LPS-induced chorioamnionitis and antenatal corticosteroids modulate Shh signaling in the ovine fetal lung

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MWK: Animal study setup & execution, animal handling, animal management, manuscript revision & approval

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ABSTRACT

Rationale Chorioamnionitis and antenatal corticosteroids mature the fetal lung functionally but disrupt late gestation lung development. Because Sonic Hedgehog (Shh) signaling is a major pathway directing lung development, we hypothesized that chorioamnionitis and antenatal corticosteroids modulated Shh signaling resulting in an altered fetal lung structure.

Methods Time-mated ewes with singleton ovine fetuses received an intra-amniotic injection of lipopolysaccharide (LPS) and/or maternal intra-muscular betamethasone 7 and/or 14 days before delivery at 120 days gestational age (GA) (term=150 days GA).

Results Intra-amniotic LPS exposure decreased Shh mRNA levels and Gli1 protein expression which was counteracted by both betamethasone pre- or post-treatment. mRNA and protein levels of fibroblast growth factor 10 and bone morphogenetic protein 4, which are important mediators of lung development, increased 2-fold and 3.5-fold respectively, 14 days after LPS exposure. Both 7 day and 14 day exposure to LPS changed the mRNA levels of elastin gene ELN and collagen type I genes Col1A1 and Col1A2 which resulted in fewer elastin foci and increased collagen type I deposition in the alveolar septa. Corticosteroid post-treatment prevented the decrease in ELN mRNA and increased elastin foci and decreased collagen type I deposition in the fetal lung.

Conclusion Fetal lung exposure to LPS was accompanied by changes in key modulators of lung development resulting in abnormal lung structure. Betamethasone treatment partially prevented the changes in developmental processes and lung structure. This study provides new insights into clinically relevant prenatal exposures and fetal lung development.
Abstract word count: 240 words

Key words: Lung development, Lung maturation, Secondary septation, Bronchopulmonary dysplasia
INTRODUCTION

Bronchopulmonary dysplasia (BPD), a disease of impaired lung development, is the most common adverse lung outcome of preterm birth (2, 30). BPD is associated with fetal lung inflammation which can be initiated by chorioamnionitis, an intra-uterine bacterial infection of the placental membranes and amniotic fluid that is often clinically silent (21). Chorioamnionitis can induce a potentially harmful inflammatory response in the immature fetal lungs which disrupts lung septation and vascular development leading to a decreased lung surface area (16).

Antenatal corticosteroids are given to mothers at risk of imminent preterm birth to induce lung maturation in the fetus which increases neonatal survival, but do not decrease BPD (4, 14). Because the incidence of chorioamnionitis is about 60% for very preterm babies, the administration of maternal antenatal corticosteroids in the presence of chorioamnionitis is common and standard of care (3). Although antenatal corticosteroids cause functional lung maturation, they also can inhibit lung development (54). As a result, a large number of premature infants are exposed \textit{in utero} to both pro- and anti-inflammatory stimuli which each alter normal fetal lung development and might predispose the infants to the development of BPD (10). The molecular mechanisms by which chorioamnionitis and antenatal corticosteroids influence these lung developmental processes are largely unknown.

Sonic Hedgehog (Shh) signaling is critical for lung development as Shh-null mice have hypoplastic lungs and die due to respiratory failure (32). During lung development, Shh expression is localized to the epithelium and activates Gli transcriptional activators Gli1, Gli2 and Gli3 (5). The Shh pathway regulates the expression of lung growth factors such
as fibroblast growth factor 10 (FGF10) and bone morphogenetic protein 4 (BMP4) which both mediate branching and myofibroblast differentiation (52).

