial sloughing was not different between Sal/Sal and LPS/Sal lambs (P>0.99).

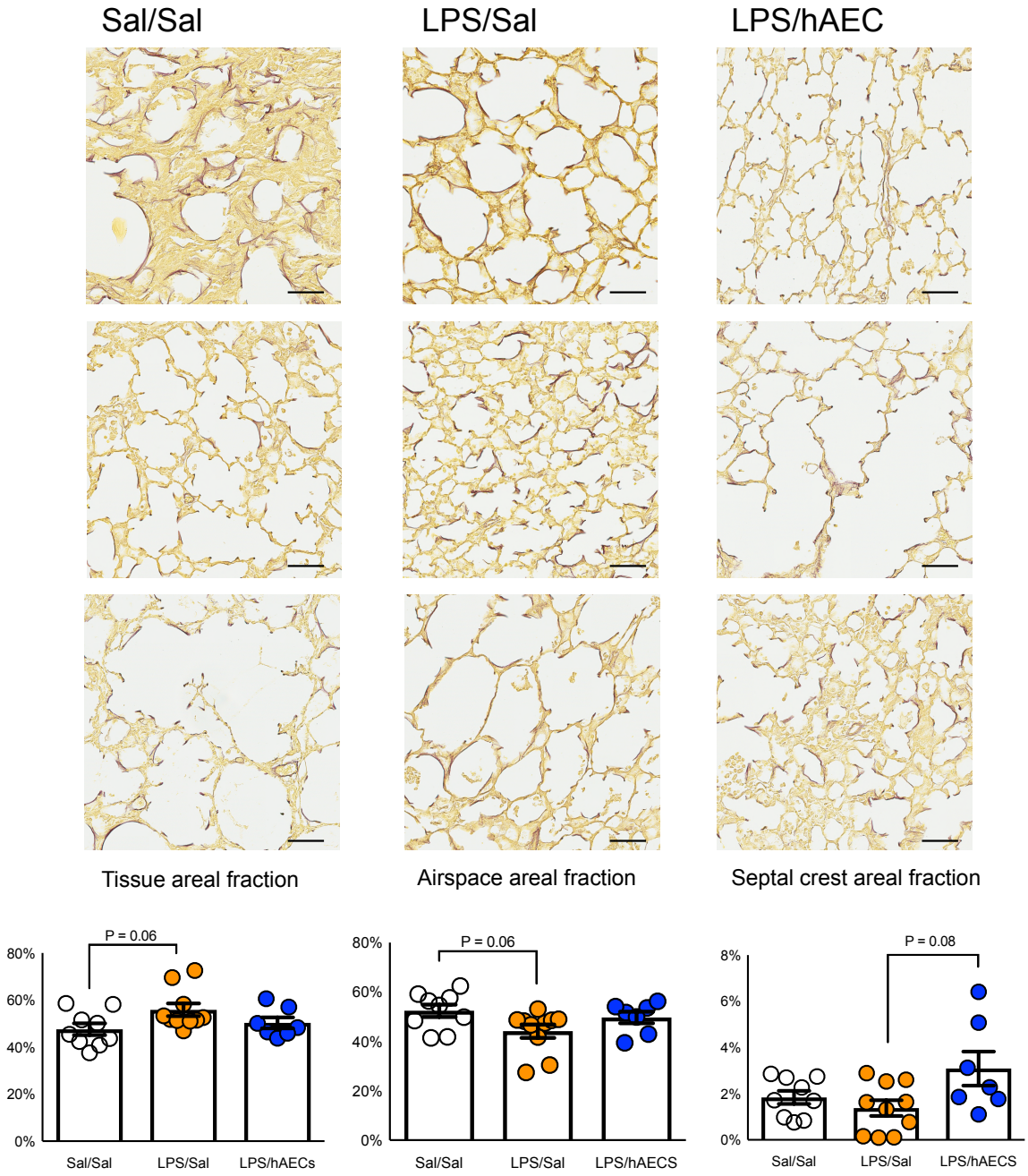

Figure 17. Representative images depicting variability in the lung parenchyma of preterm lambs within the same treatment group. Quantification of tissue, airspace and septal crest areal fractions in the lungs of preterm lambs on day 7 of life. Sal/Sal preterm lambs were exposed pre- and postnatally to saline, LPS/Sal preterm lambs were exposed prenatally to LPS and postnatally to saline and LPS/hAECs preterm lambs were exposed prenatally to LPS and postnatally to human amnion epithelial cells (hAECs). Elastin is stained in black and tissue in yellow. All images are taken at 20X magnification. Scale bar = 50 μm.

Lung inflammation and cell characteristics

The average number of CD45+ cells in the lungs was variable and not different between groups (Sal/Sal: 93.1 ± 11.3 cells/ROI; LPS/Sal: 139.7 ± 23.4 cells/ROI and LPS/hAECs: 121 ± 13.0 cells/ROI; P>0.05; Figure 18). The number of CD163+ cells in the lungs was not different between Sal/Sal (36.8 ± 7.4 cells/ROI), LPS/Sal (55.9 ± 9.0 cells/ROI) and LPS/hAEC (57.5 ± 14.2) lambs (P>0.05; Figure 18).

The areal density of Ki67+ cells within the lung tissue and airspaces was greatest in LPS/Sal lambs compared to Sal/Sal lambs (P=0.02), and tended to be increased relative to LPS/hAEC lambs (H(2) = 10.3, P=0.06; mean ranks: 10.7 for Sal/Sal, 19.5 for LPS/Sal and 8.6 for LPS/hAECs; Figure 19). Ki67+ cells were often clumped within the airspaces of LPS/Sal lambs (Figure. 19). The areal density of Ki67+ cells in the airspaces of LPS/Sal lambs was higher than Sal/Sal lambs (P=0.049), but not different to LPS/hAEC lambs (H(2) = 7.1, P>0.99; mean ranks: 7.8 for Sal/Sal, 16.1 for LPS/Sal and 15.7 for LPS/hAECs; Figure 19).

The areal density of α-SM actin in the lungs was not different between Sal/Sal (38.2 ± 3.6 %) and LPS/Sal (28.0 ± 6.7 %; P=0.61) groups, but tended to be lower in LPS/hAEC lambs (20.1 ± 4.8 %; P=0.07; Figure 20).

