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Intraamniotic LPS modulates expression of antimicrobial peptides in the fetal sheep lung

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Abstract

Background—Damage-associated molecular patterns (DAMPs) and antimicrobial peptides (AMPs) are components of pulmonary innate immunity and tissue repair. We hypothesized that DAMPs and AMPs would increase in response to fetal pulmonary inflammation caused by chorioamnionitis in a time-dependent manner.

Methods—Fetal sheep were exposed to intra-amniotic saline or LPS (10mg) between 5 hours and 15 days prior to preterm delivery at 125±2 days. Lung tissue mRNAs for pro-inflammatory cytokines; AMPs: myeloid antimicrobial peptide-29 (MAP29), dodecapeptide, sheep beta-defensin-1 (SBD1), sheep beta-defensin-2 (SBD2); DAMPs: IL-1α, lactoferrin, heat-shock protein-70 (HSP70), high-mobility group box protein-B1 (HMGB1), receptor for advanced glycation endproducts (RAGE) were measured by RT-qPCR. Immunohistochemistry of DAMPs and in situ hybridization of AMPs was performed.

Results—IL-1α, IL-1β, IL-6, IL-8, IL-10, MCP-1, and TNF-α mRNA increased after LPS exposure. MAP29, dodecapeptide, SBD1 and SBD2 mRNA were suppressed at 24 hours. MAP29 and dodecapeptide mRNA then increased at 8 days. Lactoferrin increased at 24 hours. There were no changes for HMGB1, HSP70 or RAGE. MAP29 and dodecapeptide localized to alveolar cells, increased 8 days after exposure to LPS.

Conclusion—AMPs are initially suppressed in the fetal lung by LPS-induced chorioamnionitis. The late induction of MAP29 and Dodecapeptide may be related to lung repair.

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Introduction

Chorioamnionitis is a frequent cause of fetal inflammation and the lung is an important organ in initiating the fetal inflammatory response (1). Chorioamnionitis also has been inconsistently associated with bronchopulmonary dysplasia (BPD), with some studies reporting no association (2), while others report a positive association (3). A recent meta-analysis showed association of increased BPD with histological chorioamnionitis (4). However, in animal models of chorioamnionitis, the lung injury observed is mild and resolves despite continued exposure to proinflammatory mediators (5, 6), suggesting participation of repair mechanisms following the initial inflammatory insult.

In fetal lambs, intra-amniotic bacterial LPS induces chorioamnionitis and lung inflammation (7). LPS signals the innate immune response by binding to the Toll-like receptor 4 (TLR4) and intraamniotic LPS increases TLR4 mRNA expression and protein in the fetal lung (8, 9). The TLRs are part of a group of receptors called pattern recognition receptors (PRRs), which recognize microorganisms through pathogen-associated molecular patterns (PAMPs), as well as non-infectious material and endogenous molecules released during cell injury called damage-associated molecular patterns (DAMPs) (8, 10).

DAMPs are constitutively present in the cytoplasm, nucleus and granules of cells such as leukocytes and epithelial cells. They can activate both innate and adaptive immunity, being quickly released by degranulation or cell necrosis in response to infection or tissue injury, and can also be induced in response to cytokines (10). Several DAMPs have been identified, including high-mobility group box-1 (HMGB1), heat shock proteins (HSP) and lactoferrin. These DAMPs are able to recruit and activate inflammatory cells, including dendritic cells and are able to interact with both TLRs and IL-1 receptor. Even though not formally considered DAMPs, IL-1α and Lactoferrin have similar characteristics being constitutively expressed and trigger inflammatory responses upon release from necrotic cells (10).

Antimicrobial peptides (AMPs) are an essential part of the innate immune response. They not only have direct antimicrobial activity but are also involved in the modulation of inflammation and healing (11). Defensins and cathelicidins, the most extensively studied AMPs in mammals, are large cationic peptides with antimicrobial and immunomodulatory activity (12). In sheep, two β-defensins have been described: β-defensin-1 (SBD1), and β-defensin 2 (SBD2) (13, 14). Several cathelicidins also have been described in sheep and the myeloid antimicrobial peptide 29 (MAP29) has been the most studied. The expression of defensins and cathelicidins are developmentally regulated with increased expression towards the end of gestation and with a subsequent postnatal decrease in different fetal tissues (13-15). However their role in the fetal inflammatory response and tissue repair remains to be further elucidated. Therefore, we hypothesized that AMPs and DAMPs would increase with pulmonary inflammation from chorioamnionitis. We report novel information regarding the time course of expression of inflammatory cytokines and expression of DAMPs and AMPs in a clinically relevant preterm animal model.

Results

Animals

All animals exposed to intraamniotic LPS or saline were alive at delivery (table 1). The pH values were low because the ewes were killed prior to delivery of the fetuses, which resulted in high fetal pCO_2 values. We previously reported decreased lymphocytes in the cord blood at 5, 12, and 24 hours and increased neutrophils at 8 days for this series of animals (16).

