


***In utero* inflammatory challenge induces an early activation of the hepatic innate immune response in late gestation fetal sheep**

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Abstract

Chorioamnionitis is associated with inflammatory end-organ damage in the fetus. Tissues in direct contact with amniotic fluid drive a pro-inflammatory response and contribute to this injury. However, due to a lack of direct contact with the amniotic fluid, the liver contribution to this response has not been fully characterized. Given its role as an immunologic organ, we hypothesized that the fetal liver would demonstrate an early innate immune response to an *in utero* inflammatory challenge. Fetal sheep (131 ± 1 d gestation) demonstrated metabolic acidosis and high cortisol and norepinephrine values within 5 h of exposure to intra-amniotic LPS. Likewise, expression of pro-inflammatory cytokines increased significantly at 1 and 5 h of exposure. This was associated with NF-κB activation, by inhibitory protein IκBα degradation, and nuclear translocation of NF-κB subunits (p65/p50). Corroborating these findings, LPS exposure significantly increased pro-inflammatory innate immune gene expression in fetal sheep hepatic macrophages *in vitro*. Thus, an *in utero* inflammatory challenge induces an early hepatic innate immune response with systemic metabolic and stress responses. Within the fetal liver, hepatic macrophages respond robustly to LPS exposure. Our results demonstrate that the fetal hepatic innate immune response must be considered when developing therapeutic approaches to attenuate end-organ injury associated with *in utero* inflammation.

Keywords

Chorioamnionitis, fetus, innate immunity, liver, macrophages

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Introduction

Chorioamnionitis accounts for high morbidity and mortality rates of the fetus and neonate, and is associated with premature birth and infection-induced disorders.^{1–5} Infection-induced inflammation is thought to underlie many of the complications associated with exposure to inflammatory stress *in utero* such as brain damage,⁶ bronchopulmonary dysplasia,⁷ and necrotizing enterocolitis.⁸ The fetal response to an inflammatory challenge is characterized by an accumulation of pro-inflammatory cytokines in the amniotic fluid (local) and the umbilical blood (systemic).^{9–13}

Reports have demonstrated that the fetal innate immune system contributes to the inflammatory

response to an intra-amniotic (IA) immune challenge. IA exposure to LPS, a TLR4 agonist, is a well-established model used to study the fetal response to a pro-inflammatory stimulus.^{14,15} It has been shown previously that direct LPS exposure to the fetal

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skin,¹⁶ gut,¹⁷ respiratory epithelium,^{18,19} and placenta results in a local pro-inflammatory response.²⁰ These findings support the hypothesis that the epithelial surfaces that are in contact with the amniotic fluid drive the inflammatory innate immune response.

It is increasingly recognized that the liver functions as a primary immune organ during early stages of development.^{21,22} Hepatic macrophages, particularly, are uniquely positioned to initiate the innate immune response to systemic insult due to their unique location outside the sinusoidal space. Here, hepatic macrophages are able to capture pathogens and antigens in the bloodstream,^{23–25} and send signals to other organs through the production of cytokines released systemically.²⁶ Surprisingly, there are no data to support the hypothesis that the fetal liver contributes to the fetal innate immune response to IA LPS challenge. Specifically, previous studies have demonstrated that, starting at 5 h following IA LPS challenge, hepatic expression of pro-inflammatory cytokines is not increased in the preterm lamb.²⁷ However, by 2 d after exposure to IA LPS, the liver does show evidence of injury through activation of acute-phase proteins,²⁸ and polymorphonuclear immune cell aggregation in preterm babies.²⁹ Thus, the immediate fetal hepatic response to an IA inflammatory challenge is relatively understudied and leaves us with little knowledge on how the fetal liver, and, specifically, the hepatic macrophages respond to a pro-inflammatory innate immune challenge.

To understand whether the liver contributes to the systemic inflammatory response, a better characterization of the temporal relationship between IA LPS exposure and the fetal response is necessary. Precise determination of the systemic response to LPS at time points immediately following IA exposure would increase our understanding of the fetal response to IA LPS challenge, and thus guide interrogation of the hepatic response. Without this information, therapeutic approaches to manipulate key components that might trigger and regulate the response to chorioamnionitis, and, thus, minimize the injury associated with infection-induced inflammation are limited.

Our research group has a well-established chronic catheterization fetal sheep model that allows us to monitor local and systemic fetal responses at different time points.^{30,31} Likewise, our model can provide sufficient liver cell populations to measure the cell-specific contribution to multiple challenges *in vitro*.^{32,33} A better understanding of the fetal-organ-specific innate immune response to inflammatory stimuli can provide novel insights into the development of the innate immune response and its role on the pathophysiology of end-organ injury in the perinatal period. The aim of the present study was, therefore, to determine

the effects of an IA LPS challenge on the early (1 and 5 h) fetal hepatic innate immune signaling and transcriptional response. We hypothesized that the fetal liver contributes uniquely to the early activation of the innate immune signaling after an *in utero* pro-inflammatory challenge in the sheep.

Materials and methods

Fetal surgical preparation

A total of 13 pregnant late-gestation Columbia-Rambouillet mixed-breed ewes (singleton pregnancies) were obtained from Nebeker Ranch (Lancaster, CA, USA), housed, and had access to food and water *ad libitum*. Fetal surgery was performed at 125 ± 1 d of gestation. Briefly, ewes were fasted 24 h before surgery and received a pre-operative dose of antibiotics (penicillin, 600,000 U IM) and non-steroidal analgesic (flunixin meglumine, 1 mg/kg). Anesthesia was induced through the administration of ketamine (20 mg/kg) and diazepam (0.2 mg/kg), and maintained with 1–3% isoflurane during the entire procedure. Our fetal chronic catheterization model has been published elsewhere.^{32,34} Briefly, at surgery, the fetus was exposed by maternal laparotomy and hysterectomy, and a set of catheters was placed in the fetus (umbilical vein and abdominal aorta), the amniotic fluid space, and in the ewe (femoral vein and artery) (Table 1). Ampicillin (500 mg) was injected into the amniotic space before closing the uterus. All catheters exited the right flank of the ewe subcutaneously and were placed into a disposable pouch. Ewes had at least 6 d of post-operative care, where they received 2 d of treatment with flunixin meglumine (2.2 mg/kg) and probiotics (10 g orally), and their health status and food/water intake were monitored constantly.

Experimental sheep model of chorioamnionitis

Figure 1 depicts our experimental design through and the administration of IA LPS. Ewes (131 ± 1 d of gestation) were allocated into two groups according to the time after IA LPS exposure (LPS 1 h, $n = 5$; LPS 5 h, $n = 8$). Animals used for this study were conscious and freestanding during the experiment. *Escherichia coli* O55:B5 LPS (L2637, Sigma-Aldrich, St. Louis, MO, USA) diluted in sterile PBS ($1 \times$, 2 ml) was administered (20 mg) in the intra-amniotic space. Fetal arterial blood samples (1 ml) were collected in heparinized tubes before (two baselines), and 1 h and 5 h (LPS 5 h group) or 15, 30, and 60 min (LPS 1 h group) post-IA LPS exposure. We measured blood gases values with the ABL 800 Flex blood gas analyzer (Radiometer, Copenhagen, Denmark), Glc and lactate with the

Yellow Springs Instrument model 2900 Select Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, OH, USA), plasma norepinephrine by HPLC (model no. 2475; Waters Corp., Milford, MA, USA), and plasma cortisol by an ovine-specific ELISA assay (Alpco Diagnostics, Windham, NH, USA). Experiments were terminated after 1 or 5 h of IA LPS exposure. At this point, ewes received a dose of diazepam (0.2 mg/kg) and ketamine (20 mg/kg) IV, fetuses were delivered by laparotomy and hysterectomy, and both ewes and fetuses were euthanized with an IV overdose of sodium pentobarbital (Fatal Plus; Bortech Pharmaceuticals, Dearborn, MI, USA). Fetal organs were dissected, snap frozen in liquid nitrogen, and stored at -80°C .