The number of pro-SP-C cells in the lungs was similar in all groups: Sal/Sal 23.6 ± 12.7 cells/ROI; LPS/Sal 19.1 ± 10.6 cells/ROI; and LPS/hAECs 20.1 ± 18.8 cells/ROI; P>0.05).

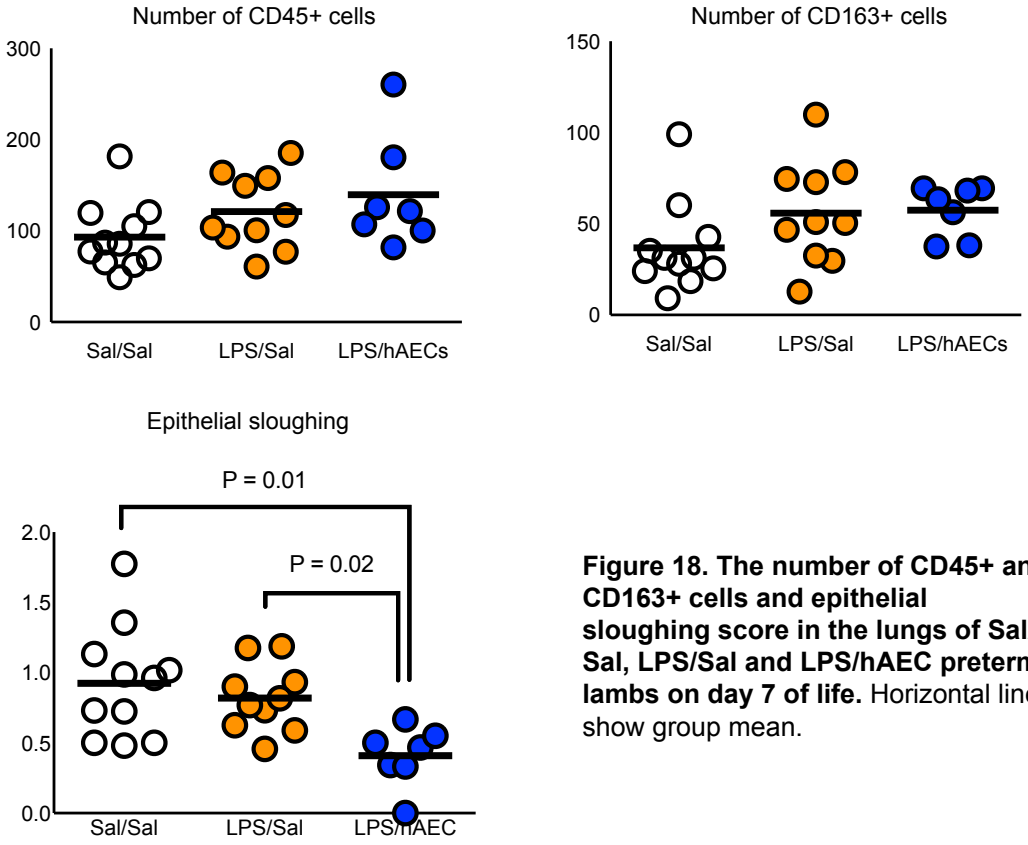

Figure 18. The number of CD45+ and CD163+ cells and epithelial sloughing score in the lungs of Sal/ Sal, LPS/Sal and LPS/hAEC preterm lambs on day 7 of life. Horizontal lines show group mean.