AMPs

The mRNA levels of the β-defensins (SBD1 and SBD2) and cathelicidins (MAP29 and dodecapeptide) decreased 24 hours after LPS exposure (Figure 1). However, the mRNA levels of the cathelicidins, had an 8-fold increased expression at 8 days. In contrast, the mRNA levels of SBD1 and SBD2 returned to levels similar to control by 8 days.

Localization of cathelicidins mRNA

In order to identify the location of the increased expression of cathelicidins at 8 days we performed in situ hybridization for cathelin-related peptide, which cross-reacts with all the sheep cathelicidins. Lung sections from control lambs expressed cathelicidin mRNAs localized to the peripheral lung. The cell type appeared to be consistent with alveolar type II cells and occasional inflammatory cells. The number of positive cells and intensity of staining increased at 8 days after IA LPS exposure (Figure 2).

Expression of DAMPs

Expression of IL-1α increased at 12 hours after LPS (Figure 3). The mRNA for lactoferrin increased 24 hours after exposure. There were no differences in the mRNA levels for HMGB1, RAGE, or HSP70.

Immunolocalization of DAMPs

To characterize the expression of DAMPs in the lung we performed immunohistochemistry for lactoferrin, HMGB1, RAGE, and HSP70 (Figure 4). The bronchial glands and epithelial cells of the bronchi immunostained for lactoferrin (Figure 4A-B). The immunostaining for HMGB1 was diffuse and intense in bronchial epithelial cells, submucosal gland epithelium, alveolar epithelium and inflammatory cells (Figure 4C-D). The immunostaining for RAGE, the HMGB1 receptor, was also in similar location as HMGB1, although the staining appeared to be less intense (Figure 4E-F). Immunostaining for HSP70 was in occasional airway epithelial cells, and even less frequently in inflammatory and vascular smooth muscle cells. There was an apparent increase in the immunostaining for HSP70 in the airway epithelial cells at 24 hours after LPS exposure (Figure 4 G-H).

Pro-inflammatory cytokines

Expression of the pro-inflammatory cytokines IL-1β, TNF-α, MCP-1, and IL-8 were increased after LPS exposure (Figure 5). Three temporal patterns of expression were detected. We found IL-6 had the earliest peak, at 5 hours followed by a subsequent decrease.

IL-1β, MCP-1 and IL-10 were significantly increased 5h-12h after exposure, peaking at 2 days. TNF-α and IL-10 were increased only at 2 days.

Discussion

We report the dynamic expression of AMPs and DAMPs with distinct temporal patterns of expression in the fetal lung following intra-amniotic exposure to LPS. To our knowledge this is the first study to report the expression AMPs and DAMPs in the preterm fetal lung in response to intrauterine inflammation. The expression of several AMPs decreased 1d after exposure to IA LPS. The finding that cathelicidins are subsequently induced in the lung following LPS exposure only after 8 days is particularly interesting given their role in lung repair. We previously reported that chorioamnionitis induced early lung injury followed by proliferation, consistent with early suppression followed by increased cathelicidin expression detected in this study (17). These results add to our understanding of how antenatal inflammation may be inconsistently associated with bronchopulmonary dysplasia depending on the time of exposure (4, 6).

Several β-defensins have been described in humans, but only two in sheep, which are characteristically expressed in epithelial cells. In humans, β-defensin 1 is constitutively expressed and not induced (18), while β-defensin 2 is induced in pulmonary epithelial cells in response to LPS following activation of nuclear factor kappa B (NF-κB) (19). In sheep, expression of SBD1 progressively increases in the bronchiolar-alveolar junction from 30 days prior to term to 15 days postnatal (13). In normal fetuses and neonatal sheep, β-defensin 2 is expressed constitutively in small amounts in the lungs (14). SBD1 was up regulated in response to viral infection (20), but suppressed in infection by gram-negative bacterial (21). Previous studies have shown a large individual variability in the developmental expression of β-defensins, which we also observed in our samples (13, 14). We demonstrate that the intraamniotic administration of the gram-negative bacterial cell wall product LPS also suppressed SBD1 and SBD2 early after exposure.

The only known human cathelicidin is LL-37, which is produced by neutrophils, macrophages and epithelial cells (22). The pulmonary expression of LL-37 can be induced by pulmonary or systemic infection, and LL-37 expression correlates with TNF-α levels (13). Of the several cathelicidins described in sheep, the myeloid antimicrobial peptide 29 (MAP29) was highly effective against common gram-positive and gram-negative bacteria (23).

Interestingly, in our study, cathelicidins and defensins were initially suppressed after LPS exposure. Given their antimicrobial role, this suppression could potentially increase fetal susceptibility to further infection from microorganisms. Suppression of the cathelicidin MAP29 was observed in pulmonary infection of neonatal lamb with gram-negative bacteria, in contrast to the previously reported induction following viral infection suggesting differential roles in bacterial or viral infections (20, 21).

