Minimal lung and systemic responses to TNF-α in preterm sheep

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CHORIOAMNIONITIS IS FREQUENTLY associated with preterm deliveries, a decreased incidence of respiratory distress syndrome, but an increased risk of bronchopulmonary dysplasia (BPD) (30, 34). Amniotic fluid aspirated from women with chronic indolent chorioamnionitis contains multiple proinflammatory mediators and white blood cells (WBC) (8, 28). These inflammatory mediators are thought to participate in the injury processes that can result in BPD and brain injury (33, 34). We have modeled subclinical indolent chorioamnionitis in fetal sheep by injecting endotoxin into the amniotic fluid of ventilated preterm lambs and decreased the mRNA for surfactant protein C but increased inflammatory responses to IL-1 and IL-6 in the bronchoalveolar lavage fluid of ventilated preterm lambs after 5 h, 2 days (d), and 7 d. In contrast, IL-1α induced inflammation and lung maturation. TNF-α given into the Airways at birth increased granulocytes in the bronchoalveolar lavage fluid of ventilated preterm lungs and decreased the mRNA for surfactant protein C but did not adversely effect postnatal lung function. An intravascular injection of IL-1α caused a systemic inflammatory response in fetal sheep, whereas there was no fetal response to infravascular TNF-α. Fetal and newborn preterm sheep are minimally responsive to TNF-α. Therefore, the presence of a mediator such as TNF-α in a developing animal does not necessarily mean that it is causing the responses anticipated from previous results in adult animals.

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Recombinant ovine cytokines. The cDNA sequences corresponding to amino acids 78–234 of TNF-α and amino acids 113–268 of IL-1α were cloned into the LIC site of vector pET-30 xα/LIC (Novagen, Madison, WI). Recombinant proteins were expressed in Escherichia coli [BL21(DE3)] by Protein Express (Cincinnati, OH). Purified fusion proteins were cleaved with factor Xa, and the cytokines were eluted from Ni-NTA columns. Cytokines were further purified, quantified by protein assay, and demonstrated to contain <1 ng of endotoxin per dose by the limulus assay. Any residual endotoxin in the cytokine is much less than the minimum dose of 1 mg, which we previously showed to be required to induce lung maturation after intra-amniotic injection in sheep, or 0.1 mg, which induces inflammation in the ventilated preterm lamb lung (13, 18).

Bioactivities of recombinant cytokines. We evaluated the in vitro bioactivity of recombinant TNF-α and IL-1α by assaying
for MAPK phosphorylation and cytokine mRNA expression. We isolated alveolar macrophages from bronchoalveolar lavage fluid (BALF) of healthy adult sheep using Percoll gradients. Macrophages were cultured overnight at 37°C in 5% CO2 in Ham’s F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 1% fetal bovine serum. More than 95% of macrophages remained viable after overnight culture as assayed by the trypan blue dye exclusion test. Cells (2 × 106 cells/ml) were incubated for 10 min with 10 or 100 ng/ml TNF-α or IL-1α. We evaluated phosphorylation of p44/42 MAPK induced by the recombinant cytokines by Western blot using phosphospecific antiphospho-ERK antibody (1:100) and anti-total p44/42 MAPK antibody (New England Biolabs, Beverly, MA). IL-1β mRNA and IL-8 mRNA expression in cultured macrophages was also determined by ribonuclease protection assays as described in assessments of inflammation after 2-h incubation with 1 or 10 ng/ml TNF-α.

Inflammatory responses of adult mouse lungs to TNF-α were evaluated to assess bioactivity in vivo. The amino acid sequences of mouse TNF-α and ovine TNF-α are 75% identical. Recombinant ovine TNF-α was given by intratracheal injection of 7.4 × 105/kg FVH-1 or FVH-100 mg/kg TNF-α was delivered and not ventilated. Because 40 min of ventilation was associated with hypercarbia and decreased lung function (20), we evaluated the response of prematurely delivered newborn lamb lung to TNF-α and IL-1α. The animal protocols were approved by the Animal Use Committees at the Cincinnati Children’s Hospital Medical Center and the Western Australian Department of Agriculture. Chorioamnionitis, lung inflammation, and lung maturation were assessed in fetal sheep that received 140 μg of TNF-α by intra-amniotic injection 5 h, 2 days (d), or 7 d before preterm delivery at 123 d of gestation (13). To compare with TNF-α, responses to 100 μg of IL-1α were evaluated 24 h and 15 d after intra-amniotic injection. We previously reported that IL-1α induced both lung inflammation and maturation 7 d after intra-amniotic injection (32). Date-bred Merino ewes were randomized to cytokine or saline injections, each given in a volume of 2 ml of saline by ultrasound-guided intraamniotic injection (14). To verify intra-amniotic rather than allantoic injection, NaCl and Cl concentration were determined on samples of fluid aspirated immediately before injection (15). The methods for animal protocols used for the present study were the same as used previously for the 7-d IL-1α group (32).