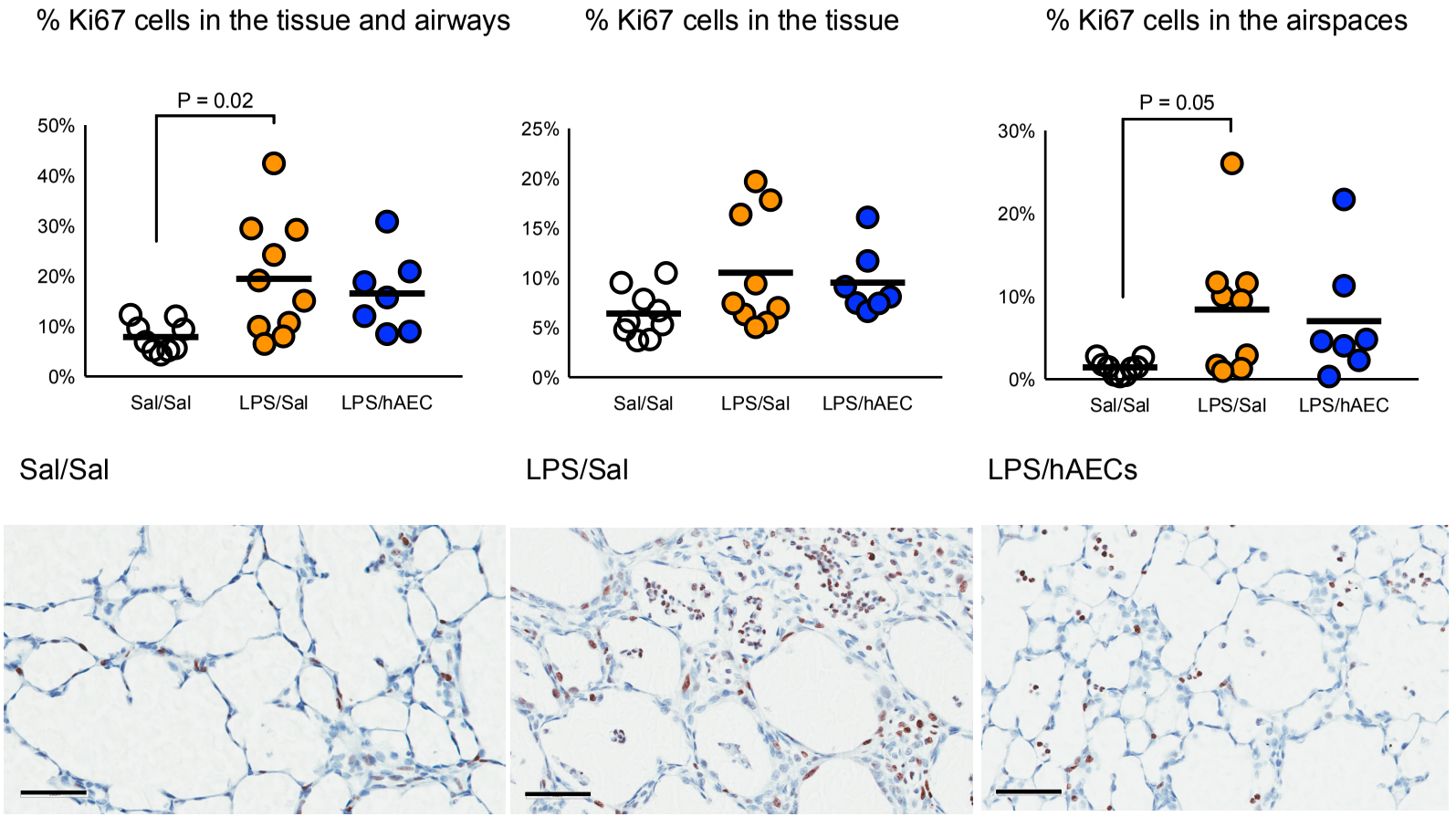

Figure 19. Proliferating cells within the tissue and airspace of the lungs, within the tissue only, or within the airspaces only of preterm lambs on day 7 of life. Representative images of the lungs of Sal/Sal, LPS/Sal and LPS/hAEC treated preterm lambs. Clumps of proliferating cells were often noted in the airspaces of animals exposed to antenatal LPS. Line represents mean. Scale bar = 50 μm.

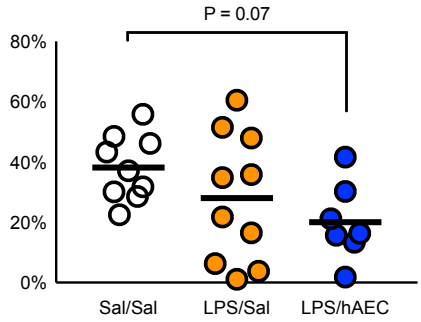

Figure 20. Proportion of myofibroblasts in the lung tissue of preterm lambs on day 7 of life. Line represents the mean.

Expression of genes in the lungs

IL-1α, IL-1β, IL-8 and *TNF* was upregulated in LPS/Sal and LPS/hAEC lambs compared to Sal/Sal lambs (P<0.05; Figure 21). LPS/hAEC lambs had increased *IL-6, IL-10, TGF-β, VEGF-A, SP-A, SP-D* and *CTGF* expression compared to Sal/Sal lambs (P<0.05, Figure 21), but not compared to LPS/Sal lambs. Other genes were not differentially expressed between groups (Table 15).

Expression of genes in the liver

Messenger RNA expression of inflammatory and vascular genes in the livers of Sal/Sal, LPS/Sal and LPS/hAEC lambs were not different (Supplementary Table 1).

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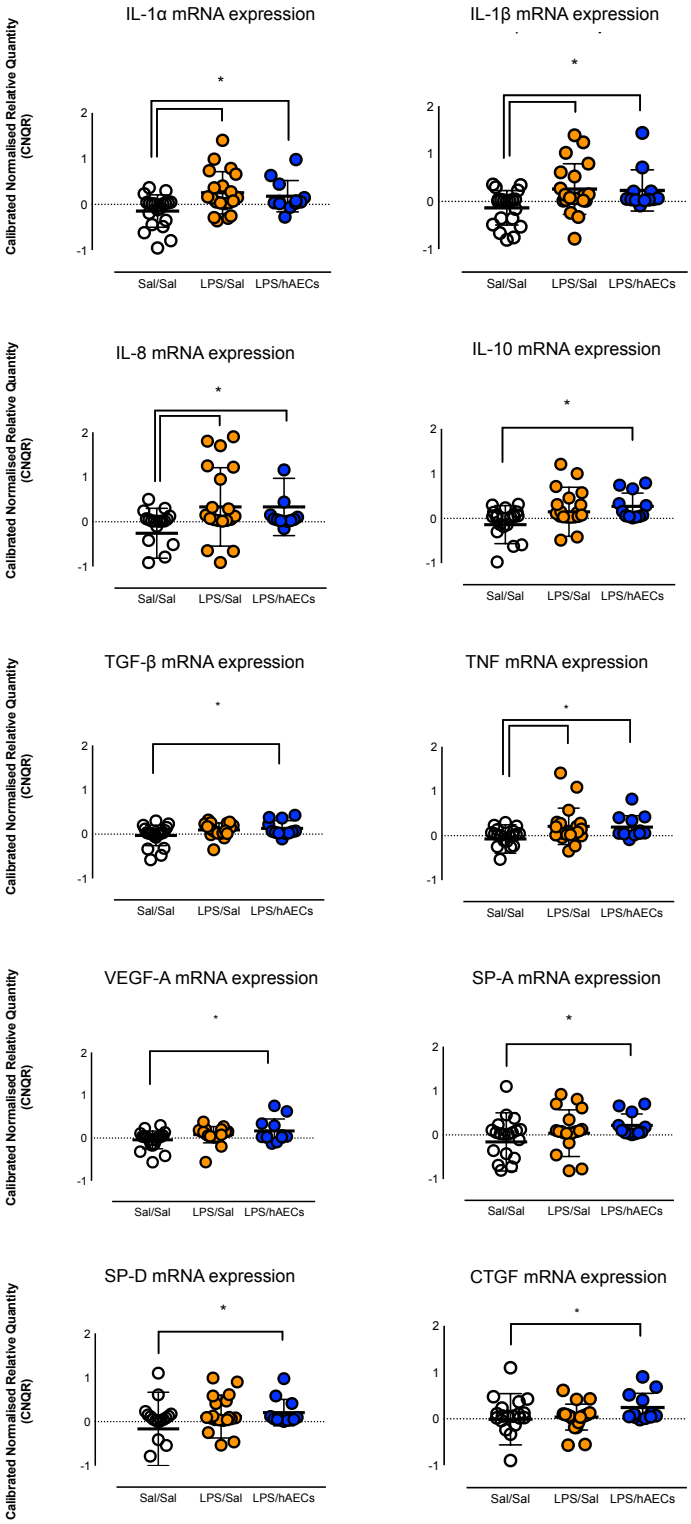

Figure 21. IL-1α, IL-1β, IL-8, IL-10, TGF-β, TNF, VEGF-A, SP-A, SP-D and CTGF mRNA expression in the lungs of Sal/Sal lambs, LPS/Sal lambs or LPS/hAEC lambs. *Signifies P<0.05 from Sal/Sal. Data are mean ± SD in duplicates. Sal/Sal group is normalised to approximately 0.00 using qBase+ software. LPS/Sal and LPS/hAEC groups are expressed as a fold change from Sal/Sal.