Both cathelicidins and defensins demonstrate important immunomodulatory function. They can directly recruit leukocytes as well as induce the expression of chemokines. Cathelicidins

also inhibit TLR4 mediated release of cytokines in dendritic cells and macrophages as well as release of TNF- from human monocytes (24, 25). The complex functions of AMPs suggest an important role in the regulation of the immune response. The early suppression we observed is potentially significant for the fine-tuning of the fetal inflammatory response to chorioamnionitis in the preterm lung.

Besides their role in modulating inflammation AMPs are also important for tissue healing following injury or inflammation. The human β-defensin 2 stimulates migration and proliferation of endothelial cells to wounds, helping wound closure (26). Specifically in pulmonary cells, neutrophil-derived human defensins enhance proliferation of epithelial cells, accelerating wound closure. This effect is mediated by activation of the EGF receptor (EGFR) and its downstream pathway (27). Defensins are also important for cell differentiation during wound healing in the lung. After injury, defensins induce expression of mucins MUC5B and MUC5A in cells of the epithelium (28). The human cathelicidin LL-37 may induce signaling pathways involved in wound closure, activating airway epithelial cells via EGFR, as well as neovascularization of wounds (29, 30). The late induction of the cathelicidins MAP29 and Dodecapeptide observed in our study could be involved in the process of tissue healing following inflammation. The in situ hybridization showed that the late upregulation of cathelicidins originated both in epithelial as well as in inflammatory cells, demonstrating the potential role of both these cell types in lung repair. Consistent with our results, the fetal lung exposed to intraamniotic LPS initially demonstrates injury – increased apoptosis with decreased septation and microvascular injury that recover despite continuous or repeated exposure to LPS (6, 17, 31).

Regarding the DAMPs, we found that expression of IL-1α was increased 12 hours after LPS exposure, while IL-1β expression peaked later at 2 days. IL-1α is expressed constitutively in epithelial and mesenchymal cells and both its precursor and mature forms are active transcription regulators (32). In cell injury induced by hypoxia, IL-1α is responsible for early recruitment of neutrophils in response to inflammation with little effect on recruitment of macrophages or monocytes, while IL-1β seems to be more important for maintenance of the inflammatory response and recruitment of macrophages (33). Our findings are consistent with the role described for early recruitment of inflammatory cells. Potentially, IL-1α has a similar role in the fetal inflammatory response being an early mediator of chemotaxis followed by later expression of IL-1β.

The expression of another DAMP, lactoferrin was increased at 24 hours. Epithelial cells and neutrophils can produce lactoferrin, and we observed it localized to the bronchial glands. Lactoferrin has antimicrobial properties by both chelating iron and disrupting bacterial cell membrane (34). Lactoferrin has the ability to bind LPS preventing further activation of the inflammatory response and production of pro-inflammatory cytokines (35). The increased expression of lactoferrin in response to chorioamnionitis has the potential to increase the innate ability to kill invading microorganisms while limiting local inflammatory response and damage. The staining pattern observed was consistent with what has been reported in the literature, however, no difference was observed in the immunostaining, which could be due to the rapid secretion of the peptide from the bronchial glands.

Even though there was no difference in the mRNA level for HSP70, we observed increased immunostaining at 12 and 24 hours after LPS exposure. This difference was seen only in the airway epithelium and the conflicting results might be related to the localization of HSP70 as we observed previously with a volutrauma injury (36). Besides, the discrepancy between immunostaining and the mRNA quantitation could be due to post-transcriptiona regulations, since the mRNA for HSP70 has been shown to be subject to microRNA regulation leading to supression of translation and polyadenylation leading the increased translation. HSP70 appears to have cytoprotective role in the lung epithelium against LPS and hypoxia-mediated injury (37). Expression of HMGB1 and its receptor RAGE in the lung were not affected by LPS exposure.

Among the pro-inflammatory cytokines IL-6 and MCP-1 had the earliest increase in expression at 5 hours after LPS exposure, while the remainder cytokines peaked at 2 days. This response pattern suggests a role of IL-6 and MCP-1 in the initiation of pulmonary inflammation in chorioamnionitis. We previously reported that MCP-1 is expressed in the lung mesenchymal cells potentially suggesting a role for these lung cells in the initiation of lung inflammation (38). In contrast to the early induction of IL-6 and MCP-1, TNF-α and IL-10 mRNAs were only increased at 2 days, suggesting that these cytokines play a secondary role in modulating the lung inflammation after LPS induced chorioamnionitis.

