

# Intra-amniotic LPS modulates expression of antimicrobial peptides in the fetal sheep lung

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**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 lipopolysaccharide (LPS) (10 mg) between 5 h and 15 d prior to preterm delivery at  $125 \pm 2$  d. Lung tissue mRNAs for proinflammatory cytokines; AMPs: myeloid AMP-29 (MAP29), dodecapeptide, sheep  $\beta$ -defensin-1 (SBD1), and sheep  $\beta$ -defensin-2 (SBD2); and DAMPs: interleukin (IL)-1 $\alpha$ , lactoferrin, heat-shock protein-70 (HSP70), high-mobility group box protein-B1 (HMGB1), and receptor for advanced glycation endproducts (RAGE) were measured by reverse-transcriptase quantitative polymerase chain reaction. Immunohistochemistry of DAMPs and *in situ* hybridization of AMPs was performed.

**RESULTS:** IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IL-10, MCP-1, and tumor necrosis factor (TNF)- $\alpha$  mRNA increased after LPS exposure. MAP29, dodecapeptide, SBD1, and SBD2 mRNA were suppressed at 24 h. MAP29 and dodecapeptide mRNA then increased at 8 d. Lactoferrin increased at 24 h. There were no changes for HMGB1, HSP70, or RAGE. MAP29 and dodecapeptide localized to alveolar cells, increased 8 d 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.

**C**horioamnionitis 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, with some studies reporting no association (2), while others report a positive association (3). A recent meta-analysis showed association of increased bronchopulmonary dysplasia 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 lipopolysaccharide (LPS) induces chorioamnionitis and lung inflammation (7). LPS signals the innate immune response by binding to the Toll-like receptor 4 (TLR4) and intra-amniotic 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, which recognize microorganisms through pathogen-associated molecular patterns, as well as noninfectious 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 interleukin (IL)-1 receptor. Even though not formally considered DAMPs, IL-1 $\alpha$  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  $\beta$ -defensins have been described:  $\beta$ -defensin-1 (SBD1), and  $\beta$ -defensin 2 (SBD2) (13,14). Several cathelicidins also have been described in sheep and the myeloid AMP 29 (MAP29) has been the most studied. The expression of defensins and cathelicidins are developmentally regulated with increased expression toward 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

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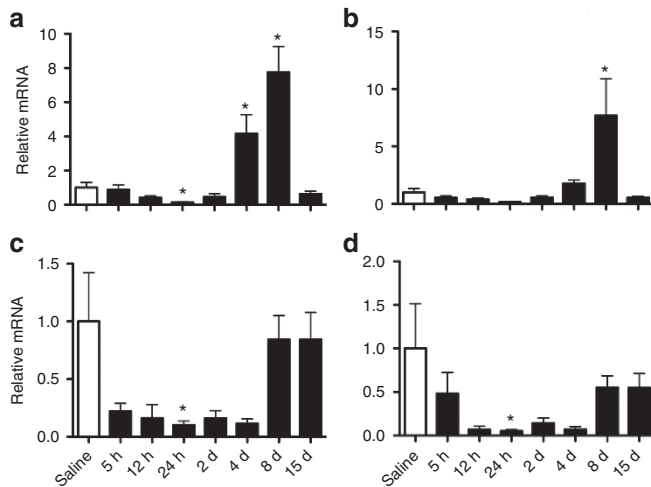
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tissue repair remains to be further elucidated. Therefore, we hypothesized that AMPs and DAMPs would increase with pulmonary inflammation from chorioamnionitis. We report

novel information regarding the time course of expression of inflammatory cytokines and expression of DAMPs and AMPs in a clinically relevant preterm animal model.

