Outside-in?

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ABSTRACT

BACKGROUND: The preterm birth syndrome (delivery before 37 weeks' gestation) is a major contributor to the global burden of perinatal morbidity and mortality. The aetiology of preterm birth is complex, multifactorial, and likely dependent, at least in part, on the gestational age of the fetus. Intrauterine infection is frequent in preterm deliveries occurring before 32 weeks' gestation; understanding how the fetus responds to pro-inflammatory insult will be an important step towards early preterm birth prevention. However, animal studies of infection and inflammation in prematurity commonly use older fetuses possessing comparatively mature immune systems.

OBJECTIVE: Aiming to characterise acute fetal responses to microbial agonist at a clinically-relevant gestation, we used 92d gestational age fetuses (62% of term) to develop a chronically catheterized sheep model of very preterm pregnancy. We hypothesised that any acute fetal systemic inflammatory responses would be driven by signalling from the tissues exposed to *E. coli* lipopolysaccharide introduced into the amniotic fluid.

STUDY DESIGN: 18 ewes carrying a single fetus at 92d gestation had recovery surgery to place fetal tracheal, jugular, and intraamniotic catheters. Animals were recovered for 24h before being administered either: intraamniotic *E.coli* lipopolysaccharide (LPS; n=9); or sterile saline (n=9). Samples were collected for 48h before euthanasia and necroscopy. Fetal inflammatory responses were characterised by microarray analysis, quantitative PCR and ELISA.

RESULTS: Intraamniotic LPS reached the distal trachea within 2h. LPS increased tracheal fluid interleukin-8 within 2h and generated a robust inflammatory response characterised

by interleukin-6 signalling pathway activation, up-regulation of cell proliferation, but no increases in inflammatory mediator expression in cord blood RNA.

CONCLUSIONS: In very preterm sheep fetuses, LPS: **i)** stimulates inflammation in the fetal lung and fetal skin; and **ii)** stimulates a systemic inflammatory response that is not generated by fetal blood cells. These data argue for amniotic fuid-exposed tissues playing key role in driving acute fetal and intrauterine inflammatory responses.

KEY WORDS: preterm birth, infection, inflammation, sheep, fetus.

INTRODUCTION

Preterm birth (PTB; delivery before 37 weeks' completed gestation) is a multi-origin syndrome[1] that results in excess of 1 million perinatal deaths each year[2, 3]. The preterm deliveries at highest risk of death and significant disability are those occurring at or below 32 weeks of gestation, most commonly in association with intrauterine infection[4] and chorioamnionitis[5]. Goldenberg and colleagues have suggested that between 25 and 40% of preterm births are due to infection, noting that the true rate may in fact be higher due to spuriously negative microbial culture results[6]. An increased risk of adverse neonatal outcomes is also independently associated with an elevated cord blood plasma interleukin (IL)-6 level (>11pg/mL), termed the fetal inflammatory response syndrome or FIRS[7].

In the presence of microbial agonist, innate immune receptors (including the TOLL-, NOD-, and RIG-I-like receptor families) in fetal and maternal tissues drive the expression and release of pro-inflammatory mediators including IL-1α/β, IL-6, IL-8 and tumour necrosis factor (TNF)-α[1, 4, 8]. Intrauterine inflammation is considered key to both the premature activation of the pathways of parturition and a number of the diseases (e.g. cerebral palsy) associated with prematurity[8, 9]. As such, interventions that resolve both intrauterine infection and inflammation may prevent microbially-associated PTB and its sequelae. A number of investigators have studied responses to intrauterine infection and inflammation in primate[10, 11], sheep[12, 13], and rodent models[14, 15] of pregnancy. However, much remains to be understood with regards the tissues responsible for driving intrauterine inflammation. Of note, a significant number of previous studies in this field have been undertaken in late-gestation pregnancies[4].

We aimed to chronically catheterise fetal sheep to characterise the acute intrauterine inflammatory response to *E.coli* lipopolysaccharide (LPS) in very preterm fetuses. Noting that LPS does not cross cell-cell barriers[16], we hypothesised that any acute fetal systemic inflammatory responses would be driven by signalling from the tissues exposed to the amniotic fluid (AF).

