Intra-amniotic LPS and antenatal betamethasone: inflammation and maturation in preterm lamb lungs

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Kuypers E, Collins JJ, Kramer BW, Ofman G, Nitsos I, Pillow JJ, Polglase GR, Kemp MW, Newnham JP, Gavilanes AW, Nowacki R, Ikegami M, Jobe AH, Kallapur SG. Intra-amniotic LPS and antenatal betamethasone: inflammation and maturation in preterm lamb lungs. *Am J Physiol Lung Cell Mol Physiol* 302: L380–L389, 2012. First published December 9, 2011; doi:10.1152/ajplung.00338.2011.—The proinflammatory stimulus of chorioamnionitis is commonly associated with preterm delivery. Women at risk of preterm delivery receive antenatal glucocorticoids to functionally mature the fetal lung. However, the effects of the combined exposures of chorioamnionitis and antenatal glucocorticoids on the fetus are poorly understood. Time-mated ewes with singleton fetuses received an intra-amniotic injection of lipopolysaccharide (LPS) either preceding or following maternal intramuscular betamethasone 7 or 14 days before delivery, and the fetuses were delivered at 120 days gestational age (GA) (term = 150 days GA). Gestation matched controls received intra-amniotic and maternal intramuscular saline. Compared with saline controls, intra-amniotic LPS increased inflammatory cells in the bronchoalveolar lavage and myeloperoxidase, *Toll-like receptor 2 and 4* mRNA, PU.1, CD3, and Foxp3-positive cells in the fetal lung. LPS-induced lung maturation measured as increased airway surfactant and improved lung gas volumes. Intra-amniotic LPS-induced inflammation persisted until 14 days after exposure. Betamethasone treatment alone induced modest lung maturation but, when administered before intra-amniotic LPS, suppressed lung inflammation. Interestingly, betamethasone treatment after LPS did not counteract inflammation but enhanced lung maturation. We conclude that the order of exposures of intra-amniotic LPS or maternal betamethasone had large effects on fetal lung inflammation and maturation.

chorioamnionitis; lung inflammation; surfactant; bronchopulmonary dysplasia; respiratory distress syndrome

CHORIOAMNIONITIS, DEFINED AS inflammation of the fetal membranes, complicates up to 70% of preterm deliveries before 30 wk of gestation (16). The epidemiological associations of preterm infants exposed to chorioamnionitis are fetal systemic inflammation and lung, brain, and gastrointestinal injury (17, 42). Lung inflammation may initiate a progressive injury that results in bronchopulmonary dysplasia (26). However, the exposure of the fetal lung to inflammation associated with chorioamnionitis also increases surfactant proteins and lipids with salutary effects on respiratory distress syndrome (1, 30, 36).

Antenatal corticosteroids are a standard treatment given to mothers at risk of imminent preterm birth to induce lung maturation (41). Lung maturation induced by corticosteroids results from the simultaneous induction and suppression of multiple genes that have the net effects of increasing surfactant lipids, surfactant proteins, and thinning of the lung mesenchyme to increase the potential air spaces (23, 46, 48). Clinical studies and experimental evidence suggest that antenatal corticosteroids are also efficacious in the setting of chorioamnionitis (18, 39, 40). The delivery of many women given corticosteroids is delayed for several days to weeks (5), and chorioamnionitis is clinically silent in a majority of women (1). Therefore, the combined exposures of antenatal corticosteroids and chorioamnionitis are common in the preterm fetus, but the order of exposures can vary.

We reported previously that simultaneous exposure of the preterm sheep fetus to antenatal corticosteroids and lipopolysaccharide (LPS) suppressed the pulmonary inflammation for 1 to 2 days after the exposure but amplified the inflammatory response to chorioamnionitis 5 to 15 days after the exposure (28, 33). However, it is not known whether the order of exposure is important in mediating lung inflammation and maturation. Therefore, we aimed to study the interactive effects of chorioamnionitis and antenatal corticosteroids on the inflammatory and maturational response in the fetal lungs in a clinically relevant preterm sheep model of chorioamnionitis (32). We tested the hypothesis that the order of exposure to maternal betamethasone and intra-amniotic LPS will differentially impact lung surfactants and inflammatory responses in the preterm fetus. Fetal sheep were exposed in utero to intra-amniotic LPS or antenatal maternal intramuscular betamethasone, with an interval of 7 days between the two interventions. Furthermore, we asked whether the order of exposure to antenatal corticosteroids and intra-amniotic LPS altered fetal lung outcomes.

