Endotoxin-induced maturation of monocytes in preterm fetal sheep lung

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Groups of three to seven time-mated ewes received saline or 10 mg of endotoxin (Escherichia coli 055:B5) in saline by intra-amniotic injection for intervals from 1 to 14 days before operative delivery at 124 days of gestational age. Monocytic cells from lung tissue were recovered using Percoll gradients. Monocytic cells consistent with macrophages were identified morphologically and by myosin heavy chain class II expression. An increase in macrophages was preceded by induction of granulocyte-macrophage colony-stimulating factor in the lung and subsequent activation of the transcription factor PU.1. The production of IL-6 by monocytes/macrophages in response to endotoxin challenge in vitro increased 7 and 14 days after exposure to intra-amniotic endotoxin. Recombinant TNF-α induced IL-6 production by lung monocytic cells exposed to intra-amniotic endotoxin but not in control cells. Monocytic phagocytosis of apoptotic neutrophils also increased 7 and 14 days after exposure to intra-amniotic endotoxin. Intra-amniotic endotoxin induced lung monocytes to develop into functionally mature cells consistent with macrophages. These findings have implications for lung immune responses after exposure to chorioamnionitis.

Materials and Methods

Animals. All animals were studied in Western Australia with approval from the animal care and use committees of the Cincinnati Children’s Hospital and the Western Australian Department of Agri-

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culture. Time-mated Merino ewes with singleton fetuses were randomly assigned in groups of three to seven animals to receive a single dose of 10 mg of endotoxin (E. coli 055:B5; Sigma, St. Louis, MO) by intra-amniotic injection for intervals of 1 to 14 days before cesarean delivery at 124 days of gestational age as outlined in Fig. 1. The control group included animals of the same gestational age that received intra-amniotic saline injections (preterm controls). Alveolar macrophages from healthy adult sheep were obtained by bronchoalveolar lavage (BAL) as adult controls. Lung compliance was assessed by the gas volume at 40 cmH\textsubscript{2}O pressure (15). The blood monocyte function was previously reported for some of the groups of animals (24).

Monocyte/macrophage isolation and culture. Following vascular perfusion with Hank’s balanced salt solution (HBSS) of the right lower lobe to remove blood, this lobe was minced thoroughly into fine pieces. Enzymatic digestion was not performed to minimize contamination with lung cells. The minced lung was shaken in HBSS at +37°C for 1 h. The lung suspension was then passed through a 100-μm mesh filter, and the resulting cell suspension was centrifuged twice to recover the cells. The cells were then layered over a two-step Percoll gradient (1.085 and 1.046 g/ml; Sigma–Aldrich) and centrifuged at 400 g for 40 min at 4°C. Monocytic cells were recovered from the interface between the two different Percoll densities, and the cell concentration was adjusted to 2.5 × 10\textsuperscript{6} cells/ml. Macrophages were identified by fluorescence-activated cell sorting (FACS) light-scattering properties (33).

Immunohistochemistry. Cytospins of cells from fetal lung suspension and from the mononuclear cell fractions from the Percoll gradients were stained with the Pappenheim stain to differentiate inflammatory cells. The cells also were stained with an antibody against pan-cytokeratin (Dako, Copenhagen, Denmark) and against prosurfactant protein C to detect epithelial and alveolar type II cell contamination (27). The transcription factor PU.1 was detected on lung sections (5 μm) and for cells after Percoll purification by immunohistochemistry using a polyclonal antibody (sc-225; Santa Cruz Biotechnology). After deparaffinization and rehydration, antigen retrieval was carried out with citric acid buffer, pH 6.0 (19). Endogenous peroxidase activity was blocked with methyl alcohol-hydrogen peroxide. Nonspecific interactions were inhibited with 2% goat serum during both primary and secondary antibody incubation. Slides were incubated overnight at 4°C in a humidified chamber with primary mouse antibodies in blocking solution. Immunostaining was visualized using the Vectastain ABC peroxidase Elite kit to detect the antigen-antibody complexes (Vector Laboratories). The antigen detection was enhanced with nickel-diaminobenzidine, followed by incubation with Tris-cobalt to give a black precipitate. The nuclei were counterstained with Nuclear Fast Red for photomicroscopy.