Each ewe was sedated with ketamine (1 g im) and xylazine (25 mg im) followed by spinal anesthesia (2% lidocaine, 3 ml). The fetal head was exposed through maternal midline abdominal and uterine incisions, and amniotic fluid was collected. The fetus was sedated (10 mg/kg ketamine im), and after administering local anesthesia (2% lidocaine sc), we performed a tracheotomy and secured a 4.5-mm endotracheal tube in place. Lung fluid was aspirated by syringe, the animals were delivered, and the umbilical cord was cut. The animals that were not ventilated received a lethal dose of pentobarbital by intravenous injection. After delivery, lambs were weighed.

Premature newborn lambs with antenatal exposure of the intra-amniotic cytokines 7 or 15 d before delivery were ventilated for 40 min to evaluate lung function as described previously (13). Temperature was maintained at 39°C with an overhead warmer and plastic wrap. An arterial catheter was advanced to the level of the descending aorta via an umbilical artery, and lambs were anesthetized with pentobarbital sodium (15 mg/kg). Animals were placed on pressure-limited infant ventilators set to deliver 100% oxygen at a tidal volume of 5 ml/kg, pressure-limited V̇E of 10 breaths per min, and a respiratory rate of 40 breaths per min. Tidal volume was monitored continuously with a neonatal respiration monitor (Acutronic, Baar, Switzerland). Arterial carbon dioxide partial pressure (PaCO2) was measured every 10 min, and PIP was adjusted to maintain adequate ventilation. Other ventilator settings were not altered during the study. The target PaCO2 was 45–50 mmHg; however, animals were permitted to become hypercarbic when the target PaCO2 was not achieved with the maximum V̇E of 40 cmH2O and/or maximum tidal volume of 10 ml/kg. Compliance was calculated by dividing tidal volume by ventilatory pressure (P/PIP-PEEP) and then normalized to body wt in kg (15). Ventilation efficiency index (VEI), an index that integrates ventilation with respiratory support, was calculated according to the formula VEI = 3,800/(PxFxPaCO2), where 3,800 is a carbon dioxide production constant, P is ventilatory pressure, and F is the ventilation rate (26). At 40 min, postdelivery animals were deeply anesthetized with pentobarbital sodium. We degassed the lungs by clamping the endotracheal tube for 5 min. The chest was opened, and the lung was inflated to 40 cmH2O for 1 min and lung volume (VLT) was determined (13).

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Ventilated premature newborn lamb lung responses to TNF-α. To evaluate the response of prematurely delivered newborn lamb lung to TNF-α, we delivered premature lambs at 130-d gestation age by Cesarean section as described above. Before the initiation of ventilation, lambs were given 100 mg/kg surfactant (Survanta; Abbott Laboratories, Columbus, OH) mixed with 50 μg of TNF-α intratracheally or an equal volume of saline (11). Lambs were ventilated for 6 h as described above, except FIO2 was changed to maintain the target PaO2 of 100–200 mmHg (18).

Systemic effects of TNF-α and IL-1α on fetal sheep. Fetal sheep had intravascular catheters placed at 110-d gestation in the systemic effects of TNF-α and IL-1α. At 117 d of gestation, a 50-μg bolus intravenous injection of TNF-α (n = 3) or IL-1α (n = 3) was infused with continuous monitoring of blood pressure and heart rate and collection of fetal blood for pH, blood gas, and lactate measurements for 8 h after the infusion. The results were compared with animals monitored similarly for 8 h after a saline injection (n = 4).