Table 15. Relative expression of genes in the lungs of preterm lambs.

Gene of interest	Experimental group		
	Sal/Sal	**LPS/Sal**	**LPS/hAEC**
IL-4	0.02 ± 0.12	0.09 ± 0.15	0.25 ± 0.14
IL-6	-0.12 ± 0.12	0.21 ± 0.14	0.31 ± 0.21*
MMP9	-0.00 ± 0.09	0.13 ± 0.07	0.16 ± 0.08
MMP12	0.10 ± 0.07	0.07 ± 0.12	-0.18 ± 0.09
CCL2	-0.06 ± 0.09	0.19 ± 0.09	0.14 ± 0.12
PECAM	-0.01 ± 0.05	0.06 ± 0.04	0.15 ± 0.07
VEGF-R1	-0.00 ± 0.16	0.05 ± 0.06	0.11 ± 0.09
Angiopoietin-1	0.03 ± 0.12	0.09 ± 0.06	0.10 ± 0.08
SP-B	-0.12 ± 0.23	0.08 ± 0.09	0.10 ± 0.06
SP-C	0.08 ± 0.09	0.02 ± 0.06	0.01 ± 0.05
EGR1	0.01 ± 0.14	0.13 ± 0.07	0.06 ± 0.15
CYR61	-0.01 ± 0.18	0.08 ± 0.06	0.15 ± 0.09

Data are mean ± SD. Expression is a fold change from Sal/Sal group. *Signifies P<0.05 compared to Sal/Sal.
IL = interleukin; TNF = tumour necrosis factor; TGF = transforming growth factor; MMP = matrix metallopeptidase; CCL = C-C motif ligand; PECAM = platelet endothelial cell adhesion molecule; VEGF (R) = vascular endothelial growth factor (receptor); SP = surfactant protein; EGR = early growth response protein; CTGF = connective tissue growth factor; CYR = cysteine-rich angiogenic inducer.

Discussion

We present the first study to analyse the impact of a single dose of hAECs in ventilated preterm neonatal lambs at 1 week of life. Lambs were managed in accordance with contemporary clinical practice, aimed at early weaning from mechanical ventilation. Despite a slight increase in the proportion of time spent on MV in hAEC-treated lambs compared to Sal/Sal lambs, hAEC-treated lambs had improved lung morphology.

The administration of hAECs enhanced the lung immune response but reduced lung injury in preterm lambs. LPS-induced lung inflammation is decreased by hAECs in fetal sheep.[129] Mechanical ventilation and concomitant hAEC administration in fetal sheep and preterm lambs have slightly different impacts on ventilation-induced lung injury. Ventilation *in utero*

causes lung inflammation, which is reduced by simultaneous hAEC administration.[130] Human AECs, delivered via the endotracheal tube at initiation of ventilation, augment lung inflammation and tend to decrease lung compliance in preterm lambs at 2 hours and 2 days of life.[131, 204] In those studies, delivery of hAECs through the endotracheal tube may have increased lung resistance through the clumping of cells at the airways.[131, 204] However, we saw similar reductions in static lung compliance in lambs receiving hAECs compared to saline-exposed lambs, despite IV cell administration and 7-day outcomes. A lack of compliance may imply fluid-filled lungs, consistent with hAEC- and LPS-treated preterm lambs having increased lung weights. The combination of antenatal LPS and postnatal ventilation predisposes to significant lung injury in the postnatal period[32] and a single prophylactic hAEC dose may enhance the pulmonary immune response rather than overcome pulmonary inflammation, as is observed in fetal sheep receiving hAECs.

We did not routinely screen lambs for postnatal sepsis but infection was confirmed or suspected in two Sal/Sal lambs (culture positive), one confirmed and one culture-negative LPS/Sal lamb and three LPS/hAEC lambs (no cultures performed), of which the causes of postnatal sepsis were unknown but were likely multifactorial. Potential contributors to sepsis in preterm lambs and preterm infants are previously reported: indwelling arterial and venous catheters, immature immune regulation, intolerance of feeds causing gastrointestinal disease or poor aseptic routines.[206-210]

Preterm lambs were delivered at a GA that would be fatal without intervention with MV. Thus, weaning from MV was difficult and many preterm lambs were reintubated following periods of extubation. Studies in preterm baboons have associated prolonged MV and repeated reintubations with increased risk of microbial colonisation and inflammation.[211] Neonates intubated for longer than 4 days have an 80 % increased risk of infection.[212] Reintubations may explain suspected sepsis in preterm lambs in our study.

Lambs receiving hAECs spent a slightly increased proportion of time on MV compared to Sal/Sal lambs, but this was not different to LPS/Sal lambs. There were no differences in time intubated (i.e. MV and ET CPAP) between groups. Thus, successful de-escalation of respiratory support was difficult in the presence of antenatal inflammation, similar to infants with chorioamnionitis who have increased likelihood of becoming ventilator-dependent.[20] Many lambs were extubated intermittently in the first few days of life and this coincided with gradual respiratory alkalosis (elevated $PaCO_2$, HCO_3^- and BE), although within target ranges. Resolution of respiratory alkalosis was difficult if lambs were spontaneously breathing (i.e.

extubated), as ventilation parameters could not be adjusted appropriately. The most prominent cases of respiratory alkalosis were in Sal/Sal lambs, and this group spent the least amount of time on MV. All lambs were exposed to antenatal betamethasone, which may more effectively reduce respiratory distress in Sal/Sal lambs compared to LPS-exposed lambs. Antenatal betamethasone and intervention with MV was necessary to ensure survival of control (Sal/Sal) lambs, and we controlled for any influence of betamethasone on respiratory distress by exposing all lambs to antenatal corticosteroids. However, whilst antenatal betamethasone matures the lungs, when administered prior to IA LPS the lung parenchyma is thicker (less mature)[159, 168] and LPS-induced lung inflammation is only temporarily supressed by betamethasone in fetal sheep.[159] Thus, increasing postnatal ventilation requirements in LPS-exposed lambs may be due to an inability of antenatal betamethasone to reduce LPS-induced lung inflammation by day 7 of life.

