Chorioamnionitis induced fetal gut injury is mediated by direct gut exposure of inflammatory mediators or by lung inflammation

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Abstract

Intraamniotic exposure to pro-inflammatory agonists causes chorioamnionitis and fetal gut inflammation. Fetal gut inflammation is associated with mucosal injury and impaired gut development. We tested whether this detrimental inflammatory response of the fetal gut results from a direct local (gut derived), or an indirect inflammatory response mediated by the chorioamnion/skin or lung, since these organs are also in direct contact with the amniotic fluid.

The gastrointestinal tract was isolated from the respiratory tract and the amnion/skin epithelia by fetal surgery in time-mated ewes. Lipopolysaccharide (LPS) or saline (controls) was selectively infused in the gastrointestinal tract, trachea or amniotic compartment at 2 or 6d prior to preterm delivery at 124d gestation (term 150d).

Gastrointestinal and intratracheal LPS exposure caused distinct inflammatory responses in the fetal gut. Inflammatory responses could be distinguished by the influx of leukocytes (MPO+, CD3+ and FoxP3+ cells), TNF-α and IFN-γ expression, and differential upregulation of mRNA levels for TLR 1,2,4,6. Fetal gut inflammation after direct intestinal LPS exposure resulted in severe loss of the tight junctional protein ZO-1 and increased mitosis of intestinal epithelial cells. Inflammation of the fetal gut after selective LPS instillation in the lungs caused only mild disruption of ZO-1, loss in epithelial cell integrity and impaired epithelial differentiation. LPS exposure of the amnion/skin epithelia did not result in gut inflammation or morphological, structural and functional changes.

Our results indicate that the detrimental consequences of chorioamnionitis on fetal gut development are the combined result of local gut and lung mediated inflammatory responses.
Introduction

A frequent association with preterm delivery is chorioamnionitis, a bacterial infection of the amniotic fluid, fetal membranes and placenta (13, 28, 30). Chorioamnionitis is associated with an increased risk for poor postnatal developmental outcomes (3, 4, 15, 32). The inflammatory response to chorioamnionitis is postulated to be the proximal cause of injury to fetal organs which increase the risk of periventricular leukomalacia (6) necrotizing enterocolitis (3, 4), and bronchopulmonary dysplasia (34, 37). Since the fetus swallows and aspirates the amniotic fluid, infection of the amniotic fluid exposes the premature lungs and intestine directly to bacteria and inflammatory products. In addition, the fetal skin and the amniotic epithelium are exposed to the contaminated amniotic fluid.

We have used a preterm fetal sheep model of chorioamnionitis, induced by bacteria or their pro-inflammatory components, to characterize the effects of chorioamnionitis on fetal organ development. The earliest organ inflammation after LPS exposure was detected in the amnion/chorion (23). A fetal inflammatory response in the lung was initiated within a few hours after intraamniotic LPS delivery (21, 23) In addition, 2 d after intra-amniotic LPS administration, proinflammatory cytokines and chemokines were detected in the ovine fetal skin (25, 41).

Importantly, the pulmonary and cutaneous inflammatory responses resulted in systemic inflammation of the fetus (21, 24, 27). In contrast to these early responses, we recently observed no signs of inflammation in the fetal intestine 2 days following intraamniotic injection of LPS or live Ureaplasma parvum (38, 39). However, 7 and 14 days after intraamniotic injection of LPS or U. parvum, there was an inflammatory response in the preterm gut (38, 39). Therefore, an important question is whether gut
inflammation is induced by direct contact with swallowed LPS or the result of indirect inflammation in other fetal organs. The delayed inflammatory response in the fetal intestine impaired the development of the intestinal immune system and barrier function (38-40). The mechanisms underlying disrupted gut development in premature neonates in the course of chorioamnionitis remain largely unknown. Therefore we asked if the inflammatory response to chorioamnionitis in the fetal gut results from a local (gut derived) or fetal inflammatory response syndrome (FIRS) mediated by the fetal lung, chorioamnion or the skin. We surgically isolated the fetal gastrointestinal (GI) tract from the respiratory tract and the skin/chorioamnion and evaluated intestinal inflammation and development following lipopolysaccharide (LPS) administration either into the trachea, the gastrointestinal tract or the amniotic fluid.