**Table 1.** Physiological variables 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       |
| 5 h    | 6 | 3.1±0.2      | 2/4       | 7.1±0.1       |
| 12 h   | 7 | 2.8±0.2      | 5/2       | 7.0±0.1       |
| 24 h   | 7 | 2.8±0.6      | 4/3       | 7.1±0.1       |
| 2 d    | 6 | 2.9±0.3      | 3/3       | 7.1±0.2       |
| 4 d    | 5 | 3.0±0.1      | 2/3       | 7.1±0.2       |
| 8 d    | 7 | 3.0±0.4      | 2/5       | 7.2±0.1       |
| 15 d   | 7 | 2.9±0.2      | 3/4       | 7.2±0.1       |



**Figure 1.** Time-dependent changes in the expression of antimicrobial peptides (AMPs) in preterm fetal sheep lung after intra-amniotic lipopolysaccharide (LPS) and mRNA expression of the antimicrobial peptides (a) myeloid AMPs (MAP29), (b) dodecapeptide, (c) sheep  $\beta$ -defensin (SBD1), and (d) SBD2 by reverse-transcriptase quantitative polymerase chain reaction using Taqman probes were analyzed. The AMP expression decreased at 24 h after LPS. The expression of MAP29 and dodecapeptide increased at 4–8 d, whereas SBD1 and SBD2 returned to levels similar to controls. \* $P < 0.05$  compared to saline.

## RESULTS

### Animals

All animals exposed to intra-amniotic LPS or saline were alive at delivery (Table 1). The pH values were low because the ewes were killed prior to the delivery of the fetuses, which resulted in high fetal  $p\text{CO}_2$  values. We previously reported decreased lymphocytes in the cord blood at 5, 12, and 24 h and increased neutrophils at 8 d for this series of animals (16).

### AMPs

The mRNA levels of the  $\beta$ -defensins (SBD1 and SBD2) and cathelicidins (MAP29 and dodecapeptide) decreased 24 h after LPS exposure (Figure 1). However, the mRNA levels of the cathelicidins, had an eightfold increased expression at 8 d. In contrast, the mRNA levels of SBD1 and SBD2 returned to levels similar to control by 8 d.

### Localization of Cathelicidins mRNA

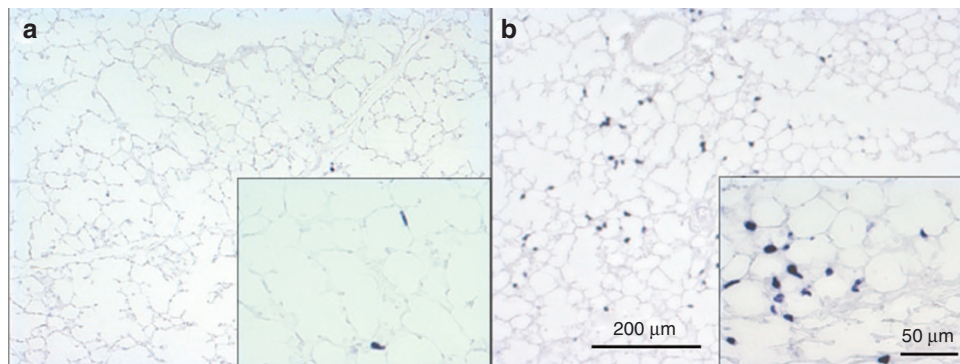
In order to identify the location of the increased expression of cathelicidins at 8 d, we performed *in situ* hybridization for cathelin-related peptide, which crossreacts with all the sheep cathelicidins. Lung sections from control lambs expressed cathelicidin mRNAs localized to the peripheral lung. 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 d after IA LPS exposure (Figure 2).

