IL-8 signaling does not mediate intra-amniotic LPS-induced inflammation and maturation in preterm fetal lamb lung

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Kallapur SG, Moss TJ, Auten RL, Nitsos I, Pillow JJ, Kramer BW, Maeda DY, Newnham JP, Ikegami M, Jobe AH. IL-8 signaling does not mediate intra-amniotic LPS-induced inflammation and maturation in preterm fetal lamb lung. Am J Physiol Lung Cell Mol Physiol 297: L512–L519, 2009. First published July 2, 2009; doi:10.1152/ajplung.00105.2009.—Preterm infants exposed to chorioamnionitis and preterm sheep fetuses exposed to intra-amniotic (IA) LPS have lung inflammation, increased IL-8 levels, and lung maturation. We tested the hypothesis that IL-8 signaling mediates IA LPS-induced lung inflammation and lung maturation. Two strategies were used: 1) we tested if IA injection of recombinant sheep IL-8 (rsIL-8) induced fetal inflammation and 2) if IL-8 signaling was blocked by a novel CXCR2 receptor blocker, nicotinanilide thioglycolate methyl ester (NTME). To test effects of IL-8 in the fetus, rsIL-8 was given intravascularly (50 μg) at 124 ± 1 day of gestation (term = 150 days). A separate group of sheep was given IA rsIL-8 (100 μg) and delivered 5 h to 7 days later at 124 ± 1 day of gestation. After confirming efficacy of the CXCR2 inhibitor, effects of IL-8 blockade were tested by injecting fetal sheep intramurally with NTME (10 mg) before IA injection of Escherichia coli LPS (10 mg). Sheep fetuses were delivered 1 or 7 days after injections at 124 ± 1 day of gestation. IA rsIL-8 induced a modest fivefold increase in bronchoalveolar lavage (BAL) monocytes and neutrophils and increased lung monocyte hydrogen peroxide generation. However, rsIL-8 did not induce lung maturation. Intravascular rsIL-8 did not change fetal cardiovascular variables, blood pH, or blood leukocyte counts. Inhibition of CXCR2 decreased IA LPS-induced increases in BAL proteins at 1 day but not at 7 days. NTME did not significantly decrease IA LPS-induced BAL leukocyte influx and lung cytokine mRNA expression. Inhibition of CXCR2 did not change IA LPS-induced lung maturation. IL-8 signaling does not mediate LPS-induced lung inflammation and lung maturation.

Prematurity; respiratory distress syndrome; bronchopulmonary dysplasia; fetal inflammatory response syndrome; CXCR2

CHORIOAMNIOTIS, defined as inflammation of the fetal membranes, complicates up to 70% of preterm deliveries before 30 wk of gestation (12). The epidemiological associations of chorioamnionitis are fetal inflammation (13, 35), a decreased incidence of respiratory distress syndrome (2, 39), and an increased risk of brain injury, necrotizing enterocolitis, and bronchopulmonary dysplasia (1, 2, 13, 38). While the mechanisms of organ injury associated with chorioamnionitis remain undetermined, components of the fetal inflammatory response are thought to play a major role in the pathogenesis (35). A number of cytokines, notably IL-1, IL-6, IL-8, and TNFα, have been implicated in the pathogenesis of chorioamnionitis-induced fetal inflammation (3, 40, 45). In one study, IL-8 was implicated in the pathogenesis of chorioamnionitis induced by Ureaplasma species, the most common microbe cultured in women with chorioamnionitis (42).

IL-8 belongs to the C-X-C family of chemokines with a diverse range of biological functions that includes chemotaxis, generation of reactive oxygen species, cell adhesion, release of azurophil granules, and promotion of angiogenesis (14). Although IL-8 is frequently recognized as a neutrophil-specific chemokine, it also modulates the function of a variety of inflammatory cells including T and B lymphocytes, basophils, NK cells, eosinophils, and monocytes (33). IL-8 signals via the G protein-coupled receptors CXCR1 and CXCR2. IL-8-mediated degranulation is attributed to signaling via CXCR1, whereas chemotaxis is mediated by CXCR2 (19). Besides IL-8, other chemokines such as GROα and β, NAP-2, ENA-78, and GCP-2γ also utilize CXCR2 for signal transduction (19). Therefore, blockade of the CXCR2 receptor has the advantage of simultaneously blocking related chemokines. Inhibition of CXCR2 significantly reduced hyperoxia-mediated lung inflammation in neonatal rats (4, 44). A series of small-molecule compounds, nicotinanilides, are known to be potent inhibitors of neutrophil chemotaxis (8). Nicotinanilide thioglycolate methyl ester (NTME) is a specific CXCR2 inhibitor with biological activity in the nanomolar range. Upon entry in neutrophils, NTME is cleaved by intracellular esterases to its corresponding acid, nicotinanilide thioglycolic acid (NTA), which mediates the biological activity (Maeda, unpublished observations).