Materials and methods

Animals

This study was approved by the animal ethics committee of the University of Western Australian, Perth, WA, Australia and the Children's Hospital Medical Center, Cincinnati, OH. Fetal lambs were allocated at random to fetal surgery and LPS (Escherichia coli 055:B5; Sigma Chemical, St. Louis, MO) or saline exposure for either 2 d or 6 d as defined below and in Table 1. LPS was infused by an osmotic pump over 24h (Alzet Cupertino, CA) and the doses used were as follows: 10mg for IA infusion, 5mg for gut infusion and 1mg for tracheal infusion. These reduced lung and gut infusion doses were based on previous data (26) and the presumed fractional volumes of amniotic fluid that contact the gut and lung. Fetal surgery was performed using strict aseptic precautions. Ewes received pre-medication with intra-
muscular (IM) injections of Buprenorphine (0.02 mg/kg) and Acepromazine (0.01 mg/kg), at least 30 minutes before induction of anesthesia with an intravenous bolus of Midazolam (0.25 mg/kg) and Ketamine (5 mg/kg). After intubation, general anesthesia was maintained with 1-2% isoflurane. A second surgery was performed at 124d GA ± 2 d to deliver the fetal sheep, 2 or 6 d after the initial surgery. The ewe and fetus were euthanized with an intravenous bolus of pentobarbitone (100 mg/kg).

The pulmonary and systemic inflammation in these animals was recently reported (24).

Isolation of the fetal lung: An incision was made in the cartilage ring just below the cricoid cartilage for the insertion of two occlusive catheters. One catheter was connected to a 2 liter collection bag that was sited in the amniotic cavity to collect fetal lung fluid. A second catheter was attached to a mini-osmotic pump (secured in a sub-dermal pocket in the fetal neck) for delivery of 1 mg LPS (or saline for controls) to the distal trachea over a 24-hour period. The trachea was occluded around the catheters and ligated above the catheter site. Thus we separated the fluid exchange in fetal lung from the amniotic fluid. Therefore LPS administered to the fetal lung did not reach the amniotic fluid. We also ligated the esophagus so that amniotic fluid could not be swallowed.

Fetal gastro-intestinal tract isolation: The trachea was cannulated to drain fetal lung fluid to a bag as for the lung surgery. A catheter attached to a mini-osmotic pump was passed via a small incision in the esophagus into the stomach. The osmotic pump was secured in a sub-dermal pocket in the neck and delivered 5 mg LPS (or saline for controls) into the fetal stomach over 24h. The esophagus was ligated above the catheter insertion site to achieve LPS exposure to the GI tract only.
Fetal snout occlusion: The trachea was cannulated to drain fetal lung fluid to a bag and the esophagus was ligated as for the other surgery groups. The snout was occluded securely with a sterile, size 6 surgical glove (Ansell, Iselin, NJ). A mini-osmotic pump was sutured to a hind limb for the intraamniotic delivery of 10 mg LPS (or saline for controls) over a 24h period. This procedure allowed us to expose only the fetal skin and chorioamnion to LPS, specifically with no exposure of LPS to the oral and nasal epithelium, the tonsils, the lung or the GI tract.

Intra-amniotic: The surgical procedures were identical to those for the lung, gut and fetal snout occlusion (‘IA Ocln’) groups with the exception that the trachea, esophagus and fetal snout were not occluded (sham surgery). Thus these animals were able to freely swallow or aspirate amniotic fluid. A mini-osmotic pump was sutured to a hind limb to deliver 10 mg LPS (or saline for controls) into the amniotic fluid over 24h.

Antibodies
The following antibodies were used: rabbit antibodies against human MPO (catalogue # A0398, Dakocytomation, Glostrup, Denmark, 1:500) and CD3 (catalogue # A0452, Dakocytomation, 1:200), Phospho-Histone H3 (pHistone-H3) (catalogue # sc-101679, Santa Cruz Biotechnology, Santa Cruz, CA, 1:1000) and Zonula Occludens protein 1 (ZO-1) (catalogue # 617300, Invitrogen, San Francisco, CA, 1:100); monoclonal antibody against human FoxP3 (catalogue # 14-7979-82, eBioscience, San Diego, CA, 1:250); guinea pig antibody against human Kruppel-like factor 5 (KLF5) (1:2000) (kindly provided by Dr. Jeffrey Whitsett, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH), human Ki-67, catalogue # M7240, Dako.
1:100), biotin conjugated goat anti-mouse (catalogue # E0433, Dakocytomation, 1:200,), biotin conjugated swine anti-rabbit (catalogue # E0353, Dakocytomation, 1:200), Texas red conjugated goat anti-rabbit (catalogue # 4050-07, ITK Diagnostics SouthernBiotech, 1:100), peroxidase conjugated goat anti-rabbit (catalogue # 111-035-045, West Grove, PA, USA, 1:200) and biotin conjugated goat anti-guinea pig (catalogue # 106-065-003, Jackson ImmunoResearch West Grove, PA, USA, 1:200).