RNA extraction and quantitative real time-PCR

We used the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) to extract mRNA from LPS-exposed fetal liver (1 h, $n=5$; 5 h, $n=7$), lung (1 h, $n=5$; 5 h, $n=7$), cotyledon (1 h, $n=5$; 5 h, $n=8$), skin (1 h, $n=5$), and control tissues (liver, lung, and cotyledons, $n=4$; skin $n=3$) (CON) that were subjected to the same instrumentalization except for IA LPS administration.

Table 1. Catheters placement and their purpose in our fetal ovine inflammatory challenge model.

Origin	Catheter placement	Purpose
Fetal	Abdominal aorta	Fetal systemic condition assessment
	Umbilical vein	LPS quantification
Maternal	Femoral artery	Maternal systemic condition assessment

mRNA was converted into cDNA using the Verso cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). Primers were designed on the *Ovis aries* genomes for SYBR green, and included IL-1 α (*IL1A*), IL-1 β (*IL1B*), IL-6 (*IL6*), and IL-8 (*IL8*), and TNF- α (*TNF*) (Table 2). We used the ovine reference gene 40S ribosomal protein S15 (*RPS15*) to normalize RT-qPCR results, as previously described.³⁵ cDNA samples (20 ng) and 5 μM of each primer were assayed in duplicate using FastStart Essential DNA Green Master Kit (Roche, Pleasanton, CA, USA) in a Lightcycler 96 (Roche, Pleasanton, CA, USA). RT-qPCR conditions were as follows: 95 $^{\circ}\text{C}$ for 5 min, followed by 40 cycles of amplification at 95 $^{\circ}\text{C}$ for 10 s and 60 $^{\circ}\text{C}$ for 30 s; and melting curve from 60 $^{\circ}\text{C}$ to 95 $^{\circ}\text{C}$. Data are expressed as a fold change relative to the mean in the CON group based on absolute values by using standard curve calculations of pooled cDNA as described previously.³⁶

Hepatic and pulmonary nuclear and cytoplasmic protein extraction

LPS-exposed fetal liver (1 h, $n=5$; 5 h, $n=4$) lung (1 h, $n=4$; 5 h, $n=4$), and control tissues ($n=4$) (CON) were homogenized using the Bullet Blender (Next Advance, Troy, NY, USA), and protein lysates were collected and kept in T-PER buffer (ThermoFisher Scientific). Fetal hepatic cytosolic and nuclear extracts were prepared with the NE-PER kit (ThermoFisher Scientific).

Western blot analysis

Both LPS and CON cytosolic and nuclear extracts were electrophoresed on a 4–12% polyacrylamide gel (Invitrogen, Carlsbad, CA, USA) and proteins were

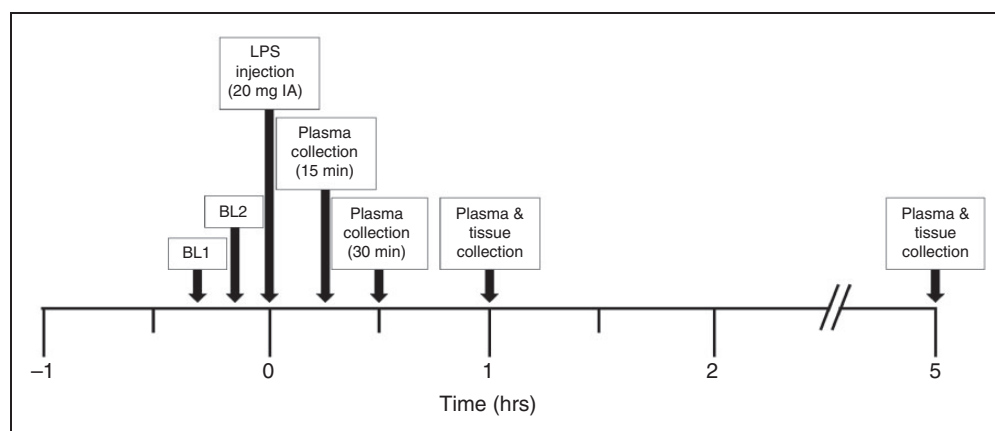


Figure 1. Experimental IA LPS design and timeline. After surgery, ewes (131 ± 1 d) were allocated into two groups according to the time after IA LPS exposure (LPS 1 hr, $n=5$; LPS 5 hrs, $n=8$). BL: baseline measurements.

Table 2. Primers used for real-time qPCR analyses.

Gene symbol	Gene name	Forward primer	Reverse primer	Probe
<i>18S</i>	18S Ribosomal RNA	GATCCATTGGAGGGCAAGTCT	GCAGCAACTTTAATATACGCTATTGG	SYBR Green
<i>CD11B</i>	Integrin Subunit Alpha M	TTTCCGGCTGCTGGCCTGTG	ACTCTCTGAGGGCCGCTGGG	SYBR Green
<i>IL1A</i>	Interleukin 1 alpha	GATGACCTGGAAGCCATTGC	ATGTGCTGATCTGGGCTTGAT	SYBR Green
<i>IL1B</i>	Interleukin 1 beta	ACGAACATGTCTTCCGTGAT	ACCAGGGATTTTTGCTCTCT	SYBR Green
<i>IL6</i>	Interleukin 6	ACCTGGACTTCTCCAGAAC	TTGAGGACTGCATCTTCTCC	SYBR Green
<i>IL8</i>	Interleukin 8	ATGAGTACAGAACTTCGA	TCATGGATCTTGCTTCTC	SYBR Green
<i>PEPCK</i>	Phosphoenolpyruvate Carboxykinase	TGTCCGAGGAGGATTTTGGAG	ATGCCAATCTTGGACAGAGG	SYBR Green
<i>RPS15</i>	40S Ribosomal protein S15	ATCATTCTGCCCGACATGGTG	CGGGCCGGCCATGCTTTACG	SYBR Green
<i>TNF</i>	Tumor necrosis factor	CTGGGCAGGTCTACTTTGGG	GAAGGGGATGAGGAGGGTCT	SYBR Green

transferred to an Immobilon-P membrane (MilliporeSigma, Burlington, MA, USA) and blotted with Abs against the NF- κ B subunits p50 (1:1000, Abcam, Cambridge, MA, USA, Cat. no. ab32360, RRID: AB_776748) and p65 (1:1000, Cell Signaling, Danvers, MA, USA, Cat. no. #6956, RRID: AB_10828935), and NF κ B inhibitory protein I κ B α (1:1000, Cell Signaling, Cat. no. #4814, RRID: AB_390781). All Abs used in this study were incubated at 4°C overnight. For loading controls in our assays, we used β -actin (1:1000, Cell Signaling, Cat. no. #3700, RRID: AB_2242334) for cytosolic extracts; and lamin B (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA, Cat. no. SC-6217, RRID: AB_648158) for nuclear extracts. Immunoblots were imaged with the Li-Cor Odyssey[®] Fc imaging system (Li-Cor, Lincoln, NE, USA) and densitometric analysis was conducted using Image Studio version 4.0 (Li-Cor).

Plasma 3-hydroxytetradecanoic acid detection

3-Hydroxytetradecanoic acid is the most common fatty acid part of lipid A, the lipid component of the LPS innermost region, and responsible for the toxicity of Gram-negative bacteria.³⁷ We had enough remaining plasma from three critical time points (baseline, 15 min post-LPS exposure, 30 min post-LPS exposure) from four fetuses to test for 3-hydroxytetradecanoic acid levels. Plasma samples collected from the umbilical vein were base hydrolyzed and extracted, and the extracts were analyzed using LC-MS/MS performed on a Waters Acquity M-Class UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer (Waters Corp.) using the methods reported by Pais de Barros et al.³⁸ Peak areas of 3-hydroxytetradecanoic acid were detected and integration of peak area was performed manually using Masslynx (Waters Corp.). Total 3-hydroxytetradecanoic acid peak areas detected

were the sum of the free (after hydrolysis) and bound (intact) LPS forms.