We hypothesized that chorioamnionitis and/or antenatal corticosteroids modulate Shh signaling to alter fetal lung structural development. We evaluated this signaling pathway after LPS-induced chorioamnionitis in a 120 days gestational age (GA) preterm lamb model during a stage of early alveolar septation. Fetal sheep were exposed in utero to intra-amniotic lipopolysaccharide (LPS) from gram negative bacteria and/or antenatal betamethasone, a corticosteroid used clinically to induce lung maturation (28, 41). We correlated Shh signaling components with markers for lung damage (heat shock protein (HSP)70, cell proliferation (Ki67) and changes in the lung structural proteins elastin and collagen, which are crucial for alveolar septation (9, 25, 49).
METHODS

Animal model and sampling protocol
All studies were approved by the Animal Ethics Committees at The University of Western Australia and Cincinnati Children`s Hospital Medical Center (animal ethics protocol RA/3/100/830). The experimental design of this study was published previously (29). Time-mated Merino ewes with singleton fetuses were randomly assigned to one of six treatment groups to receive an intra-amniotic (IA) injection of lipopolysaccharide (LPS) (10 mg *Escherichia Coli* 055:B5, Sigma Chemical, St. Louis, MO, USA) and/or an intra-muscular injection of betamethasone (Beta) (Celestone Soluspan, Schering-Plough, North Ryde, New South Wales (NSW), Australia, 0.5 mg/kg maternal weight) and/or an equivalent injection of saline for control animals at 107 days and/or 114 days GA. All ewes in this study received a single intra-muscular injection of 150 mg medroxyprogesterone acetate (Depo-Provera, Kenral, NSW, Australia) at 100 days GA to prevent preterm birth induced by betamethasone treatment. Lambs were surgically delivered at 120 days GA (term = 150 days GA) and euthanized after birth. Lung tissue from the right lower lobe (RLL) was snap frozen and the right upper lobe (RUL) was inflation-fixed in 10% buffered formalin for 24 hours.

RNA extraction and real-time PCR
Total RNA was extracted from frozen lung tissue of the RLL using the SV Total RNA Isolation system (Z3100, Promega, Madison, USA) according to the manufacturer's instructions. Genomic DNA contamination was removed by treatment with RQ1 DNase (M610A, Promega) and the RNA was tested for the presence of genomic *GAPDH*, a housekeeping gene. Briefly, PCR amplification for the detection of genomic DNA was
performed with DNA Taq Polymerase (M124B, Promega) at 95°C for 5 minutes followed by 40 cycles at 95°C for 30 seconds, 55°C for 45 seconds and 72°C for 30 seconds. Total RNA was used as a template. PCR products were analyzed on a 1.5% agarose gel. Total RNA was reverse transcribed with the First Strand cDNA synthesis kit (4379012001, Roche-Applied, Mannheim, Germany) according to manufacturer’s instructions using anchored oligo-primers. Primers for real-time PCR (RT-PCR) were constructed based on published ovine or bovine cDNA sequences (Table 1). Dilution experiments were performed to ensure similar PCR amplification efficiency of the primers. RT-PCR reactions were performed in duplicate with the LightCycler 480 SYBR Green I Master mix (4707516001, Roche-Applied) on a LightCycler 480 Instrument according to the manufacturer’s instructions. RT-PCR results were normalized to cyclophilin A, a housekeeping gene, and mean fold changes in mRNA expression were calculated by the ΔΔCt-method (33).

**Protein extraction and enzyme-linked immunosorbent assay (ELISA) of HSP70**

Frozen RLL lung tissue was homogenized (PRO Quick Connect Generators part no. 02-07095; PRO Scientific Inc., Oxford, CT) in ice-cold RIPA buffer (R0278, Sigma Aldrich) containing 0.1% protease inhibitors (p9599, Sigma Aldrich) and subsequently centrifuged at 12x RCF for 5 minutes at 4°C (31). HSP70 was measured with an R&D DuoSet ELISA development kit (human/mouse/rat total HSP70: DYC1663, R&D Systems, Minneapolis, MN, USA) according to manufacturer’s instructions. HSP70 protein concentrations were calculated per kilogram bodyweight.
Immunohistochemistry