METHODS

Animal model and sampling protocol. All studies were approved by the Animal Ethics Committees at The University of Western Australia and Cincinnati Children's Hospital Medical Center. Time-mated ewes with singleton fetuses were randomly assigned to one of six treatment groups to receive an intra-amniotic injection of lipopolysaccharide (LPS) (10 mg *Escherichia coli* 055:B5, Sigma Chemical, St. Louis, MO), intramuscular injection of betamethasone [Celestone Soluspan,

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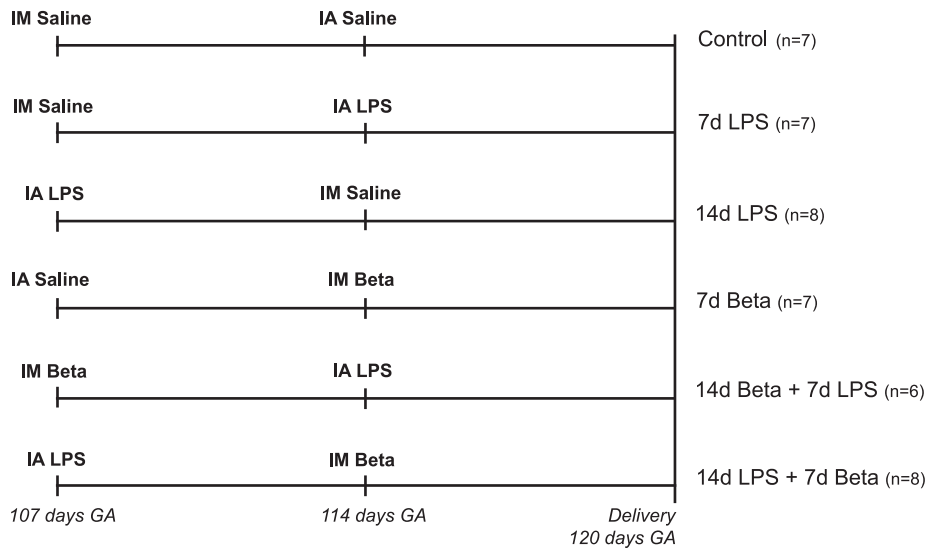
Fig. 1. Study design. Pregnant ewes received an intra-amniotic (IA) injection of lipopolysaccharide (LPS) and/or an intramuscular (IM) injection of betamethasone (Beta) and/or an equivalent injection of saline for control animals at 107 days and/or 114 days gestation (GA). Lambs were delivered preterm by cesarean section at 120 days GA (term = 150 days GA). 7d, 7-day exposure; 14d, 14-day exposure.

Schering-Plough, North Ryde, New South Wales (NSW), Australia, 0.5 mg/kg maternal weight], or an equivalent injection of saline (controls) at 107 days and/or 114 days GA in different permutations and combinations (Fig. 1). All ewes in this study received a single intramuscular injection of 150 mg medroxyprogesterone acetate (Depo-Provera, Kenral, NSW, Australia) at 100 days gestational age to prevent preterm birth induced by betamethasone treatment (24). Lambs were surgically delivered at 120 days GA (term = 150 days GA) and euthanized with 100 mg/kg pentobarbital. The fetus was weighed and fetal cord blood was collected. Following opening of the chest, a deflation air pressure-volume curve was measured from static inflation of the lung to 40 cmH_2O airway pressure (25). The lungs were removed, separated, and weighed prior to a bronchoalveolar lavage of the left lung with 0.9% NaCl (25). The bronchoalveolar lavage fluid (BALF) was used for differential cell counts and surfactant measurements. Lung tissue from the right lower lobe (RLL) was snap frozen and the right upper lobe (RUL) was inflation fixed in 10% buffered formalin with 30 cmH_2O pressure for 24 h.

