Lipopolysaccharide increases alveolar type II cell number in fetal mouse lungs through Toll-like receptor 4 and NF-κB

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Prince, Lawrence S., Victor O. Okoh, Thomas O. Moninger, and Sadis Matalon. Lipopolysaccharide increases alveolar type II cell number in fetal mouse lungs through Toll-like receptor 4 and NF-κB. Am J Physiol Lung Cell Mol Physiol 287: L999–L1006, 2004; doi:10.1152/ajplung.00111.2004.—Chorioamnionitis is a major cause of preterm delivery. Infants exposed to inflammation in utero and then born preterm may have improved lung function in the immediate postnatal period. We developed a mouse model of chorioamnionitis to study the inflammatory signaling mechanisms that might influence fetal lung maturation. With this in vivo model, we found that Escherichia coli lipopolysaccharide (LPS) increased the number of alveolar type II cells in the fetal mouse lung. LPS also increased type II cell number in cultured fetal lung explants, suggesting that LPS could directly signal the fetal lung in the absence of maternal influences. Using immunostaining, we localized cells within the fetal mouse lung expressing the LPS receptor molecule Toll-like receptor 4 (TLR4). Similar to the signaling pathways in inflammatory cells, LPS activated NF-κB in fetal lung explants. Activation of the TLR4/NF-κB pathway appeared to be required, as LPS did not increase the number of type II cells in C3H-Tlr4null mice, a congenic strain containing a loss of function mutation in Tlr4. In addition, the sesquiterpene lactone parthenolide inhibited NF-κB activation following LPS exposure and blocked the LPS-induced increase in type II cells. On the basis of these data from our mouse model of chorioamnionitis, it appears that LPS specifically activated the TLR4/NF-κB pathway, leading to increased type II cell maturation. These data implicate an important signaling mechanism in chorioamnionitis and suggest the TLR4/NF-κB pathway can influence lung development.

Many premature infants are exposed to inflammation before delivery. Chorioamnionitis is the most common identifiable cause of preterm labor and premature rupture of the amniotic membranes (10). Infants born to mothers with chorioamnionitis have increased morbidity and mortality (12). Despite the negative effects of chorioamnionitis on neonatal outcomes, premature infants exposed to chorioamnionitis have improved lung function in the immediate perinatal period (13, 30). These infants are less likely to display clinical signs of surfactant deficiency than premature infants not exposed to chorioamnionitis. One possibility that might explain this clinical observation is that inflammatory mediators present in chorioamnionitis can accelerate fetal lung maturation. Experimental data in animals support this hypothesis, as exposure to cytokines and bacterial endotoxin increased surfactant synthesis in neonatal rabbits and fetal sheep (5, 7, 21, 22). By understanding how inflammatory signals influence lung development, we may learn more about the basic developmental mechanisms in the fetal lung.

During fetal life, the lung continuously aspirates amniotic fluid during normal breathing movements (8, 23). Microbial particles and substances in the amniotic fluid therefore have access to the fetal airway. Within the airway, infectious particles can activate Toll-Like receptors (TLRs) on the epithelial cell surface. TLR4 is the receptor for lipopolysaccharide (LPS) from gram-negative bacteria (3). TLR activation leads to nuclear NF-κB translocation and production of the innate immune response. In a sheep model of chorioamnionitis, injection of bacterial LPS into the amniotic fluid increases surfactant production and lung growth (21). Separation of the amniotic fluid prevents changes in the lung following amniotic LPS injection (22). Therefore, LPS required direct interaction with the fetal lung to alter development. We wanted to better understand the molecular mechanisms by which inflammatory signaling could influence lung development. To take advantage of the powerful genomic and molecular tools available, we developed a murine model of chorioamnionitis. With this model, we tested the hypothesis that LPS increases alveolar type II cell number through directly activating the TLR4/NF-κB pathway in fetal mouse lung.