Primary hepatocyte and hepatic macrophage cell culture studies

Primary hepatocytes and hepatic macrophages were isolated from normal late gestation fetal sheep using previously described methods.^{30,39} Briefly, a portion of the right lobe of the fetal liver was perfused and digested with collagenase. Hepatocytes were separated from the total mixture of digested cells by centrifugation at 100 g. Hepatocytes were plated in complete DMEM medium with 1.1 mM Glc supplement with 2 mM glutamine, 2.2 mM lactate, 1 mM pyruvate, 1 \times non-essential amino acids, 100 U/ml penicillin-streptomycin, 1 nM insulin, 100 nM dexamethasone, and 10% FBS on collagen coated Primaria (BD Falcon) plates (Thermo Fisher Scientific). After a 4 h attachment period, cells were washed, and the medium was replaced with serum-free DMEM plus 0.2% BSA (SF). For hepatic macrophage isolations, the supernatant after centrifugation at 100 g containing all non-parenchymal cells was filtered and spun at 800 g to pellet non-parenchymal cells. Cells were resuspended in 24% Histodenz (Sigma-Aldrich) and gradients were prepared and spun at 1500 g for 20 min. Cells at the interface were collected, washed, and plated on tissue culture plates in complete DMEM with 55 μ M 2-mercaptoethanol. Both cell types were incubated at 37°C and 5% CO₂. After 1–2 h attachment, non-adherent cells were aspirated and media was replaced on adherent cells, representing hepatic macrophages.⁴⁰ Both cell types were incubated at 37°C and 5% CO₂. Hepatocytes incubated in SF media and hepatic macrophages incubated in complete medium with 2-mercaptoethanol were studied 24 h after isolation. A total of four isolated cell groups (hepatocytes and hepatic

Table 3. Maternal and fetal artery blood gas and metabolite values at baseline, and 1- and 5 h post intra-amniotic LPS (IA LPS) administration ($n=8$). Data are shown as means \pm SEM. Asterisks (*) indicate statistical difference ($P < 0.05$) vs. baseline.

	pH (units)	pCO ₂ (mmHg)	HCO ₃ ⁻ (mmol/l)	O ₂ content (mmol/l)	Glc (mg/dl)	Lactate (mmol/l)
Maternal values: (Femoral artery)						
Baseline	7.46 \pm 0.02	35.7 \pm 1.5	24.6 \pm 1.5	5.5 \pm 0.3	65.6 \pm 2.9	0.69 \pm 0.90
1 h	7.46 \pm 0.02	37.8 \pm 1.5	26.4 \pm 1.5	5.6 \pm 0.4	65.1 \pm 3.0	0.73 \pm 0.94
5 h	7.48 \pm 0.02	35.9 \pm 1.5	25.7 \pm 1.5	5.7 \pm 0.4	60.2 \pm 2.9	1.57 \pm 0.90
Fetal values: (Abdominal aorta)						
Baseline	7.37 \pm 0.02	52.8 \pm 1.5	29.0 \pm 1.4	3.1 \pm 0.3	16.8 \pm 2.9	2.24 \pm 0.90
1 h	7.34 \pm 0.02	55.6 \pm 1.6	28.9 \pm 1.5	2.6 \pm 0.4	17.8 \pm 3.1	2.21 \pm 0.99
5 h	7.25 \pm 0.02*	57.6 \pm 1.5*	24.3 \pm 1.4*	2.4 \pm 0.3	15.2 \pm 2.9	9.21 \pm 0.90*

macrophages) were incubated with SF media (basal) or LPS (100 ng/ml) for 3 h of treatment and then harvested for RNA isolation and gene expression. Purity assessment was performed by measuring gene expression of the hepatocyte (phosphoenolpyruvate carboxykinase, *PEPCK*) and hepatic macrophage (integrin subunit alpha M, *CD11B*) fractions (Table 2). Gene expression was normalized to 18s ribosomal RNA (*18S*) and the RT-qPCR conditions were the same as described in the section *RNA extraction and quantitative real time-PCR*. Results were expressed relative to the average basal expression in hepatocytes from the cell preparations.

Statistical analyses

We used the Mixed or NPARIWAY Procedure of SAS/STAT 9.3[®] (SAS Institute Inc., Cary, NC, USA) for data analysis. For blood gas, endocrine, western blot, and ELISA data, the statistical analysis involved a one-way ANOVA with repeated measures (baseline, 1 h, and 5 h post exposure). Appropriate covariance structures were used by selecting the best fit statistics. Cell culture data RT-qPCR was analyzed as one-way ANOVA with four treatments (hepatocytes \pm LPS, hepatic macrophages \pm LPS). Differences between means were obtained using the Fisher's least significant difference test. Hepatic WB and RT-qPCR data values failed to follow the normal distribution and thus were analyzed by the Mann-Whitney *U* nonparametric test. Significance was declared at $P < 0.05$.

Results

IA LPS challenge induces systemic inflammatory stress in the fetus within 5 h of exposure

First, we sought to determine the time course whereby IA LPS challenge produced a systemic response in the fetus. Most reports describe metabolic alterations such

as acidosis, hyperlactatemia, and hypoglycemia from a period starting at 6 h to d after exposure in LPS-exposed fetuses,⁴¹ neonates,^{42,43} and adults,^{44,45} but the early timing of this acute response in the fetus is not yet elucidated. Due to the lack of sufficient data describing the initial effects of IA LPS *in utero*, we measured the fetal systemic response at 1- and 5-h post-exposure to confirm whether an IA LPS challenge produced a similar systemic metabolic feature in an earlier time course. We determined fetal blood gases chemistry, Glc, and lactate parameters in the maternal and fetal abdominal aorta at early time points (0–5 h, Table 3). An acute inflammatory challenge produced a significant decrease in fetal pH and an increase in pCO₂ values in the fetal abdominal aorta at 5 h compared with baseline ($P < 0.05$). Furthermore, IA LPS-challenged fetuses showed a marked reduction in plasma bicarbonate levels in the abdominal aorta at 5 h after exposure ($P < 0.05$). Plasma lactate concentrations showed an approximate 5-fold increase in IA LPS exposed fetuses compared with control ($P < 0.001$). Although Glc has been reported to increase during LPS challenges in adults,⁴⁶ there were no differences in maternal/fetal plasma Glc levels at these acute time points. Furthermore, we did not detect changes in maternal pH, pCO₂, bicarbonate, or oxygen content values after LPS challenge.

IA LPS challenge activates fetal stress responses characterized by an increase in cortisol and norepinephrine levels

Once we observed acute fetal metabolic acidosis as a response to IA LPS, we then determined whether IA LPS activates systemic fetal neuroendocrine stress responses. IA LPS-challenged fetuses (Figure 2) showed a significant increase in plasma norepinephrine and cortisol levels at 5 h post stimulus compared with baseline ($P < 0.01$). This suggests IA LPS activates

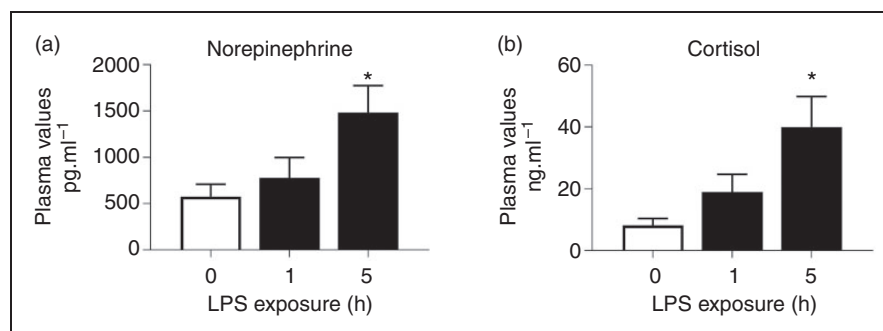


Figure 2. IA LPS activates fetal stress responses systemically. Fetal abdominal aorta plasma (A) norepinephrine (pg/ml) and (B) cortisol (ng/ml) were measured at baseline, and 1 and 5 hours post IA LPS challenge (n=8). Data are shown as means \pm SEM. Asterisks (*) indicate statistical difference ($P < 0.05$) vs. baseline.

robust and acute stress responses along with acidemia in the fetus.