Processing of lungs. We removed the lungs from the chest, weighed each lung, and used the left lung for bronchoalveolar
lavage (BAL) by infusing and withdrawing a sufficient volume of saline at 4°C to fully distend the lungs three times with five separate saline volumes (13). The five BALF were pooled. Tissue from the right middle lobe was used for a dry-wet wt ratio measurement. Tissue from the right lower lobe was frozen in liquid nitrogen for later analysis.

**Assessments of inflammation.** Amniotic fluid was incubated for 30 min at 37°C with 20 mg/ml N-acetyl-L-cysteine, 1 U/ml neuraminidase, and 20 U/ml hyaluronidase (Sigma) to reduce the high viscosity. Cells were isolated from aliquots of amniotic fluid and BALF by centrifugation at 500 g for 10 min, and the pellets were resuspended in PBS. After total cell counts by trypan blue exclusion to identify live cells, differential cell counts were performed on cytospin preparations stained with Diff-Quick (Dade Behring, Düningen, Switzerland). Without liquefying the amniotic fluid, we could not collect cells by centrifugation for cell counts.

A capture ELISA assay was used to measure IL-1α concentration in amniotic fluid. An IgG fraction prepared from rabbit anti-sheep IL-1α antiserum was used for the primary antibody, and guinea pig anti-sheep IL-1α was the secondary antibody as described previously (17). In brief, RNA transcripts of ovine interleukins (IL-1α, IL-6, IL-8) and ovine ribosomal protein L32 as a reference RNA were synthesized with [32P]UTP (Life Sciences Products, Boston, MA) using SP6 or T7 polymerase (Promega, Madison, WI). Aliquots of 10 μg of RNA were hybridized with excess radiolabeled probes for cytokines and L32 at 55°C for 18 h. Single-stranded RNA was digested with RNase A/RNase T1. Protected fragments were electrophoresed on a 6% polyacrylamide-8-mol urea sequencing gel and visualized by autoradiography. The protected bands were quantified on a PhosphorImager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Saturated phosphatidylcholine.** Lipids were extracted from aliquots of the BALF with chloroform-methanol. Saturated phosphatidylcholine (Sat PC) was isolated from lipid extracts by neutral alumina column chromatography after exposure to osmium tetroxide (23). Sat PC was quantified by phosphorus assay (3).

**Surfactant protein mRNA.** The mRNA for surfactant protein (SP)-A, SP-B, and SP-C were measured using S1 nuclease, the protected fragments were resolved on 6% polyacrylamide-8-mol urea sequencing gels, visualized by autoradiography, and quantified.

**Statistical analyses.** Unless otherwise stated, values are given as means ± SE. Normally distributed data were compared between control and treated groups by one-way ANOVA, and post hoc pairwise comparisons were made using Dunnett’s procedure. For data not normally distributed, global comparisons were made by Kruskal-Wallis ANOVA on ranks, and post hoc pairwise comparisons were made using Dunn’s procedure. Statistical significance was accepted for P < 0.05.

**RESULTS**

**Bioactivity of recombinant TNF-α and IL-1α.** Using macrophages isolated from adult sheep BALF, we induced phosphorylation of p44/42 ERK MAPK similarly by the recombinant ovine TNF-α and IL-1α (Fig. 1A). IL-1β mRNA and IL-8 mRNA were induced in cultured sheep macrophages by 2-h incubation with TNF-α (Fig. 1B). TNF-α increased IL-1β mRNA (2.2-fold with 1 ng/ml TNF-α and 6.7-fold with 10 ng/ml TNF-α) and IL-8 mRNA (4.1-fold with 1 ng/ml TNF-α and 6.0-fold with 10 ng/ml TNF-α). Therefore, the recombinant ovine cytokines were bioactive for cells from adult sheep when tested in vitro.

Lungs from adult mice responded to intratracheal instillation or intravascular injections of TNF-α or endotoxin with increased total cell counts in BALF, which were primarily monocytes (Fig. 2). The cell responses to TNF-α and endotoxin were similar. In contrast to the intratracheal endotoxin, TNF-α increased the IL-1β in the mouse lungs eightfold above control levels (P < 0.05). Thus recombinant sheep TNF-α was biologically active when tested in adult mice.