We present a novel report of the time course expression of pro- and anti-inflammatory cytokines in a model of chorioamnionitis. Our study provides insight on the expression of AMPs and DAMPs in the early and late pulmonary responses to intra-uterine inflammation. The early suppression of both cathelicidins and defensins could potentially increase fetal susceptibility to further infection. The late induction of cathelicidins MAP29 and dodecapeptide could be related to their role in tissue repair following inflammatory injury.

Methods

Animals

The Animal Care and Use Committees of the Cincinnati Children's Hospital Medical Center and University of Western Australia approved the study protocol. All procedures involving animals were performed at The University of Western Australia (Perth, Australia). Time-mated Merino ewes with singleton fetuses were randomly assigned to groups for exposure to either 10mg of intra-amniotic LPS from *Escherichia coli* (Sigma Aldrich, St Louis, MO) diluted in 2 mL of sterile saline or 2 mL of sterile saline as control. This dose of LPS has been shown to induce chorioamnionitis and fetal inflammatory response (39). The inflammatory response of the fetal thymus was reported previously for this series of animals (16).

Intraamniotic LPS or saline was dosed at one of the following time points: 5 hours, 12 hours, 24 hours, 2 days, 4 days, 8 days or 15 days with ultrasound guidance (39) prior to preterm delivery at 125 ± 2 days. We used 5 to 7 animals per group. Ewes were euthanized with 100mg/kg of intravenous pentobarbital with rapid surgical delivery of the fetus (16). Fetal lungs for mRNA analysis were quickly dissected and snap frozen. For histological

analysis the right upper lobe of the lung was inflated fixed with 10% buffered formalin at a pressure of 30 cmH$_2$O, and further processed to embed in paraffin (40).

Relative mRNA Quantitation

Total RNA was isolated from frozen lungs after homogenization with TRIzol (Invitrogen, Carlsbad, CA) as previously describe (40). Reverse transcription was performed using Verso cDNA kit (Thermo Scientific, Waltham, MA) to produce single-strand cDNA. The genes IL-1β, TNF-α, MCP-1, Il-6, IL-8, IL-10, IL-1α, SBD1, SBD2, MAP29, Dodecapeptide, Lactoferrin, HMGB1, RAGE, and HSP70 were amplified using the cDNA template and sheep-specific primers along with Taqman probes (Applied Biosystems, Foster City, CA). The mRNA expression for each gene was normalized to the mRNA for the ribosomal protein 18s as internal standard. Final data were expressed as fold increase over the control value.

Immunohistochemistry

Sections from formalin fixed tissues in paraffin blocks were deparaffinised and rehydrated before microwave-assisted antigen retrieval in citric acid buffer at pH 6.0. Endogenous peroxidase activity was blocked with CH$_3$OH/H$_2$O$_2$ treatment and were blocked with 2% BSA in PBS. Sections were incubated overnight at 4°C with the primary antibody diluted in 2% serum in PBS(7). We used the following primary antibodies: Lactoferrin (Abcam, Cambridge, MA, dilution 1:50), HMGB1 (R&D Systems, Minneapolis, MN, dilution 1:50), RAGE (AbD Serotec, Oxford, UK, dilution 1:1000), HSP70 (Biogenex, San Ramon, CA, dilution 1:50). Sections were then washed and incubated with the appropriate species-specific secondary antibody diluted 1:200 in 2% serum for 2 hours at room temperature. After further washing, antigen:antibody complexes were visualized using a Vectastain ABC peroxidase kit (Vector Laboratories Inc., Burlingame, CA). Antigen detection was enhanced with nickel-diaminobenzidine, followed by incubation with TRIS-cobalt. Slides were counterstained with Nuclear Fast Red for photomicroscopy.

In situ hybridization

In situ localization of mRNA was performed with digoxigenin-labeled anti-sense sheep riboprobes for cathelin-related peptide that cross-reacts with all sheep cathelicidins (Forward: TGT GGC TCC TGC TGC TGG GAT TA; Reverse: TAT GAT GCG ACA TAT TCG GGC TGC). Digoxigenin-labeled riboprobes (sense and anti-sense) were synthesized from cDNA templates using DIG RNA labeling kits (Roche Diagnostics, Germany) and diluted in hybridization buffer to a final concentration of 1 μg/ml. The sections were pretreated with 4% paraformaldehyde, treated with proteinase K, and hybridized with the probe overnight at 61°C, based on GC content. Sections were washed with formamide, treated with RNase A (100 μg/ml), and then blocked with 10% horse serum. Following incubation overnight at 4°C with anti-digoxigenin antibody (Roche Diagnostics), the slides were developed with nitro blue tetrazolium- 5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics) in the absence of ambient light using dark boxes. The slides were monitored for color development, and then stopped with Tris-EDTA buffer. Controls for specificity of ribo-probe binding were done with the use of the homologous (sense) probe.

Statistical analysis

Values are expressed and depicted in graphs as means ± standard deviation. All analysis were performed using the software GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). mRNA expression levels were compared using one-way ANOVA and each group was individually compare to the control group using t test. Values of $p < 0.05$ were considered significant.