Surfactant proteins and cytokine mRNA quantitation. Total RNA was isolated from frozen lung tissue of the RLL by a modified Chomzynski method, and mRNA quantitation was performed by real-time PCR (27). Gene expression was measured for surfactant proteins and cytokines: *SP-A*, *SP-B*, *SP-C*, *IL-1β*, *IL-6*, *IL-8*, *MCP-1*, and *Serum amyloid A3*. The mRNA was reverse transcribed to yield a single-strand cDNA (verso cDNA kit, Thermo Scientific), which was used as a template with primers and TaqMan probes (Applied Biosystems, Carlsbad, CA) specific to sheep sequences (29). The values for each cytokine were normalized to the internal 18S rRNA. Data were expressed as fold increased over control values.

Toll-like receptor mRNA quantitation. For the Toll-like receptor mRNA measurements, total RNA was extracted from frozen lung tissue of the RLL by using the SV Total RNA Isolation system (Z3100, Promega, Madison, WI) according to the manufacturer's

instructions. Genomic DNA contamination was removed by treatment with RQ1 DNase (M610A, Promega), and the RNA was tested for the presence of genomic GAPDH. Briefly, PCR amplification for the detection of genomic DNA was performed with *Taq* DNA polymerase (M124B, Promega) at 95°C for 5 min followed by 40 cycles at 95°C for 30 s, 55°C for 45 s, and 72°C for 30 s. Total RNA was used as a template. PCR products were analyzed on 1.5% agarose gels. Total RNA was reverse transcribed with the First Strand cDNA synthesis kit (4379012001, Roche-Applied, Mannheim, Germany) according to manufacturer's instructions by use of anchored oligo primers. Primers for real-time PCR (RT-PCR) were constructed based on published ovine or bovine cDNA sequences (Table 1). Dilution experiments were performed to ensure similar PCR amplification efficiency of the primers. RT-PCR reactions were performed in duplicate with the LightCycler 480 SYBR Green I Master mix (4707516001, Roche-Applied) on a LightCycler 480 Instrument according to the manufacturer's instructions. RT-PCR results were normalized to cyclophilin A, a household gene, and mean fold changes in mRNA expression were calculated by the $\Delta\Delta Ct$ method (37).

Surfactant Sat PC and SP-D. Pulmonary surfactant protein D (SP-D) in the BALF was measured with a sandwich ELISA using rabbit anti-ovine SP-D as coating antibody and guinea pig anti-ovine SP-D a secondary antibody (19, 20). Saturated phosphatidylcholine (Sat PC) was isolated from the BALF by organic solvent extraction followed by oxidation with osmium tetroxide and recovery from neutral alumina, followed by a phosphorus measurement (7).

Immunohistology. The following antibodies were used to identify different cell types: CD3 for T cells, myeloperoxidase (MPO) for activated neutrophils and monocytes, PU.1 for maturation marker for myeloid cells, precursor form of surfactant protein C (SP-C; pro-SP-C) for maturation marker for alveolar type II cells, thyroid transcription factor-1 (TTF-1) for alveolar type II cells, Foxp3 for regulatory T cells. Paraffin-embedded RUL lung sections (4 μm, trans-

Table 1. *Primers used for RT-PCR*

Gene		Sequence (5′-3′)	Amplicon Size	T^m	Accession Code (RefSeq)
TLR2	Fw	GGCTGTAATCAGCGTGTTCA	160 bp	64°C	NM_001048231.1
	Rv	GATCTCGTTGTCGGACAGGT			
TLR4	Fw	GAGAAGACTCAGAAAAGCCTTGCT	200 bp	65°C	NM_001135930.1
	Rv	GCGGGTTGGTTTCTGCAT			
TLR9	Fw	CCCTGGAGAAGCTGGACAT	175 bp	60°C	NM_001011555.1
	Rv	GACAGGTCCACGAAGAGCAG			

T^m, melt temperature; RefSeq, reference sequence; Fw, forward; Rv, reverse.