**Responses of fetal sheep to intra-amniotic TNF-α and IL-1α.** Control animals had very few inflammatory cells in amniotic fluid, and intra-amniotic TNF-α did not increase cell numbers (Fig. 3A). In contrast, intra-amniotic IL-1α induced a striking increase in monocytes, lymphocytes, and neutrophils within 24 h (Fig. 3B). The neutrophils decreased at 7 d and returned to control values by 15 d. However, the monocytes and lymphocyte numbers remained elevated in amniotic fluid 15 d after the intra-amniotic IL-1α exposure.

**Fig. 1.** A: Western blot of cell lysate protein from macrophages from bronchoalveolar lavage (BAL) of adult ewes. Phosphorylation of p44/42 MAPK occurred 10 min after exposure to 10 ng/ml or 100 ng/ml recombinant sheep IL-1α or TNF-α. B: increased IL-1β mRNA and IL-8 mRNA in cultured sheep alveolar macrophages 2 h after incubation with 1 ng/ml TNF-α or 10 ng/ml TNF-α.
Intra-amniotic TNF-α did not increase IL-1α in amniotic fluid at 5 h, 2 d, or 7 d as measured by ELISA. In contrast, after intra-amniotic injection of 100 μg of IL-1α, the amniotic fluid levels of IL-1α were 12 ± 2 ng/ml at 24 h, 0.4 ± 0.2 ng/ml at 7 d, and not detectable at 15 d.

Intra-amniotic TNF-α increased monocytes in BALF within 5 h, but there were no significant increases in neutrophils at 5 h or 2 d (Fig. 3C). Compared with the ventilated controls, intra-amniotic TNF-α significantly increased lymphocytes and neutrophils at 7 d, although these increases were small. In contrast, intra-amniotic IL-1α resulted in large increases in monocytes, lymphocytes, and neutrophils in BALF within 24 h, and these cells remained elevated relative to the ventilated control values 15 d after the intra-amniotic IL-1α (Fig. 3D). Cell numbers in BALF from ventilated lambs 15 d after intra-amniotic IL-1α were lower than after the 7-d exposure interval. The fetal lung had a detectable but minimal inflammatory cell response to TNF-α but a large response to IL-1α.

Proinflammatory cytokine mRNA levels for IL-1β, IL-6, and IL-8 in the lung were not increased 5 h or 2 d after intra-amniotic TNF-α compared with the saline controls (Fig. 4). Ventilation for 40 min increased these cytokine mRNAs similarly for both the saline control and the 7-d TNF-α groups. Intra-amniotic TNF-α had no effect on IL-1β, IL-6, or IL-8 mRNA expression in the fetal lung.

TNF-α had no effect on the arterial pH and blood gas values, compliance, VEI, V40, or the amount of Sat PC in BALF for the lambs used for the assessments of lung maturation after 40 min of ventilation (Table 1, Fig. 5). In contrast, blood gas values after 40 min of ventilation for the animals treated with intra-amniotic IL-1α were significantly better than for the control lambs. The IL-1α was associated with the need for less ventilatory pressure, improved respiratory system compliance and VEI, and large increases in V40. Intra-amniotic IL-1α also increased the amount of Sat PC recovered by BAL. Cord plasma cortisol and dry-wet lung wt ratios were not affected by any of the antenatal treatments. Intra-amniotic TNF-α did not increase surfactant protein mRNAs for SP-A, SP-B, or SP-C, and SP-B mRNA was decreased 7 d after the intra-amniotic TNF-α (Fig. 6). Expression of SP-A mRNA and SP-B mRNA was in-
increased eight- and sixfold, respectively, 24 h after intra-amniotic IL-1α injection and returned to control levels at 15d.

Systemic effects of cytokines in fetal sheep. Chronically catheterized fetal sheep were given bolus intravenous doses of 50 μg of TNF-α or IL-1α. Intravenous TNF-α had no effect on fetal blood gas pH values or blood lactate levels (Fig. 7). Mean blood pressures, heart rates, and WBC also did not change. In contrast, the 50-μg dose of IL-1α decreased PO2 and pH and increased blood lactate. Heart rate increased from a control value of 184 ± 19 beats/min to 235 ± 17 beats/min at 1 h (P < 0.05). The WBC count fell from 5.0 ± 1.1 × 10⁶ to 0.46 ± 0.03 × 10⁶ cells/ml (P < 0.05) at 4 h. IL-1α given by intravascular infusion caused a shock-like syndrome in the fetus.