IA LPS challenge activates the fetal innate immune response in the liver at 1 and 5 h post exposure

The acute and early effects of IA LPS on the fetal innate immune response have not been fully described. To test this, we determined the effects of LPS on the fetal lungs, cotyledons (fetal placental side), and skin since these tissues have shown to produce inflammatory responses as a consequence of an IA LPS exposure.^{16,47,48} Furthermore, we decided to study the fetal liver since it has been reported to contribute to the innate immune response after an inflammatory stimulation in the perinatal period,⁴⁹ and harbor a unique type of macrophage cells that have a pro-inflammatory phenotype due to their condition as sentinels in the fetal bloodstream.^{22,50} First, we assessed the expression of primary innate immune response genes (*TNF*, *IL1A*, and *IL1B*)⁵¹ at 1 h post IA LPS. Figure 3 shows the IA LPS effects on the fetal liver, lung, placenta, and skin innate immune gene expression at 1 h post stimulus. IA LPS-exposed fetuses showed a significant up-regulation of hepatic primary immune response genes (Figure 3a) at 1 h compared with control ($P < 0.05$). The fetal lung and skin showed a significant up-regulation of *IL1B* (Figure 3b and 3d) at 1 h vs. control, with no differences in the expression of *IL1A* and *TNF*. There were no differences in the expression of tested genes observed in the fetal cotyledon exposed to IA LPS (Figure 3c). We then decided to determine whether these transcriptomic effects continue at 5 h post IA LPS exposure. IA LPS produced a significant up-regulation of hepatic (Figure 4a) *IL1A* ($P < 0.05$), *IL1B* ($P < 0.05$), *TNF* ($P < 0.05$), *IL6* ($P < 0.05$) and *IL8* ($P < 0.05$), and a significant increase in expression of *IL1A* ($P < 0.05$)

and *IL6* ($P < 0.01$) (Figure 4b) in the lung compared with control. In contrast, IA LPS did not produce any gene expression changes in the placenta innate immune response profile transcriptome (Figure 4c). These results demonstrate that the fetal hepatic innate immune response is both rapid and robust in response to an IA inflammatory challenge.

IA LPS-induced hepatic and pulmonary pro-inflammatory cytokine are associated with NF- κ B activation

The transcription factor NF- κ B is intrinsic among all living species and has a key role in the response to various infectious stimuli.^{52,53} It is well recognized that this transcription factor regulates the expression of many pro-inflammatory genes downstream of LPS-mediated TLR4 signaling.⁵³ Following LPS exposure, NF- κ B dimer pairs (e.g., p50 and p65) are translocated to the nucleus due to degradation of the cytosolic inhibitory protein I κ B α .^{52,54} Based on this, we decided to evaluate whether IA LPS induced changes in the levels of the cytosolic protein levels of the NF- κ B inhibitory protein, I κ B α and whether it induced nuclear translocation of the NF- κ B subunits p65 and p50 in target organs that demonstrated up-regulation of key pro-inflammatory cytokines.

Figure 5 indicates the hepatic (Figure 5a–d) and pulmonary (5e–f) cytosolic I κ B α and nuclear p65/p50 protein levels in fetuses exposed to IA LPS compared with a control group at 1 h and 5 h. Hepatic cytosolic I κ B α protein levels decreased significantly 1 h after exposure ($P < 0.05$) and showed no difference vs. control at 5 h. Hepatic nuclear NF- κ B subunits p65 and p50 showed a similar pattern with an increase in protein expression at 1 h post IA LPS ($P < 0.05$) and returned to control values 5 h after exposure.

Fetal lung cytosolic I κ B α protein expression showed a similar significant reduction at 1 h post IA LPS

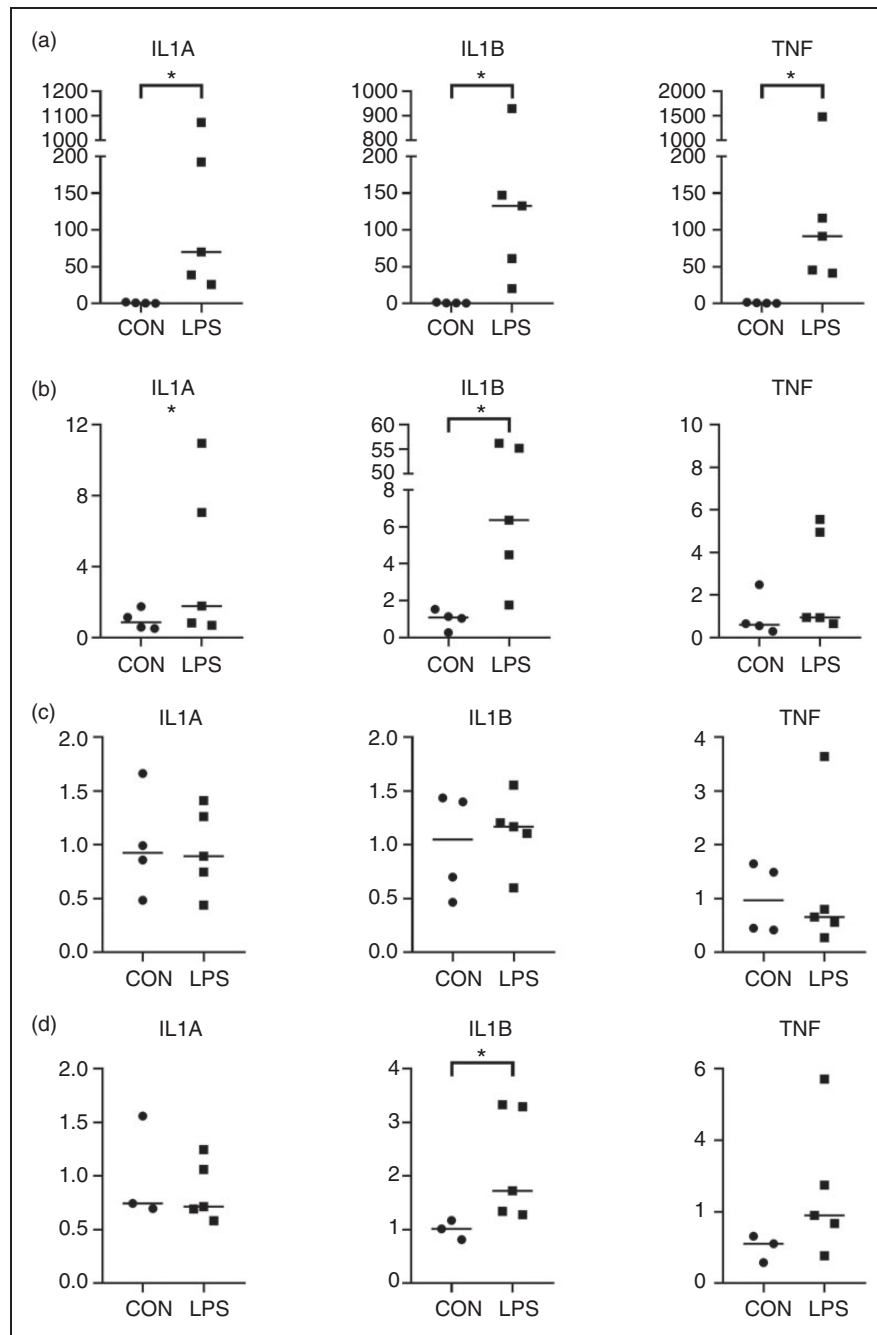


Figure 3. Fetal innate immune transcriptomic response to IA LPS at 1 hour after IA LPS. (A) Liver, (B) lung, (C) placenta, and (D) skin gene expression levels for innate immune response markers at 1-hour post IA LPS ($n=5$) (LPS) vs. control ($n=4$; skin $n=3$) (CON) groups. Ovine RPS15 was used as a reference gene. Values are represented as fold change compared with CON based on absolute values. Asterisks (*) indicate statistical difference ($P < 0.05$) vs. CON by the Mann-Whitney test.