Table 2. *Variables at birth*

| Treatment Group | Fetal Outcomes | | | Birth Weight, kg | Cord Blood, pH | Sex | Lung Wt/Body Wt, g/kg |
	Fetal Death	Abortion	Alive				
Control	1		7	2.7 ± 0.2	7.24 ± 0.03	3/4	32.7 ± 0.9
7d LPS			7	2.5 ± 0.1	7.26 ± 0.02	3/4	33.6 ± 1.2
14d LPS			8	2.5 ± 0.1	7.27 ± 0.02	5/3	37.1 ± 1.8
7d Beta	2	1	7	2.7 ± 0.2	7.20 ± 0.04	4/3	36.7 ± 0.8
14d Beta + 7d LPS		3	6	2.7 ± 0.1	7.25 ± 0.04	1/5	36.2 ± 2.4
14d LPS + 7d Beta	1	1	8	2.1 ± 0.1*	7.25 ± 0.03	4/4	39.9 ± 2.7

Data are expressed as means ± SE. *$P < 0.05$ vs. controls by 1-way ANOVA with Tukey's post hoc test. LPS, lipopolysaccharide; Beta, betamethasone; GA, gestational age; Wt, weight; M, male; F, female; 7d, 7-day exposure; 14d, 14-day exposure.

verse) were stained for CD3 (DAKO A0452, Dakocytomation, Glostrup, Denmark), MPO (DAKO A0398, Dakocytomation), PU.1 (sc-352, Santa Cruz Biotechnology, Santa Cruz), Foxp3 (14–7979, eBioscience, San Diego), TTF-1 (WRAB-1231, Seven Hills Biore-agents, Cincinnati, OH) or pro-SP-C (WRAB-9337, Seven Hills Bioregeants). Briefly, the sections were deparaffinized in an ethanol series and endogenous peroxidase-activity was blocked by incubation with 0.3% H_2O_2 in 1 × phosphate buffered saline (PBS, pH 7.4) or methanol (for TTF-1 and pro-SP-C). Antigen retrieval was performed by incubating the sections in heated citrate buffer (10 mM, pH 6.0) for 30 min. To block nonspecific binding, the slides were incubated with 20% normal goat serum (NGS) in PBS (for MPO and Foxp3), 5% bovine serum albumin in PBS (for CD3 and PU.1) or 2% NGS in PBS (for TTF-1 and pro-SP-C). Sections were incubated overnight at 4°C with the diluted primary antibody (CD3 1:200, MPO 1:500, PU.1 1:400, Foxp3 1:30, TTF-1 1:100, pro-SP-C 1:1,500). After incubation with a goat-anti-mouse biotin labeled (for Foxp3) (DAKO E0433, Dakocytomation) or swine-anti-rabbit biotin-labeled secondary anti-body (DAKO E0353, Dakocytomation), immunostaining was en-hanced with Vectastain ABC Peroxidase Elite kit (PK-6200, Vector Laboratories, Burlingame, CA) and stained with nickel sulfate-di-aminobenzidine. Subsequently, the sections were rinsed in Tris-saline and incubated with Tris-cobalt. After counterstaining with 0.1% Nuclear Fast Red, the sections were washed, dehydrated, and cover-slipped.

Since the air space expansion and tissue characteristics were different in the different groups, we measured tissue area in the lung sections. Blinded measurements of tissue fractions (expressed relative to the total lung area) were performed in five random nonoverlapping fields (×20 objective) for each animal and for at least four animals per group using the color threshold function of the program MetaMorph v6.1r0 (Molecular Devices/Universal Im-aging, Sunnyvale, CA). The average measurement from each lamb was used to compute a group average. The tissue fractions in different experimental groups were normalized to the control group average to obtain a correction factor. For example, if the tissue fraction in an experimental group was 1.2 times control, then the cells counted per microscopic field were divided by 1.2. Thus the expressed cell counts per microscopic field incorporated this cor-rection factor.