MATERIALS AND METHODS

Animals: Animal studies were approved by The University of Western Australia's Animal Ethics Committee (approval RA/3/100/1289). 18 date-mated ewes with singleton fetuses at 92d gestational age (GA) had aseptic recovery surgery to place catheters into the fetal jugular vein, trachea and two catheters into the AF as described previously[17]. After a 24h recovery, animals were assigned at random to receive either: **i)** 10mg *E.coli* LPS (O55:B5; Sigma Aldrich, St. Loius, MO) in 2mL sterile saline via intraamniotic catheter (n=9); or **ii)** 2mL sterile saline via intraamniotic catheter (n=9).

Amniotic fluid (AF), tracheal fluid (TF) and fetal plasma (FP) were serially sampled immediately before and 2, 4, 8, 12, 24 and 48h after treatment administration. Animals were euthanised with an intravenous bolus (100mg/kg) of pentobarbitone at 48h. Fetal cord blood was collected for blood chemistry analysis, and for RNA extraction using PAXgene Blood RNA Tubes (PreAnalytiX GmbH, Switzerland) in accordance with manufacturer's instructions. Fetal tissues were snap frozen in liquid nitrogen for subsequent analysis. 6-9 animals were analysed per group.

Tissue RNA preparation: RNA was isolated from fetal tissues (internal groin skin, spleen, lung right lower lobe, frontal cortex), and mechanically disrupted with a Precellys homogeniser

(Bertin Technologies, Rockville, MD) in 1mL TRIzol (Life Technologies, Carlsbad, CA) in accordance with manufacturers' instructions. RNA was treated with Turbo-DNase (Life Technologies) as previously reported and quantified using a QUBIT fluorometer (Life Technologies).

Quantitative PCR: Sheep-specific probes and PCR primer sets were used to perform quantitative PCR reactions for IL-1β, IL-6, IL-8, TNF-α, MCP-2, IL-10 and IL-13 using an EXPRESS One-Step SuperScript qRT-PCR Kit (all Life Technologies) containing 125 ng RNA template in a final volume of 20 µL, following manufacturer's instructions. Cycling conditions and primer/probe sets were as described previously[18]. Cq values were normalised against 18s rRNA and expressed as fold changes relative to saline control values.

Limulus Assay: The concentration of LPS in the AF and TF was determined using Pierce LAL Chromogenic Endotoxin Quantitation Kits (Life Technologies) in accordance with manufacturer's instructions. Plates were read at a wavelength of 405 nm on a Anthos 2010 Standard Plus plate reader (Biochrom Ltd. Cambridge, UK).

ELISA: Quantification of IL-6 and IL-8 protein concentrations in AF, TF and FP samples were performed using in-house ELISAs as previously described[19]. TNF-α and MCP-1 levels were assayed using ELISA VetSets (Kingfisher Biotech, Saint Paul, MN) according to the manufacturer's instructions with one modification; coated wells were incubated with samples overnight at 4 °C. For IL-8 quantification, samples were diluted 1:10 in assay buffer. Plates were read at a wavelength of 450 nm on an Anthos 2010 Standard Plus plate reader (Biochrom Ltd. Cambridge, UK). For each experiment, samples and standards were assayed in duplicate.

Microarray: Comparative transcriptomic analysis (Ovine Gene 1.0 ST 8 x 15K Array; Affymetrix, Santa Clara, CA) was performed on arterial cord blood RNA collected at necroscopy from 4 LPS-exposed and 4 saline-exposed fetuses selected at random. All RNA samples underwent quality control assessment for purity and integrity and had a RIN value of between 6.5 and 8.8. For each sample, 100 ng of total RNA was processed using a WTPlus Kit (Affymetrix) following manufacturer's instructions. Fragmented, labelled single-stranded DNA was hybridised in a GeneChip hybridisation oven 640 at a final concentration of 23 ng/µL using a GeneChip Hybridization, Wash and Stain Kit (both Affymetrix) in accordance with manufacturer's instructions. Arrays were washed and stained on a GeneChip Fluidics Station 450 and scanned on a GeneChip 3000 7G+ scanner and using GeneChip Command Console Software (all Affymetrix), following manufacturer's instructions. Microarray data were pre-processed using the robust multi-array average (RMA) algorithm, background correction, quartile normalisation and gene-level probe set summation[20]. Genes differentially expressed between the two groups were identified by Significance Analysis of Microarrays (SAM) analysis with a false discovery rate (FDR) of 0.1 using BRBArrayTools Version 4.4 software[21]. Gene networks were generated by Ingenuity Pathway Analysis (IPA) software (Qiagen, Redwood City, CA).