In fetal sheep, intra-amniotic (IA) injection of LPS causes chorioamnionitis and lung inflammation and induces lung maturation (5, 18). IA LPS robustly induces IL-8 expression in the chorioamnion and fetal lung (27). IA injection of Ureaplasma, commonly associated with prematurity, also induces IL-8 expression and lung maturation in fetal sheep (31). However, the specific role of IL-8 in IA LPS-induced lung/systemic inflammation or lung maturation is not known.

We tested the hypothesis that IL-8 mediates IA LPS-induced pulmonary inflammation and lung maturation. Using a gain of function strategy, we tested if IA injection of recombinant sheep IL-8 (rsIL-8) induced lung inflammation and lung maturation. To test loss of function, NTME was used to block the
CXCR2 receptor in preterm fetal lambs exposed to IA Escherichia coli LPS.

MATERIALS AND METHODS

Animals. The animals were studied in Western Australia with approval from the animal care and use committees of the Cincinnati Children's Hospital (Cincinnati, OH), the Department of Agriculture and Food (Western Australia), and the University of Western Australia. Some of the animals used in this study (control and 7-day LPS group in Table 2) were also used in a previously reported study (26). In separate protocols, time-mated Merino ewes with singleton fetuses were randomly assigned to different study groups of 4–10 animals (Tables 1 and 2). A separate group of preterm lambs (n = 3 for IL-8, n = 2 for saline controls) had fetal arterial catheterization for the intravascular study. There were no fetal deaths. The ewes were killed with a penetrating captive bolt or heavily anesthetized with ketamine and medetomidine followed by the delivery of the fetus. The fetuses were given lethal intravascular doses of pentobarbital.

IL-8 and NTME. Full-length sheep IL-8 (36) was cloned, and rsIL-8 was custom synthesized by Protein Express (Cincinnati, OH) using bacterial expression followed by removal of residual endotoxin. NTME (Syntrix Biosystems, Auburn, WA) is an allosteric inhibitor of CXCR2 signaling. To test efficacy of rsIL-8 and NTME in vitro, sheep neutrophils were recovered by Percoll density gradient separation of blood followed by osmotic red blood cell lysis (22). The final purity was >87% live neutrophils. The efficacy of rsIL-8 (300 nM) to prime the neutrophil respiratory burst was tested by measuring reactive oxygen species generation. Neutrophils (1 × 10^5 cells/well) were incubated with 2 μM H_2DCFDA (Invitrogen, Carlsbad, CA) in KRPG buffer for 30 min at 37°C along with rsIL-8. Phorbol myristate acetate (PMA) was then added to a final concentration of 10 ng/ml, and fluorescence was measured (excitation 480 nm, emission 530 nm) at 30 min with a plate reader equipped with dual monochromators (Safire; TECAN, Vienna, Austria). To test the efficacy of CXCR2 inhibitors (NTME and NT), inhibition of rsIL-8-induced chemotaxis was measured using a modified Boyden chamber (Neuroprobe #101-3, Gaithersburg, MD) as described (10). Sheep neutrophils were incubated with 2 μM calcine acetoxyethyl succinate (Invitrogen) and CXCR2 inhibitors at the designated concentrations for 30 min at 37°C. Purified sheep neutrophils were then added at a concentration of 3 × 10^5/ml to the upper chamber (30 μl volume). The lower chamber contained rsIL-8 (50 nM, 29 μl volume) with the CXCR2 inhibitor. Chemotaxis through a 3-μm polycarbonate membrane was allowed for 30 min at 37°C. After wiping the top surface of the membrane, the migrated neutrophils were quantified by measuring calcine fluorescent intensity (excitation 490 nm, emission 520 nm) in the cells on the undersurface of the membrane with the plate reader.