Immunohistochemistry

Immunohistochemistry was performed on terminal ileal tissue as described (38). We evaluated the terminal ileum since this region of the gastrointestinal tract is most vulnerable to injury and intestinal pathologies such as necrotizing enterocolitis in the preterm. Formalin fixed paraffin embedded samples (3 µm) were incubated with the primary antibody of interest. After washing, sections were incubated with the appropriate secondary conjugated antibody. CD3, FoxP3, KLF5 and pHistone-H3 antibodies were detected with the streptavidin-biotin system (Dakocytomation) and the MPO antibody was detected using a peroxidase conjugated secondary antibody. Positive staining for MPO and CD3 was visualized with 3-amino-9-ethylcarbazole (AEC, Sigma); nuclei were counterstained with haematoxylin. Immunoreactivity for FoxP3, KLF5, Ki-67 and pHistone-H3 was visualized using nickel-DAB. The number of cells exhibiting immunostaining were counted per single (MPO, CD3 and pHistone-H3, 200x) or three high power fields (FoxP3, 100x). Stained sections were scored by 3 investigators who were blinded to the experimental conditions.

Immunofluorescence
Immunofluorescence was performed and interpreted as described (38). Briefly, ileal cryo sections embedded in OCT (3 µm) were incubated with anti-ZO-1 followed by Texas Red conjugated goat anti rabbit antibody (Jackson, West Grove, PA) and 2 min incubation with 2',6-amino-2-phenyl indole (DAPI). The ZO-1 distribution was recorded at a magnification of 200× using the Metasystems Image Pro System (black and white charge-couple device camera; Metasystems, Sandhausen, Germany) mounted on a Leica DM-RE fluorescence microscope (Leica, Wetzler, Germany).

Cytokine and TLR mRNA Quantitation

Total RNA was isolated from terminal ileal tissue by Trizol/chloroform extraction. mRNA quantitation was performed using real-time PCR. Total RNA was reverse transcribed using oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Life Technologies) according to the supplier's recommendations. cDNA was used as a template with primers and Taqman probes (Life Technologies, Carlsbad CA) specific to sheep sequences. The values for each cytokine or TLR were normalized to the internal 18S rRNA value. Data were expressed as fold increase over the control values.

Enzyme-Linked Immunosorbent Assay (ELISA)

Differences of plasma Intestinal-Fatty Acid Binding Protein (I-FABP) levels were determined by Enzyme-Linked ImmunoSorbent Assay (ELISA) to measure intestinal mucosal cell damage (10, 39). A 96-well plate was coated overnight at 4 °C with 5 µg of anti-human I-FABP mAb. A standard titration curve was developed by serial dilution of a known quantity of human recombinant I-FABP protein. Fetal plasma samples were diluted at least 4-fold and incubated for 1 h at room temperature. Next,
the plates were incubated with biotin-conjugated anti-human I-FABP pAb (2 μg/ml), followed by washing and incubation with streptavidin peroxidase for 1 h. After adding 3,3′,5,5′-tetramethyl-benzidine (TMB) substrate, the reaction was stopped with acid and the OD was determined at 450 nm. Plasma Haptoglobin levels were measured by ELISA following manufacturer instructions (Cat.no.E-10HPT; ICL, Portland, OR, USA).

Circulatory endotoxin levels

Total circulating endotoxin was determined in fetal plasma by a chromogenic Limulus Amoebocyte Lysate (LAL) assay following the manufactures instructions (Genscript, Piscataway, NJ).

Statistical analysis

Data are presented as mean and standard deviation. The number of cells with immunostaining for MPO, CD3, FoxP3 and pHistone-H3 were counted per high power field. Since the data were not distributed normally, Mann-Whitney non-parametric U-tests were used for between-group comparisons. In addition, time dependent TLR changes were separately analysed by a two way ANOVA with Bonferroni test for post hoc analysis. Statistical calculations were made using SPSS 15.0 for Windows (SPSS, Chicago, IL) and differences were considered statistically significant at p<0.05.
Results

Systemic inflammation following the LPS exposures

We first determined whether selective LPS exposure of the gut, lung, skin and chorioamnion resulted in a systemic inflammatory response. The acute phase plasma protein haptoglobin is a marker for systemic inflammation (17). Relative to controls, 2 d LPS exposure resulted in significantly increased concentrations of plasma haptoglobin in ‘Gut’, ‘Lung’, ‘IA Ocln’ and ‘IA’ groups (Figure 1A). Although variable, haptoglobin levels in all groups were not different from control values when lambs were exposed to endotoxin for 6 d (Figure 1B).