GM-CSF. Lung expression of GM-CSF mRNA was detected using RT-PCR. Sheep GM-CSF was cloned into pGEM-T vector (Promega, Madison WI) from endotoxin-exposed fetal sheep lung cDNA by PCR using primers complementary to the published sheep GM-CSF sequence (NCBI accession no. X 55991) (35). Identity of ovine GM-CSF was confirmed by double-stranded sequence comparison with the published sequence. Total RNA was extracted from fetal lung tissue by using a modified Chomczynski method (22). GM-CSF was amplified with reverse transcriptase, followed by PCR using sheep-specific primers. The amplified GM-CSF band was initially resolved on an agarose gel, followed by transfer to a nitrocellulose membrane using a Southern blot procedure. To confirm the identity of the PCR bands, the nitrocellulose membrane was reacted overnight with a 32P-labeled radioactive sheep GM-CSF DNA probe, followed by increasing stringency of washes. The bands were then visualized using autoradiography. GM-CSF protein was detected by an ELISA procedure using a high-sensitivity sandwich ELISA assay developed for human GM-CSF (kit HISGM0; R&D Systems, Minneapolis, MN). This kit detects sheep GM-CSF with a lower sensitivity compared with human GM-CSF. Sheep GM-CSF protein was quantified using recombinant sheep GM-CSF standards (kind gift of Dr. Gary Entrican, Edinburgh, UK).

Expression of CD14, Toll-like receptor 4, and myosin heavy chain II. Monocytic cells were exposed in vitro to 100 ng/ml E. coli endotoxin for 6 h. Sheep-specific CD14 endotoxin receptor and myosin heavy chain (MHC) class II antibodies (both obtained from VMRD, Pullman, WA) and a species cross-reacting anti-Toll-like receptor-4 (TLR-4) antibody (BD Biosciences, San Jose, CA) were used to measure surface receptors by FACS analysis using appropriate secondary antibodies. Control staining was performed with isotype antibodies (VMRD) and with secondary antibody alone to evaluate background fluorescence. Cells (monocytes and macrophages) were identified on the basis of forward and side scatter properties on FACS.

IL-6 ELISA. IL-6 was measured in the cell culture medium of cultured monocytes with an ovine-specific ELISA (28) after stimulation with endotoxin or recombinant bioactive ovine TNF-α (15).

Induction of apoptosis and phagocytosis of apoptotic neutrophils. Functional assay of monocytic cell phagocytosis was measured as the ability of these cells to phagocytose apoptotic neutrophils (31, 39). Neutrophils were isolated from the blood of adult ewes using a Percoll gradient (26). Contaminating erythrocytes were removed by osmotic lysis (26). Neutrophil apoptosis was induced by 30 min of UV radiation followed by 3 h of culture (13). Apoptotic cells were stained with fluorescence-labeled annexin V. Necrotic cells were stained with

<table>
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<th>Groups</th>
<th>n</th>
<th>Birth Weight, kg</th>
<th>V40, ml/kg</th>
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<tbody>
<tr>
<td>Adult</td>
<td>8</td>
<td>2.7±0.1</td>
<td>7.9±1.5</td>
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<tr>
<td>Preterm saline control</td>
<td>7</td>
<td>2.4±0.2</td>
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<tr>
<td>Endotoxin (1 day)</td>
<td>7</td>
<td>2.9±0.1</td>
<td>5.5±0.5</td>
</tr>
<tr>
<td>Endotoxin (3 days)</td>
<td>6</td>
<td>2.8±0.1</td>
<td>8.6±1.7</td>
</tr>
<tr>
<td>Endotoxin (4 days)</td>
<td>3</td>
<td>2.9±0.1</td>
<td>8.2±1.6</td>
</tr>
<tr>
<td>Endotoxin (7 days)</td>
<td>6</td>
<td>2.7±0.1</td>
<td>17.1±3.1*</td>
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<tr>
<td>Endotoxin (14 days)</td>
<td>6</td>
<td>2.9±0.1</td>
<td>21.9±2.2*</td>
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</table>