The administration of hAECs following antenatal inflammation resulted in similar alveolar wall thickness to saline-exposed lambs, but tended to increase septal crest areal fraction. Septal crests are the last structures to form during normal lung development, with a significant proportion of septal crests developing up to 2 years postnatally.[213] Thus, the development of septae indicate lung maturity. Failure to develop septae indicates inhibition of alveolarisation.[214] Hislop suggested that impaired alveolar development causes infant mortality in preterm infants with and without BPD.[215] Septal crest areal fraction in the lungs of LPS/hAEC lambs was ~3 % of lung tissue. In contrast, previous studies in naïve fetal sheep at 118[153] and 128 d GA[216] estimated the average septal crest areal fraction at 11 % and 8.5 %, respectively. Thus, we believe that the combination of antenatal inflammation and postnatal respiratory support likely has greater influence on septation in the lungs than previously appreciated. Indeed, septation is inhibited in fetal sheep exposed to LPS[21] and in chronically ventilated preterm lambs (~5 %) vs. term lambs (~15 %).[217]

Other postnatal events, such as hyperoxia, can inhibit alveolar septation in mice, but septal crest number increases in mice exposed to hyperoxia and treated with hAECs.[133] Septation changed without changes in tissue and airspace areal fractions in mice, consistent with our findings. Thus, MV with or without hyperoxia likely suppresses septation, and this is partially reversed by hAECs. Additionally, we noted hAEC-treated preterm lambs had reduced epithelial sloughing in their lungs, another indicator that hAECs have protective effects. One other study observed reduced epithelial sloughing in hAEC-treated, ventilated preterm lambs at 2 h of age.[131] Improved lung morphology (increased septation and reduced epithelial

sloughing) in LPS/hAEC lambs indicates partial reversal of BPD-like lung pathology at day 7 of life.

Alveolar hypoplasia and altered alveolar development are coupled with dysmorphic vasculature in preterm infants with severe BPD, where vessels are distanced from the alveolar walls.[11] Thinning of the lung parenchyma to be in close proximity to the vasculature is essential for respiration. VEGF is involved in the formation of vessels[218] and VEGF-A mRNA was increased in the lungs of LPS/hAEC lambs. Low VEGF in tracheal aspirates is reported in infants who were exposed to chorioamnionitis and develop BPD and in infants who die from severe BPD.[219] Although we did not analyse the architecture of vessels in the lungs, elevated VEGF indicates improved lung morphology. Indeed, VEGF is localised to the tips of septal crests in term baboons.[220] Preterm lambs receiving hAECs in our study had increased VEGF mRNA and a tendency for increased septal crests on day 7 of life, supporting a pro-reparative effect if hAECs. Despite these improvements in lung morphology (that should aid in independent respiration) we did not see effects on respiratory parameters in LPS/hAEC lambs.

The increased expression of surfactant proteins and cytokines in the lungs of hAEC-treated lambs suggests an enhanced inflammatory response. Elevated SP-A and SP-D expression with hAEC administration supports stimulation of the pulmonary immune system, as SP-A and SP-D have immuno-modulatory functions.[221] *IL-1β, TNF* and *TGF-β* are all upregulated in hAEC-treated lambs, and these cytokines all upregulate VEGF,[222] which could be another explanation for elevated VEGF in these lambs. *TGF-β* is increased in aspirates of infants with BPD, and increasing *TGF-β* is indicative of increasing BPD severity. *TGF-β* activates fibroblasts in the lungs in response to injury, causing fibrosis,[223] which is consistent with reduced static lung compliance in hAEC-treated lambs. However, we did not observe any changes in lung fibrosis between groups, as evidenced by a lack of difference in picrosirus red and total elastin areal density in the lungs. Alternatively, the tendency for lower myofibroblast expression in LPS/hAEC lambs suggests a gradual resolution of the immune response and reversal of lung inflammation as myofibroblast expression decreases with cessation of normal wound healing.[224]

The peak inflammatory response of the fetal lungs to IA LPS occurs two days after injection.[148] Thus, at the time of delivery, preterm lambs in this study likely had maximal lung inflammation. The number of hAECs used in this study (30 million) was lower than previously used in fetal sheep (90-180 million)[129, 130] and preterm lambs (180 million)[131] but was higher

than that used in a Phase I (safety) trial of hAECs (1 million cells/kg) in preterm infants with established BPD.[140] Our cell dose is consistent with that of a planned Phase II dose-escalation trial that will administer between 2-30 million hAECs/kg to preterm infants at risk of BPD (ACTRN: 1261800092029p). Higher and/or repeated doses of hAECs should be explored preclinically and should take into consideration prenatal inflammation, which is predictive of BPD.

Limitations

We observed differences in animal wellbeing between the two years over which these experiments were conducted. Lambs delivered in the second year were considerably more difficult to care for. We are unsure why year of delivery influenced outcomes, but this may be due to a number of factors, including: differing LPS potencies between years, different persons performing experiments, different numbers of lambs delivered between years (all Sal/Sal lambs delivered in one year), or a subconscious gradual caution to wean lambs or subconscious change in neonatal care/staff.

We chose to undertake a pragmatic study design which may have contributed to heterogeneous lung architecture within and between groups, as no lamb was treated identically. Variability within our datasets may have limited our ability to demonstrate statistical significance. However, it was important to focus on outcomes relevant to a clinical study, hence our emphasis on duration of ventilation (as opposed to parameters during ventilation) and lung inflammation and injury on day 7 of life. Our study design using prophylactic hAEC therapy allowed for endpoint outcomes, not temporal comparison of ventilation variables or systemic responses.