Paraffin embedded RUL lung sections (4 µm, transverse) were stained for Ki67 (M7240, DAKO, Denmark), Gli1 (ab49314, Abcam, Cambridge, UK) and BMP4 (sc-6896, Santa Cruz Biotechnology). Briefly, the sections were deparaffinized in an ethanol series and endogenous peroxidase-activity was blocked by incubation with 0.5% H2O2 in 1x phosphate buffered saline (PBS, pH 7.4). Antigen retrieval was performed by incubating the sections in heated citrate buffer (10 mM, pH 6.0) for 30 minutes. In order to block aspecific binding, the slides were incubated with 20% normal goat serum (NGS) in PBS. Sections were incubated overnight at 4°C with the diluted primary antibody (Ki67 1:50, Gli1 1:500, BMP4 1:500). After incubation with the appropriate secondary antibody, immunostaining was enhanced with Vectastain ABC peroxidase Elite kit (PK-6200, Vector Laboratories, Burlingame, USA) and stained with nickel sulfate-diaminobenzidine (NiDAB). Subsequently, the sections were rinsed in Tris/saline and incubated with Tris/cobalt. After counterstaining with 0.1% Nuclear Fast Red, the sections were washed, dehydrated and coverslipped. All slides were stained at the same time under the same conditions.

Evaluation was performed by light microscopy (Axioskop 40, Zeiss, Germany) with LeicaQWin Pro v.3.4.0 software (Leica Microsystems, Germany). Alveolar Ki67 and Gli1 staining was scored by blinded observers with a semi-quantitative scoring system: 1, little staining; 2, some staining; and 3, heavy staining. BMP4 staining was semi-quantitatively scored in three representative bronchioli using Image J software (Rasband, W.S., Image J US National Institutes of Health, Bethesda, Maryland, USA) and represented as a percentage of the entire bronchiole surface area.
**Elastin and collagen staining**

Elastin and collagen stainings were performed each on 4 paraffin sections of the RUL per animal (4 µm, transverse). For the visualization of elastin, the sections were deparaffinized in an ethanol series and incubated in Hart`s staining solution (70% ethanol, 10% Weigerts Resorcine-Fuchsine (2E 030, Chroma, Münster, Germany) and 2% hydrochloric acid) overnight at room temperature. After rinsing with water, the sections were incubated in 0.25% acetic acid for 3 minutes at room temperature. Subsequently the sections were washed and dehydrated. For the detection of collagen fibers, the sections were deparaffinized and incubated in 0.2% phosphomolybdic acid for 5 minutes. Sections were placed in a Sirius Red solution for 90 minutes in the dark. After rinsing with 0.01 M HCl for 3 minutes, the sections were washed, dehydrated and coverslipped. Evaluation was performed by light microscopy (Zeiss, Axioskop 40) with LeicaQWin Pro v.3.4.0 software. The number of elastin foci and the percentage of collagen fibers in the total lung surface area were quantified using 4 paraffin sections per animal, 12 representative images per section across septa at 200x magnification by a blinded observer using specialized LeicaQWin Pro v.3.4.0 software.

**Data analysis**

Results are given as means ± standard error of mean (SEM). The groups were compared using one-way ANOVA with Dunnett’s or Tukey’s test for post-hoc analysis as appropriate. Statistical analysis was performed by GraphPad Prism v5.0. Significance was accepted at p<0.05.
RESULTS

Lung damage and cell proliferation

Characteristics of the animals and the pulmonary inflammatory and maturation response to LPS-induced chorioamnionitis and/or antenatal corticosteroids were reported previously (29). Lung injury due to the exposure to LPS was assessed by measurement of heat shock protein 70 (HSP70) in the lung tissue. HSP70 protein expression was not increased in any of the experimental groups compared to control (Figure 1A). To assess cell proliferation, lung tissue was stained for Ki67, a marker of mitotic cells. There were increased proliferating cells, which by morphologic evaluation could be discerned as immune cells, 7 and 14 days after the exposure to LPS (Figure 1B). Representative images are shown for controls (Figure 1C) and 7 day LPS exposed lungs (Figure 1D).