V40, lung gas volume measured at 40 cmH\textsubscript{2}O. *P < 0.05 vs. preterm control.
propidium iodide, and the relative cell populations were determined by flow cytometry (44). After overnight culture, monocytic cells were washed with PBS and new culture medium was added. Apoptotic neutrophils ($2 \times 10^6$ cells/ml) were added to the adherent monocytic cells in culture dishes, incubated for 3 h at 37°C, and washed with ice-cold PBS containing 0.5 mM EDTA. Cells in the culture dishes were fixed and stained for myeloperoxidase, which is predominantly distributed in neutrophils allowing for detection of phagocytosed apoptotic neutrophils (45). Phagocytosis was evaluated by counting apoptotic vesicles in 300 monocytic cells per well as previously described (26).

**Data analysis.** Results are means ± SE. Comparisons between endotoxin-treated groups and untreated controls were performed by analyses of variance with Student-Newman-Keuls tests used for post hoc analyses. Statistical significance was accepted at $P < 0.05$.

**RESULTS**

**Physiological variables at birth.** The animals of different groups had similar birth weights (Table 1). Consistent with our previous results, intra-amniotic endotoxin induced increases in lung gas volumes at 7 and 14 days after exposure (Table 1).

**Inflammatory cells from lung tissue.** Differential counts of cells recovered from the dissociated lung were performed before Percoll density separation. The cells recovered from control lambs were primarily monocytes with very few macrophages, neutrophils, or lymphocytes. Lungs from endotoxin-exposed animals had increased inflammatory cells (Table 2). Following endotoxin exposure, neutrophils increased from $<10^5$ cells/kg body wt to $\sim 2 \times 10^5$ cells/kg at the time intervals studied. Percoll separation yielded monocytic cells with a purity of 94 ± 2% (Fig. 2). Monocyte numbers increased by an order of magnitude or more at 1, 3, and 7 days (Fig. 3). These cells were negative for pan-cytokeratin, excluding epithelial cell contamination, and for prosurfactant protein C, excluding alveolar type II cell contamination (11, 27) (data not shown).

**Endotoxin induction of lung macrophages.** The lung cells purified on Percoll gradients in the control animals predominantly distributed with the forward and side scatter characteristics shown as gate 1 cells (Fig. 3A). In contrast, the FACS dot plot demonstrated a distinct cell population consistent with lung macrophages in the intra-amniotic endotoxin-exposed fetal lungs (shown as gate 2 cells, Fig. 3B).

The absolute numbers of cells in both gate 1 and gate 2 FACS distribution increased three- and fourfold, respectively, 7 days after exposure to intra-amniotic endotoxin. Furthermore, the percentage of cells in gate 2 distribution as a fraction of all cells isolated from Percoll increased twofold 7 days after exposure to intra-amniotic endotoxin (Fig. 3D). Apart from different scatter properties, the expression of MHC class II was also higher in gate 2 cells (310 ± 25 mean fluorescence units) compared with gate 1 cells (95 ± 13 mean fluorescence units) at all time points studied.

**GM-CSF expression.** Control lambs had no detectable GM-CSF mRNA expression in the lung (Fig. 4A). Intra-amniotic endotoxin induced a 50- to 75-fold increase in lung GM-CSF mRNA expression at 1 and 2 days with a return to control levels by 4 and 7 days after intra-amniotic endotoxin exposure. The tissue compartment in which GM-CSF protein expression was induced was determined using ELISA. GM-CSF protein was not detectable in BAL, lung homogenate, amniotic fluid, or plasma from control lambs (Table 3). Intra-amniotic endotoxin induced large increases in GM-CSF protein only in the amniotic fluid and the fetal lungs (BAL and lung homogenate) but not in the fetal plasma. Thus a local (lung and chorioamnion) but not a systemic induction of GM-CSF occurred in response to intra-amniotic endotoxin.