Treatments and procedures. To test the systemic effects of IL-8, fetal lambs had fetal arterial and venous catheters placed surgically (26). After the recovery period, fetal lambs at 124 ± 1 day gestational age (GA) had arterial catheter connected to a pressure transducer to continuously measure blood pressure and heart rate. An intravenous bolus infusion of rsIL-8 (50 μg/fetus, ~20 μg/kg) or saline was given. Serial samples of fetal arterial blood (2.5 ml) were drawn for measurement of blood pH and PCO_2, white blood cell counts, plasma glucose, and lactate for 24 h after injection.

All animals were delivered at 124 ± 1 day of gestational age. *P < 0.05 vs. controls. †P < 0.05 vs. corresponding LPS group. NTME, nicotinanilide thiglycolate methyl ester, a CXCR2 receptor blocker; IM, intramuscular.

For the CXCR2 inhibitor studies, fetal lambs received 10 mg (~4 mg/kg) of NTME in 1 ml of DMSO or 1 ml of DMSO as the vehicle by fetal intramuscular injection 3 h before IA LPS (10 mg E. coli LPS; 055:B5; Sigma, St. Louis, MO). The animals that were delivered 1 day of gestational age were ventilated without prior surfactant treatment (24). For the CXCR2 inhibitor studies, fetal lambs received 10 mg (~4 mg/kg) of NTME in 1 ml of DMSO or 1 ml of DMSO as the vehicle by fetal intramuscular injection 3 h before IA LPS (10 mg E. coli LPS; 055:B5; Sigma, St. Louis, MO). The animals that were delivered 1 day of gestational age were ventilated without prior surfactant treatment (24). For the CXCR2 inhibitor studies, fetal lambs received 10 mg (~4 mg/kg) of NTME in 1 ml of DMSO or 1 ml of DMSO as the vehicle by fetal intramuscular injection 3 h before IA LPS (10 mg E. coli LPS; 055:B5; Sigma, St. Louis, MO). The animals that were delivered 1 day of gestational age were ventilated without prior surfactant treatment (24).
day later received only one injection of NTME. The animals delivered 6–7 days after the initial injection received an additional 10 mg of NTME in DMSO or DMSO only by fetal intramuscular injections 2 and 4 days after the first dose. Some animals received NTME in DMSO only to assess any effects of the drug. All fetuses were surgically delivered at 124 ± 1 day of gestation.

Tissue processing at delivery and blood counts. For bronchoalveolar lavage (BAL), the left lung was instilled with normal saline to total lung capacity followed by withdrawal, and the procedure was repeated three times (18). BAL fluids (BALF) were pooled and used for cell counts and saturated phosphatidylcholine analysis. BALF cell counts were expressed as total cells recovered from the lavage normalized to body weight. A portion of the right lower lobe of the lung was snap-frozen for RNA extraction. Automated total white blood cell counts and differential counts were performed with correction for nucleated red blood cells.

Lung monocyte culture and hydrogen peroxide generation. Following vascular perfusion of the right lower lobe with HBSS to remove blood, the fetal lung was chopped thoroughly into fine pieces and incubated in HBSS with shaking at 37°C for 1 h (29). The lung suspension was then gently passed through a 100-μm mesh filter, and the suspension was washed twice with PBS. Cells from the suspension were then layered using trypsin blue to evaluate viability and then plated in culture dishes using media supplemented with 10% heat-inactivated fetal calf serum (Sigma Chemical). After incubation at 37°C for 2 h, nonadherent cells were removed, and plates were washed twice with PBS. To estimate the number of monocytes, cells were scraped from the culture dishes, and differential cell counts were performed on cytospin preparations stained with DiffQuick (Baxter Healthcare, McGaw Park, IL). The adherent cell population was 90 ± 3% monocytes. Production of hydrogen peroxide by the cultured lung monocytes was measured with an assay based on the oxidation of ferrous iron (Fe2+) to ferric iron (Fe3+) by hydrogen peroxide under acidic conditions (Bioxytech H2O2-560 assay; OXIS International, Portland, OR).