Evaluation was performed by light microscopy (Axioskop 40, Zeiss) with LeicaQWin Pro v.3.4.0 software (Leica Microsystems). MPO-, CD3-, PU.1-, and pro-SP-C-positive cells were counted in three representative high-power fields at ×200 magnification by a blinded observer and averaged per animal. Because FoxP3-positive cells were unevenly distributed throughout the lung tissue, sections were scored for positive signal for Foxp3 with a semiquantitative scoring system by a blinded observer: 1, little staining; 2, light staining; 3, heavy staining. For TTF-1, a computer-aided manual count of five random fields at ×200 magnification per animal was performed, using the computer program MetaMorph (Image-Pro Plus v7.0). The MPO, CD3, and TTF-1 cell counts per microscopic field incorporated the correction factor for tissue fraction. The SP-C-
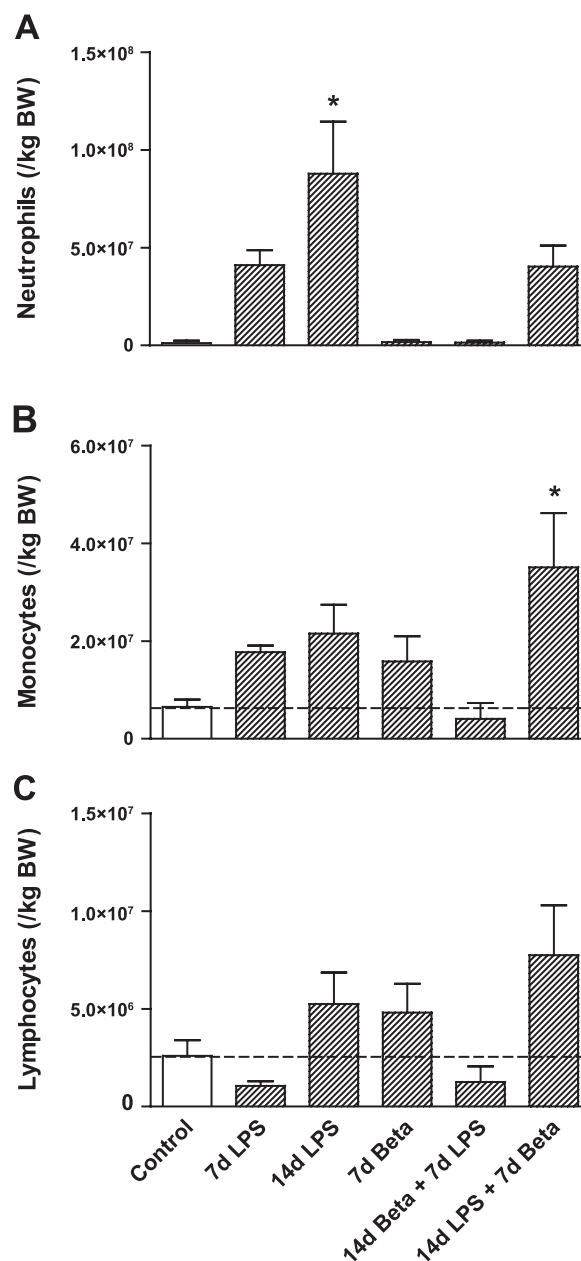
Fig. 2. Differential cell count of the bronchoalveolar lavage. *A*: neutrophil levels increased 14 days after the exposure to LPS. *B*: combined exposure to LPS for 14 days and betamethasone for 7 days increased monocytes in the bronchoalveolar lavage. *C*: lymphocyte count did not differ in any of the treatment groups compared with controls. *$P < 0.05$ vs. controls by 1-way ANOVA with Tukey's post hoc test. BW, body weight.
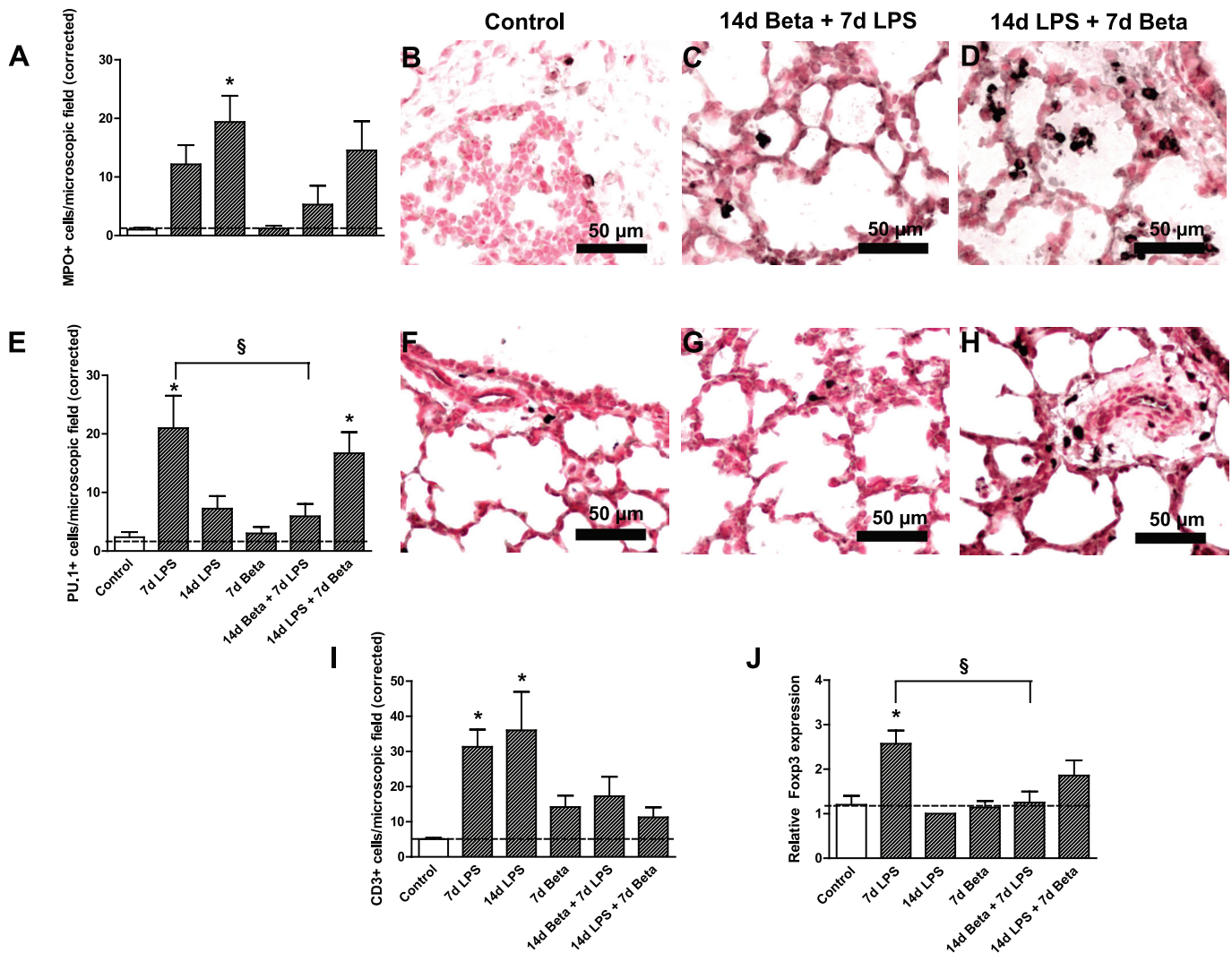