**PU.1 expression.** The transcription factor PU.1 is downstream of GM-CSF signaling in monocyte to macrophage transformation in the lung (3, 39). Cells recovered from Percoll were stained for PU.1. The percentage of PU.1-positive cells

**Table 2. Neutrophils and lymphocytes recovered in cell suspensions after exposure to intra-amniotic endotoxin**

<table>
<thead>
<tr>
<th>Groups</th>
<th>$n$</th>
<th>Neutrophils, $\times 10^6$/kg</th>
<th>Lymphocytes, $\times 10^6$/kg</th>
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</thead>
<tbody>
<tr>
<td>Preterm saline control</td>
<td>7</td>
<td>N.D.</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Endotoxin (1 day)</td>
<td>7</td>
<td>0.8 ± 0.3*</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Endotoxin (3 days)</td>
<td>6</td>
<td>2.7 ± 0.3*</td>
<td>3.2 ± 0.4*</td>
</tr>
<tr>
<td>Endotoxin (7 days)</td>
<td>6</td>
<td>2.1 ± 0.3*</td>
<td>0.5 ± 0.1*</td>
</tr>
<tr>
<td>Endotoxin (14 days)</td>
<td>6</td>
<td>1.8 ± 0.2*</td>
<td>0.9 ± 0.2*</td>
</tr>
</tbody>
</table>

N.D., not detectable. *$P < 0.05$ vs. preterm control.

**Fig. 2.** A: cytopsin of cell suspension recovered from lung before separation on a Percoll gradient after 3 days of intra-amniotic endotoxin exposure. B: cells from the same suspension after recovery from a Percoll gradient. (Pappenheim staining; magnification, ×200).
increased from 4% in preterm controls to 30% 7 days after exposure to intra-amniotic endotoxin (Fig. 4B). Immunohistochemistry of lung sections indicated PU.1 expression in monocytes and neutrophils as identified by nuclear morphology after 2 days of endotoxin-induced chorioamnionitis (Fig. 5, A–D and B, inset). However, 4 and 7 days after endotoxin, PU.1 expression appeared to be confined to monocytes and macrophages in the lung (see large PU.1-positive cells in Fig. 5D, inset).

Immunophenotypic characterization of lung monocytes/macrophages. We measured CD14, TLR-4 and MHC class II expression by the cultured cells after stimulation with 100 ng/ml endotoxin in vitro. CD14 expression was not different between lung monocytc cells from control preterm lambs and adult animals (Fig. 6). CD14 expression increased 14 days after intra-amniotic endotoxin exposure. TLR-4 expression was decreased in cells from the preterm lungs compared with adult alveolar macrophages. TLR-4 expression increased in lung monocytc cells 14 days after intra-amniotic endotoxin (Fig. 6B). MHC class II expression was higher in adult macrophages than in preterm control monocytc cells (Fig. 6C). MHC class II expression increased at 7 and 14 days after intra-amniotic endotoxin.

IL-6 production induced by endotoxin and recombinant TNF-α. Adult macrophages secreted more IL-6 than lung monocytc cells from preterm control lungs after in vitro challenge with endotoxin (Fig. 7A). Fetal lung monocytc cell IL-6 secretion increased at 7 and 14 days after intra-amniotic endotoxin exposure in response to in vitro endotoxin challenge. TNF-α induced little IL-6 production by preterm control lung monocytc cells (Fig. 7B). After exposure to intra-amniotic endotoxin, a robust production of IL-6 by lung monocytc cell was induced 7 and 14 days after endotoxin exposure.