Quantitative PCR reactions to validate microarray findings were performed on samples submitted for microarray analysis using the Roche Universal Probe Library (Roche Diagnostics Australia Pty Ltd, NSW, Australia) in accordance with manufacturer's instructions. The four most differentially upregulated targets by SAM analysis were selected for analysis: CCNB1 (Right Primer: *cctctggaaaaggctcctg*; Left Primer: *ccttaacaggctcgggttc*; Universal Probe: 36 Amplicon Size (nucleotides, nt): 73); MT2A (Right Primer:

ggatcccaactgctcctg; Left Primer: *gcgcacttgcaatctttg*; Universal Probe: 92; Amplicon Size (nt): 78); TCN1 (Right Primer: *ttgttgggattaagagtcaaagg*; Left Primer: *ttatgtcttctttgatttgtccactc*; Universal Probe: 150; Amplicon Size (nt): 63); TOP2A (Right Primer: *gaccattatcaatttggctcaga*; Left Primer: *ggctgcaaaaggttcagatt*; Universal Probe: 44; Amplicon Size (nt): 60). Cq values were normalised against GAPDH (Right Primer: *ggcctccaaggagtaaggtc*; Left Primer: *tctcttcctctcgtgctcct*; Universal Probe: 23; Amplicon Size (nt): 60); and β-Actin (Right Primer: *ggacggaccctcacacatac*; Left Primer: *gtgagaagcctgccaacg*; Universal Probe: 70; Amplicon Size (nt): 61).

Statistical Analysis: All qPCR values are mean ± standard deviation (SD). Statistical tests were performed on dCq values and data are reported as fold change vs. control. All ELISA values are median ± interquartile range (IQR). Statistical tests were performed using SPSS Statistics for Windows, Version 20.0 (IBM Corporation, Armonk, NY.). Variance and distribution were assessed for normality. Mean differences between normally distributed data were tested for significance with one-way ANOVA using a *p* value of 0.05. Tukey's test was used to perform multiple *post-hoc* comparisons. Between-group differences in non-parametric data were tested for significance with Kruskal-Wallis one-way ANOVA, with a *p* value of 0.05 accepted as significant. Rank-sum tests were used to perform multiple post-hoc comparisons were performed with the *p* value corrected for *n* multiple comparisons where appropriate.

RESULTS

Clinical observations: All surgeries were successfully completed and there were no fetal deaths prior to scheduled euthanasia. No significant differences were identified in birth weight, arterial cord blood pH, pO_2, pCO_2, lactate, glucose or white blood cell counts (total or differential) between treatment and control groups. None of the study animals had meconium and there was no increase in the presence of fetal edema or ascites as a result of LPS exposure. Compared to Saline Control, the concentration of γ-glutamyltransferase (a marker of oxidative stress) in fetal cord blood plasma collected at euthanasia was significantly increased ($p<0.05$) at necroscopy in animals exposed to LPS for 48h (Table 1).

Endotoxin quantification: For animals randomised to LPS-exposure, the endotoxin levels in both AF and TF were below the assay limit of detection (0.1EU/mL) immediately before LPS administration. Endotoxin concentrations in the AF and TF exceeded 1EU/mL by 2h and remained above 1EU/mL at all subsequent time points to 48h.

ELISA: All values are median (25^{th} - 75^{th} centile). Statistical comparisons are relative to saline control samples collected contemporaneously at matched time points. There were no significant increases in TNF-α concentrations in AF, TF or FP at any time point (data not shown). IL-6 concentrations were significantly increased in AF from LPS-exposed fetuses at 12h, 24h, and 48h, relative to Saline Controls. IL-6 concentrations were significantly increased in TF from LPS-exposed fetuses at 24h, and 48h, relative to Saline Control. IL-6 concentrations were significantly increased in FP from LPS-exposed fetuses at 12h, relative to Saline Control (Table 2).