Cytokine mRNA quantitation. Total RNA was isolated from the snap-frozen lung samples using a modified Chomzynski method (27). Ten micrograms of total RNA was used for IL-1β, IL-6, IL-8, and serum amyloid A3 quantitation using RNase protection analysis as previously described (27, 41). Briefly, solution hybridization was performed for 16 h using a molar excess of αP32 UTP-labeled riboprobes. Unhybridized single-strand RNA was digested with RNase A/T1 (Pharmingen, San Diego, CA). RNase was then inactivated, and protected RNA was precipitated using the RPA III inactivation buffer (Ambion, Austin, TX). The ribosomal protein mRNA L32 was used as an internal control (23). The protected fragments were resolved on 6% polyacrylamide 8 mol/l urea gels, visualized by autoradiography, and quantified on a Phosphorimager using Image-Quant v1.2 software (Molecular Dynamics, Sunnyvale, CA).

Evaluation of lung maturation. Surfactant lipids and lung compliance were measured to evaluate lung maturation. Saturated phosphatidylcholine (Sat PC) was isolated from chloroform-methanol (2:1) extracts of BALF by neutral alumina column chromatography after exposure of lipid extracts to osmium tetroxide (30). Sat PC was quantified by phosphorus assay (6, 30). Lung compliance was evaluated by measuring the deflation limb of a pressure-volume curve with the chest open (27).

Fig. 2. Intra-amniotic (IA) rsIL-8 infusion induced mild lung inflammation but did not change lung gas volumes. Time course following IA rsIL-8 injection. Bronchoalveolar lavage fluid (BALF) cells expressed per kg body weight unventilated lambs 5 h or 2 days after rsIL-8 exposure (A) and ventilated lambs 7 days after rsIL-8 exposure (B). Hydrogen peroxide generation by lung monocytes expressed per kg body weight unventilated lambs (C) and lambs after 40-min ventilation (D). Saturated phosphatidylcholine (Sat PC) concentration in BALF normalized to body weight (E) and deflation limb pressure-volume curves (F) 7 days after exposure. N = 4–8 animals/group; C, control; *P < 0.05 vs. control.
Plasma NTME and NTA levels. The drug was extracted from sheep plasma using ethyl acetate buffer. Plasma NTME (mol wt 320.9) and its active metabolite NTA (mol wt 306.9) were measured by LCMS, tandem mass spectroscopy using C18 cartridges. The mobile phase utilized the following buffers: phase A, 10 mM ammonium acetate; phase B, 100% methanol. NTME and its corresponding acid, NTA, were detected on the basis of unique daughter ion peaks. These compounds were quantitated using internal standards (synthetic compound) with similar molecular weight and solubility profiles.

Data analysis. Results are given as means ± SE. Comparisons among three or more groups were performed by ANOVA with Student-Newman-Keuls tests used for post hoc analyses. Comparison of two groups was done by a nonparametric t-test (Mann-Whitney U-test). Statistical significance was accepted at P < 0.05.

RESULTS

Validation of rsIL-8 and NTME. The rsIL-8 stimulated adult sheep neutrophils to produce oxidants equivalently to PMA alone in vitro (Fig. 1A). As expected, the combination of rsIL-8 and PMA further stimulated oxidant production, consistent with a priming effect. rsIL-8 also increased neutrophil chemotaxis, and this effect was blocked by the pro-drug NTME and its active metabolite acid NTA at concentrations of 50–200 nM (Fig. 1B). Therefore, the rsIL-8 and CXCR2 blockers were bioactive for adult sheep neutrophils.

Responses to IA rsIL-8. rsIL-8 was given by IA injection to test its potential to cause lung inflammation in the fetus. Early lung inflammation was assessed after 5-h and 2-day exposures, whereas the late inflammatory and lung maturation effects were measured 7 days after the IA rsIL-8. The lambs delivered 7 days after exposure were mechanically ventilated for 40 min to evaluate lung function and to assess possible synergy between the IL-8 and ventilation for lung inflammation. The IA rsIL-8 increased the amount of protein recovered in BALF (Table 1). No neutrophils and very few monocytes were detected in the BALF of control lambs (Fig. 2A). The rsIL-8 recruited 44 × 10^4/kg monocytes and 3 × 10^5 neutrophils/kg to the air spaces by 5 h. Two days after the IA rsIL-8 exposure, the airway leukocyte counts were similar to those at 5 h. These cell counts are ~100 times lower than the inflammatory cells induced by IA LPS or IL-1α (27). After 40 min of mechanical ventilation, BALF monocyte counts in control lambs increased 10-fold to 7 × 10^5/kg with smaller increases in neutrophils. Preterm lambs exposed to rsIL-8 7 days before delivery had a further 30-fold increase in BALF neutrophils to 94 × 10^4/kg and no increases in BALF monocyte counts compared with the ventilated control lambs. Hydrogen peroxide generation by lung monocytes was highest at 5 h in response to IA rsIL-8 and decreased 2 days after exposure (Fig. 2C). An increase in hydrogen peroxide production was also evident for monocytes exposed 7 days previously to rsIL-8, followed by brief mechanical ventilation (Fig. 2D). The IA IL-8 induced modest increases in protein and inflammatory cells in BALF, and the monocytes were activated as they produced more hydrogen peroxide than control cells. This inflammatory response to IA rsIL-8 did not increase Sat PC in BALF or improve the pressure-volume curve of the fetal lung, indicating no maturational effect (Fig. 2, E and F).