Fig. 3. Characterization of inflammatory cells in the fetal lung tissue. *A*: quantitation of myeloperoxidase (MPO)-expressing cells in lung sections per microscopic field corrected for the tissue fraction (see METHODS for details). Representative photomicrographs of immunostaining against myeloperoxidase using lung sections from controls (*B*), 14d betamethasone + 7d LPS (*C*), and 14d LPS + 7d betamethasone (*D*). MPO-positive cells increased in the lung tissue 14 days after LPS exposure compared with controls. *E*: quantitation of PU.1 expressing cells in lung sections per microscopic field. Representative photomicrographs of immunostaining against PU.1 using lung sections from controls (*F*), 14d betamethasone + 7d LPS (*G*), and 14d LPS + 7d betamethasone (*H*). LPS exposure for 7 days and combined exposure to LPS for 14 days and betamethasone for 7 days increased the number of PU.1-positive cells in the fetal lung. Betamethasone treatment 7 days before the LPS exposure prevented this increase. *I*: number of CD3-positive cells increased significantly 7 and 14 days after LPS exposure compared with controls. *J*: Foxp3 expression increased 7 days after LPS exposure compared with controls. Betamethasone treatment before the LPS exposure prevented this increase. *$P < 0.05$ vs. controls; §$P < 0.05$ between experimental groups by 1-way ANOVA with Tukey's post hoc test.