($P < 0.05$) along with an increase in nuclear p65 protein expression ($P < 0.05$), with both values returning to control levels 5 h after. There were no differences observed in the nuclear p50 levels in the lung. These results demonstrated that the fetal liver is capable of mounting a robust NF- κ B-mediated innate immune response activation as early as 1 h post IA LPS.

3-Hydroxytetradecanoic acid can be detected in the fetal circulation after IA LPS challenge

Having observed a systemic (blood gases and endocrine) and local (hepatic innate immune activation) fetal response to an IA LPS exposure, we hypothesized that this is a consequence of LPS presence the fetal

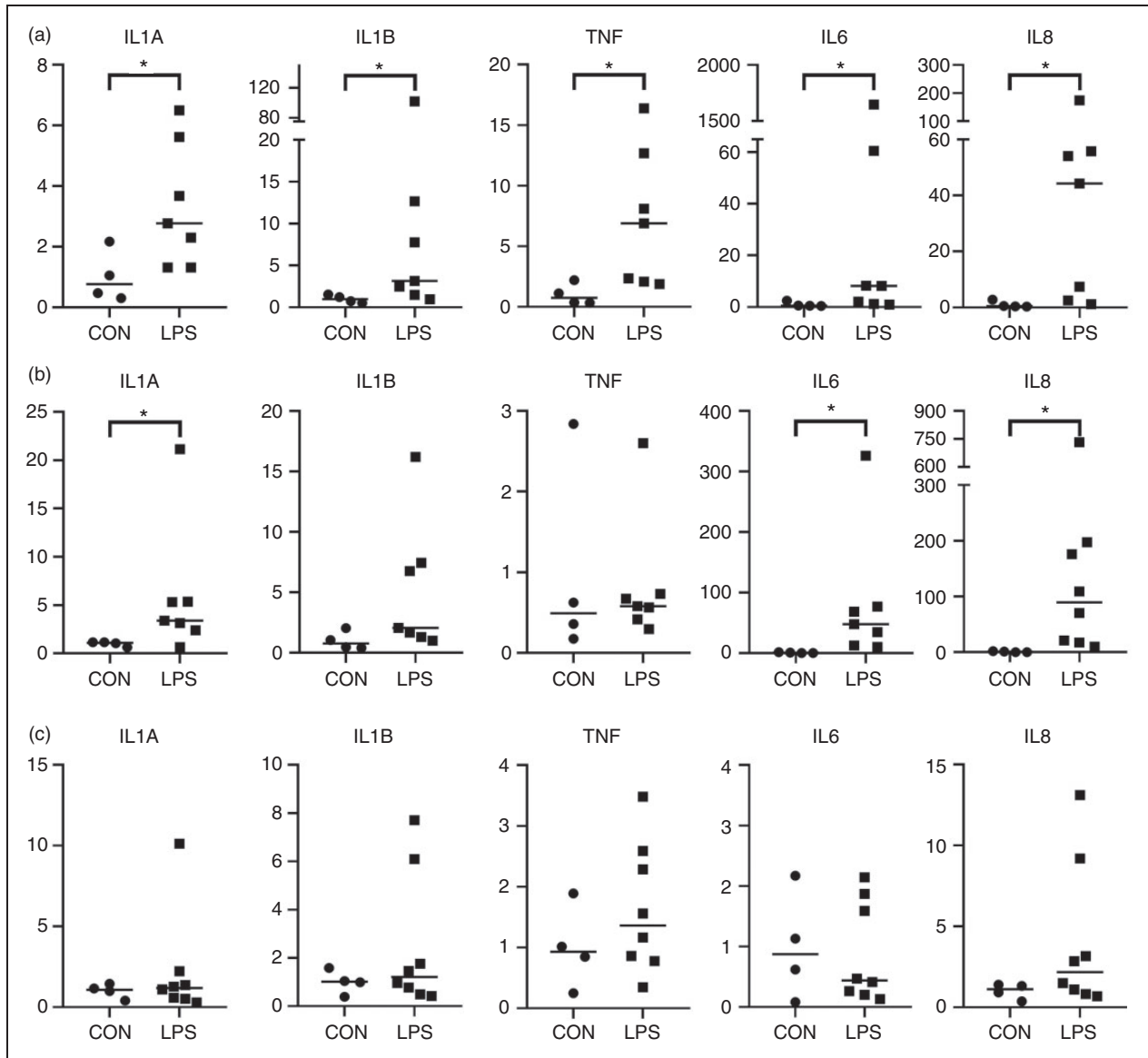


Figure 4. Fetal innate immune transcriptomic response to IA LPS at 5 hours after IA LPS. (A) Liver (n=7), (B) lung (n=7), and (C) placenta (n=8) gene expression levels for innate immune response markers between 5-hour post IA LPS (LPS) vs. control (n=4) (CON) groups. Ovine RPS15 was used as a reference gene. Values are represented as fold change compared with CON based on absolute values. Asterisks (*) indicate statistical difference ($P < 0.05$) vs. CON by the Mann-Whitney test.

circulation. For this, we sought to detect fetal systemic 3-hydroxytetradecanoic acid (main metabolite from *E. coli* LPS) levels from the umbilical blood at earlier time points (15- and 30-min post exposure). Figure 6 shows the fetal plasma 3-hydroxytetradecanoic acid integrated peak intensities from four LPS 1-h animals at different time points. We found that two animals showed a raise in 3-hydroxytetradecanoic acid intensity peaks at 15 min post IA LPS, with values returning to basal levels 30 min post exposure. Similarly, in a third animal, intensity rose at 15 min post IA LPS, but, rather than decreasing at 30 min, the intensity continued to rise. Finally, the last fetus started with higher

3-hydroxytetradecanoic acid intensity peaks in the umbilical vein before IA LPS (0 min). While the reason for this is not clear, one possibility is subclinical infection. These data indicate that intact *E. coli* LPS or its main metabolite (3-hydroxytetradecanoic acid) can be detected systemically in the fetus as early as 15 min following an IA exposure.