LPS in fetal plasma could not be detected within 2 d or 6 d post LPS treatment in any of the experimental groups (data not shown). Systemic inflammation in the 2 d groups after selective LPS exposure is therefore most likely not the result of LPS absorbance and distribution via the fetal blood.

Inflammation in the fetal ileum following selective gut, pulmonary, cutaneous or intraamniotic lipopolysaccharide (LPS) exposure

Small numbers of cells expressing myeloperoxidase (MPO), an enzyme expressed by activated neutrophils and monocytes, were detected in the ileum from preterm control animals. The number of infiltrating MPO positive cells did not change after 2 d LPS exposure in any compartment (Figure 2A). Infusion of LPS in either the lung or the gut for 6 d resulted in increased numbers of MPO expressing cells in the ileum (Figure 2B). As described previously (38), intraamniotic endotoxin exposure for 6 d resulted in an influx of MPO expressing cells (Figure 2B) in the fetal ileum whereas
occlusion of the snout before intraamniotic endotoxin injection did not result in an increased influx of MPO+ cells (Figure 2B).

Similar to MPO expression, the number of CD-3 expressing lymphocytes was not altered in any of the 2 d exposure groups (Figure 2C). After 6 d of LPS treatment, increased CD3+ T-cells were detected in the fetal ileum in the gut, lung and IA groups exposed to LPS. CD3+ cells in the ‘IA Ocln’ group remained unchanged when compared with saline treated control animals (Figure 2D).

Regulatory T-cells (Tregs) are a subset of T-cells that have potent anti-inflammatory effects and can be identified by the expression of the transcription factor FoxP3 (12, 16). When compared to control animals, ileal FoxP3 positive cells tended to decrease at 2 d after LPS exposure in the gut (p=0.10) or in the lung (p=0.14) (Figure 2E). Remarkably, at 6 d after LPS infusion in the GI tract, the number of FoxP3+ cells returned to control levels in the ‘gut’ group, whereas these cells remained decreased in the ileum after 6 days of LPS infusion in the ‘6d lung’ group (p=0.09) (Figure 2F).

Intraamniotic endotoxin exposure for 2 d resulted in a significant decrease of FoxP3 expressing cells (Figure 2E) which normalised within 6 d after IA endotoxin injection (Figure 2F). The number of ileal FoxP3+ cells in the ‘IA LPS Ocln’ group remained unchanged when compared with saline treated control animals at 2 and 6 d (Figure 2E+F).

When compared to controls, increased ileal TNF-α mRNA levels were only detected in the IA LPS group (Figure 3A) whereas IL-1b, IL-6 and IFN-γ mRNA did not increase in any of the 2 d exposure groups (Figure 3 C, E, G). TNF-α, IL-1b and IFN-γ mRNA levels in the fetal ileum increased after selective gut LPS exposure for 6 d (Figure 3 B, D, H). In contrast to the responses after selective gut exposure, only a modest increase of TNF-α and IL-1b in the fetal ileum after 6 d of intratracheal LPS
infusion was seen (Figure 3 B) whereas no significant increase for IFN-γ mRNA levels was detected (Figure 3 D). Compared to controls, ileal IL-6 mRNA levels decreased after 6 d of IA LPS exposure with no change in the other 6 d LPS exposed groups (Figure 3 F).

Consistent with the other inflammatory indicators, no significant changes in either TNF-α, IL-1β, IL-6 or IFN-γ mRNA levels were measured in the fetal ileum in the ‘IA Ocln’ group when compared to the controls (Figure 3 A-D).

TLRs in the fetal ileum are differently regulated after intratracheal or gastrointestinal LPS exposure

Ileal TLR1 mRNA levels did not change after 2 d exposures in any group (Figure 4A). However, compared to age matched saline treated animals, ileal TLR1 mRNA expression decreased after 6 d of selective LPS exposure to the gut, the lung or the amniotic cavity but not in the ‘IA Ocln’ group (Figure 4B). Time dependent changes of TLR1 mRNA between day 2 and day 6 were significantly different (Table 2). TLR1 mRNA levels in the fetal ileum decreased at 6d of exposure in the “IA”, “gut” and “lung” LPS groups.