METHODS

Antibodies and animals. Goat polyclonal anti-TLR4 (M-16) and rabbit antithyroid transcription factor-1 (TTF-1, H-190) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antisurfactant protein A (SP-A) and antisurfactant protein C (SP-C) were purchased from Research Diagnostics (Flanders, NJ). Rabbit antisurfactant protein B (SP-B) was obtained from Chemicon (Temecula, CA). BALB/cJ and C3H-Tlr4null mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were housed in pathogen-free facilities and mated overnight with embryonic day (E) 0 defined as the day following vaginal plug. All animal procedures and protocols were reviewed and approved by the Animal Care Review Committees at both institutions.

Fetal mouse lung explant culture. E16 mice were euthanized by pentobarbital sodium injection. The fetal lungs were then dissected away from adjacent tissues. Lung tissue was minced into 0.5- to 1-mm3 cubes with fine-tipped scissors under a stereomicroscope. The pieces of lung were placed onto 24-mm clear polyester membrane supports (Transwell, 0.4-μM pore size; Corning, Corning, NY). Culture medium (DMEM) was added only to the basal compartment. The
explicating were cultured in a humidified atmosphere of 95% air-5% CO₂ at 37°C.

In vivo chorioamnionitis model. For the establishment of chorioamnionitis, pregnant mice were injected with phenol-extracted, iron exchange-purified LPS from *Escherichia coli* (055:B5, Sigma L4524; Sigma-Aldrich, St. Louis, MO). E15 mice were anesthetized by pentobarbital sodium injection (50 mg/kg ip). The abdominal wall was infiltrated with 0.1–0.2 ml of bupivacaine. A 1-cm midline abdominal incision allowed externalization of the uterine horns. A fine-tipped pulled glass pipette was used for direct intra-amniotic injection of 5 µl of sterile, endotoxin-free saline or LPS (20 ng/µl) into the amniotic sac of each fetus. After internalization of the uterus, the abdominal wall was sutured in two layers. Mice were returned to their cages, given food and water ad libitum, and monitored for signs of pain or distress. Euthanasia 24–72 h following surgery allowed procurement of amniotic fluid, uterine wall with membranes, placenta, and intact lungs.

During the development of this protocol, we determined that 100 pg/fetus of LPS allowed delivery at term and survival of >95% of the fetal mice. Doses of 5 ng or higher were associated with increased miscarriage and fetal death. Injection of <10 pg/fetus LPS did not consistently cause histological inflammation or elevate cytokines in the amniotic fluid. The number of fetuses in each litter did not seem to correlate with survival or inflammatory response.

**Immunohistochemistry.** Formalin-fixed tissue was paraffin-embedded, sectioned, and affixed to glass slides. In addition to standard immunohistochemical techniques, we routinely performed antigen recovery in sodium citrate (10 mM, pH 6) and quenching of endogenous peroxidase activity using 3% H₂O₂ in methanol (20). Immunostaining was detected using avidin-biotin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA) and diaminobenzidine. Images were captured under a Zeiss Axiovert microscope coupled with a Micropublisher charge-coupled device (CCD) camera (Q Imaging, Burnaby, BC, Canada). For type II cell determination, control, saline-injected, and LPS injected-litters were euthanized at E18. Six separate litters were used for each condition. Three fetal lungs from each litter were processed and stained with the indicated antibodies against surfactant proteins or TTF-1. The investigators recognized the possibility of nonspecific inflammation arising from the injection procedure. However, injection with sterile, endotoxin-free saline did not recruit inflammatory cells to the uterus and elevate cytokines within the amniotic fluid. Twenty-four hours following injection, the placenta, amniotic membranes, and uterine wall were fixed, sectioned, and stained with hematoxylin and eosin. Microscopy revealed inflammatory cells along the uterine wall of LPS-exposed mice (Fig. 1B). We recognized the possibility of nonspecific inflammation arising from the injection procedure. However, injection with sterile, endotoxin-free saline did not recruit inflammatory cells (Fig. 1A). LPS disrupted the normal placental structure and increased fibrin deposition, consistent with inflammatory changes (Fig. 1D) (2). No changes were seen in the placentas of saline-injected controls (Fig. 1C).