Endotoxin challenge activates fetal hepatic macrophages in vitro

Having determined that an IA LPS exposure induces a robust innate immune response in the fetal liver, we

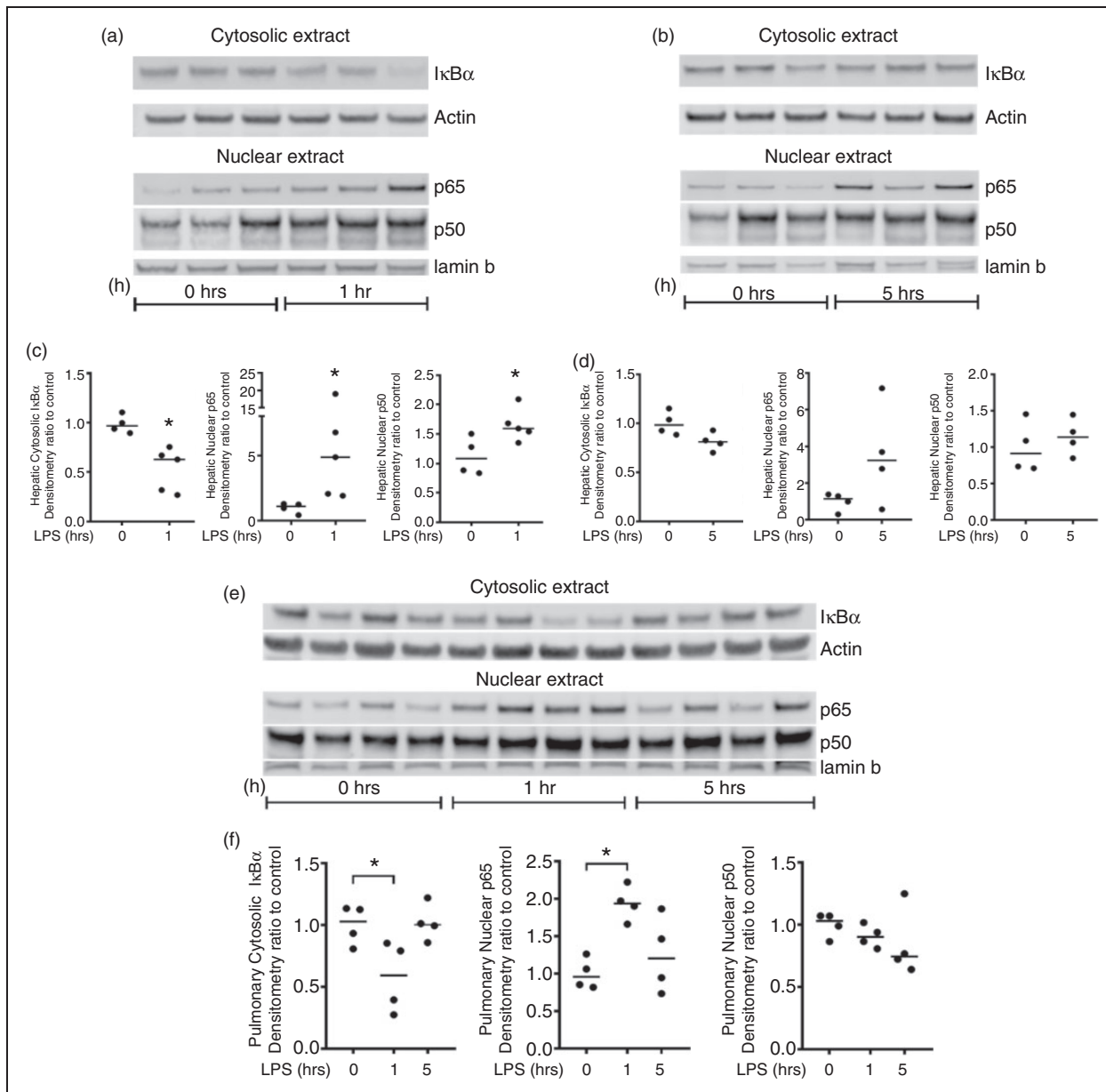


Figure 5. IA LPS induces NFκB activation in the fetal liver and lungs. (A, B) Representative Western Blot images showing hepatic (A, B) and pulmonary (E) cytosolic IκBα and nuclear NFκB subunits p65 and p50 at 1 hr and 5 hrs post IA LPS exposure compared with control (0 hrs). Actin and lamin b were used as loading controls for the cytosolic and nuclear extracts, respectively. Densitometry ratio to control (n=4) (0 hrs) of hepatic (C, D) and pulmonary (F) cytosolic IκBα and nuclear NFκB subunits p65 and p50 following LPS exposure at 1 hr (n=5) and 5 hrs (n=4). Asterisks (*) indicate statistical difference (P < 0.05) vs. control (0 hrs) by the Mann Whitney test.

next sought to determine whether there was a particular hepatic cell type responsible for this activation. Hepatic macrophages have been described as pro-inflammatory, with high phagocytic and cytokine producing activity due to their location inside the sinusoid space and their direct contact with the fetal circulation.^{25,50} Likewise, human hepatocyte cell lines have been shown to initiate and amplify acute inflammatory

response when stimulated.⁵⁵ However, the acute innate immune response activation of fetal hepatocytes and/or hepatic macrophages have never been described. We collected fetal hepatocytes and hepatic macrophages from control animals and exposed them to LPS (100 ng/ml, 3 h) or basal media and measured the gene expression levels for innate immune response markers *in vitro* (Figure 7). Importantly, LPS exposure

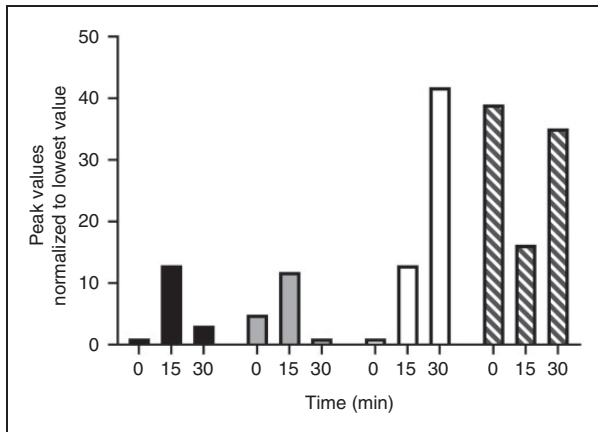


Figure 6. Fetal plasma 3-hydroxytetradecanoic acid integrated peak intensities after IA LPS exposure. 3 umbilical vein plasma samples per animal (baseline, 15 and 30 minutes; total = 12 samples) were base hydrolyzed and extracted using LC-MS/MS. Each color represents a different fetus exposed to IA LPS. Values are represented as average peak values normalized to the lowest peak. Peak quantification was used by the method described in Pais de Barros et al.

induced significant up-regulation of pro-inflammatory innate immune response markers (*IL1A*, *IL1B*, *TNF*, *IL6*, and *IL8*) in fetal hepatocytes and hepatic macrophages. Of note, LPS-induced pro-inflammatory gene expression was significantly higher in hepatic macrophages when compared with fetal hepatocytes. These results demonstrate that both the fetal hepatocyte and the fetal hepatic macrophage respond to LPS-exposure by up-regulating pro-inflammatory gene expression. Furthermore, the fetal hepatic macrophage mounts a more robust innate immune transcriptional response to LPS exposure when compared with similarly exposed hepatocytes.

Discussion

The present work aimed to evaluate the acute fetal response to inflammatory challenge (IA LPS), with main focus on the fetal hepatic innate immune response. Our study found that a single dose of IA LPS caused acute physiologic systemic disturbances