### Expression of DAMPs

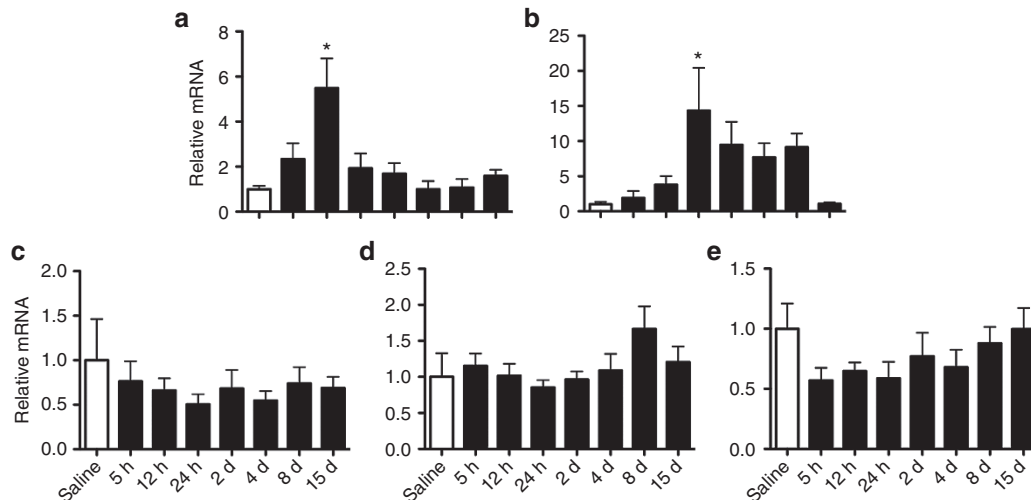
Expression of IL-1 $\alpha$  increased at 12 h after LPS (Figure 3). The mRNA for lactoferrin increased 24 h after exposure. There were no differences in the mRNA levels for HMGB1, receptor for advanced glycation endproducts (RAGE), or HSP70.

### Immunolocalization of DAMPs

To characterize the expression of DAMPs in the lung, we performed immunohistochemistry for lactoferrin, HMGB1,



**Figure 2.** Cellular localization of antimicrobial peptides (AMPs) in preterm fetal sheep lung after intra-amniotic lipopolysaccharide (LPS). Representative photomicrographs of *in situ* hybridization for cathelin-related peptide. mRNA signal localized to alveolar cells with increased staining at 8 d after LPS exposure. (a) Control; (b) 8 d after intra-amniotic LPS (magnification  $\times 100$  for main frame and  $\times 400$  for insert).



**Figure 3.** Expression of damage-associated molecular patterns (DAMP) mRNAs in preterm fetal sheep lung after intra-amniotic lipopolysaccharide. Expression of the antimicrobial DAMP mRNAs by reverse-transcriptase quantitative polymerase chain reaction using Taqman probes (Applied Biosystems) was analyzed. (a) IL- $\alpha$  expression peaked at 12 h, (b) lactoferrin peaked at 24 h, (c) high-mobility group box protein-1 (HMGB1), (d) receptor for advanced glycation endproducts (RAGE), (e) heat-shock protein-70 (HSP70). (\* $P < 0.05$  compared to saline).

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 h after LPS exposure (Figure 4g,h).

#### Proinflammatory Cytokines

Expression of the proinflammatory cytokines IL-1 $\beta$ , tumor necrosis factor (TNF)- $\alpha$ , 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 h followed by a subsequent decrease. IL-1 $\beta$ , MCP-1, and IL-10 were significantly increased 5–12 h after exposure, peaking at 2 d. TNF- $\alpha$  and IL-10 were increased only at 2 d.

#### 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 1 d after exposure to IA LPS. The finding that cathelicidins are subsequently induced in the lung following LPS exposure only after 8 d 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  $\beta$ -defensins have been described in humans, but only two in sheep, which are characteristically expressed in epithelial cells. In humans,  $\beta$ -defensin 1 is constitutively expressed and not induced (18), while  $\beta$ -defensin 2 is induced in pulmonary epithelial cells in response to LPS following activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) (19). In sheep, expression of SBD1 progressively increases in the bronchiolar-alveolar junction from 30 d prior to term to 15 d postnatal (13). In normal fetuses and neonatal sheep,  $\beta$ -defensin 2 is expressed constitutively in small amounts in the lungs (14). SBD1 was upregulated 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  $\beta$ -defensins, which we also observed in our samples (13,14). We demonstrate that the intra-amniotic 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- $\alpha$  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