Acknowledgments

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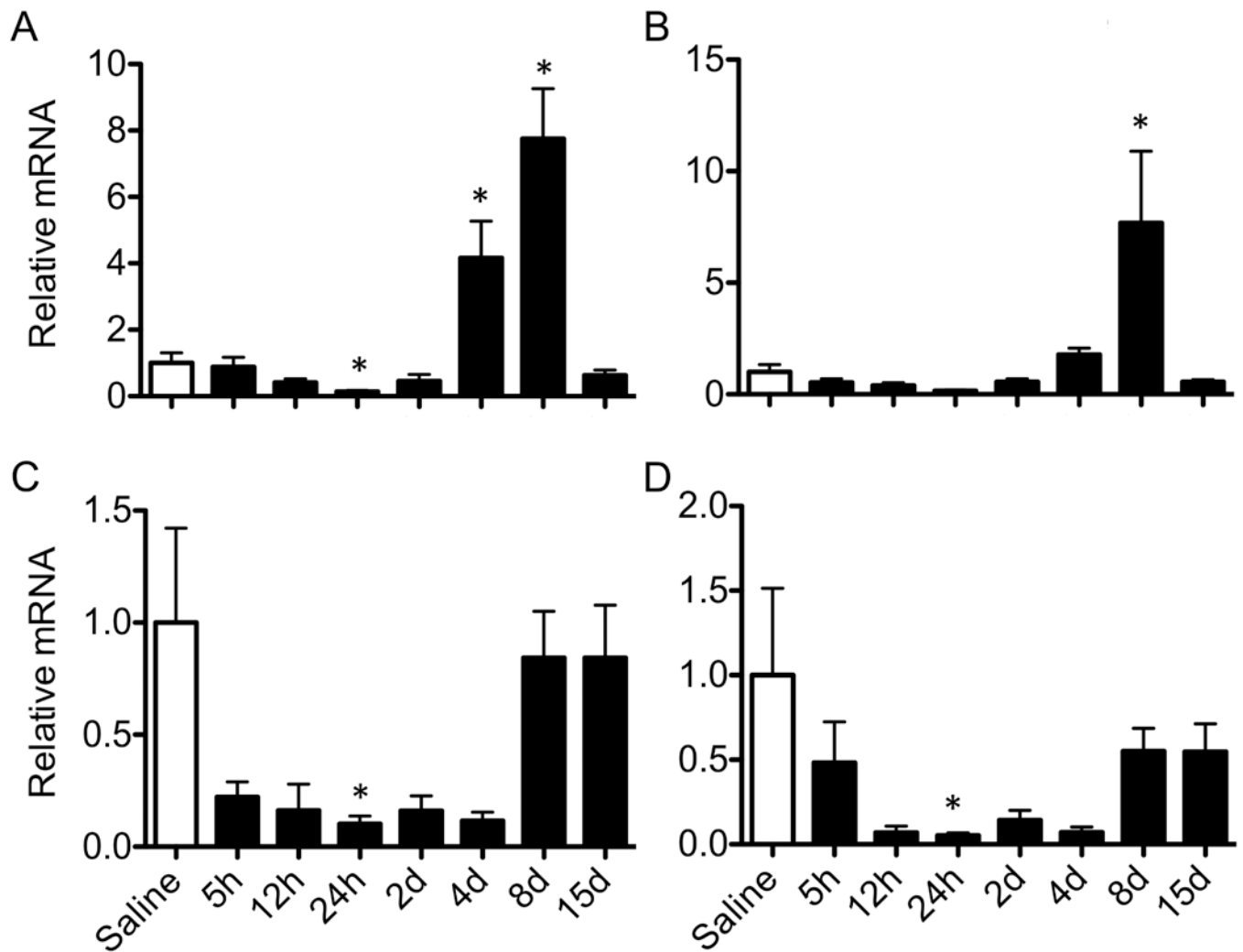
Figure 1. Time-dependent changes in the expression of antimicrobial peptides (AMPs) in preterm fetal sheep lung after IA LPS