Gut TLR2 mRNA levels in the fetal ileum selectively increased at 2 d of LPS exposure to the lung (Figure 4C) and decreased after 6 d of lung, IA or ‘IA Ocln exposure when compared to matched saline treated animals (Figure 4D). In contrast, ileal TLR2 mRNA levels remained unaltered after 2 or 6 d of selective LPS exposure to the gut (Figure 4C+D). Ileal TLR2 mRNA levels significantly decreased between 2 and 6d of endotoxin exposure in the “IA” and “lung” LPS groups (Table 2). Compared to age matched saline treated animals, ileal TLR4 mRNA increased after 6 d of selective LPS exposure to the gut but decreased after 6 d of selective IA or lung LPS
exposure with no change in the ‘IA Ocln’ group (Figure 4F). Time dependent changes in the fetal ileum of TLR4 mRNA between day 2 and day 6 were significantly different (Table 2). TLR4 mRNA levels decreased at 6d of exposure in the “gut” and “lung” LPS groups.

TLR6 mRNA in the fetal ileum increased after 2 d of selective LPS exposure to the gut when compared to age matched control animals (Figure 4G). Compared to all groups, ileal TLR6 mRNA levels were reduced at 6 d after intraamniotic or intratracheal injection of LPS (Figure 4H). Time dependent changes of TLR6 mRNA in the fetal ileum between day 2 and day 6 were significantly different (Table 2). TLR6 mRNA levels decreased at 6d of exposure in the “IA”, “gut” and “lung” LPS groups.

Intestinal epithelial differentiation and proliferation

We stained ileal tissue for Kruppel like factor-5 (KLF5) to gain insight into whether selective LPS exposure was associated with impaired proliferation and migration of intestinal epithelial cells (Figure 5A-I). KLF5 regulates mucosal healing through its effects on epithelial proliferation, differentiation, and cell positioning along the crypt radial axis (23). Consistent with previous reports, constitutive KLF5 expression was high in intestinal epithelial cells located in the lower to middle crypt region (9). The number of KLF5+ cells was only reduced after selective endotoxin exposure of the lung for 2 d (Figure 5E). In these animals, KLF5 expression was restricted to the bottom of the crypts (Figure 5E).

To determine whether the reduced number of KLF5 expressing enterocytes was paralleled by changes in cell proliferation, we evaluated Ki67 and phospho Histone-H3 by immunohistochemical staining. The number of proliferating and mitotic cells in
the fetal ileum increased after selective gut exposure to endotoxin for 2 d compared to control animals (Figure 6 A, B). Consistent with the reduced number of KLF5+ cells, proliferating cell numbers did not increase after selective infusion of LPS in the lung (Figure 6 A, B). Interestingly, increased proliferation was seen after 6 d intraamniotic LPS (Figure 6 C, D). However, the number of proliferating and mitotic cells remained unchanged in the ‘IA LPS Ocln group’ when compared with saline treated control animals (Figure 6 C, D). Importantly, the increase of proliferating cells in the fetal ileum after selective gut exposure to LPS for 2 d and intraamniotic LPS exposure for 6 d did not result in significant changes of the villus lengths when compared to the other groups (data not shown).

Gut wall integrity loss following selective local gut or pulmonary LPS exposure

Consistent with our previous reports in preterm sheep fetuses (38, 40), the epithelial tight junction protein Zona occludens-1 (ZO-1) staining was fragmented in premature saline treated control animals of 125d GA (Figure 7A). This pattern remained unchanged after 2 d of LPS exposure in all surgical groups (Figure 7B-E). In contrast, fetuses selectively exposed to LPS for 6 d in the gut, the lung or amniotic cavity had a further fragmented tight junctional distribution with the most severe disturbance in the intraamniotic and gut exposed groups (Figure 7F-I). The ZO-1 distribution was not changed in the ‘IA LPS Ocln’ group when compared with saline treated control animals (Figure 7I).

Mucosal damage was further assessed by circulating intestinal fatty acid binding protein (I-FABP) levels since this cytosolic protein is present in enterocytes and rapidly released into the systemic circulation upon cell damage (5). The concentration of I-FABP in the fetal circulation was significantly elevated (P<0.05) only in 6 d LPS
l lung exposed animals compared with saline controls (Figure 8B) and there was no change in any of the 2 d groups (Figure 8A).

Discussion

The pathways to gut inflammation and postnatal intestinal pathologies in preterm infants following exposure to chorioamnionitis are unknown. We previously reported that proinflammatory agonists in the amniotic cavity induce fetal gut inflammation and an injury response (38-40). We demonstrate in this study that LPS infusion in the amniotic cavity and selective infusion of LPS in the gut induced fetal gut inflammation and mucosal injury. This response is consistent with an inflammatory reaction induced by direct contact between LPS and gut epithelium. We report the unanticipated novel finding that selective LPS exposure in the fetal lung also resulted in fetal gut inflammation and subsequent mucosal injury, which is not the result of direct LPS contact with the gut epithelium or systemic translocation of LPS in the blood. Interestingly, when fetal swallowing was abolished by snout occlusion, amniotic infusion of LPS did not result in gut inflammation or injury. Therefore our experiments demonstrated that the gut responses to chorioamnionitis require either contact of the pro-inflammatory agonist to the GI mucosa or the lung epithelium.