IL-8 concentrations were significantly increased in AF from LPS-exposed fetuses at 4h, 8h, 12h, 24h, and 48h, relative to Saline Control. IL-8 concentrations were significantly increased in TF from LPS-exposed fetuses at 2h, 4h, 12h, 24h, and 48h, relative to Saline Control. IL-8 concentration was significantly increased in FP from LPS-exposed fetuses at 12h, relative to Saline Control (Table 3).

MCP-1 concentrations were significantly increased in AF from LPS-exposed fetuses at 4h, 8h, 12h, 24h, and 48h, relative to Saline Control. MCP-1 concentrations were significantly increased in TF from LPS-exposed fetuses at 8h, 12h, 24h, and 48h, relative to Saline Control. MCP-1 concentrations were significantly increased in FP from LPS-exposed fetuses at 4h, 8h, 12h, 24h, and 48h , relative to Saline Control (Table 4).

Quantitative PCR: Results are presented in Figure 1. At 48h, increases in cytokine and chemokine mRNA were greatest in the fetal lung (Figure 1A), with statistically significant increases in IL-1β (20.7±20 vs. 1.1±0.5), IL-6 (5.9±5.5 vs. 1.1±0.4), IL-8 (66.4±79.5 vs. 1.0±0.3), TNF-α (13.7±13.8 vs. 1.1±0.4), MCP-2 (83.7±87.3 vs. 1.5±1.7), and IL-10 (3.5±1.6 vs. 1.1±0.4) mRNA expression. Smaller, statistically significant increases were detected in fetal skin (Figure 1C) IL-1β (3.4±1.7 vs. 1.2±0.7) and IL-8 (4.6±2.9 vs. 1.3±0.8) and fetal spleen (Figure 1D) IL-1β (1.9±0.6 vs. 1.1±0.4) and TNF-α (2.1±0.7 vs. 1.1±0.5) mRNA expression. There was no change in cytokine / chemokine expression in fetal cortex tissue (Figure 1B; IL-6, IL-8, MCP-2, IL-13) or RNA collected from fetal arterial cord blood (Figure 1E; IL-1β, IL-8, TNF-α; IL-6 and MCP-2 were not detectable in either control or LPS-exposed groups).

Microarray: Using a SAM analysis with a false discovery rate of 0.1, 63 genes were found to be significantly differentially expressed between LPS- and saline-exposed groups. Array data

has been deposited with NCBI under series entry GSE72240. In keeping with the results of qPCR analyses (above), levels of RNA transcripts for key immunomodulatory cytokines (IL-1α, IL-1β, IL-6, IL-8, IL-10 IL-17A, IL-18) associated with PTB and fetal injury were not differentially regulated (either increased or decreased) in circulating RNA collected from arterial cord blood at 48h. Figure 2 displays summaries for three signalling pathways identified as being significantly upregulated by Ingenuity Analysis. RNA transcript for Cyclin B (CCNB1) was found to be differentially regulated in response to LPS exposure. CCNB1 plays an important role in regulating the cell cycle, though its interaction with cyclin-dependent kinase 1, allowing transition from G2 phase to mitosis. The CCNB1 promoter is upregulated by a number of factors including 17-β estradiol, insulin-like growth factor and prolactin-releasing hormone. The overexpression of CCNB1 is associated with a number of cancers in humans, and may be indicative of resistance to radiotherapy and chemotherapies in breast and neck cancers [22]. Quantitative PCR was used to validate statistically significant increases in four transcripts (CCNB1, MT2A, TCN1 and TOP2A) identified as being differentially upregulated in LPS-exposed fetal blood RNA by SAM analysis, relative to saline-exposed control samples. Relative to saline control, all four transcripts assessed (CCNB1, 2.4 fold increase, p=0.028; MT2A, 2.7 fold increase, p=0.043; TCN1, 2.4 fold increase, p=0.030; and TOP2A, 2.5 fold increase, p=0.028) were significantly upregulated, although at a slightly lower magnitude than that identified in the microarray analysis. Pathway profiles suggest the activation of canonical signalling pathways (IL-6 signalling, cellular proliferation, viral infection response) associated with cell cycle control and cellular proliferation. These data point to a central role for CCNB1 in these pro-proliferative response pathways. Ingenuity Pathway Analysis (IPA) Upstream Regulator (UR) analytic was used to predict upstream transcriptional regulators that could explain the observed changes in gene expression in the

LPS vs. saline-control samples. Interestingly, signalling activation was characteristic of a cellular response to viral infection; a number of key regulatory molecules were predicted as highly probable upstream signalling nodes, including IL6 and CDKN2A (both $p < 0.001$). CDKN2A encodes the tumour-suppressor protein p16, which exerts a negative influence on cell cycling by preventing the transition from G1 phase to S phase [23].