Conclusion

We show that a single dose of hAECs does not reduce ventilation duration in preterm lambs exposed to antenatal inflammation and postnatal respiratory support. Prophylactic hAECs reduced lung injury and enhanced the pulmonary immune response in preterm sheep at day 7 of life, suggesting hAECs may be a viable prophylactic therapy for lung immaturity in preterm infants.

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Supplementary Table 1. Relative expression of genes in the liver of preterm lambs

Gene of interest	Experimental group		
	Sal/Sal	LPS/Sal	LPS/hAEC
IL-1α	-0.04 ± 0.08	0.14 ± 0.13	0.15 ± 0.12
IL-1β	-0.05 ± 0.06	0.13 ± 0.10	0.07 ± 0.23
IL-4	-0.06 ± 0.15	0.10 ± 0.22	-0.09 ± 0.14
IL-6	-0.06 ± 0.15	0.10 ± 0.22	0.76 ± 0.70
IL-8	-0.11 ± 0.12	0.33 ± 0.19	0.15 ± 0.31
IL-10	-0.10 ± 0.11	0.34 ± 0.16	0.04 ± 0.25
TNF	-0.12 ± 0.08	0.24 ± 0.12	0.07 ± 0.14
TGF-β1	0.01 ± 0.05	-0.03 ± 0.05	0.01 ± 0.05
MMP-9	-0.02 ± 0.07	0.09 ± 0.06	0.08 ± 0.14
MMP-12	0.28 ± 0.20	-0.05 ± 0.13	0.08 ± 0.16
CCL2	-0.06 ± 0.09	0.02 ± 0.15	0.01 ± 0.18
PECAM	0.05 ± 0.04	-0.05 ± 0.05	0.02 ± 0.05
Hepcidin	0.06 ± 0.08	0.03 ± 0.05	0.12 ± 0.04
SAA	0.09 ± 0.10	0.11 ± 0.11	-0.25 ± 0.28
VEGF-A	0.07 ± 0.05	-0.08 ± 0.06	0.05 ± 0.04

Data are mean ± SEM. IL = interleukin; TNF = tumour necrosis factor; TGF = transforming growth factor; MMP = matrix metallopeptidase; CCL = C-C motif ligand; PECAM = platelet endothelial cell adhesion molecule; SAA = serum amyloid A; VEGF = vascular endothelial growth factor.

Chapter 6

Preterm birth occurs in almost 10 % of all live births, and is often preceded by chorioamnionitis.[2] The most common complication of preterm birth in infants born before 28 weeks GA is the chronic inflammatory lung disease bronchopulmonary dysplasia (BPD).[2] BPD is diagnosed based on the requirement for respiratory support for at least 28 days postnatal age.[16] Improvements in clinical care and management have reduced the incidence of all other co-morbidities in preterm infants, but the incidence of BPD is increasing.[2] BPD is predominately characterised by lung inflammation and abnormal lung development.[225, 226] The lungs of infants who died from BPD have simplified lung structure and a lack of alveolarisation.[10, 15] Similar lung morphology is noted in preterm lambs exposed to antenatal inflammation[129] or ventilation.[131]

I conducted separate studies to investigate the impact of two anti-inflammatory therapies, postnatal dexamethasone or human amnion epithelial cells (hAECs), on ventilation requirements, lung inflammation and structure in 1-week-old preterm lambs. Studies in this thesis involving preterm lambs take into account multifactorial contributors to BPD, including prematurity, antenatal and postnatal inflammation and contemporary neonatal care.

The use of high-dose postnatal dexamethasone is associated with neurological deficit,[227] resulting in reluctance for its clinical use and therefore difficulty in conducting appropriate trials in neonates. Chapter 3 describes the first study of postnatal dexamethasone in preterm animals at lower doses than previously used in neonatal care. Chapter 4 broadens our understanding of hAEC behaviour in different culture temperatures. In Chapter 5, I report the first studies to assess the use of hAECs beyond 48 h of life in preterm lambs with BPD-like lung pathology.

This general discussion will summarise the studies I undertook and provide an extended critique not possible in peer reviewed publications.

Human AECs are not influenced by changes in culture temperature

I investigated the viability and migration of hAECs in culture at temperatures ranging from 33-39 ºC. There were four reasons for this: 1) lambs have higher body temperature (39 ºC) than

humans (37 ºC) and cells were obtained from human placentae, 2) preterm infants are at risk of hypothermia due to high body surface area to weight ratio, allowing for rapid heat loss (< 36 ºC)[186], 3) preterm infants with suspected brain injury may be subjected to therapeutic hypothermia (< 33 ºC) and 4) preterm infants are susceptible to sepsis, a symptom of which is fever (>38 ºC).[187] Human AEC viability and migration were not altered by culture temperatures ranging from 33-39 ºC, confirming their efficacy for use in sheep and other species. Whilst hAEC viability decreased with increasing duration of culture this was independent of culture temperature and did not impact macrophage phagocytic activity. Studies in Chapter 4 confirmed the efficacy of hAEC use in preterm lambs and imply that the effects of hAECs are reproducible at temperatures likely to be encountered clinically. Additionally, hAECs may be viable and beneficial to preterm infants with co-morbidities that involve alterations in body temperature, such as sepsis.

The impact of antenatal inflammation on ventilator requirements and lung morphology

The relationship between chorioamnionitis and BPD is complex, but generally chorioamnionitis reduces the incidence of RDS without reducing rates of BPD.[18] Infants exposed to histological chorioamnionitis are more likely to become ventilator-dependent and, hence, be diagnosed with BPD.[18] Antenatal inflammation in fetal sheep results in lung morphology similar to infants who have died from BPD,[21, 28, 29] suggesting some prenatal origin for BPD development in infants exposed to chorioamnionitis.

Preterm lambs exposed to antenatal inflammation do not have reduced ventilator requirements at 3 hours of age,[32] in contrast to reduced ventilator requirements at 1 hour of life[21] and reduced RDS incidence seen in preterm infants exposed to chorioamnionitis. Whist I found no alteration in longer-term ventilator requirements in preterm lambs exposed to antenatal inflammation, these experiments ended on day 7 of life and more longer-term studies are required.