The gut inflammation/injury in our experiments occurred due to local effects of LPS, since LPS was not detected in the plasma after infusion in any of the fetal compartments. This is in line with previous results demonstrating that 1-5 µg of LPS given intravascularly causes profound hypotension in preterm sheep (11) (Gisslen et al Innate Immun 2013 in press) but doses of up to 100 mg LPS given intraamniotically (>10,000 fold higher dose) are tolerated without hypotension (18).
Taken together these results strongly suggest that the IA LPS does not cross into the systemic circulation.

Gut inflammation induced by LPS selectively infused in different fetal compartments was qualitatively different with distinct temporal patterns. The response of the fetal gut after tracheal LPS infusion could be clearly distinguished from the intestinal response after LPS exposure of the GI tract by the lymphocyte influx and the high ileal TNF-α and IFN-γ mRNA levels in the ‘LPS-gut’ animals. This different pattern suggests that the inflammatory response of the fetal gut differs depending on the site of the inflammatory stimulus and the origin of the activated immune cells (33, 36).

This is consistent with earlier findings showing that resident and circulating cells that orchestrate the inflammatory response of the fetal gut will be different depending on the origin of the inflammatory process. Such involvement of other immune cells could result in a different TLR repertoire expression and concomitant innate immune sensing (19). In keeping with these reports, the levels of TLR mRNAs in the fetal gut following LPS exposure to the gut or the lung were different in our study. CD3+ cells and MPO+ cells increased 6d after selective LPS exposure to the gut, lung or amniotic cavity. However, a more detailed analysis of the CD3+ cells and the FoxP3 positive Treg cells might reveal differences in subsets of lymphocytes based on site of origin of pro-inflammatory stimulus. In addition, a functional analysis of lamina propria located CD4+ CD25high CD127low Treg cells would be ideal to determine the immunosuppressive capacity of these cells. Unfortunately, such experiments are hampered by a lack of suitable antibodies for sheep.

Another reason for the observed differences of the inflammatory characteristics could be the timing of the onset of the inflammatory process. We
previously reported that LPS exposure of the fetal lung induced a rapid local and
dystemic inflammatory response within hours after exposure (27) whereas amniotic
LPS exposure did not induce gut inflammation within 48h (38).

The relatively high levels of TNF-α and IFN-γ after direct LPS exposure were
paralleled by severe loss of ZO-1 in the fetal ileum whereas a slight increase of TNF-
α and IFN-γ mRNA such as detected in the 'lung LPS' fetuses was associated with a
milder loss of ZO-1 in the fetal gut. Consistent with these observations, this tight
junctional protein that has a crucial role in paracellular barrier sealing was previously
shown to be disrupted by both proinflammatory cytokines (7). Remarkably, loss in
epithelial cell integrity, as indicated by increased plasma intestinal fatty acid binding
protein (I-FABP) levels, was only observed in pulmonary LPS exposed fetuses but
not after direct LPS contact with the fetal gut. These findings indicate that LPS
exposure of the fetal lung or direct gut exposure to LPS via amniotic fluid swallowing
results in different characteristics of gut inflammation, which determine the level of
epithelial and or tight junctional integrity loss.

In this study we demonstrated that LPS exposure to the fetal gut resulted in
increased mitosis of cells in the intestinal mucosa. Our findings are supported by data
from germ-free animals and in vitro experiments showing that mitosis and
proliferation of intestinal epithelial cells are substantially increased following exposure
to bacteria (2, 35) or LPS in particular (31).

Despite the inflammatory and injury response in the fetal gut after lung LPS
exposure, there were no changes in the number of proliferating cells in the crypts in
this group compared to the controls. The altered distribution of the pro-proliferative
regulator KLF5 could be a mechanistic explanation for the apparent lack of increased
mitosis after pulmonary LPS exposure, since the number of cells expressing KLF5,
become restricted to the lowest crypt region within 2 d after pulmonary LPS exposure. However, since KLF5 is known to be regulated in response to stress stimuli such as tissue damage or LPS exposure (8, 29), it remains unclear why the distribution of KLF5 in the fetal intestine is specifically influenced following intratracheal LPS instillation and not after intestinal LPS exposure.