COMMENT

The present work describes the successful development of a chronically catheterized sheep model to study inflammatory responses generated by very preterm fetuses. This study presents three key findings pertinent to advancing our understanding of the inflammatory pathophysiology that underpins the fetal inflammatory response syndrome in very preterm fetuses:

i) when administered to the AF, LPS rapidly (within 2h of administration) entered the TF. We, and others, have previously shown the fetal lung to be a key organ in driving systemic fetal inflammatory responses[12]. These data similarly suggest that microorganisms gaining access to the intrauterine environment (i.e. by ascending infection[1] / focal breach in the fetal membranes[24]) will rapidly gain access to the fetal lung;

ii) relative to Saline Control, significant increases in soluble pro-inflammatory mediators were identified in TF as early as 2h (IL-8) and in AF as early as 4h (IL-8, MCP-1) after intraamniotic LPS administration. The data suggest that the magnitude and speed with which changes occur in each compartment (AF, TF, FP) may be cytokine/chemokine-dependent. Concentrations of MCP-1 and IL-6 in AF and TF were similar across sampling points. The highest concentration of IL-6 was identified in the FP at 12h, after which time the concentration returned to baseline. Concentrations of IL-8 were substantially higher in the AF and TF than FP. Increases in cytokine mRNA expression in tissues collected at autopsy were

greatest in the fetal lung. Together, these data suggest an important role for the lung in driving intraamniotic and systemic fetal inflammatory responses to microbial invasion of the AF in early pregnancy; and

iii) the acute systemic fetal inflammation mediated by intraamniotic LPS administration was rapidly detectable in FP (MCP-1 by 4h, IL-6 and IL-8 by 12h). An absence of cytokine/chemokine RNA expression changes in whole arterial blood collected at euthanasia, very modest changes in spleen cytokine mRNA expression, and limited changes in markers of liver inflammation, oxidative stressand injury (AST, γGT, GLDH) together suggest that the acute systemic fetal inflammatory response to LPS is driven by fetal tissues exposed to the amniotic environment.

Given the importance of inflammation to preterm labour and fetal injury, there is significant interest in developing and testing anti-inflammatory interventions to complement antibiotic therapies in the setting of infection-associated preterm birth. To date, investigators have used a variety of pharmacological approaches in an attempt to regulate intrauterine inflammation[13, 25-27].

We characterised the acute fetal responses to intraamniotic endotoxin exposure in chronically catheterized fetal sheep at 62% of gestation to model early gestational inflammatory responses. These data are of importance for the development of interventions to identify and target the tissues that respond to intrauterine inflammatory stimulation. Moreover, because fetal immunological capacity is a function of gestational development[4, 28], the use of gestation-appropriate fetuses is an important consideration in

infection-associated PTB model system selection. We and others have previously investigated fetal lung responses to LPS fetuses at approximately 80% of gestation[29]. In the present study, we demonstrate that the very preterm fetal lung also has the capacity to mount a robust pro-inflammatory response to microbial agonist. The fetal skin has also been suggested as a potential pro-inflammatory organ in humans[30] and we have previously characterised its responses to LPS[31], and preterm birth associated organisms such as *Ureaplasma parvum*[32] and *Candida albicans*[19] in mature fetuses. In the present study, LPS exposure yielded modest increases in fetal skin cytokine mRNA expression, notably IL-8, in keeping with earlier reports. These data reinforce the ability of the fetus to mount a robust pro-inflammatory response to microbial agonist from an early GA. From a model perspective, significant increases in FP IL-6, and an IL-6 driven signalling response in fetal cord blood cells reinforces the utility of the sheep as a model system for intrauterine infection in human pregnancy[33].