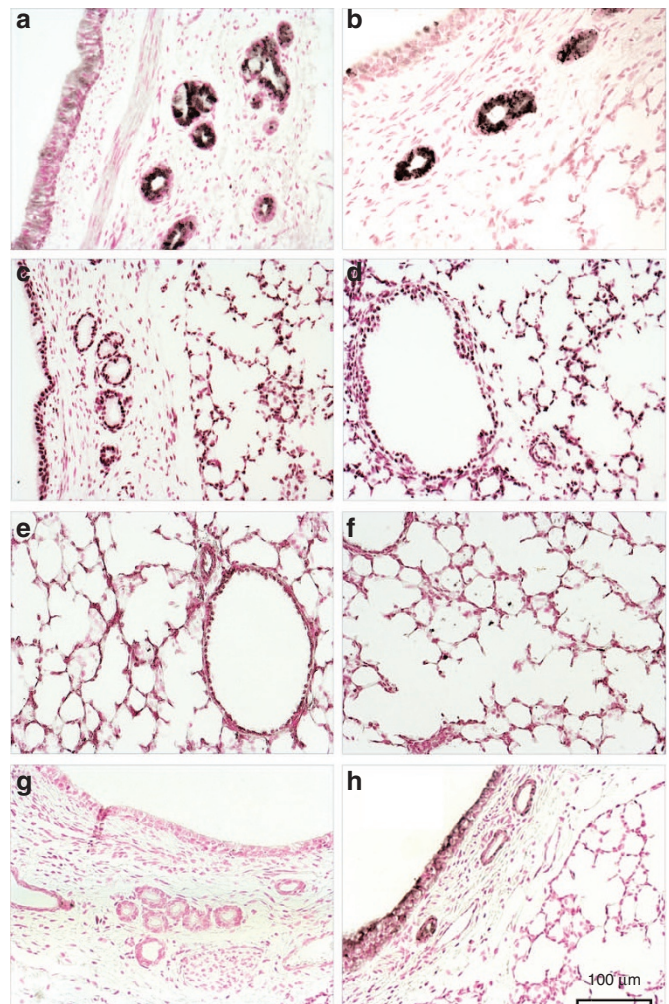
in pulmonary infection of neonatal lamb with Gram-negative bacteria, in contrast to the previously reported induction following viral infection suggesting differential roles in bacterial or viral infections (20,21).

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- $\alpha$  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  $\beta$ -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 epidermal growth factor receptor 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 epidermal growth factor receptor, 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 intra-amniotic 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-1 $\alpha$  was increased 12 h after LPS exposure, while IL-1 $\beta$  expression peaked later at 2 d. IL-1 $\alpha$  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-1 $\alpha$  is responsible for early recruitment of neutrophils in response to inflammation with little effect on recruitment of macrophages or monocytes, while IL-1 $\beta$  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-1 $\alpha$  has a similar role in the fetal inflammatory response being an early mediator of chemotaxis followed by later expression of IL-1 $\beta$ .

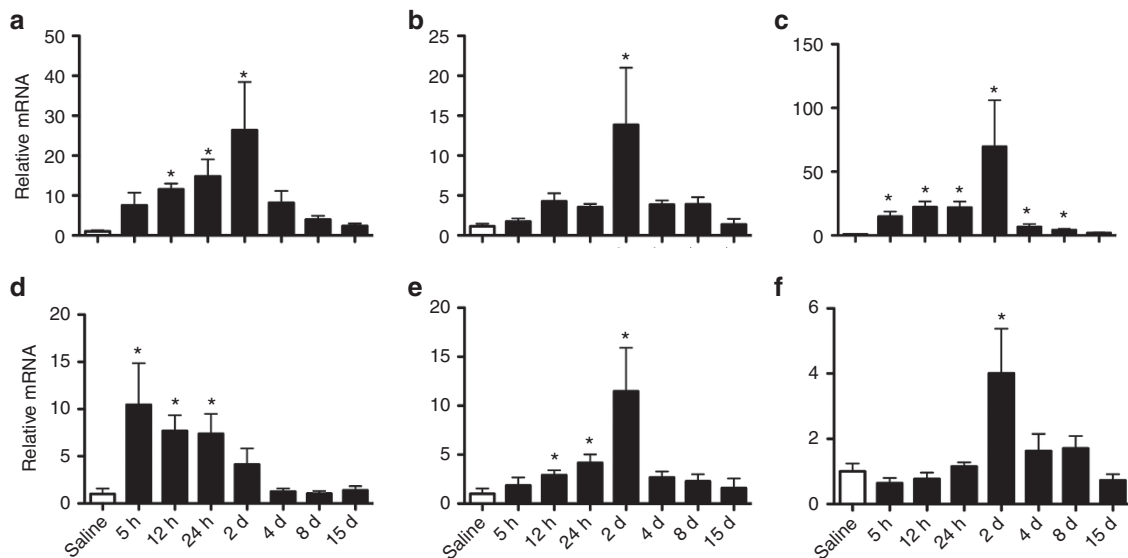
The expression of another DAMP, lactoferrin was increased at 24 h. Epithelial cells and neutrophils can produce lactoferrin, and we observed it localized to the bronchial glands. Lactoferrin