Since preterm fetal sheep have an inadequate gut barrier, LPS translocation and systemic inflammation might be expected to occur after gut exposure to LPS. However, no endotoxin could be detected in the plasma following gastrointestinal LPS delivery. Consistently, in the current study and in a recent report from Kemp et al., selective gut exposure to LPS only results in a mild systemic response as shown by modestly increased circulating haptoglobin and amyloid A3 levels (24). Importantly, despite the increase of these acute phase proteins, no other systemic inflammatory markers including MCP-1, IL-6 and IL-8 were detected in the Kemp et al. study. Based on these combined findings, it is unlikely that a gastrointestinal mediated systemic reaction could be responsible for the detrimental inflammatory response of the fetal gut.

The systemic inflammatory response of the fetus, induced by lung or chorioamnion/cutaneous LPS exposure, was more prominent when compared to the mild systemic effects following LPS delivery in the GI tract. Pulmonary, or amniotic LPS but not GI tract LPS induced leucocytosis, neutrophilia, elevated circulatory MCP-1 levels and inflammation of the fetal liver and spleen (24).

Interestingly, the systemic inflammatory response of the fetus following LPS exposure of the fetal skin/amniotic compartment (‘IA OcIn’ group) did not provoke an inflammatory response and adverse outcomes of the fetal gut. Furthermore, Kemp et al. showed that systemic inflammation after fetal skin/amnion exposure did not result
in fetal lung inflammation, indicating that exposure of the skin/amnion did not result in a multi-organ systemic fetal inflammatory response (24).

The mechanisms by which pulmonary LPS exposure causes changes of the fetal intestine are thus not clear. We speculate that the lymphatics mediate the effects since the lymphatic channels between the gut and the lung communicate. In this regard, we previously demonstrated that the posterior mediastinal lymph node responds with a doubling of weight, and increased trafficking of activated neutrophils and monocytes as a result of intrauterine lung inflammation (20). We recently demonstrated that IA LPS injection decreased T-regs and increased interleukin-17 producing cells in the posterior mediastinal lymph node of fetal rhesus macaques (22). Interestingly, these profound changes in the local lymph nodes were not paralleled by changes in the blood cells, showing compartmentalization of the FIRS responses after chorioamnionitis. These results highlight the peculiar characteristics of the fetal immune response in FIRS and provide a better understanding of the higher rate of severe neonatal morbidity such as necrotising enterocolitis after chorioamnionitis (1, 14).

In summary, our findings suggest that the detrimental consequences of chorioamnionitis on fetal gut development (38, 40) are the combined result of local gut derived and pulmonary driven systemic inflammatory responses. Moreover, the distinct inflammatory responses in the fetal gut following indirect or direct LPS exposure interfere with different segments of the developing fetal intestine. This study contributes to an understanding of the mechanisms of local and compartment specific systemic inflammation and organ injury responses after chorioamnionitis.
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“Disclosure”

The authors declared no conflict of interest.


Figure 1
The concentration of circulating Haptoglobin was measured by ELISA and groups were compared with the Mann-Whitney test. Haptoglobin plasma levels significantly increased in the 2d ‘IA Ocln’, 2d ‘gut’ and the 2d ‘lung’ LPS groups (A). No significant changes in circulating Haptoglobin levels were detected after 6 d of LPS exposure (B). *P < 0.05 vs controls using a Mann-Whitney non-parametric U-test.

Figure 2
For each experimental group, MPO (A-B), CD3 (C-D) or FoxP3 (E-F) positive cells were counted in the fetal ileum per high-power field and the average value of the sum of 3 representative areas is given. Groups were compared with the Mann-Whitney test. *P < 0.05 vs control using a Mann-Whitney non-parametric U-test.

Figure 3
Quantitative real-time PCR assays in the fetal ileum using ovine specific primers and Taqman probes. The values for TNF-α (A,B), IL-1b (C,D), IL-6 (E,F) and IFN-γ (G,H) were normalized to 18s rRNA and groups were compared with the Mann-Whitney test. The mean mRNA signal in controls was assigned to a value of 1. Mean fold changes in mRNA expression of all other experimental groups were expressed relative to controls. *P < 0.05 vs control using a Mann-Whitney non-parametric U-test.
Figure 4
Quantitative real time PCR mRNA expression levels of TLRs in the fetal ileum using sheep specific primers and Taqman probes. TLR mRNA values were normalized to 18s rRNA and groups were compared with the Mann-Whitney test. The mean mRNA signal in controls was set to 1 and levels at each time point were expressed in a relative manner. *P < 0.05 vs control using a Mann-Whitney non-parametric U-test.