positive cells were expressed relative to TTF-1-positive cells to assess whether changes were due to increased expression in each of the alveolar type II cell vs. increased numbers of alveolar type II cells in different groups.

Data analysis. Results are given as means ± SE. The groups were compared by one-way ANOVA with Tukey's test for post hoc analysis as appropriate. Statistical analysis was performed by Graph-Pad Prism v5.0. Significance was accepted at $P < 0.05$.

Table 3. *Cytokine and acute-phase reactant expression in the fetal lung*

Treatment Group	IL-1β mRNA	IL-6 mRNA	IL-8 mRNA	MCP-1 mRNA	Serum Amyloid A3 mRNA
Control	1.0 ± 0.2	1.0 ± 0.3	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.7
7d LPS	3.5 ± 0.5*	0.9 ± 0.1	4.7 ± 0.8	3.7 ± 0.4	29 ± 10*
14d LPS	3.1 ± 0.6	1.1 ± 0.2	4.2 ± 1.5	3.8 ± 0.9	12 ± 8.0
7d Beta	1.2 ± 0.3	1.0 ± 0.3	1.0 ± 0.4	1.3 ± 0.3	1.2 ± 0.5
14d Beta + 7d LPS	1.8 ± 0.4	0.8 ± 0.1	2.1 ± 0.8	1.7 ± 0.5	4.3 ± 3.0
14d LPS + 7d Beta	3.2 ± 1.5	1.6 ± 0.8	4.3 ± 2.3	5.0 ± 3.2	31 ± 26

Data are expressed as means ± SE of the fold increase of cytokine relative to controls. *$P < 0.05$ vs. controls by 1-way ANOVA with Tukey's post hoc test.

RESULTS

Description of animals. The experiments were prospectively designed to test the interactions of antenatal corticosteroids and intra-amniotic LPS exposure. Despite the medroxyprogesterone acetate treatment, animals exposed to maternal betamethasone experienced fetal losses, such that we reassigned animals from the group that only received betamethasone 14 days before delivery to other treatment groups because our priority was to test the interaction of betamethasone and LPS. The intra-amniotic LPS injections were not associated with fetal losses, but fetal deaths identified by ultrasound and fetal losses were frequent in the betamethasone-treated groups (Table 2). All animals had comparable birth weights except for a lower birth weight for animals with combined exposure to LPS 14 days and betamethasone 7 days before delivery. Cord blood pH values and lung-to-body-weight ratios were comparable across all groups.

Lung inflammation. Pulmonary inflammation resulting from LPS and the anti-inflammatory effect of betamethasone exposures were assessed by differential cell counts on the BALF (Fig. 2). Neutrophil levels were modestly increased 7 days after the exposure to LPS and significantly increased 14 days after LPS exposure (Fig. 2A). This increase did not occur in lambs exposed to maternal betamethasone 7 days after intra-amniotic LPS. In this group, however, there was a 10-fold increase in monocytes, which was not seen in the groups exposed to LPS only (Fig. 2B). Lymphocyte numbers did not differ in experimental groups compared with controls (Fig. 2C).

Lung inflammation was further characterized by identifying markers for activation of inflammatory cells (Fig. 3). MPO-positive cells were significantly increased 14 days after LPS exposure (Fig. 3, A–D). Betamethasone pretreatment 7 days before the LPS exposure prevented the increase of MPO-positive cells in the fetal lung. However, betamethasone given 7 days after LPS only partially inhibited the influx of LPS-mediated MPO-positive cells. PU.1 expression, which is a transcription factor that indicates maturation of monocytes (34), increased 7 days after LPS exposure in the fetal lung (Fig. 3, E–H). This increase in PU.1-positive cells could be prevented by pretreatment with betamethasone 7 days before the LPS exposure. However, exposure to 14 days of LPS followed by betamethasone for 7 days increased PU.1-expressing cells in the lung. LPS exposure for either 7 or 14 days increased the number of CD3-positive cells in the fetal lung (Fig. 3I). The groups exposed to both betamethasone and LPS did not show a significant increase in CD3-positive cells compared with controls, indicating that betamethasone pre- and posttreatment both countered the LPS-induced influx of CD3-positive cells. Foxp3-positive cells only increased in the lung tissue 7 days after LPS exposure (Fig. 3J), which was inhibited by betamethasone pretreatment.