Changes in Shh signaling after intra-uterine LPS exposure

Shh mRNA levels decreased to less than 25% of control value after 7 and 14 days of LPS exposure (Figure 2E). Betamethasone pre- or post-treatment prevented the decrease in Shh mRNA. In addition, we analyzed the expression of Gli1 and Gli2, which are components of the Shh pathway. Gli1 mRNA expression had a similar decreased expression at 7 and 14 days following LPS exposure (Figure 2F). Gli1 protein expression was mainly detected in the bronchiolar and alveolar epithelium in controls (Figure 2A). Exposure to LPS for 7 or 14 days selectively decreased Gli1 protein expression in the alveolar epithelium (Figure 2B). Betamethasone pre- or post-treatment again prevented this decline (Figure 2G). Representative images are shown for controls (Figure 2A), 14 day LPS exposed lungs (Figure 2B), 7 day betamethasone exposed lungs (Figure 2C) and 14 day LPS and 7 day betamethasone exposed lungs (Figure
Glil2 mRNA expression had similar trends towards declines after LPS exposure (Figure 2H).

Levels of FGF10 and BMP4, which are two important Shh regulated mediators of lung development, were also assessed. Both FGF10 and BMP4 mRNA increased 14 days after LPS exposure, by 2-fold and 3.5 fold respectively (Figure 3A and 3B). Exposure to betamethasone after LPS exposure lowered FGF10 and BMP4 mRNA. BMP4 protein expression was mainly localized in the bronchial epithelial cells, which corresponds with recent data obtained in adult lung tissue (35, 42). Immunohistochemical analysis of BMP4 expression in bronchioli revealed that BMP4 was decreased 7 days after LPS exposure, and showed a trend towards increased expression at 14 days after LPS exposure (Figure 3C). Treatment with betamethasone before LPS exposure prevented the decrease in BMP4 levels seen after 7 day LPS exposure only. Treatment with betamethasone 7 days after the LPS exposure decreased BMP4 levels.

Expression of lung structural proteins

Elastin foci were quantified in lung sections as an assessment of secondary septation. Representative images are shown for control (Figure 4A), 7d LPS (Figure 4B), 14d LPS (Figure 4C), 7d Beta (Figure 4D), 14d Beta + 7d LPS (Figure 4E) and 14d LPS + 7d Beta (Figure 4F) lambs. The number of elastin foci decreased in the lungs of LPS exposed groups lambs (Figure 4G). Pre-treatment with betamethasone minimized the decrease in elastin foci. Post-treatment with betamethasone after LPS exposure increased elastin foci in the fetal lung. ELN mRNA first decreased by 50% 7 days after LPS exposure followed by a 50% increase 14 days after LPS exposure compared to controls (Figure 4H). Betamethasone pre-treatment followed by 7 day LPS exposure
increased *ELN* mRNA by 50% compared to controls. No change in *ELN* mRNA was detected in the 14d LPS + 7d Beta animals.

Representative images of the collagen deposition in the fetal lungs are shown for controls (Figure 5A), 7d LPS (Figure 5B), 14d LPS (Figure 5C), 7d Beta (Figure 5D), 14d Beta + 7d LPS (Figure 5E) and 14d LPS + 7d Beta (Figure 5F) lambs. *Col1A1* mRNA increased more than 2-fold after combined 14 day Beta and 7 day LPS exposure (Figure 5H). Collagen type I deposition increased after 14 days of LPS exposure (Figure 5G). In contrast, 14 day LPS exposure followed by betamethasone post-treatment resulted in a significant decrease of collagen type I deposition. Betamethasone pre-treatment followed by 7 day LPS exposure increased collagen type I deposition in the fetal lung. In contrast, 14 day LPS exposure followed by betamethasone post-treatment significantly decreased mRNA levels of *Col1A1* similar to the collagen deposition in the lung. mRNA levels of collagen type I gene *Col1A2* decreased to 50% after 14 days of LPS exposure irrespective of betamethasone post-treatment (Figure 5I). Interestingly, 7 day betamethasone exposure only also decreased *Col1A2* mRNA by around 40%. Further computerized morphometric analyses are reported elsewhere and did not show differences (29).
DISCUSSION