Having observed inflammatory changes in the placenta and uterus, we next tested whether LPS could increase cytokine levels in the amniotic fluid. Elevated cytokines in the amniotic fluid might expose the fetal mice and particularly the fetal lungs to an inflammatory environment. We measured IL-1β and TNF-α concentrations in the amniotic fluid of saline-injected and LPS-injected mice 24 h following exposure and from E16 mice that did not undergo a surgical procedure. LPS increased concentrations of each cytokine in the amniotic fluid compared with control mice (Fig. 1, E and F), whereas saline injection did not. These findings collectively demonstrated that LPS injection specifically induced chorioamnionitis in timed pregnant mice.

**RESULTS**

**Intra-amniotic injection of LPS causes chorioamnionitis in mice.** We established a murine model of chorioamnionitis to study how inflammatory signals alter fetal lung development. E15 BALB/cJ mice received 100 pg/fetus of *E. coli* LPS via intra-amniotic injection. This dose of LPS did not cause significant mortality or fetal loss. We tested whether LPS injection would specifically recruit inflammatory cells to the uterus and elevate cytokines within the amniotic fluid. Twenty-four hours following injection, the placenta, amniotic membranes, and uterine wall were fixed, sectioned, and stained with hematoxylin and eosin. Microscopy revealed inflammatory cells along the uterine wall of LPS-exposed mice (Fig. 1B). We recognized the possibility of nonspecific inflammation arising from the injection procedure. However, injection with sterile, endotoxin-free saline did not recruit inflammatory cells (Fig. 1A). LPS disrupted the normal placental structure and increased fibrin deposition, consistent with inflammatory changes (Fig. 1D) (2). No changes were seen in the placentas of saline-injected controls (Fig. 1C).

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test this hypothesis, we measured type II cell number in fetal mouse lungs by immunohistochemistry. Using an antibody against SP-A, we stained fetal mouse lung sections at E18, 3 days following injection of LPS or saline into the amniotic fluid. As shown in Fig. 2, LPS increased the number of SP-A-positive alveolar cells lining the distal airways. LPS also increased the number of cells expressing TTF-1, a marker of alveolar type II cells (Fig. 2). Similar data were obtained using antibodies against SP-B and SP-C (data not shown). LPS...
therefore increased the number of surfactant-expressing alveolar type II epithelial cells within the fetal mouse lung.

LPS might directly signal the epithelia of the fetal lung, or it could elicit a systemic inflammatory response leading to increased type II cell number. We tested whether LPS could induce similar responses in fetal mouse lung explants removed from the systemic circulation. Fetal mouse lung explants cultured for 2–4 days developed alveolar structures closely resembling E18 fetal mouse lungs. Transmission electron microscopy of the explants showed alveolar type II cell differentiation and surfactant secretion (Fig. 3, A and B). We cultured E16 fetal lung explants for 3 days in serum-free media in the presence and absence of LPS (250 ng/ml). Adding LPS to the media increased the number of cells expressing SP-B (Fig. 3D). We obtained similar results using antibodies against SP-A, SP-C, and TTF-1 (not shown). These data indicate that LPS might increase type II cell number by directly interacting with fetal lung tissue.

LPS activates the TLR4/NF-κB pathway in fetal lung. Previous studies in other tissues suggest that LPS initiates an innate immune response through TLR4 (1, 3). If LPS can increase alveolar type II cells by directly signaling the fetal lung, then cells within the fetal lung might express TLR4. To identify these potentially LPS-responsive cells, we stained sections of fetal mouse lung tissue using an antibody against TLR4. We detected immunostaining for TLR4 both in the airways and in distal epithelia and mesenchyme (Fig. 4A). Columnar epithelial cells lining the airway expressed TLR4 at the apical and basal surfaces (Fig. 4A). The distal air spaces and adjacent mesenchyme labeled with slightly less intensity (Fig. 4B). Nonimmune rabbit IgG gave no detectable staining (not shown). We also detected TLR4 expression in E16 fetal lung explants using immunofluorescence. In explants cultured for 3 days, antibodies against TLR4 labeled epithelial and mesenchymal cells in the distal air spaces (Fig. 4C). On the basis of these results, it is possible that cells within the proximal and distal lung express TLR4 and respond to LPS.