From a treatment-development perspective, two of study findings are of particular relevance. Firstly, the speed at which inflammatory changes became detectable in the TF, AF and FP following LPS administration is of great interest. The hypothetical therapeutic window for administering anti-inflammatory therapies to the fetus to prevent preterm labour and brain/lung injury remains unknown, but is likely dependent on the infecting organism and host susceptibility. Previous studies have demonstrated neurological injury in association with intrauterine inflammation[34]. The present study was not designed to assess neurological injury, although it is interesting to note an absence of inflammatory changes in a limited panel of mediators measured in the fetal cortex at 48h. Developing anti-inflammatory therapies for PTB prevention is likely an important element of our quest to

prevent PTB and improve perinatal outcomes. However, the speed with which (<2h) the fetus responded to agonist in the present study, along with previous work demonstrating pathological changes in the fetal brain following exposure to even sub-clinical doses of LPS[35], serve to highlight the urgent need for better tests to identify women at risk of intrauterine infection and PTB.

Secondly, and of particular interest, is the nature of the response presumably mounted by circulating immunocytes to acute fetal inflammation. Surprisingly, this response was characterised by inhibition of cell-cycle control, proliferation, but an absence of *de novo* inflammatory mediator expression. In keeping with previous animal studies, quantitative or qualitative changes in immunocyte populations were not detected after 48h of LPS exposure in this study. It is, however, tempting to speculate that the pro-proliferative signalling state identified in the present study is in keeping with qualitative and quantitative changes in immunocyte populations previously identified in LPS-exposed sheep fetuses by 7d[29]. Appropriate targeting of anti-inflammatory therapies is an important consideration as immunocyte proliferation and differentiation plays a crucial role in augmenting the host response to infection. *Ureaplasma* spp. are the microorganisms most commonly isolated from cases of early PTB; data from clinical studies suggest that perhaps up to 30% of very early preterm infants are cord-blood culture positive for *Ureaplasma spp*[36-39]. In these cases, the presence of systemic microbial agonist argues for the need for anti-inflammatory agents to reach the fetal circulation. However, it appears that in the majority of cases, infection (and thus pro-inflammatory agonist) is restricted to the AF. In these cases, risking systemic fetal immune-modulation may be unnecessary, especially if it were to inhibit immunocyte proliferation, which is crucial in allowing the fetus to protect itself from microbial invasion.

There are a number of factors to consider when interpreting the data presented above. Sheep provide a well-validated model with which to study fetal inflammatory responses to microbial agonist; however it is important to note that unlike a number of species (including rodents and non-human primates), administration of LPS in early-mid pregnancy does not induce the onset of labour [31]. Secondly, there are well documented differences between species with regards to sensitivity to LPS[40], and it is possible that a more vigorous fetal response, or analysis outside of the 48h window used in this study, may both lead to the identification of de novo cytokine/chemokine production by cells in the blood. Lastly, the present study models fetal responses to microbial agonist (LPS) in the AF. It would be of particular interest to determine the effect of alternative models of infection, such as acute trophoblast infection (with and without AF involvement) on fetal inflammatory responses.

CONCLUSIONS

Developing interventions to control the pathological inflammation associated with intrauterine infection is likely an important step in our efforts to prevent PTB and fetal injury. The availability of gestation-appropriate animal models, such as that described in the present study, will likely play a critical role in this work. In successfully developing a chronically catheterized sheep model of early pregnancy, our data demonstrate that the very premature fetus rapidly generates a robust inflammatory response in response to intraamniotic LPS exposure. In the absence of bacteraemia, acute systemic inflammation appears to be driven by amniotic-fluid exposed tissues; fetal blood cells (presumably immunocytes) respond to systemic inflammation by entering a pro-proliferative state but do not contribute to the acute production of inflammatory mediators.

This study advances our understanding of fetal responses to microbial agonist at a gestational age which is frequently associated with very early preterm birth. This study underscores the need to appropriately target anti-inflammatory therapies to fetal tissues exposed to the agonist-containing AF, and highlights the likely importance of the fetal lung. The speed at which substantial intrauterine inflammation occurs and the comparatively subtle nature of acute fetal systemic inflammatory responses also reinforces the urgent need to seek improved means of identifying women at risk of intrauterine infection.