LPS exposure leads to changes in Shh signaling in the fetal lung

In the context of developmental biology research, less is known about later fetal lung development than early organogenesis. Later lung development is, however, an area of human biology where clinical care interfaces with development since survival after very preterm birth at 60% of gestation is now frequent. We used an animal model with similarities to late gestation human lung development to test two very common clinical exposures, chorioamnionitis and antenatal steroids. We used 7 and 14 day intra-uterine periods of exposure based on our previous findings of a delay in alveolar development after 7 and 14 days of LPS-induced inflammation (25, 54). Here we show that fetal lung exposure to LPS-induced inflammation (29) is accompanied by changes in the Shh pathway, which is crucial for early lung development. In addition, we demonstrated that a maternal intra-muscular injection of betamethasone attenuated the effects of LPS on this developmental pathway. We therefore provide some molecular insights into the observational data from clinical practice that maternal corticosteroids are beneficial despite the inflammation of chorioamnionitis (20).

Exposure to intra-amniotic LPS has been shown to cause severe lung inflammation and damage leading to structural changes in the fetal lung which mimic pulmonary changes seen in BPD patients (26). Although in this study we did not measure an increased expression of HSP70, an indicator of oxidative-stress mediated lung damage, previous work from our group showed that LPS-induced chorioamnionitis causes fetal lung injury as early as 5h after the exposure (26). Tissue remodeling was further characterized by
increased proliferation seen up to 14 days after the LPS exposure and maturation of alveolar type II cells (26, 29).

As evidence is accumulating that the Shh pathway is involved during aberrant lung development and disease (24, 51), we asked if intra-amniotic LPS exposure altered Shh signaling in the fetal lung. Recently, LPS was shown to down-regulate Shh in vitro in pulmonary microvascular endothelial cells (55). In our study, LPS strongly down-regulated mRNA levels of \textit{Shh} and its signaling components \textit{Gli1} and \textit{Gli2} in the ovine fetal lung. LPS exposure also selectively decreased Gli1 protein expression in the distal epithelial tips where Shh signaling in the lung is mainly localized (57). Shh expression can be induced by retinoic acid (40), which is decreased in babies who develop BPD (44). The down-regulation of Shh is in line with a previous study from our group, in which LPS-induced chorioamnionitis decreased retinoic acid in fetal sheep lungs (25). Therefore, the LPS-induced decrease of retinoic acid may have decreased \textit{Shh} mRNA.

The decreases in mRNA and protein expression of Shh signaling pathway components were accompanied by 2- to 3-fold increases in \textit{FGF10} and \textit{BMP4} expression 14 days after LPS exposure, following a slight decrease 7 days after LPS exposure. The initial decrease in \textit{FGF10} expression, which has also been measured in the lung tissue of infants with BPD (8), might be due to the activation of TLR2 and 4 by LPS, which can suppress FGF10 through binding of NF-κB to the FGF10 promoter (7). As FGF10 induces BMP4 expression in the developing lung (52), the inhibition of FGF10 may indirectly decrease BMP4 expression. The continuous suppression of \textit{Shh} measured at
7 and 14 days after LPS exposure, which normally down-regulates FGF10 (6), may have caused FGF10 and BMP4 levels to rise.

The changes in these pivotal developmental pathways were accompanied by changes in mRNA levels and deposition of structural proteins which are known to direct alveolar septation (9, 25, 49). In a normally developing lung, focal expression of elastin identify sites for alveolar budding (13). In the lungs of the 7 day LPS exposed lambs, mRNA levels of \( ELN \) and the numbers of elastin foci decreased. Persistent exposure to LPS did not only result in less elastin foci but also increased collagen deposition along the alveolar wall. These observations of dysregulated elastin and collagen deposition in the fetal lung are consistent with ventilation-induced (1, 9, 12) and inflammation-induced (25, 27) animal models of BPD and histology reports of BPD patients (46, 48). Although Shh signaling has been implicated in the activation of fibroblasts and production of extracellular matrix (ECM) proteins such as collagen (19, 23, 51), Shh seems to act mainly through regulation of FGF10 to direct ECM deposition in the developing lung. Shh expression at the pulmonary epithelial tips controls FGF10 expression which in turn controls bud size and shape (37). Both overexpression and inhibition of FGFs lead to inhibition of lung branching and alterations in ECM protein expression (15, 38, 45).