Fetal responses to intravascular IL-8. Intravascular rsIL-8 was given to preterm fetal lambs to assess systemic effects over 24 h after infusion. Using regression analysis, we compared the trends in controls vs. rsIL-8-infused animals for changes in physiological variables. Heart rate or blood pressure did not change over the 8 h following the rsIL-8 infusion (Fig. 3). Blood pH and lactate did not change for 24 h after infusion. Compared with rsIL-8-infused animals, blood glucose levels were higher in the controls throughout the experimental period. However, there were no differences between the two groups in trends for blood glucose. Total peripheral blood white blood count and neutrophil count also did not change in response to intravascular rsIL-8 infusion (Fig. 4). Thus, there were no fetal responses to intravascular rsIL-8.

Effect of CXCR2 inhibitor on IA LPS. IA LPS induces a rapid and high expression of IL-8 in fetal sheep (27). We assessed the response to blockade of the CXCR2 receptor by fetal treatments with NTME (Table 2). We dosed the receptor
blocker based on its ability to inhibit chemotaxis of neutrophils from adult sheep (Fig. 1) and measurements of blood levels of NTME or NTA with a target of minimum plasma drug level of 100 nmol. The pro-drug (NTME) was barely detectable in fetal plasma 1 day after treatment. In contrast, the plasma levels of the metabolite NTA were $549 \pm 59$ nmol at 1 day ($n = 10$) and $578 \pm 3$ nmol at 6 days ($n = 9$). Interestingly, the corresponding amniotic fluid levels after drug exposure were $1,539 \pm 226$ nmol at 1 day ($n = 5$) and $1,465 \pm 170$ nmol at 6 days ($n = 11$). The threefold higher level in the amniotic fluid compared with the plasma suggests that the drug is cleared by renal excretion with a slower clearance from the amniotic fluid. There were no apparent fetal effects of the fetal injections of NTME in DMSO only.

Consistent with our previous results (27), neutrophils and monocytes in BALF increased 1 and 7 days after IA LPS (Fig. 5, A and B). Pretreatment with NTME did not significantly decrease leukocyte recruitment to the BALF by LPS. Previous experiments showed maximum proinflammatory cytokine mRNA expression 1–2 days after IA endotoxin with a return to control levels after 4–7 days (27). Consistent with our previous experiments (24, 41), IA LPS induced IL-1$\beta$ ~25-fold, IL-6 and IL-8 ~8-fold, and the acute phase reactant serum amyloid A3 100-fold compared with controls (Fig. 5, C–F). Pretreatment with NTME did not decrease LPS-induced lung cytokine and acute phase reactant mRNA expression. IA LPS caused leukopenia at 1 day and leukocytosis 7 days after injection (Table 3), and NTME did not alter these systemic responses to IA LPS at 1 day but did attenuate LPS-induced increases in blood monocyte counts at 7 days. IA LPS induced a large lung maturation response as indicated by increases in Sat PC in BALF and increases in lung gas volumes (Fig. 6). Similar to the LPS-only group, the LPS + NTME animals also had increased Sat PC and high lung volumes, indicating that IL-8 signaling did not mediate LPS-induced lung maturation.

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**Fig. 4.** Intravascular rsIL-8 infusion did not change blood leukocyte counts. Time course of peripheral blood total white blood cell (WBC) count and neutrophil count after a single fetal intravascular injection of 50 μg of rsIL-8/fetus.