**Figure 4.** Cellular localization of damage-associated molecular patterns (DAMPs) in preterm fetal sheep lung after intra-amniotic lipopolysaccharide (LPS). Representative photomicrographs showing immunostaining for the following: lactoferrin depicting staining of bronchial glands and bronchial epithelial cells in control (a) and 24 h after LPS exposure (b); high-mobility group box protein-B1 (HMGB1) with diffuse and strong nuclear staining of alveolar cells in control (c) and 24 h after LPS exposure (d); receptor for advanced glycation endproducts (RAGE) showed weaker staining of alveolar cells in control (e) and strong staining of inflammatory cells 24 h after LPS exposure (f); heat-shock protein-70 (HSP70) showing immunostaining in the epithelial airway cells in the control group (g), with increased staining at 24 h after LPS exposure (h).

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 proinflammatory 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 h



**Figure 5.** Different temporal patterns of expression of proinflammatory cytokines/chemokines in preterm fetal sheep lung after intra-amniotic lipopolysaccharide. mRNA expression of the cytokines/chemokines by reverse-transcriptase quantitative polymerase chain reaction using Taqman probes was analyzed. (a) IL-1 $\beta$ , (b) TNF- $\alpha$ , (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- $\alpha$  and IL-10. The expression for IL-1 $\beta$ , IL-8, and MCP-1 started 5–12 h after intra-amniotic lipopolysaccharide with a peak at 2 d. \* $P < 0.05$  compared to saline.

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-transcriptional regulations, since the mRNA for HSP70 has been shown to be subject to microRNA regulation leading to suppression 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 proinflammatory cytokines IL-6 and MCP-1 had the earliest increase in expression at 5 h after LPS exposure, while the remainder cytokines peaked at 2 d. 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- $\alpha$ , and IL-10 mRNAs were only increased at 2 d, 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 intrauterine 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 10 mg 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).

Intra-amniotic LPS or saline was dosed at one of the following time points: 5 h, 12 h, 24 h, 2 d, 4 d, 8 d, or 15 d with ultrasound guidance (39) prior to preterm delivery at  $125 \pm 2$  d. We used five to seven animals per group. Ewes were euthanized with 100 mg/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<sub>2</sub>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 described (40). Reverse transcription was performed using Verso cDNA kit (Thermo Scientific, Waltham, MA) to produce single-strand cDNA. The genes IL-1 $\beta$ , TNF- $\alpha$ , MCP-1, IL-6, IL-8, IL-10, IL-1 $\alpha$ , 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 deparaffinized and rehydrated before microwave-assisted antigen retrieval in citric acid buffer at pH 6.0. Endogenous peroxidase activity was blocked with CH<sub>3</sub>OH/H<sub>2</sub>O<sub>2</sub> treatment and were blocked with 2% bovine serum albumin in phosphate-buffered saline. Sections were

incubated overnight at 4 °C with the primary antibody diluted in 2% serum in phosphate-buffered saline (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 1:1,000), 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 h at room temperature. After further washing, antigen:antibody complexes were visualized using a Vectastain ABC peroxidase kit (Vector Laboratories, 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 crossreacts with all sheep cathelicidins (forward: TGT GGC TCC 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, Mannheim, 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-ethylene-diamine-tetraacetic acid 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 ± SD. 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 compared to the control group using t-test. Values of  $P < 0.05$  were considered significant.

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