mRNA expression of the antimicrobial peptides **A:** MAP29, **B:** Dodecapeptide, **C**: SBD1, **D:** SBD2 by RT-qPCR using Taqman probes was analyzed. The AMP expression decreased at 24h after LPS. The expression of MAP29 and Dodecapetide increased at 4-8d while SBD1 and SBD2 returned to levels similar to controls. *p<0.05 compared to saline.
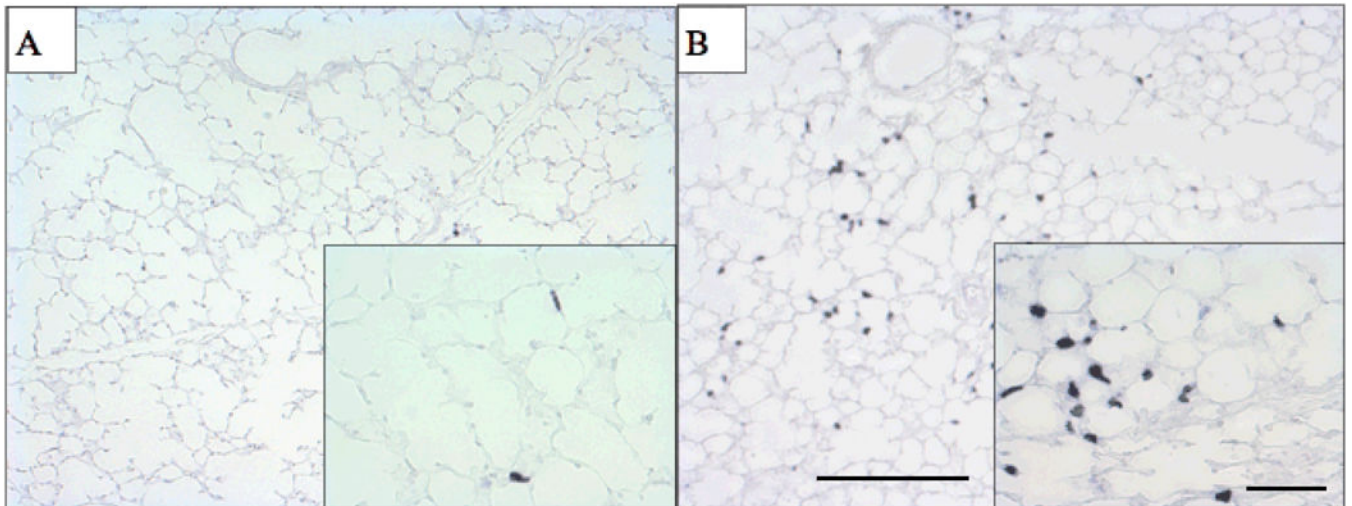
Figure 2. Cellular localization of AMPs in preterm fetal sheep lung after IA LPS
Representative photomicrographs of in situ hybridization for cathelin-related peptide.
mRNA signal localized to alveolar cells with increased staining at 8 days after LPS
exposure. **A**: Control **B**: 8 days after intraamniotic LPS (magnification 100x for main frame
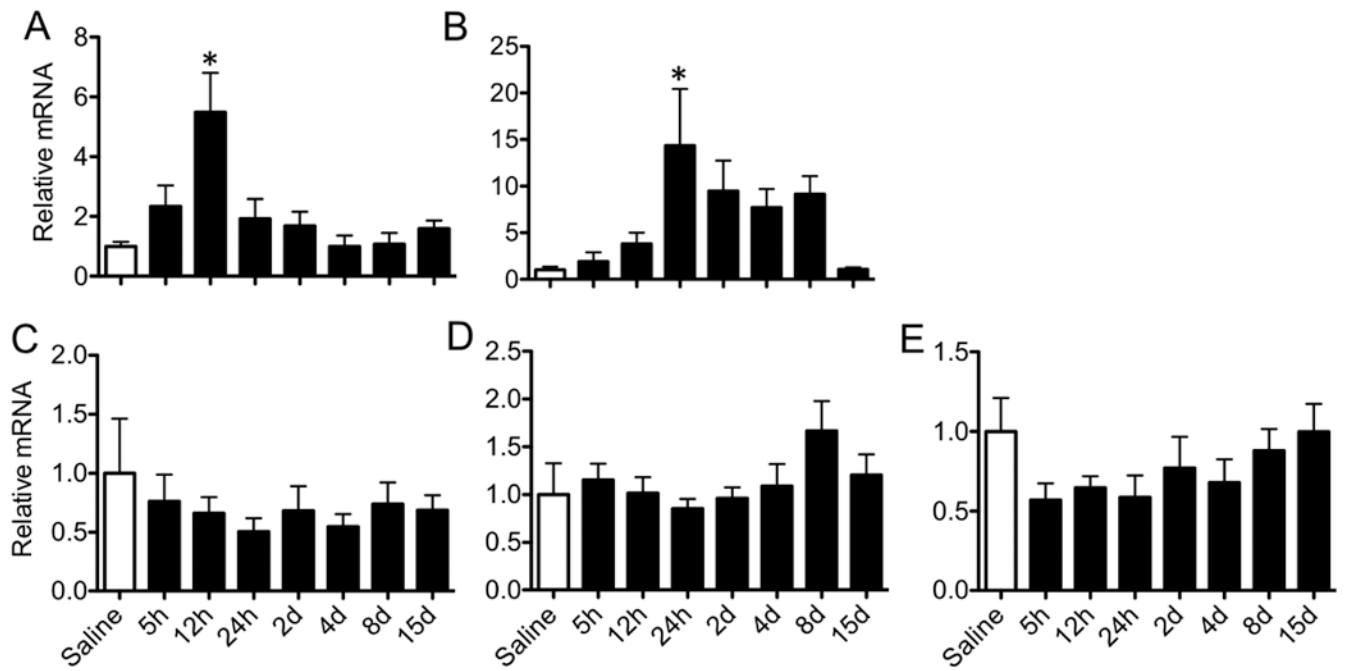and 400X for insert).