**Fig. 5.** CXCR2 inhibitor did not decrease IA LPS-induced lung inflammation. BALF neutrophils (A) and monocytes (B) expressed per kg body weight 2 and 7 days after exposures. Quantification using RNase protection assays for IL-1$\beta$ (C), IL-6 (D), IL-8 (E), and serum amyloid A3 (SAA3) mRNA (F) from fetal lung normalized to L32 (ribosomal protein mRNA) 1 day after exposures. The mean mRNA signal in control animals was given the value of 1, and levels at each time point were expressed relative to controls. NTME did not significantly decrease LPS-induced lung inflammation. $N = 5$–8 animals/group, *$P < 0.05$ vs. control.
**DISCUSSION**

IA LPS induces lung inflammation, lung maturation, and lung remodeling in preterm fetal sheep (20, 21). We tested the role of IL-8 in the mediation of lung injury and maturation in a model resembling human disease. The experiments are unique because the animals are preterm fetuses, and the route of administration of rsIL-8 or LPS is IA. In this experimental system, the proinflammatory mediator(s) contacts the airway epithelium to elicit fetal responses (32, 37). An additional feature of this experiment is that airway exposure to LPS or rsIL-8 occurs in the absence of other confounding stimuli such as hyperoxia or stretch from ventilation. Using IA injection of the agonist IL-8 and a CXCR2 receptor blocker, we demonstrate that IL-8 is not an important mediator of LPS-induced lung inflammation or lung maturation in fetal sheep.

Although preterm lambs have a robust recruitment of neutrophils and monocytes to the air space in response to IA LPS (25), the importance of individual cytokines to lung inflammation is not understood. We evaluated the role of IL-8 in fetal inflammation because IL-8 is a potent neutrophil chemotactic factor in the adult (33). In our experiments, IA rsIL-8 induced a mild neutrophil and monocyte influx in the air space. Compared with 10 mg of IA LPS or 15 µg of IA rsIL-1α, the 100 µg of rsIL-8 recruited only 1% as many inflammatory cells to the air spaces at 1–2 days. This very modest lung inflammatory response to IA rsIL-8 parallels the lack of lung inflammation induced by rsTNFα in the fetal sheep (17). These results demonstrate that responses to individual cytokines may differ greatly between the fetus and the adult. Despite the modest inflammatory response to IL-8, airway proteins increased and monocytes from the fetal lung generated more hydrogen peroxide following IL-8 exposure in vivo, demonstrating that the IL-8 was modulating some aspects of lung inflammation. We recently reported that IA LPS induced maturation of blood and lung monocyte function (28). A 7-day interval between IA rsIL-8 and delivery increased neutrophil recruitment in the air spaces following brief mechanical ventilation. Thus, although the overall inflammatory response was not large, the more subtle effects on monocytes and neutrophils could contribute to the pathophysiology of lung injury following preterm delivery.

Vascular endothelium expresses the IL-8 receptor (33). An early increase in serum IL-8 predicted later mortality in pediatric septic shock patients and bronchopulmonary dysplasia in preterm infants (34, 43). We therefore tested the effects of intravascular injection of rsIL-8 in the fetal sheep. Consistent with the minimal inflammation induced by the IA injection, intravascular injection also did not induce acute physiological changes in fetal cardiovascular status. In contrast, we recently demonstrated that 10 µg of IL-1α given by intravascular injection was lethal for the sheep fetus (26).

In a number of different animal models, IL-8 is a primary mediator of neutrophil influx. In an LPS-induced pleurisy model in rabbits, administration of an anti-IL-8 antibody reduced neutrophil influx (7). In CXCR2-deficient mice, neutrophil recruitment was impaired in response to *E. coli*-induced pyelonephritis (11). The CXCR2 inhibitor SB-265610 (3 mg/kg) attenuated hyperoxia-mediated lung neutrophil influx in neonatal rats (4, 44). Both SB-265610 and NTME (the inhibitor used in the present study) inhibited IL-8-induced sheep neutrophil chemotaxis in a similar dose-concentration range with similar IC50 values (Auten, unpublished data). The concentration of NTA (an active metabolite of NTME) achieved in the plasma in the present study was many fold in excess of the concentration needed to inhibit neutrophil chemotaxis in vitro. Multiple studies using different models of injury (LPS, infection, hyperoxia) have demonstrated that IL-8 signaling via CXCR2 is important for mediating neutrophil influx (4, 7, 11, 44). In the present experiments in fetal sheep, CXCR2 inhibition transiently decreased IA LPS-induced airway protein leak but did not reduce recruitment of neutrophils and monocytes to the lung or reduce lung cytokine expression.