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TABLES

Group	Delivery Weight (g)	# Male Fetuses	Arterial CB pH	Arterial CB pO_2	Arterial CB pCO_2	WBC 10^9/L	CB AST U/L	CB GGT U/L	CB GLDH U/L	CB TB µmol/L
Saline Control (n=9)	956.0±164.0	6/9	7.15±0.04	14.23±3.2	85.8±8.0	0.93±0.4	16.8±3.8	8.4±3.3	2.6±1.6	10.1±4.0
LPS (n=9)	850±117.0	6/9	7.13±0.04	13.0±4.5	91.0±6.7	1.1±0.8	16.6±4.9	15.4±7.2*	5.5±7.0	12.3±2.6

Table 1. Fetal delivery data. LPS, lipopolysaccharide (endotoxin) from *E.coli*; CB, fetal arterial cord blood; WBC, total white blood cell count; AST, aspartate aminotransferase; GGT, γ-glutamyltranspeptidase; GLDH, glutamate dehydrogenase; TB, total bilirubin. *, $p>0.05$.

		IL-6		
TIME	**TREAMENT**	**AF**	**TF**	**FP**
0 h	**SALINE**	0.0 (0.0 - 21.8)	84.1 (29.3 - 111.3)	0.0 (0.0 - 18.1)
	LPS	0.0 (0.0 - 103.0)	107.5 (50.7 - 180.6)	17.8 (0.0 - 64.9)
2 h	**SALINE**	0.0 (0.0 - 65.4)	79.7 (67.5 - 141.2)	99.0 (0.0 - 217.3)
	LPS	0.0 (0.0 - 71.5)	47.8 (17.3 - 109.3)	3.8 (0.0 - 33.0)
4 h	**SALINE**	0.0 (0.0 - 3.4)	22.0 (5.3 - 139.6)	15.3 (0.0 - 132.1)
	LPS	42.5 (0.0 - 120.4)	136.0 (32.9 - 174.8)	165.7 (0.0 - 219.2)
8 h	**SALINE**	0.0 (0.0 - 89.7)	41.1 (19.0 - 88.0)	26.2 (0.0 - 95.3)
	LPS	89.6 (47.8 - 165.0)	158.2 (127.4 - 201.3)	256.5 (26.6 - 757.7)
12 h	**SALINE**	0.0 (0.0 - 25.4)	98.1 (25.0 - 139.0)	0.0 (0.0 - 34.0)
	LPS	**102.7 (66.4 - 421.3)***	129.5 (120.8 - 193.4)	**1159.7 (300.1 - 1342.3)***
24 h	**SALINE**	0.0 (0.0 - 147.0)	50.0 (0.0 - 134.4)	95.3 (56.8 - 314.7)
	LPS	**186.8 (145.8 - 619.0)***	**334.7 (225.5 - 406.9)***	69.4 (44.2 - 405.4)
48 h	**SALINE**	0.0 (0.0 - 37.4)	100.4 (55.2 - 126.6)	0.0 (0.0 - 88.88)
	LPS	**406.3 (343.5 - 601.0)***	**412.2 (170.0 - 527.8)***	254.3 (53.4 - 431.2)

Table 2. IL-6 protein concentrations (pg/mL) in amniotic fluid (AF), tracheal fluid (TF) and fetal plasma (FP). h, hour; *, $p<0.05$ vs. control.