**Betamethasone and lung development**

Antenatal corticosteroids are routinely administered to mothers who are at risk of preterm birth to mature the fetal organs (4). A secondary benefit may be suppression of inflammation (4). Antenatal steroids also reduce adverse neonatal outcome after preterm birth associated with chorioamnionitis (20), which constitute the majority of early
gestational preterm births (4). The effect of these combined pro- and anti-inflammatory stimuli on pathways that are crucial for the developing lung are however unknown.

Recently, we showed that betamethasone treatment before the LPS exposure suppressed fetal lung inflammation by an unknown priming or conditioning mechanism of the fetal immune system (29). As such little pulmonary damage was inflicted by the exposure to LPS and no changes in the developmental pathways which we studied were detected. Very little is known about the effect of maternal corticosteroids on Shh, FGF10 and BMP4. Corticosteroids can inhibit Shh-mediated neural development and as such can have a detrimental effect on the neonatal developing brain (22). We found that maternal corticosteroids alone did not change the expression of these factors in the fetal lung in comparison with controls. Moreover, the effects of LPS on these factors were neutralized by maternal betamethasone, irrespective if betamethasone was given 7 days before or after LPS.

At the lung structural level, corticosteroid treatment before LPS exposure could mitigate the decrease in elastin foci and increase in \textit{ELN} mRNA levels. Corticosteroid post-treatment even increased elastin foci which is in line with previous reports showing that corticosteroids can stimulate tropoelastin production in a dose dependent manner (34, 39) most likely through at transforming growth factor (TGF)-\(\beta\)3 mediated mechanism (56). Here we show that corticosteroid treatment can counteract the negative effects of the LPS exposure on elastin deposition.
Furthermore, treatment with only betamethasone 7 days before delivery decreased Col1A2 mRNA levels. Whether this resulted in a decreased collagen deposition in a later stage remains to be investigated. However, combined exposure to 7 day Beta treatment with 14 day LPS exposure did decrease collagen deposition. Several studies have shown beneficial effects of corticosteroid treatment on attenuating fibrotic processes following lung injury (17, 36, 50). Preterm infants at risk of BPD patients showed a significant reduction in markers of collagen synthesis after prenatal corticosteroid treatment up to 7 days after administration (18, 43). Surprisingly, corticosteroid treatment before the inflammatory stimulus stimulated collagen deposition although little pulmonary inflammation was noticed in these animals (29). Brenner et al. demonstrated in vitro that corticosteroids do not uniformly suppress the fibrotic activity of lung fibroblasts in vitro. This could explain our contradictory results and the diverse outcomes of lung morphology and function of BPD patients after corticosteroid treatment (11).

In this study, we modeled a common clinical exposure to provide new insights into the effects of exposures on fetal lung development (47, 54). However, there are limitations, as exposures at different time points, for different intervals and to a single dose or repeated doses of corticosteroids during fetal development may have different outcomes. The effects of pro- and anti-inflammatory stimuli on the developing lung are clearly complex. It remains to be determined whether the observed changes are the result of direct or indirect effects of LPS and betamethasone with these developmental pathways. Based on these results, intervention studies using inhibitors of the Shh pathway (e.g. cyclopamine (53)) would be helpful. The developing lung is plastic in that it is continually changing over gestation. Because chorioamnionitis is often clinically
silent, the duration of exposure of the lung to chorioamnionitis and the subsequent inflammatory response are unknown. It is generally unknown whether antenatal maternal corticosteroids are administered before or after the onset of chorioamnionitis (4).

In conclusion, this report shows that LPS-induced chorioamnionitis can disturb Shh signaling during early alveolar lung development, which is partially mitigated by betamethasone exposure. Therefore, this report provides some insights into the complicated interactions that can alter lung structure during the maturation phase of lung development where clinical intervention may already occur.
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REFERENCES


FIGURE LEGENDS

Figure 1: **Lung injury and cell proliferation.** A: Protein levels of Heat Shock Protein (HSP)70 did not change in homogenates of LPS and/or betamethasone (Beta) exposed fetal lungs. B: The number of Ki67-positive cells in the alveoli increased after LPS exposure. Pre- and particularly post-treatment with Beta partially prevented this increase. Alveolar Ki67 expression in controls (C) and 7d LPS exposed animals (D). BW – bodyweight. * p<0.05 versus controls using a one-way ANOVA with Tukey’s post hoc test.