Figure 3. Expression of DAMP mRNAs in preterm fetal sheep lung after IA LPS

Expression of the antimicrobial DAMP mRNAs by RT-qPCR using Taqman probes was performed. **A:** IL-α expression peaked at 12 hours, **B:** lactoferrin peaked 24 hours, **C:** HMGB1, **D:** RAGE, **E:** HSP70. (*p<0.05 compared to saline).
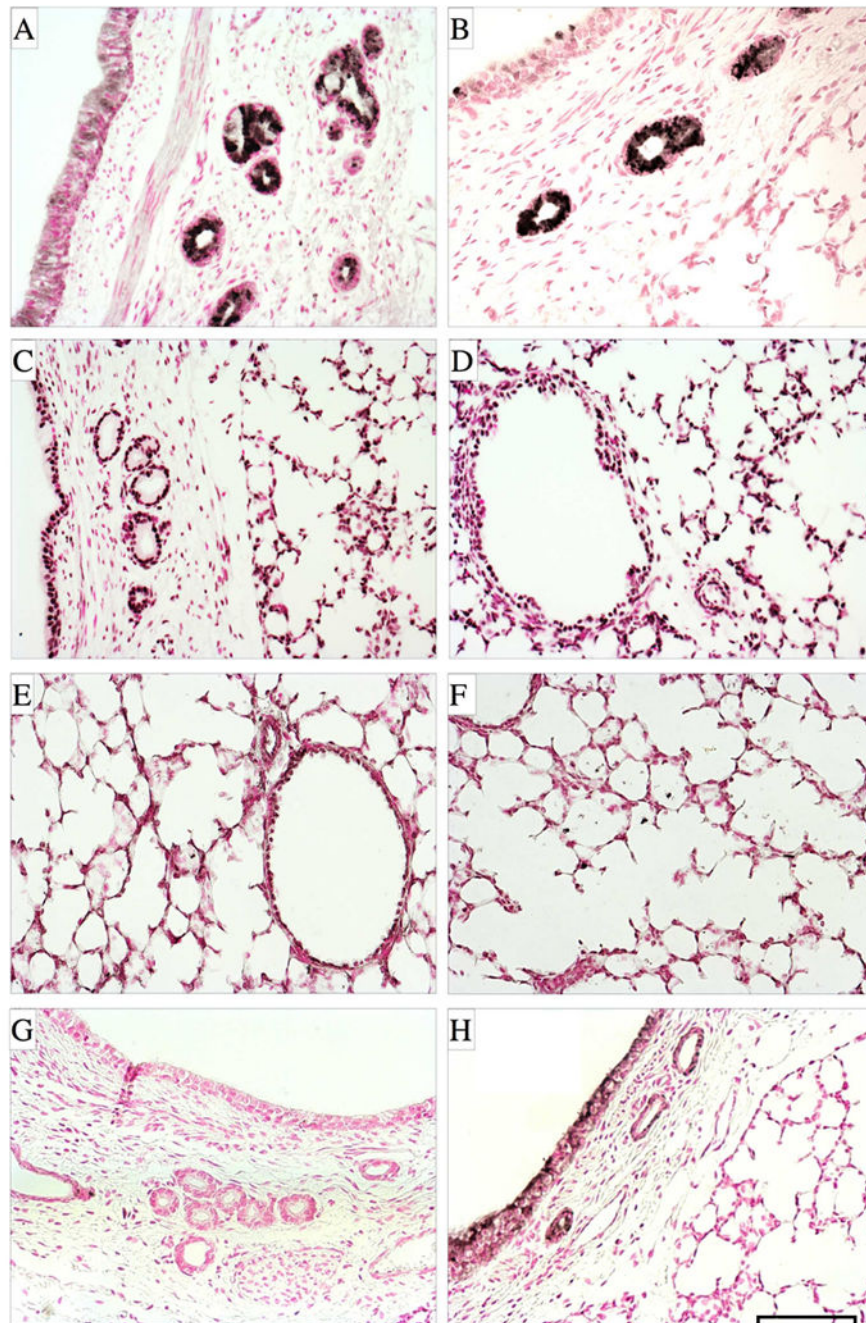
Figure 4. Cellular localization of DAMPs in preterm fetal sheep lung after IA LPS
Representative photomicrographs showing immunostaining for the following: Lactoferrin
depicting staining of bronchial glands and bronchial epithelial cells in control (A) and 24
hours after LPS exposure (B) HMGB1 with diffuse and strong nuclear staining of alveolar
cells in control (C) and 24 hours after LPS exposure (D) RAGE showed weaker staining of
alveolar cells in control (E) and strong staining of inflammatory cells 24 hours after LPS
exposure (F); HSP70 showing immunostaining in the epithelial airway cells in the control
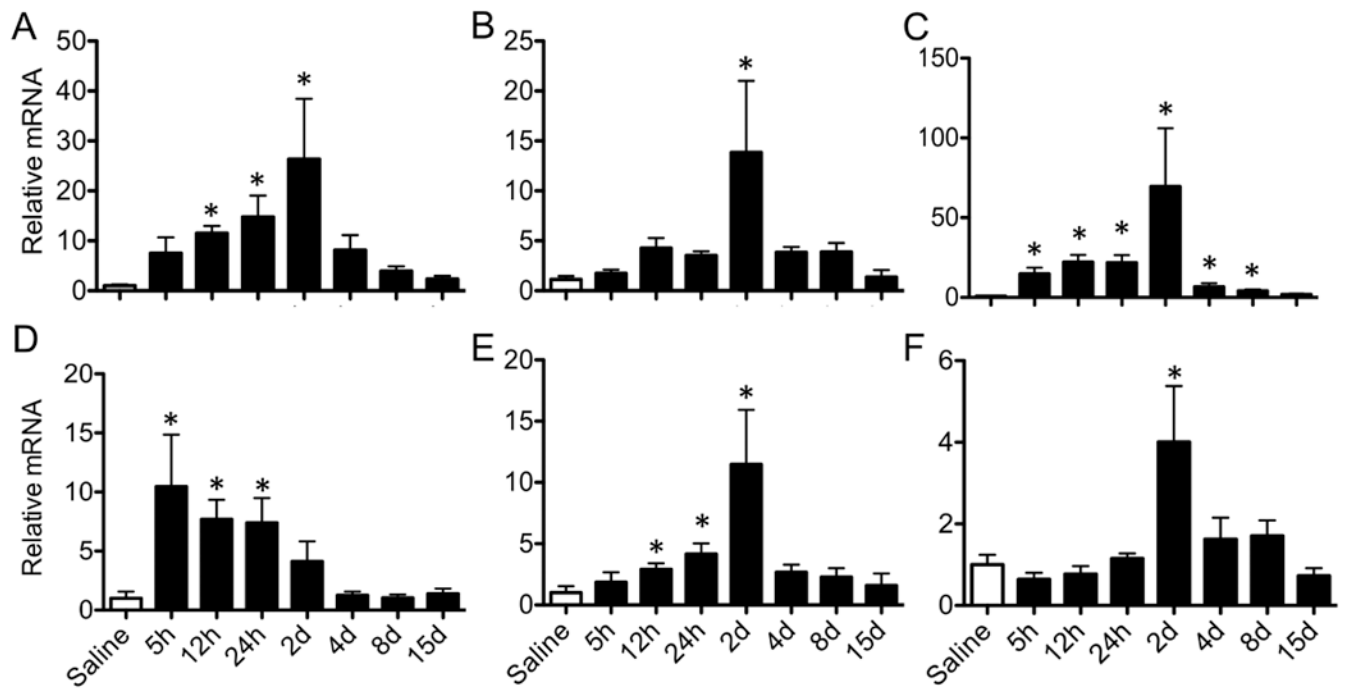group (G), with increased staining at 24h after LPS exposure (H).