The impact of antenatal LPS on respiratory drive

Although there was no difference in respiratory support required by LPS/Sal lambs compared to Sal/Sal lambs, LPS-exposed lambs were more difficult to care for. They had more difficultly suckling feeds, were less interactive, were less physically active in their sling and

were breathing independently less frequently. These traits are consistent with impairment of independent respiration in LPS-exposed lambs.

Infection and inflammation can cause apnoea and dysregulation of the autonomic nervous system,[228, 229] which may in part explain a lack of weaning from MV in LPS-exposed preterm lambs in our studies. Signalling downstream from LPS-TLR4 binding promotes expression of pro-inflammatory cytokines, IL-1β, IL-6 and TNF, which may inhibit neuronal signalling at the respiratory centres of the brainstem.[230] Over-expression of IL-1β in the brainstem results in reduced breathing frequency and apnoeic episodes in newborn rats.[231] Antenatal inflammation induces IL-1β expression[230] and may cause dysregulation or damage to the brainstem, thus suppressing spontaneous breathing in preterm neonates exposed to chorioamnionitis.

Antenatal inflammation alters lung architecture and induces lung inflammation and surfactant production[21] which undoubtedly affects ventilation requirements in preterm infants. However, the impact of antenatal inflammation on spontaneous breathing is not well understood, despite reports of increased BPD incidence in infants exposed to chorioamniontis.[20, 25] We had difficultly weaning all preterm lambs exposed to IA LPS, regardless of the postnatal intervention, and weaning from MV is not possible without spontaneous respiration.

For the dexamethasone study, the only indication of different respiratory requirements was a lower delivered MAP compared to LPS/Sal lambs. Higher MAP in LPS/Sal lambs should be considered with caution as outliers requiring increased FiO_2 suggests partial atelectasis; these few animals may have influenced comparisons between groups. For the hAEC study, we did not see differences in ventilator requirements between LPS/Sal and LPS/hAEC lambs, indicating no effect of hAECs in driving independent ventilation. All lambs exposed to antenatal LPS were equally difficult to wean from ventilation.

Antenatal LPS alters lung parenchyma at day 7 of life

The impact of antenatal LPS on lung morphology has been studied extensively in fetal sheep, with little comment on the influence of antenatal LPS on lung structure after birth. Antenatal LPS thins the lung parenchyma and inhibits septation and normal alveolarisation in fetal sheep.[21, 42] In my experiments, preterm lambs who were exposed to antenatal LPS and cared for until day 7 of life had different responses. Lung morphology was essentially opposite to what has been described in fetal sheep; lung parenchyma was thicker and septation was inhibited. Whilst antenatal LPS inhibits septation in both fetal sheep and

preterm lambs, the combination of antenatal LPS and postnatal ventilation appears to exacerbate inflammation and thicken lung tissue (likely caused by hyperproliferation of cells in the lungs). We suggest that antenatal LPS predisposes to further lung injury and exacerbates ventilation-induced lung injury in preterm lambs, consistent with clinical data suggesting that histological chorioamnionitis predisposes to BPD development.[27] The lung morphology of human infants exposed to chorioamnionitis who then develop BPD has not been described.

Tapered low-dose dexamethasone reduces lung inflammation and alters lung morphology in preterm lambs following antenatal inflammation

Postnatal dexamethasone effectively reduced lung inflammation, without inducing significant lung maturation in preterm lambs. Dexamethasone-treated preterm lambs had fewer leukocytes and macrophages in their lungs but this did not coincide with altered expression of inflammatory genes, which were intermediately expressed, compared to saline-exposed and LPS-exposed lambs. The lungs of dexamethasone-treated preterm lambs had similar tissue, airspace and septal crest areal fractions than Sal/Sal lambs. There are no clinical studies that address the impact of postnatal dexamethasone in the setting of chorioamnionitis.

Lung injury and inflammation were reduced in preterm lambs receiving tapered low-dose postnatal dexamethasone, consistent with clinical observations,[57-59] but inconsistent with clinical observations linking reduced lung inflammation with reduced ventilator requirements.[55] This was surprising as dexamethasone is used to facilitate weaning from ventilation in the NICU.[232] There are a number of possible explanations for the lack of reduced respiratory support in dexamethasone-treated lambs. First, LPS- and ventilation-induced inflammation was not overcome by our dosing of postnatal dexamethasone, resulting in similar ventilation requirements between LPS/Sal and LPS/Dex lambs. Second, the tapered dose of dexamethasone used may have had little effect by day 7 of life. Twice-daily dexamethasone without tapering may have been more effective in weaning preterm lambs from ventilation, but would have exceeded clinically safe low-dose ranges. Third, species differences in preterm lambs and preterm infants may result in postnatal dexamethasone being less effective in weaning preterm lambs from MV than reported clinically. Fourth, dexamethasone may have been ineffective for weaning from MV despite

changes in lung morphology because spontaneous breathing is suppressed by antenatal inflammation. A reduction in ventilation requirements relies on a combination of factors including activation of respiratory centres within the brain stem and hence spontaneous breathing, lung maturation, surfactant production and patent airways.

Human AECs augment lung inflammation and alter lung morphology in preterm lambs following antenatal inflammation

Administration of a single dose of hAECs increased the expression of both pro- and anti-inflammatory cytokines in the lungs of preterm lambs following antenatal inflammation. Previous studies suggest hAECs reduce lung and brain inflammation following IA LPS,[129, 182] and thus reduce the inflammation associated with BPD-like lung pathology.[136, 233] Anti-inflammatory cytokines were increased in the lungs of hAEC-treated lambs, although there was no reduction in pro-inflammatory cytokines associated with IA LPS and postnatal ventilation, in our studies.

Lung morphology and gene expression profiles in hAEC-treated lambs indicated a pro-reparative response of hAEC therapy, despite a lack of treatment effect on ventilation requirements. The impact of hAECs after antenatal inflammation in our study was contrary to studies in fetal sheep and in newborn mice, where hAECs reduced lung inflammation.[129, 133] However, hAECs have not been applied in the setting of contemporary neonatal care or when antenatal inflammation and postnatal ventilator-induced inflammation are present. Although hAECs improved lung morphology they were ineffective for weaning from ventilation.