Phagocytosis of apoptotic neutrophils. The phagocytosis of apoptotic neutrophils was decreased in preterm control monocytc cells compared with adult macrophages (Fig. 7C). The number of phagocytosed apoptotic neutrophils in lung monocytc cells 14 days after exposure to intra-amniotic endotoxin increased more than twofold compared with preterm control.
Fetal exposure to chorioamnionitis is a risk factor for developing bronchopulmonary dysplasia, a disease with high morbidity and mortality in preterm infants (20, 40). However, paradoxically, exposure to antenatal inflammation also results in clinical lung maturation with increased airway surfactant pools and improved lung mechanics (18). The common factor to both fetal lung injury and lung maturation is lung inflammation (20). Our group recently demonstrated that recruitment of inflammatory cells to the fetal lung was required for intra-amniotic endotoxin to induce lung maturation (21). We now report a novel finding that exposure of the fetal lung to chorioamnionitis matures mononuclear cells to become macrophage-like cells, which are normally present in very low numbers in the preterm fetal lung and normally only appear after term birth.

Resident alveolar macrophages play an important role in innate immune responses, monocyte trafficking, and modulation of inflammation in the lung (29). In this study we have demonstrated that upon exposure to an inflammatory stimulus from the amniotic fluid, monocytes in the fetal lung mature morphologically to macrophage-like cells. The dynamic pools of the mononuclear and the macrophage-like cells that appear in the fetal lung upon exposure to LPS are probably determined by two linked processes: recruitment of blood monocytes and monocyte maturation to macrophage. These lung macrophages express MHC class II and the transcription factor PU.1, which are consistent with immunophenotypic maturation. Furthermore, these lung cells are capable of IL-6 production when challenged with endotoxin or TNF-α in vitro, indicating functional changes consistent with maturation. Although macrophages were present in FACS analysis 3 days after intra-amniotic endotoxin exposure, the functional changes were evident only 7 days after exposure.

Our group previously reported modulation of peripheral blood monocyte function in these fetal lambs exposed to intra-amniotic endotoxin (24). Three days after exposure to intra-amniotic endotoxin, the peripheral blood monocytes had decreased oxidant and IL-6 responses to endotoxin challenge in vitro. However, blood monocytes increased IL-6 production and had oxidant responses to exogenous endotoxin 14 days after intra-amniotic endotoxin. In this study, the lung monocytic cells had increased IL-6 production and expression of MHC class II to exogenous endotoxin 7 days after exposure to chorioamnionitis, consistent with maturation.

The fetus is not normally exposed to infection and inflammation in the amniotic cavity. Although even very immature fetuses can recruit neutrophils and monocytes to the lung, multiple components of innate host defenses are deficient in fetuses relative to adults (15, 32). The preterm fetal lung contains almost no macrophages or granulocytes, and the macrophages in the fetal sheep do not respond to TNF-α (15).
Normal host defense proteins such as SP-A and SP-D also are present in very low amounts (25). Despite deficiencies in multiple aspects of innate immune function in the fetus and the fetal lung relative to the adult, this study demonstrates that the functional capabilities of the lung monocytes can be changed by antenatal exposure to inflammation. The clinical implication of our study is that exposure to chorioamnionitis can augment macrophage-mediated pulmonary inflammatory responses to other stimuli, leading to increased lung injury responses. Current research on macrophage biology emphasizes the concept of macrophage subdivision into alternatively activated, classically activated, or type II-activated macrophages (7). On this basis, endotoxin and GM-CSF may shape the environmental cues and inflammatory activity of different monocyte/macrophage populations in preterm lamb. Whether prematurely induced macrophages can be protective to the fetus by augmenting innate immune responses is not known. Epidemiological and experimental studies suggest that exposure of the fetus to chorioamnionitis can augment postdelivery lung injury responses to mechanical ventilation (14, 43). Ventilation of the already inflamed preterm lung may contribute to bronchopulmonary dysplasia (43).