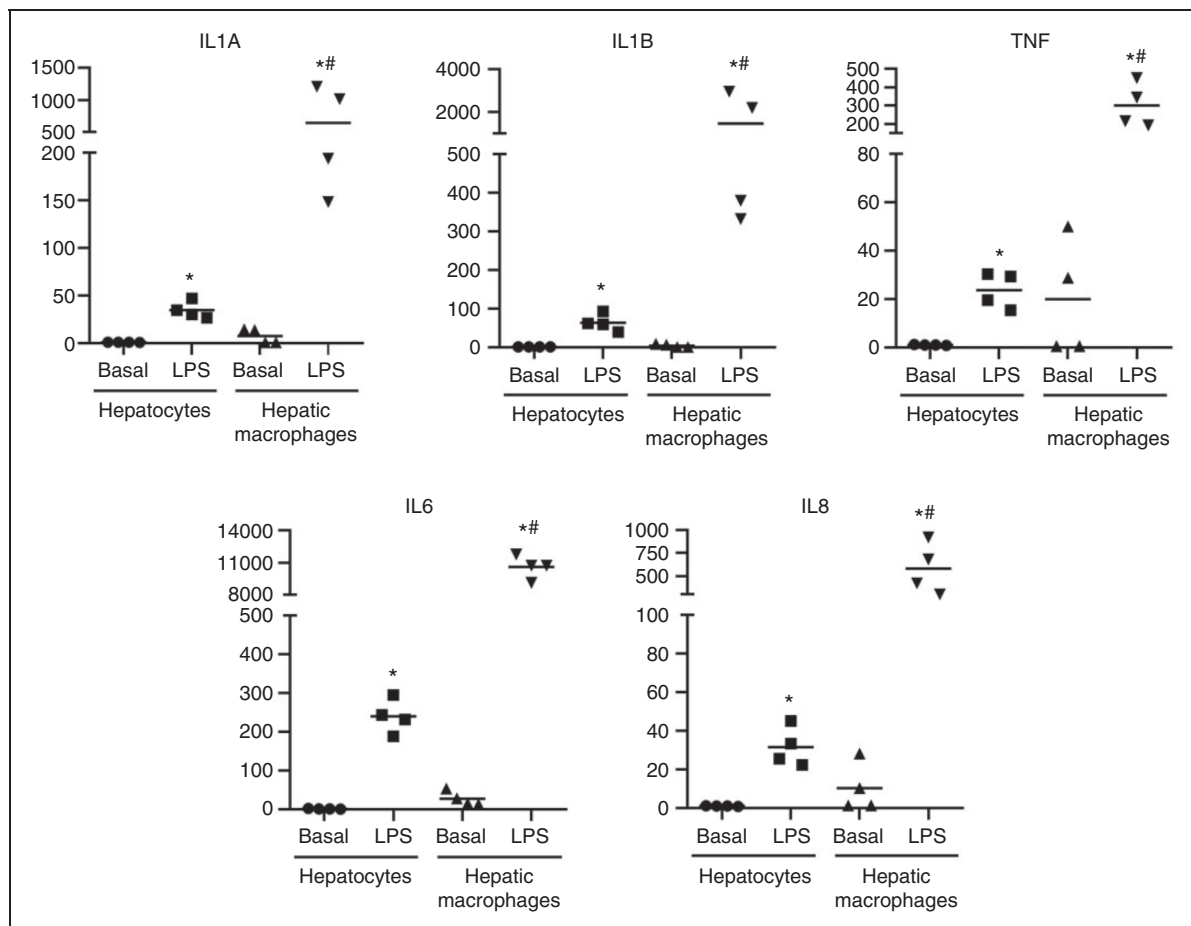


Figure 7. LPS exposure activates pro-inflammatory cytokine gene responses in fetal hepatocytes and hepatic macrophages in-vitro. Cell cultures (n=4) were exposed to serum-free DMEM plus 0.2% BSA media (Basal) or LPS (100 ng/ml) for 3 hrs. 18S was used as a reference gene. Values are represented as fold change (mean) compared with Basal Hepatocytes based on absolute values. $P < 0.05$ vs. Basal Hepatocytes (*) and LPS Hepatocytes (#).

within 5 h of exposure. This observation led us to interrogate the fetal innate immune response within this early time frame. We found that the fetus was able to mount an early and robust pro-inflammatory innate immune response, with the most activation noted in the liver and with some activation in the fetal lung and skin, all starting within 1 h of exposure. Furthermore, the early LPS-induced hepatic NF- κ B activation contributes to this response. We also observed that hepatic macrophages isolated from fetal sheep respond robustly to LPS exposure, providing evidence that this cell might contribute to the fetal innate immune response. This study shows for the first time three major and unique findings when fetuses are exposed to an IA inflammatory challenge (IA LPS): 1) the late gestation fetus is capable of producing an early and systemic innate immune response, 2) the fetal liver contributes to the activation of the NF κ B-mediated innate immune signaling, and 3) the fetal hepatic macrophages are able to mount a robust innate immune response when exposed to LPS *in vitro*.

Our study focused specifically on measuring the contribution of the hepatic early innate immune response as well as the systemic effects after an *in utero* pro-inflammatory challenge. We reasoned that taking advantage of our chronic catheterization model and early time point measurements would provide a better assessment of physiological data as well as interorgan and intervascular innate immune crosstalk coming from multiple tissue beds. We found that, systemically, IA LPS induced fetal metabolic acidosis and hyperlactatemia at 5 h post exposure. These findings are consistent with previous reports demonstrating metabolic disturbances in the fetus exposed to IA LPS. Several reports describe a rise in lactate and CO₂ values hours after an inflammatory stimulus in fetuses and neonates.^{42,56–58} Fetal acidemia might have occurred in response to IA LPS due to a partial reduction in placental perfusion,^{42,58} or alterations in the fetal metabolic activity after the inflammatory insult.⁵⁷ Fetal Glc and oxygen values were not impaired after IA LPS exposure, due possibly to a maintained nutrient supply to fetal tissues,^{57,59} or to an acidemia-dependent effect on the fetal hemodynamic response by increasing blood pressure or heart rate demonstrated in other studies.^{58,60}

Likewise, we found that IA LPS induced the activation of fetal stress responses characterized by an increase in circulating plasma cortisol and norepinephrine. Studies have shown a rise in cortisol and norepinephrine hormone concentrations after LPS administration in adult models of endotoxemia.^{61–63} However, the effects of IA LPS on fetuses have not been fully elucidated. Our results are supported by the work of Nitsos et al., who described an acute

mild increase in cortisol concentrations in younger fetuses (~120 gestational d) exposed to IA LPS.⁵⁷ Our data shows higher cortisol values, and this might have occurred due to the older age of our fetuses when exposed to IA LPS since hypothalamic-pituitary-adrenal (HPA) axis activity is reported to increase towards the end of gestation due to a reduction in negative feedback sensitivity.⁶⁴ While different studies have characterized the localized and systemic fetal inflammation following exposure to IA inflammatory challenge, very little data exist on the acute hepatic innate immune response.

This study shows that IA LPS exposure produced a unique robust activation of the hepatic innate immune transcriptomic machinery (*IL1A*, *IL1B*, and *TNF*) starting at 1 h post exposure with a lesser extent at 5 h. The pro-inflammatory response was also observed with less intensity in fetal lung at 1 h (*IL1B*) and 5 h (*IL1A*, *IL6*, and *IL8*) post IA LPS, and in fetal skin (*IL1B*) 1 h after challenge. Likewise, activation of the fetal hepatic and pulmonary innate immune response to IA LPS involves NF- κ B-signaling. We observed LPS-induced cytosolic NF- κ B inhibitory protein I κ B α and the nuclear accumulation of the transcriptionally active subunits p65 (liver and lung) and p50 (liver). Although previous studies have described the fetal innate immune system as naive and limited,^{65–67} our main findings in this study indicate an opposite perspective. Our data demonstrate that the fetal liver contributes to the early innate immune response to IA LPS through the activation of NF- κ B signaling. Previous reports did not observe any hepatic innate immune response activation to IA LPS,²⁷ or describe any degree of hepatic inflammation and injury,^{29,68} in fetuses exposed to an inflammatory challenge. However, these latter studies either focused on the hepatic response d or wk after exposure and after pre-term delivery,^{27,29} or did not address/observe changes in the transcriptional machinery response.^{68,69} Our findings provide a critical relevance to the scientific field since the association between the early fetal hepatic immune response to an *in utero* inflammatory challenge has been poorly described. The presence of active and robust NF- κ B-mediated perinatal hepatic innate immune response responses to LPS is supported by our previous findings. We observed that, in neonatal (P0) mice, intraperitoneal (IP) LPS induced a robust hepatic innate immune response activation characterized by an early up-regulation of pro-inflammatory genes,^{49,70} and degradation of cytosolic NF- κ B inhibitory proteins I κ B α and I κ B β .⁴⁹ Furthermore, we found that the fetal lung and skin showed an early activation of the innate immune signaling after IA LPS exposure, although not as robust as the fetal liver when compared with their respective controls. Fetal lung and skin

innate immune activation after IA LPS is supported by Kemp et al., who reported up-regulation of pulmonary *IL1B* and *IL8* genes,¹⁸ and high IL-8 concentrations in alveolar lavage,⁴⁷ after IA LPS exposure. Likewise, Kemp et al. described that the skin and chorion respond to IA LPS by recruiting immune cells and up-regulating inflammatory cytokines in the fetus and preterm lamb.^{16,71} Although some reports have suggested the possible role of the placenta in the up-regulation of pro-inflammatory genes and immune cells infiltration in clinical and experimental models of chorioamnionitis,^{41,72–75} we did not observe a placental immune response activation. It is possible that placental innate immune response to IA LPS might occur at later time points, as previously indicated by different groups.^{14,41,72}