The suppression of independent breathing observed in LPS/Sal lambs was not influenced by hAEC administration on the first day of life. Human AECs likely act via paracrine effects involving the polarisation of macrophages from an M1 to M2 phenotype and by suppression of T cells,[233] both of which would reduce inflammation in the lungs, but the effect of hAECs on systemic or cerebral inflammation is rarely reported.[182, 234, 235] Elevated inflammatory cytokines, IL-1β, IL-6 and TNF, all likely supress neuronal signalling in the brainstem,[231, 236] which may suppress spontaneous breathing. These cytokines were upregulated in the lungs of preterm lambs exposed to IA LPS and postnatal hAECs, but we did not measure levels in the brain.

Limitations and future directions

The pragmatic study design used for the experiments described in Chapters 3 and 5, in combination with relatively small sample sizes, likely increased variability between subjects and made it difficult to identify statistically significant differences between dexamethasone and hAEC treatments and control groups. Our study design aimed to replicate normal clinical practice, limiting our ability to identify differences in ventilator requirements between interventions in preterm lambs, because many were extubated for at least some time during the first few days of life. Our decision to use tapered low-dose postnatal dexamethasone was important as clinically dexamethasone is used to wean preterm infants off ventilation. We only analysed the proportion of time spent on MV, as increasing ventilator requirements are the main impetus for clinical use of postnatal corticosteroids. We appreciate that current descriptions of BPD can occur in preterm infants on minimal respiratory support (CPAP or HHF), who are oxygen dependent with more subtle lung injury, but it is unlikely these infants would receive postnatal corticosteroids as they are extubated. The impact of tapered low-dose postnatal dexamethasone on the duration of MV and lung inflammation were the main outcomes for our Chapter 3 studies, as these are most commonly linked to moderate and severe BPD.[27, 145]

Antenatal corticosteroids are routinely administered to pregnant women at risk of delivering preterm, to induce lung maturation and reduce immediate respiratory distress,[3] which is similarly seen in sheep studies.[49] All lambs in my studies were exposed to antenatal betamethasone, which alone has similar maturational effects on lung parenchyma and inflammation as IA LPS.[42, 157] However, antenatal betamethasone may partially explain a lack of differing MV requirements between lambs in our studies, as antenatal betamethasone prior to IA LPS initially supresses LPS-induced lung maturation but later (~ 5 d after LPS injection) increases lung inflammation.[159] The administration of postnatal dexamethasone or hAECs in our studies may have been insufficient to overcome this late surge in LPS-induced lung inflammation and thus, ventilation requirements may have increased with time in LPS-exposed lambs.

The additional influence of year of delivery on respiratory outcomes is an important observation, without consideration of which erroneous conclusions may have been made. Whilst identification of influential covariates are considered in clinical studies involving preterm birth, chorioamnionitis and BPD,[18, 27, 237, 238] animal studies do not often adjust their statistical analyses accordingly. Sheep studies of preterm birth, complications of preterm

birth and therapeutics for preterm birth-related morbidities often do not consider influential covariates, aiming to control the influence of these variables within their experimental design. Unlike the genetic similarity of lab rodents, sheep are genetically dissimilar and therefore often have greater variability.[39, 220, 239] We suggest that future studies, particularly those using large animals with more pragmatic and translational study designs (i.e. *pro re nata* ventilation or medications), should consider the influence of maternal, fetal and postnatal factors in their analyses and should increase their sample sizes.

Lung morphology of preterm lambs was similar to that reported for infants who died from BPD, although we acknowledge that our 7-day study period likely represents a pre-BPD phenotype. Preterm lambs at one week of age are equivalent to human infants at ~2 weeks of age,[240] which is not the age of clinical diagnosis of BPD. However, some preterm lambs were dependent on respiratory support, which is a key determinant of BPD severity. Future studies should take into account the 28 days (or equivalent) required to diagnose BPD and include rescue interventions as opposed to prophylactic interventions. Such extended studies should include animals exposed to antenatal inflammation and naïve animals to better elucidate the influence of antenatal inflammation in suppression of respiratory drive and BPD development.

For Chapter 3 we used dexamethasone as this is the most common glucocorticoid used in the NICU. However, hydrocortisone is also used in NICUs, particularly in Europe.[241] The exploration of different glucocorticoids for weaning from ventilation, inducing lung maturation and reducing lung inflammation is an important consideration. Neurological outcomes are essential when studying the use of glucocorticoids in the preterm infant. Although this thesis focuses on lung outcomes, neurological outcomes from the same lambs are being assessed by other investigators. Additionally there is some evidence supporting inhaled glucocorticoids for weaning infants from ventilation.[242] It was not possible to address the use of inhaled glucocorticoids in these studies as preterm lambs were intubated, infrequently breathing and anatomically have a long oro-nasal cavity which may influence drug delivery.

For Chapter 5, the administration of hAECs occurred 7 days before the end of experiment and this may have influenced our results. Mapping the response to hAEC administration by assessing lung injury and inflammation at various time points, or by assessing plasma cytokines throughout the experiment, is an important consideration that we have considered for subsequent studies. If hAECs are used clinically for BPD, and BPD is diagnosed as progressive ventilator dependence, prophylactic hAEC therapy may be unlikely. We are

currently investigating and comparing prophylactic and rescue hAEC therapy and mapping the inflammatory response to later administration of hAECs in preterm lambs.

Unfortunately we cannot directly compare the results of the animal studies in Chapters 3 and 5 because the dosing and timing of dose(s) were different. We did not investigate the combination of postnatal dexamethasone and hAEC therapies. It is likely that preterm infants at risk of developing BPD will require a number of interventions to prevent lung inflammation and BPD progression. How these interventions might interact or synergistically prevent lung inflammation and BPD is worth exploring. My experiments show that postnatal dexamethasone reduces lung inflammation whilst hAECs improve lung morphology. Thus, the combined use of postnatal dexamethasone and hAECs may be effective.

In conclusion, my studies are the first to investigate dexamethasone or hAECs as anti-inflammatory therapeutics for BPD in an experimental setting that accurately models contemporary neonatal care. Whilst neither dexamethasone nor hAECs altered ventilator requirements in preterm lambs exposed to antenatal inflammation, both treatments influenced different aspects of lung inflammation and injury associated with BPD.

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