Responses of ventilated premature newborns to intra-tracheal TNF-α. Prematurely delivered newborns at 130-d gestation were given TNF-α mixed with surfactant in the airways before first breath, and lung responses to TNF-α were measured after 6-h ventilation (Table 2). Intratracheal TNF-α did not affect pH, blood gas, ventilatory pressure, compliance, VEI, or V40 values measured from the pressure-volume curves. There were significant increases in neutrophil numbers in BALF (Fig. 8). IL-1β and IL-8 mRNA in lung tissue and in BALF cells were significantly increased by intratracheal TNF-α after 6-h ventilation (Fig. 9). TNF-α mRNA was also increased in lung tissue in the TNF-α-exposed group. The mRNA for SP-C was decreased by the 6-h TNF-α exposure with no changes detected in SP-A or SP-B mRNA (Table 2). In contrast to the lack of changes measured in lung tissue, the mRNA for cytokines IL-1β, IL-6, and IL-8 in lung tissue of animals exposed to intra-amniotic TNF-α or saline. All values are normalized to the ribosomal protein L32 reference mRNA. The value of unventilated (No Vent) control was set to 1.0. Although 40 min of ventilation (Vent) increased cytokine mRNA levels relative to the No Vent group, TNF-α had no effect.

Table 1. Ventilated animals that received intra-amniotic TNF-α, IL-1α, or saline

<table>
<thead>
<tr>
<th></th>
<th>Saline Control</th>
<th>TNF-α 7 d</th>
<th>IL-1α 7 d†</th>
<th>IL-1α 15 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>2.4 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>Cord plasma cortisol, μg/dl</td>
<td>1.05 ± 0.12</td>
<td>0.60 ± 0.20</td>
<td>1.59 ± 0.53</td>
<td>1.83 ± 0.30</td>
</tr>
<tr>
<td>Values after 40-min ventilation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.04 ± 0.05</td>
<td>7.00 ± 0.05</td>
<td>7.24 ± 0.03*</td>
<td>7.32 ± 0.04*</td>
</tr>
<tr>
<td>Pao2, mmHg</td>
<td>95 ± 12</td>
<td>96 ± 9</td>
<td>62 ± 3*</td>
<td>44 ± 4*</td>
</tr>
<tr>
<td>Paco2, mmHg</td>
<td>119 ± 31</td>
<td>75 ± 24</td>
<td>130 ± 35</td>
<td>224 ± 71</td>
</tr>
<tr>
<td>Ventilatory pressure, cmH2O</td>
<td>35.8 ± 0.3</td>
<td>36.7 ± 0.2</td>
<td>20.6 ± 1.2*</td>
<td>25.6 ± 1.1*</td>
</tr>
<tr>
<td>Dry-wet lung wt, g/g</td>
<td>0.112 ± 0.003</td>
<td>0.111 ± 0.007</td>
<td>Not done</td>
<td>0.109 ± 0.003</td>
</tr>
<tr>
<td>Sat PC in BALF, μmol/kg</td>
<td>0.21 ± 0.05</td>
<td>0.12 ± 0.02</td>
<td>9.5 ± 1.8*</td>
<td>1.85 ± 0.38*</td>
</tr>
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</table>

Values are means ± SE. Pao2, partial pressure of arterial carbon dioxide; Paco2, partial pressure of arterial oxygen; Sat PC, saturated phosphatidylcholine; BALF, bronchoalveolar lavage; d, day. *P < 0.05 vs. saline control; †data from previous report (32).
of fetal response to intra-amniotic TNF-α, intratracheal TNF-α increased several indicators of lung inflammation in ventilated premature newborns.

DISCUSSION

Studies of relationships between chorioamnionitis, preterm labor, and fetal injury have focused primarily on IL-1, IL-6, IL-8, and TNF-α as prototype early-response proinflammatory cytokines (8, 29, 34). In clinical chorioamnionitis, cytokines can be elevated for weeks or months. In general, the inflammatory and immune responses of the preterm are blunted relative to the adult because of immature cell responses, different distributions of responsive cells, and multiple other factors (22). To begin to explore how the chorioamnion and the fetus respond to single cytokines, we gave recombinant sheep IL-1α and TNF-α in high dose by intra-amniotic injection.