In cells that express TLR4 and activate inflammatory pathways, binding of LPS to TLR4 leads to activation and nuclear translocation of NF-κB (32). We wanted to determine whether a similar pathway was involved in our explant model. To measure NF-κB activation, we infected E16 fetal mouse lung explants with recombinant adenovirus expressing the luciferase gene downstream of an NF-κB response element. LPS increased luciferase gene expression in fetal lung explants, with maximal induction at 48 h following LPS exposure (Fig. 5C). Parthenolide, a sesquiterpene lactone inhibitor of NF-κB (9), inhibited the increase in luciferase activity. These data suggest LPS can activate the TLR4/NF-κB signaling pathway in fetal mouse lungs.

**TLR4/NF-κB activation increases type II cell number.** We next wanted to determine whether LPS increases type II cell number through signaling the TLR4/NF-κB pathway. To first test whether functional TLR4 was required in our in vivo chorioamnionitis model, we injected LPS into the amniotic fluid of E15 C3H-Tlr4<sup>Δp-rd</sup> mice. This congenic strain bears a loss of function mutation in the tlr4 allele on a BALB/cJ genetic background (28). In contrast to the BALB/cJ animals, injecting LPS into the amniotic fluid of C3H-Tlr4<sup>Δp-rd</sup> failed to increase the number of type II cells (Fig. 6). These results suggest that LPS signaled through TLR4 to increase the number of type II cells in fetal lung.

Consistent with TLR4 activation, we observed that LPS activated NF-κB in fetal lung explants. Using the NF-κB inhibitor parthenolide, we tested the hypothesis that NF-κB activation is required for the increase in type II cells seen following LPS exposure. Fetal lung explants were cultured for 3 days in the presence of LPS with or without parthenolide. As an additional approach to immunohistochemistry for identifi-
Our findings suggest that LPS directly signals cells within the fetal lung. We observed increased type II cells in fetal lung explants removed from both maternal and fetal circulations. Consistent with our data, experiments in sheep found that isolation of the fetal airway from the amniotic fluid prevented the changes in lung maturation seen with injection of endotoxin (22). Studies in sheep also suggested that maternal hormone production does not mediate the changes in lung development seen with endotoxin (15). Inflammation may also increase the local and systemic production of steroids or growth factors that could influence alveolar development and type II cell differentiation (31). These findings and our present data support the hypothesis that the fetal lung is capable of responding to LPS exposure in the absence of maternal influences.

Cells responsive to LPS express TLR4. We detected TLR4 expression in the epithelia and mesenchyme of fetal mouse lungs. TLR4 appeared to be required for the changes in type II cell number we observed, as LPS had no effect in C3H-TLR4<sup>−/−</sup> mice lacking functional TLR4. These experiments illustrate how our mouse model might be useful for investigating the molecular components of fetal inflammatory signaling. By studying mice containing specific targeted gene disruptions,

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**DISCUSSION**

Compared with premature infants of equal size and gestation born in the absence of inflammation, newborns exposed to chorioamnionitis have a lower incidence of respiratory distress syndrome (13, 30). Consistent with these clinical observations, newborn lambs and rabbits exposed in utero to bacterial endotoxin have increased lung compliance and larger lung volumes (5, 21, 22). We established a mouse model of chorioamnionitis to study the molecular mechanisms by which LPS could increase lung maturation. Our data show that LPS can increase the number of alveolar type II cells both in vivo and in a lung explant model. In the fetal mouse lung, LPS appears to signal through the TLR4/NF-κB pathway, similar to its action in other tissues with innate immune function. This pathway is required for the changes in type II cell number, as LPS had no effect in fetal lung explants treated with an NF-κB inhibitor or in mice lacking functional TLR4.

The proximal airway epithelia stained positive for TLR4 expression at both the apical and basolateral surfaces (A). Background staining using only the secondary antibody is shown in C. Immunofluorescence detected TLR4 expression (magenta) in E16 fetal mouse lung explants cultured for 3 days (D). Nuclei were labeled with 4′,6′-diamidino-2-phenylindole (blue).