Figure 5. Different temporal patterns of expression of pro-inflammatory cytokines/chemokines in preterm fetal sheep lung after IA LPS

mRNA expression of the cytokines/chemokines by RT-qPCR using Taqman probes was analyzed. **A:** IL-1β, **B:** TNF⟨, **C:** MCP-1, **D:** IL-6, **E:** IL-8, **F:** IL-10. Different temporal patterns of expression were noted with early expression for IL-6 and MCP-1 and later expression for TNF⟨ and IL-10. The expression for IL-1β, IL-8 and MCP-1 started 5-12h after IA LPS with a peak at 2d. (*p<0.05 compared to saline).

Table 1

Physiological variable of preterm lambs at birth.

Group	*n*	Birth weight	Sex (M/F)	Cord blood pH
Saline	6	3.0 ± 0.3	3/3	7.1 ± 0.1
5h	6	3.1 ± 0.2	2/4	7.1 ± 0.1
12h	7	2.8 ± 0.2	5/2	7.0 ± 0.1
24h	7	2.8 ± 0.6	4/3	7.1 ± 0.1
2d	6	2.9 ± 0.3	3/3	7.1 ± 0.2
4d	5	3.0 ± 0.1	2/3	7.1 ± 0.2
8d	7	3.0 ± 0.4	2/5	7.2 ± 0.1
15d	7	2.9 ± 0.2	3/4	7.2 ± 0.1