Median TNF-α levels in amniotic fluid of women with chorioamnionitis were reported to be \(~0.1\) ng/ml (34), and we gave an average dose of 140 μg, resulting in an initial concentration in the amniotic fluid in excess of 1,000 times the median concentration measured clinically. Although these estimates are imprecise, we gave a large dose of TNF-α relative to the concentrations measured in clinical samples.

TNF-α is an early response cytokine that is a potent inducer of a generalized inflammatory response. Adult sheep mount a strong inflammatory response to intravenous injections of recombinant human or ovine TNF-α (15, 21). In the adult sheep, TNF-α and endotoxin induce lung inflammation, alter lung mechanics, increase lung vascular permeability, and alter systemic hemodynamics. We anticipated that TNF-α would be as potent as IL-1α as a proinflammatory mediator in the fetal or newborn lamb and would induce effects similar to endotoxin (19). Bioactivity of the recombinant ovine TNF-α was confirmed in vitro and in vivo. The cytokine induced the phosphorylation of MAPK and proinflammatory cytokines in macrophages.
phages from adult sheep. We did not test the responses of monocytes/macrophages from immature sheep because these animals have very few cells that can be recovered by BAL. The TNF-α also induced lung inflammation in mice similarly to endotoxin and increased IL-1β in the mouse lung, demonstrating in vivo bioactivity.

We expanded our studies of IL-1α and demonstrated that it was a potent inducer of chorioamnionitis and lung inflammation within 24 h and that indicators of inflammation and lung maturation persisted for 15 d. IL-1α also caused a systemic inflammatory response when administered intravenously to fetal sheep. Therefore, the animals were clearly capable of developing the anticipated inflammatory and systemic responses to this potent proinflammatory cytokine.

Relative to the responses that we previously demonstrated for IL-1α and endotoxin, fetal sheep at 125-d gestation are quite unresponsive to TNF-α. Intra-amniotic TNF-α did not cause chorioamnionitis, but it did induce small increases in inflammatory cells in the fetal lung. The assessments of inflammation in response to TNF-α at 5 h and 2 d bracketed the time of severe inflammation induced by IL-1α. Because inflammatory responses to intra-amniotic endotoxin and IL-1α persist for weeks, we do not think that we missed an inflammatory response of the fetal lung to TNF-α. More striking increases in neutrophils in BALF were measured 6 h after intratracheal TNF-α in 130-d gestation ventilated preterm lambs, and these increases were accompanied by increases in cytokine mRNA expression in the lungs. These small responses demonstrate that the preterm sheep is not completely unresponsive to TNF-α. Nevertheless, the very modest responses demonstrate that TNF-α is not likely to be a major contributor to chorioamnionitis and inflammation of the fetal or premature newborn sheep lung. No similar information is available for the human, although TNF-α is increased in preterm infants and ventilated baboons that subsequently develop BPD (5, 16). Our study demonstrates that mediators of biological effects in the preterm cannot be extrapolated from information obtained from adult animals.

Table 2. Prematurely delivered lambs given intra-tracheal TNF-α or saline at birth followed by 6-h ventilation

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Control</th>
<th>TNF-α</th>
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<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Body wt, kg</td>
<td>3.1 ± 0.3</td>
<td>3.3 ± 0.2</td>
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<tr>
<td>Values after 6-h ventilation</td>
<td></td>
<td></td>
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<tr>
<td>pH</td>
<td>7.29 ± 0.02</td>
<td>7.31 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>PaCO2, mmHg</td>
<td>55 ± 2</td>
<td>49 ± 5</td>
<td></td>
</tr>
<tr>
<td>PaO2/FiO2</td>
<td>221 ± 55</td>
<td>202 ± 69</td>
<td></td>
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<tr>
<td>Ventilatory pressure, cmH2O</td>
<td>18.2 ± 0.6</td>
<td>22.5 ± 1.6</td>
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<tr>
<td>Compliance, ml/cmH2O·kg</td>
<td>0.42 ± 0.02</td>
<td>0.41 ± 0.03</td>
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<tr>
<td>Lung volume at 40 cmH2O, ml/kg</td>
<td>45.1 ± 2.1</td>
<td>38.9 ± 2.9</td>
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<tr>
<td>Surfactant protein mRNA</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(relative to control)</td>
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<tr>
<td>SP-A</td>
<td>1.0 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>SP-B</td>
<td>1.0 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td></td>
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<tr>
<td>SP-C</td>
<td>1.0 ± 0.1</td>
<td>0.6 ± 0.1*</td>
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</table>