		IL-8		
TIME	**TREAMENT**	**AF**	**TF**	**FP**
0 h	**SALINE**	464.0 (223.0 - 532.9)	337.7 (29.8 - 728.66)	1007. (764.8 - 1175.8)
	LPS	275.7 (175.1 - 346.1)	362.6 (326.2 - 1030.5)	1412.9 (1051.3 - 1858.1)
2 h	**SALINE**	217.6 (104.5 - 606.5)	220.9 (107.0 - 569.7)	952.4 (678.7 - 1498.3)
	LPS	432.1 (192.5 - 812.3)	**1012.0 (705.2 - 1138.3)***	1867.5 (1657.7 - 1965.6)
4 h	**SALINE**	169.6 (0.0 - 524.6)	418.8 (69.2 - 663.3)	1004.6 (874.3 - 1161.0)
	LPS	**1910.1 (1702.0 - 2529.3)***	**1904.8 (1691.5 - 2029.4)***	1246.2 (1118.6 - 1738.2)
8 h	**SALINE**	315.3 (253.5 - 795.8)	472.7 (184.9 - 802.2)	1229.2 (628.0 - 1706.6)
	LPS	**4976.2 (4283.6 - 6148.7)***	**2425.9 (2111.3 - 2551.2)***	1142.4 (512.9 - 1827.0)
12 h	**SALINE**	257.9 (68.0 - 475.3)	507.4 (212.9 - 989.3)	890.8 (427.8 - 1246.2)
	LPS	**9389.4 (8574.1 - 12020.6)***	**3265.8 (2665.2 - 5105.9)***	**2119.4 (1512.0 - 2313.3)***
24 h	**SALINE**	303.2 (150.8 - 728.4)	404.2 (133.2 - 1057.1)	796.5 (634.3 - 1140.9)
	LPS	**22430.5 (19787.1 - 27368.7)***	**13374.4 (11781.8 - 13795.3)***	1409.3 (992.6 - 1731.0)
48 h	**SALINE**	764.8 (673.2 - 2440.2)	785.7 (499.6 - 1301.4)	-
	LPS	**73963.9 (55876.0 - 89334.6)***	**43904.8 (16867.8 - 101299.6)***	-

Table 3. IL-8 protein concentrations (pg/mL) in amniotic fluid (AF), tracheal fluid (TF) and fetal plasma (FP). h, hour; *, $p<0.05$ vs. control.

		MCP-1		
TIME	**TREAMENT**	**AF**	**TF**	**FP**
0 h	**SALINE**	41.8 (21.7 - 59.6)	12.6 (6.3 - 48.9)	4.0 (0.0 - 26.0)
	LPS	95.6 (35.2 - 122.5)	29.4 (27.5 - 50.5)	12.0 (7.5 - 15.0)
2 h	**SALINE**	41.8 (26.5 - 55.3)	20.0 (4.2 - 38.5)	8.0 (5.0 - 29.0)
	LPS	77.7 (41.8 - 97.9)	79.9 (69.9 - 85.0)	31.0 (22.5 - 32.0)
4 h	**SALINE**	37.4 (19.4 - 57.7)	24.0 (2.1 - 106.5)	4.0 (3.0 - 24.0)
	LPS	**91.1 (79.6 - 120.3)***	75.7 (63.3 - 123.8)	**99.8 (83.9 - 99.8)***
8 h	**SALINE**	72.6 (37.4 - 106.8)	33.6 (11.0 - 48.9)	8.0 (2.0 - 25.0)
	LPS	**127.0 (117.0 - 151.6)***	**109.3 (74.7 - 132.4)***	**133.7 (108.8 - 142.3)***
12 h	**SALINE**	59.8 (46.3 - 88.2)	40.0 (10.5 - 50.0)	8.0 (0.5 - 18.5)
	LPS	**158.4 (111.3 - 207.7)***	**145.8 (108.0 - 148.5)***	**147.7 (79.9 - 153.7)***
24 h	**SALINE**	68.7 (64.1 - 84.4)	16.8 (12.2 - 78.5)	15.0 (5.0 - 20.5)
	LPS	**234.6 (187.5 - 241.3)***	**168.1 (147.5 - 197.6)***	**97.8 (67.4 - 127.8)***
48 h	**SALINE**	95.6 (71.0 - 132.5)	92.5 (83.8 - 117.7)	4.0 (0.0 - 12.0)
	LPS	**257.0 (247.6 - 279.4)***	**190.3 (146.3 - 248.0)***	**33.9 (30.9 - 63.9)***

Table 4. MCP-2 protein concentrations (pg/mL) in amniotic fluid (AF), tracheal fluid (TF) and fetal plasma (FP). h, hour; *, $p<0.05$ vs. control.

FIGURE LEGENDS

Figure 1.

Relative expression (fold change vs. control) of cytokines and chemokines in fetal lung (Panel A); cortex (Panel B); skin (Panel C); spleen (Panel D); and cord blood cells (Panel E). IL, interleukin; TNF, tumour necrosis factor; MCP, monocyte chemoattractant protein. *, $p<0.05$.

Figure 2.

Summary of Ingenuity Pathway Analysis of mRNA from fetal cord blood immunocytes. Panel A, IL-6 signalling cascade; Panel B, cellular proliferation cascade; Panel C, viral proliferation cascade.