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Fig. 4. Toll-like receptor (TLR) 4 was expressed in the proximal and distal fetal lung epithelia. E18 fetal mouse lungs were fixed, sectioned, and labeled with a polyclonal antibody against TLR4 (A and B). Antibody labeling was detected by diaminobenzamidine staining and bright field microscopy. The proximal airway epithelia stained positive for TLR4 expression at both the apical and basolateral surfaces (A). Background staining using only the secondary antibody is shown in C. Immunofluorescence detected TLR4 expression (magenta) in E16 fetal mouse lung explants cultured for 3 days (D). Nuclei were labeled with 4′,6′-diamidino-2-phenylindole (blue).
we might identify the molecular mechanisms of how chorioamnionitis influences fetal lung development.

Chorioamnionitis can arise from a variety of pathogens. We have used *E. coli* LPS in our model of chorioamnionitis as a potent stimulator of the innate immune system, as gram-negative bacteria may comprise a significant percentage of intrauterine infections (27). In addition, the signaling pathways activated by LPS through TLR4 have been well studied. Gram-positive and atypical pathogens can activate common innate immune pathways through TLRs and may induce similar inflammatory responses in the amniotic fluid (1). *Ureaplasma* species can be isolated in chorioamnionitis cases (17), but the chronic inflammatory response to this pathogen may prove difficult to study during the short gestation of the mouse. Cells within the placenta, amniotic membranes, and uterus can all participate in the innate immune response, secreting cytokines and chemokines including IL-8, IL-10, monocyte chemotactransport protein-1, and regulated on activation, normal T-expressed and presumably secreted (RANTES) (6, 25). In addition, fetus-derived neutrophils can occupy the amniotic fluid (18). Although we have shown the fetal lung can directly respond to endotoxin, the contribution of cells in the uterus, placenta, and amniotic membranes could alter how the fetal innate immune system responds to both microbial products and inflammatory mediators.

Intra-amniotic injection of *E. coli* LPS increased inflammatory cytokines, type II cell numbers, and lung compliance in fetal sheep (16, 21). Injection of IL-1, but not TNF-α, also increased surfactant expression, suggesting that signaling through either TLR4 or the IL-1 receptor can stimulate alveolar differentiation. IL-1β also increased surfactant gene expression in neonatal rabbits and rabbit lung explants (5, 7). Similar to the studies in sheep, TNF-α did not increase surfactant expres-
sion in fetal rabbit lungs (26). IL-1 also had a larger effect on fetal rabbit lungs effects compared with newborn animals (11). These findings further suggest inflammatory signaling can influence fetal lung development. The effects of LPS on alveolar type II cell number could also represent a response to injury in the fetal lung. Type II cells proliferate in response to injury in adult lungs. Hyperoxic injury increased type II cell numbers, possibly through the formation of reactive oxygen species and NF-κB activation (19, 29). We have not yet determined whether the effects of LPS in our system result from increased proliferation of type II cells or increased maturation of type II cell precursors. In cultured cells, NF-κB activation can increase SP-A expression through binding upstream elements in the SP-A promoter (14). Inflammatory signals and injury mechanisms could therefore both influence alveolar development.

Rounioja et al. (24) detected changes in cardiac hemodynamics and increased cytokine expression in the myocardium of fetal DBA/2 mice exposed in utero to LPS. They did not report lung inflammation or TLR4 expression in the lungs at days 15–16 of gestation. Differences in gestation and mouse strain may contribute to differences in TLR4 expression during lung development. We have detected TLR4 expression and similar increases in type II cell number following LPS exposure using both BALB/cJ and C57BL/6 mice (not shown). Our data suggest that signaling through a TLR can influence development. While we have used an inflammatory stimulus (LPS) in a model of chorioamnionitis, increased type II cell number occurred in the absence of lung neutrophil influx or gross damage to the epithelia. Activation of the NF-κB pathway in fetal lung cells may represent an additional therapeutic target for increasing the production of surfactant in premature infants.

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