Values are means ± SE. FiO2, fraction of inspired oxygen. SP, surfactant protein. *P < 0.05 vs. saline control.

flamatory responses to intra-amniotic endotoxin and IL-1α persist for weeks, we do not think that we missed an inflammatory response of the fetal lung to TNF-α. More striking increases in neutrophils in BALF were measured 6 h after intratracheal TNF-α in 130-d gestation ventilated preterm lambs, and these increases were accompanied by increases in cytokine mRNA expression in the lungs. These small responses demonstrate that the preterm sheep is not completely unresponsive to TNF-α. Nevertheless, the very modest responses demonstrate that TNF-α is not likely to be a major contributor to chorioamnionitis and inflammation of the fetal or premature newborn sheep lung. No similar information is available for the human, although TNF-α is increased in preterm infants and ventilated baboons that subsequently develop BPD (5, 16). Our study demonstrates that mediators of biological effects in the preterm cannot be extrapolated from information obtained from adult animals.

There is no specific information about how TNF-α affects the fetus outside of its presence as a component of inflammatory responses. Chronic overexpression of TNF-α in transgenic mice with the lung-specific SP-C promoter resulted in progressive fibrosing alveolitis (24). The mice that died at birth seemed to respond to TNF-α with lung injury during late gestation. Our results with sheep suggest a late gestational development of the receptors and/or cellular elements responsible for TNF-α signaling because we found no responses to intravascular infusion of fetuses with TNF-α at 118-d gestation, very minimal responses to fetal exposures at preterm delivery at 125-d gestation, and a cytokine and inflammatory cell response at 130-d gestation.

![Fig. 8. Inflammatory cells in BALF after intratracheal TNF-α followed by 6-h ventilation. Relative to control ventilated lambs, TNF-α increased the neutrophil numbers. *P < 0.05 vs. control.](http://ajplung.physiology.org/)

![Fig. 9. Steady-state mRNA levels for IL-1β, IL-8, IL-10, and TNF-α after intratracheal TNF-α injection followed by 6 h of ventilation in lung tissue (A) and cells from BALF (B). All values were normalized to the mRNA for L32 and the control values were given a value of 1.0. *P < 0.05 vs. control.](http://ajplung.physiology.org/)
We were particularly interested in the possible effects of TNF-α on the surfactant system. In the mature lung, TNF-α decreases the mRNA for both SP-B and SP-C (1, 27). The effects of IL-1α on the surfactant proteins depend on the developmental stage of the lung. In the preterm fetal sheep in vivo, IL-1α induces a persistent increase in the surfactant protein mRNAs as it does in preterm rabbit and explants of rabbit lungs (4, 7, 32). However, at later gestation and after term, IL-1α decreases surfactant protein mRNA levels. In the fetal sheep, we found a significant decrease in SP-B mRNA 7 d after intra-amniotic TNF-α. In the ventilated preterm lamb, the TNF-α decreased SP-C mRNA. These effects are consistent with the effects of TNF-α on the mature lung.

These studies were done on sheep without exposure to other factors that might mature the TNF-α response pathway, which is an important qualifier to the conclusion that fetal sheep and newborn preterm lambs respond only minimally to TNF-α. For example, chronic indolent chorioamnionitis may persist for weeks without the onset of preterm labor, and it is associated with increased fetal cortisol levels in the human (31). Women at risk of preterm delivery routinely receive antenatal glucocorticoid treatments that not only induce lung maturation but may alter fetal immune responses as well (9). A productive research area is an examination of whether antenatal inflammatory exposures and maturational treatments alter subsequent immune and inflammatory responses in the fetus. For example, we recently reported that intra-amniotic endotoxin given to sheep 30 d before preterm delivery altered the inflammatory responses to mechanical ventilation (10). Although anti-TNF-α therapies have been explored for a variety of inflammatory diseases, this study suggests that TNF-α may not be an important mediator in the preterm.

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