We next determined the role of the main two hepatic cell populations in the innate immune response to an inflammatory challenge. Our results showed that both the fetal hepatocyte and hepatic macrophage demonstrate a rapid innate immune response to LPS *in vitro*. Late gestation fetal hepatic macrophages have been reported to be prone to an M1 type phenotype due to their condition as sentinels in the bloodstream,^{24,25} and their high peroxidase and phagocytic activity.^{25,76} In our study, isolated and cultured fetal hepatic macrophages exposed to LPS *in vitro* produced a robust up-regulation of innate immune response markers (*IL1A*, *IL1B*, *TNF*, *IL6*, and *IL8*). We have previously reported that LPS-stimulated hepatic macrophages are able to produce strong inflammatory responses through the expression of *COX-2*,⁴⁹ and *MCPI* and *IP10*.^{70,77} Although we found that the hepatic macrophage innate immune response to LPS *in vitro* is early and robust, fetal hepatocytes also showed similar activation to a lesser extent. Therefore, the hepatic innate immune response to an inflammatory challenge might be regulated by activation of both main liver cell populations. Future work should be focused on manipulating the activation of these cells to ameliorate the local and systemic pro-inflammatory effects in fetuses and neonates when exposed to different inflammatory stimuli.

The exact mechanism of how IA LPS can produce an early hepatic innate immune response activation and fetal metabolic acidosis and stress remains to be determined. Our findings indicate that the hepatic innate immune transcriptomic machinery is highly up-regulated at 1 h post IA LPS exposure, which occurred earlier than the activation of the HPA axis or the development of fetal acidosis (5 h post IA LPS). We can then infer that these two systemic events might be a consequence of an early innate immune signaling, and likely involves NF- κ B activation. The HPA axis activity can be exacerbated by the action of cytokines,^{78,79} and,

thus, induce fetal hemodynamic modifications that might increase the production of lactate in some vascular areas, leading to metabolic acidosis.^{58,60}

In this study, we could not determine what activates the hepatic innate immune response in fetuses exposed to IA LPS. Besides LPS, NF- κ B-mediated activation of hepatic macrophages can be induced by primary response cytokines,^{80,81} which are secreted by other organs, as well as damage-associated molecular patterns such as lipid peroxidation and mitochondrial DNA produced by oxidative stress (TLR9-mediated NF- κ B activation).^{82,83} As a matter of fact, the current dogma indicates that IA LPS cannot reach the fetal circulation at any time point.²⁷ However, we believe that IA LPS might reach the systemic circulation by direct contact and absorption (skin),¹⁶ swallowing (lungs),¹⁹ or by portal circulation due to fenestration of the fetal gut (liver).⁸⁴ In an attempt to address this, we sought to detect LPS in fetal plasma at earlier time points (baseline, and 15 and 30 min after exposure). We measured 3-hydroxytetradecanoic acid, the most abundant component of the lipid A in *E. coli*, by LC-MS/MS. Importantly, 3-hydroxytetradecanoic acid detected by LC-MS/MS consists of both the free (detoxified LPS) and bound (intact LPS) forms. The origin of 3-hydroxytetradecanoic acid free form comes from hydrolysis of intact, and systemic presence of this metabolite has previously been shown to result from liver-resident macrophages processing of circulating systemic LPS.⁸⁵ Furthermore, hepatic macrophage-mediated LPS detoxification can release up to 40% of the lipid component (free 3-hydroxytetradecanoic acid) that could be detected by LC-MS/MS. Plasma samples were collected from the umbilical vein since it provides the central circulation of the fetus and indicates the systemic distribution of intravascular substances.^{86,87} In this study, we were able to detect 3-hydroxytetradecanoic acid (free or bound form) in the fetal circulation as early as 15 min after IA exposure. These data appear in contrast to those previously published by Kallapur et al., where LPS could not be detected in the peripheral blood of pre-term lambs after IA exposure.²⁸ However, the method for detection was not noted in that study. Furthermore, we tested umbilical vein plasma samples at earlier time points (15 and 30 min) to avoid missing LPS uptake and clearance by hepatocytes,⁸⁸ hepatic macrophages,⁸⁹ and liver sinusoidal endothelial cells.⁹⁰ In adult animals, hepatic clearance of systemic LPS is known to be extremely rapid, and has been reported to occur as early as 5 min after exposure of IP LPS in rats.⁸⁸ In total, all fetuses showed detectable levels of LPS metabolite, and three fetuses showed an increase in 3-hydroxytetradecanoic acid peak intensities 15 min after exposure. While these data are limited to four animals, by using highly

sensitive techniques (LC-MS/MS) in plasma samples at early time points, we were able to detect the presence of circulating LPS metabolites in all animals. These unique data provide novel insights to systemic fetal exposure to LPS following IA exposure, and provide the premise for developing therapeutic approaches to reduce potential local and systemic damage. However, our results are mostly descriptive and future studies are needed to confirm our findings in detecting LPS systemically minutes after IA exposure and to determine the percentage of LPS crossing the fetal circulation from the amniotic fluid compartment in different animal models. Furthermore, chorioamnionitis is the most important cause for pre-term birth since it is inversely correlated with gestational age.^{91–93} Since the role of the fetal liver during an inflammatory challenge is not fully understood, this study focused on establishing the basis of how the hepatic innate immune system responds to IA LPS in late gestation fetuses. However, our findings might not be applicable for antenatal infections that occur in early gestational ages. Therefore, future studies targeting the liver as a primary immune organ in fetuses exposed to inflammatory stimuli at different developmental time points will provide great insights in understanding the development of chorioamnionitis. Likewise, reports indicate that the degree of the innate immune response to infection can be sex-specific.^{94,95} Unfortunately, we did not have sufficient power to study the sex effects on the hepatic innate immune response to IA LPS, but determining its contribution can be relevant to study the immune function at later developmental time points.

To conclude, this study has demonstrated that an inflammatory challenge (IA LPS) induces the activation of fetal systemic (HPA axis and metabolic acidosis) and early NF- κ B-mediated hepatic innate immune responses. Furthermore, the fetal hepatic macrophages mainly activate a robust pro-inflammatory response when exposed to LPS *in vitro*. For the first time, this work provides compelling evidence that the fetal liver is an active immune organ with the ability of inducing an early and robust innate immune response activation to an *in utero* inflammatory challenge. Understanding the mechanisms of how the activation of the fetal hepatic innate immune response is associated with systemic blood gas and stress disturbances has yet to be determined. Interventional studies should aim to over-activate or inhibit the hepatic innate immune response to assess the fetal systemic effects as well as the immune activation of other peripheral organs, which will bring novel insights for the development of better therapeutic approaches to the compromised fetus/neonate during sepsis.

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Declaration of conflicting interests

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Statement of ethics

All procedures were approved by the University of Colorado Institutional Animal Care and Use Committee (101924) and performed in compliance with the United States Department of Agriculture and the American Association for Accreditation for Laboratory Animal Care at the Perinatal Research Center at the University of Colorado School of Medicine (Aurora, CO, USA).

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