Preterm fetal sheep have low lung surfactant pools, and IA LPS exposure increases surfactant protein mRNAs followed by increased airway surfactant lipid pools, leading to improve lung compliance (5, 27). In this study, IA rsIL-8 did not induce lung maturation, and inhibition of CXCR2 signaling did not inhibit IA LPS-induced lung maturation. IL-8 is known to

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**Table 3. Total and differential white blood cell counts in the peripheral blood**

<table>
<thead>
<tr>
<th>Group</th>
<th>Total WBC (10⁹/l)</th>
<th>Neutrophils (10⁹/l)</th>
<th>Lymphocytes (10⁹/l)</th>
<th>Monocytes (10⁹/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>3.0±0.2</td>
<td>0.8±0.1</td>
<td>2.1±0.2</td>
<td>0.1±0.04</td>
</tr>
<tr>
<td>IA LPS (1 day)</td>
<td>1.6±0.1*</td>
<td>0.2±0.05*</td>
<td>1.2±0.1*</td>
<td>0.02±0.01*</td>
</tr>
<tr>
<td>IA LPS + IM NTME (1 day)</td>
<td>1.3±0.2*</td>
<td>0.3±0.1*</td>
<td>0.9±0.2</td>
<td>0.04±0.02*</td>
</tr>
<tr>
<td>IA LPS (7 days)</td>
<td>9.9±3.7*</td>
<td>6.5±3.3*</td>
<td>2.7±0.3</td>
<td>0.6±0.2*</td>
</tr>
<tr>
<td>IA LPS + IM NTME (7 days)</td>
<td>6.0±0.5*</td>
<td>2.8±0.3*</td>
<td>3.1±0.5</td>
<td>0.2±0.1*†</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. control, †P < 0.05 vs. corresponding LPS group. WBC, white blood cells.

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**Fig. 6. Inhibition of CXCR2 did not alter lung maturation. Sat PC concentration in BALF normalized to body weight (A) and deflating limb pressure-volume curve (B) 7 days after exposures. The measurements after NTME were not different from the LPS group. N = 7–8 animals/group, *P < 0.05 vs. control.**
increase the expression of CD11b/CD18 on the cell surface of inflammatory cells (9, 16). We previously reported that LPS-induced inflammatory cell influx, mediated by the integrin CD18, was required for induced lung maturation (25). However, the inhibition of the IL-8 receptor CXCR2 did not inhibit LPS-induced lung maturation. Although IL-8 is highly expressed in the fetal lung in response to IA LPS or IL-1α, IL-8 alone is not an important mediator of inflammatory cell influx or lung maturation.

IL-8 can bind with high affinity to two closely related receptors, CXCR1 and CXCR2 (15). Among the IL-8 family of chemokines, only IL-8 (CXCL8) and GCP-2 (CXCL6) bind CXCR1, whereas the chemokines that can bind CXCR2 include GROα, β, γ (CXCL1, L2, L3), NAP-2 (CXCL7), and ENA-78 (CXCL5), in addition to IL-8 and GCP-2 (14, 33). We inhibited CXCR2 because this strategy was used successfully in different experimental models to inhibit neutrophil influx and inflammation (4, 44). In addition, CXCR2 inhibition will simultaneously block signaling of the other chemokines in the IL-8 family. It is possible that IL-8 may mediate LPS-induced lung inflammation via CXCR1. However, the lack of significant lung inflammation and lung maturation following IL-8 infusion suggests that IL-8 does not play a significant role in fetal inflammation induced by LPS.

Fetal inflammation induced by chorioamnionitis is postulated to be the proximate cause of fetal organ injury such as periventricular leukomalacia (1), necrotizing enterocolitis (2), or bronchopulmonary dysplasia (1, 13, 38). Therefore, inhibitors of the fetal inflammatory response are of clinical and therapeutic interest. We recently reported that IA injection of an IL-1 receptor antagonist decreased LPS-induced lung and systemic inflammation, demonstrating the importance of IL-1 signaling in the pathophysiology of chorioamnionitis-induced fetal inflammation (26). By contrast, IL-8 signaling appears to induce airway protein leak and cause subtle increases in airway leukocytes. Despite the high expression of IL-8 following IA LPS in fetal sheep and its association with chorioamnionitis and lung injury in the human, it does not seem to be a potent mediator in this fetal sheep model. Other pathways that contribute to chorioamnionitis-induced inflammation remain to be identified.

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DISCLOSURES

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