Figure 2: **Inhibition of the Sonic Hedgehog (Shh) pathway.** Gli1 expression in alveolar and bronchial tissue as seen in controls (A), 14 days after LPS exposure (B), 7 days after betamethasone (Beta) treatment (C) and after a combination of 14 days LPS followed by post-treatment with Beta (D). E: Expression of Shh was decreased after 7 and 14 days LPS exposure. Both pre- and post-treatment with betamethasone (Beta) normalized Shh mRNA levels compared to controls. F: Gli1 mRNA levels were decreased in LPS exposed lungs. Both pre- and post-treatment with Beta normalized Gli1 mRNA levels compared to controls. G: Gli1 protein expression as scored in the alveoli in lung sections decreased after 7 and 14 day LPS exposure. Pre- or post-treatment could partially attenuate this decrease. H: Levels of Gli2 mRNA in experimental groups did not differ significantly from controls. * p<0.05 versus controls and § p<0.05 between experimental groups using a one-way ANOVA with Tukey’s post hoc test.
**Figure 3: Expression of Fibroblast Growth Factor (FGF)10 and Bone Morphogenetic Protein (BMP)4.**

**A:** mRNA levels of FGF10 were increased two-fold 14 days after LPS exposure. Post-treatment with betamethasone (Beta) normalized FGF10 levels compared to controls. **B:** mRNA levels of BMP4 were increased 3.5-fold 14 days after LPS exposure. Post-treatment with Beta normalized BMP4 levels compared to controls. **C:** Immunohistochemical analysis of BMP4 expression in bronchioli decreased after 7 days of LPS exposure, but a recovery of BMP4 14 days after LPS exposure. Pre-treatment with Beta before LPS exposure prevented a drop in BMP4 levels. * p<0.05 versus controls and § p<0.05 between experimental groups using a one-way ANOVA with Tukey’s post hoc test.

**Figure 4: Altered expression of elastin.** Elastin deposition in the alveoli of control (A), 7d LPS (B), 14d LPS (C), 7d Beta (D), 14d Beta + 7d LPS (E) and 14d LPS + 7d Beta (F) lambs. **G:** The number of elastin foci per mm² tissue was decreased in LPS exposed lambs. Pre-treatment with Beta could prevent a significant decrease in elastin foci. Post-treatment with Beta after LPS exposure increased the number of elastin foci. **H:** ELN mRNA levels decreased by 50% in 7d LPS lambs but increased in 14d LPS exposed lambs. Pre-treatment with Beta before LPS exposure resulted in a 60% increase in ELN mRNA levels. Post-treatment with Beta prevented the increase in ELN mRNA. * p<0.05 versus controls and § p<0.05 between experimental groups using a one-way ANOVA with Dunnett’s post hoc test.

**Figure 5: Collagen I expression** Collagen I deposition in the alveoli of control (A), 7d LPS (B), 14d LPS (C), 7d Beta (D), 14d Beta + 7d LPS (E) and 14d LPS + 7d Beta (F)
lambs. **G:** 14 day LPS exposure and 14d Beta + 7d LPS exposure increased the percentage of collagen type I tissue in the fetal lung. Post-treatment with Beta after LPS exposure decreased collagen type I expression. **H:** mRNA levels of \( Col1A1 \) increased 2-fold after combined exposure to 14 day Beta + 7 day LPS. 14 day LPS exposure followed by Beta treatment decreased \( Col1A1 \) mRNA by 40%. **I:** \( Col1A2 \) mRNA levels decreased in 14d LPS, 7d Beta and 14d LPS + 7d Beta lambs by 50%. * p<0.05 versus controls and § p<0.05 between experimental groups using a one-way ANOVA with Dunnett’s post hoc test.
### Table 1: Primers used for RT-PCR

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