Figure 5
For each experimental group, the ileal distribution of KLF5+ cells was analyzed by immunohistochemistry. Compared to control animals (A), reduced KLF5+ cell numbers were observed in fetuses of the ‘2 d LPS lung’ group (E). The distribution of KLF5+ cells in the ‘2 d LPS IA’ (B), ‘2 d LPS Ocln’ (C), ‘2 d LPS gut’ (D), ‘6 d LPS IA’ (F), ‘6 d LPS Ocln’ (G), ‘6d LPS gut’ (H) and ‘6 d LPS lung’ (I) LPS groups did not change. Magnification 200x.

Figure 6
The numbers of proliferating and mitotic cells in the fetal ileum were measured by an immunohistochemical staining for Ki67 and phospho-Histon H3 respectively. Gut exposure to LPS for 2d increased the number of Ki67 and phospho-Histon H3-positive cells (A, B). Compared to controls, intraamniotic LPS exposure for 6 d resulted in increased Ki67 and phospho-Histon H3-positive cells (C, D). Groups were compared with the Mann-Whitney test. *P < 0.05 vs control using a Mann-Whitney non-parametric U-test.
Figure 7

ZO-1 distribution was assessed in fetal ileal tissue using an immunofluorescent staining. A fragmented ZO-1 staining was observed in premature saline treated control animals (A), and in lambs from the ‘2 d IA LPS’ (B), ‘2d LPS Ocln’ (C), ‘2 d LPS gut’ (D) or ‘2 d LPS lung’ groups (E). ZO-1 distribution was even more disturbed in the ‘6 d LPS IA’ (F), ‘6 d LPS gut’ (H) and ‘6 d LPS lung’ (I) groups. The ZO-1 distribution pattern in the ‘6d LPS Ocln’ group (G) was comparable to saline treated control animals (A). Magnification 200×.

Figure 8

Circulating I-FABP levels were measured by ELISA and groups were compared with the Mann-Whitney test. I-FABP plasma levels did not change after exposure to LPS for 2 d in each experimental group (A). Lung exposure to LPS for 6 d significantly increased I-FABP plasma levels (B). *P < 0.05 vs control using a Mann-Whitney non-parametric U-test.
Table 1: Summary of fetal surfaces exposed to intervention (either *E.coli* LPS or saline) in each surgical group.

<table>
<thead>
<tr>
<th>Procedure and site of delivery</th>
<th>Site of exposure</th>
<th>Abbreviation for group</th>
<th>Site of exposure</th>
<th>Abbreviation for group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gut isolation and stomach infusion</td>
<td>Gut LPS (n=5)</td>
<td>‘2 d LPS gut’</td>
<td>Gut LPS (n=5)</td>
<td>‘6 d LPS gut’</td>
</tr>
<tr>
<td>Lung isolation and tracheal infusion</td>
<td>Lung LPS (n=6)</td>
<td>‘2 d LPS lung’</td>
<td>Lung LPS (n=6)</td>
<td>‘6 d LPS lung’</td>
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<tr>
<td>Sham surgery and intraamniotic (IA) infusion</td>
<td>Gut, lung, skin, amniotic cavity (n=6)</td>
<td>‘2 d LPS IA’</td>
<td>Gut, lung, skin, amniotic cavity (n=6)</td>
<td>‘6 d LPS IA’</td>
</tr>
<tr>
<td>Snout occlusion and intraamniotic (IA) infusion</td>
<td>Amniotic cavity skin (n=6)</td>
<td>‘2 d LPS Ocln’</td>
<td>Amniotic cavity skin (n=6)</td>
<td>‘6 d LPS Ocln’</td>
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<tr>
<td>Combined control*</td>
<td>Saline 2 d * (n=8)</td>
<td>‘2 d Saline’</td>
<td>Saline 6 d * (n=8)</td>
<td>‘6 d Saline’</td>
</tr>
</tbody>
</table>

* Combined control group for all surgical groups.
Table 2: Relative TLR mRNA changes in the fetal ileum between the 2d and 6d post LPS treatment groups for each surgical group. Time dependent TLR changes were analysed by a two way ANOVA with a Bonferroni post hoc test.

<table>
<thead>
<tr>
<th>TLR</th>
<th>LPS IA 2d vs 6d</th>
<th>LPS Ocln 2d vs 6d</th>
<th>LPS gut 2d vs 6d</th>
<th>LPS lung 2d vs 6d</th>
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<tr>
<td>TLR1 mRNA</td>
<td>P &lt; 0.05</td>
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<td>P &lt; 0.05</td>
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<td>TLR2 mRNA</td>
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<td>P &lt; 0.001</td>
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<td>TLR4 mRNA</td>
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<td>P &lt; 0.05</td>
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<tr>
<td>TLR6 mRNA</td>
<td>P &lt; 0.05</td>
<td>ns</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
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</tbody>
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