There are important limitations of the current study. Because of the lack of cross-reacting antibodies and unavailability of species-reactive antibodies, immunophenotyping of the macrophages could not be accomplished with the resolution possible in the rodent and human. For example, we unsuccessfully tried several anti-CD68 antibodies for these sheep experiments. For the same reason, the precise transition of lung monocytes to lung macrophages could not be accurately quantified and dendritic cells were not identified. The kinetics of trafficking of blood monocytes into the lung compartment was not defined in the study. On the other hand, sheep have been frequently used to characterize primary fetal immune responses to systemic or intra-amniotic injections of antigen (8, 36). Whether the phenotype of monocyte maturation in this study is specific to LPS (TLR-4 activation) or is a more general phenomenon of inflammation from any stimulus remains to be determined. Nevertheless, the study provides novel data on the precocious maturation of lung monocytes in a clinically relevant model of chorioamnionitis.

The critical roles of GM-CSF and the transcription factor PU.1 in monocyte maturation to alveolar macrophages were demonstrated by the absence of functionally mature alveolar macrophages in transgenic mice lacking GM-CSF or PU.1 (39). A role for GM-CSF in surfactant homeostasis was also revealed in these GM-CSF knockout mice, which had abnormal accumulation of surfactant proteins and lipids in the air space (6). This abnormal surfactant accumulation resulted from defective catabolism of surfactant by the alveolar macrophages (49). The alveolar proteinosis in mice could be corrected by lung but not systemic treatment with GM-CSF (38). Consistent with the findings in adult mice, autoantibodies to GM-CSF in humans cause idiopathic pulmonary alveolar proteinosis, resulting in abnormal accumulation of surfactant proteins and the space (41). Therefore, GM-CSF produced locally in the adult lung can induce maturation of lung macrophages, and that GM-CSF is required for normal surfactant homeostasis. We report that GM-CSF protein was present in the BAL fluid and the amniotic fluid but not in the plasma of fetal sheep within 1 day of exposure to endotoxin-induced chorioamnionitis. PU.1 expression was detected in lung monocytes and neutrophils 2 days after endotoxin exposure, whereas mature functional lung macrophages were detected 7 days after endotoxin exposure. These results are consistent with the requirement for GM-CSF and PU.1 for the maturation of monocytes to macrophages in the fetal lung as has been demonstrated in mouse models. Further studies to limit GM-CSF activity in this model may help to better understand the importance of this pathway in the pathogenesis of fetal lung injury after chorioamnionitis.

Fig. 5. Intra-amniotic endotoxin induces PU.1 expression. PU.1 immunostaining was performed on 5-μm fetal lung sections. A: PU.1 expression was not detected in lungs of control animals of the same gestational age. B: PU.1 expression increased within 2 days of exposure to endotoxin-induced chorioamnionitis (IA Endo). C: PU.1 expression persisted for 4 and 7 days. Expression was localized to nuclei of mononuclear cells (filled arrows) and neutrophils (open arrows) at early time points and to mononuclear cells only (filled arrows) at 4 and 7 days. Insets in B and D show higher magnifications. Note the large PU.1 staining in mononuclear cells consistent with macrophage in inset in D. Scale bars, 50 μm.
Intra-amniotic endotoxin results in the phenotype of lung maturation as indicated by increases in surfactant lipids and lung volumes within 5–7 days in fetal sheep (22, 28). However, this is not normal lung maturation, because it is caused by inflammation and results in transient injury to the microvasculature and alveolar septation processes (27, 47). Proinflammatory cytokine mRNA expression (e.g., IL-1), acute phase reactants (e.g., SAA3), and activation of inflammatory cells (e.g., inducible nitric oxide synthase expression) are present in the fetal lung 1–4 days after exposure to intra-amniotic endotoxin (19, 22, 28, 48). Our group’s previous experiments demonstrated that recruitment of systemic neutrophils and monocytes are required for intra-amniotic endotoxin-induced lung maturation (21). The relative importance of neutrophils or monocytes to the lung maturation response is presently unknown. It is also not known whether monocyte “maturation” to macrophages is required for inflammation-induced lung maturation or whether the maturation of the surfactant system and macrophages are parallel processes in the fetal lung exposed to inflammation.

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REFERENCES