Consistent with our previous observation of maximal induction of cytokines 2 d after exposure (32, 35), the mRNA expression of *IL-1β*, *IL-6*, *IL-8*, and *MCP-1* were only modestly increased 7 days after LPS exposure compared with controls (Table 3). Combined exposure to LPS and betamethasone had no significant effect on lung cytokine mRNA expression. The mRNA levels of *Serum amyloid A*, an acute-phase reaction protein expressed in the lung and liver (49), increased 7 days after the LPS exposure compared with controls. Beta-
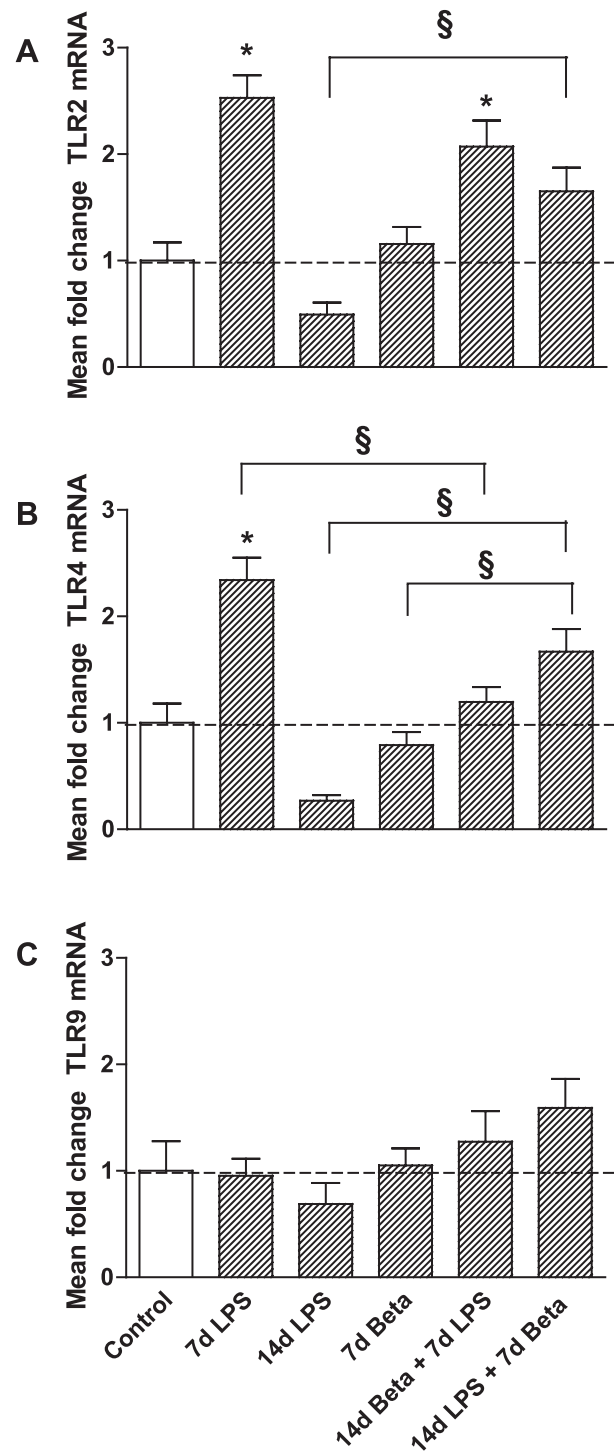

Fig. 4. Expression of Toll-like receptors (*TLR*) 2, 4, and 9. *TLR2* (*A*) and *TLR4* (*B*) mRNA expression were upregulated 7 days after LPS exposure but returned to baseline 14 days after exposure to LPS. Betamethasone treatment before LPS exposure prevented increased *TLR4* expression, but not *TLR2* expression. *TLR2* and *TLR4* mRNA levels increased in the lungs of lambs that received betamethasone after LPS exposure, although these levels were not higher than levels measured in controls. *C*: *TLR9* was not differently expressed in experimental groups compared with controls. *$P < 0.05$ vs. controls; §$P < 0.05$ between experimental groups by 1-way ANOVA with Tukey's post hoc test.

Table 4. *Markers of lung maturation*

Treatment Group	SP-A mRNA	SP-B mRNA	SP-C mRNA	TTF-1+cells/HPF
Control	1.0 ± 0.4	1.0 ± 0.3	1.0 ± 0.6	514 ± 84
7d LPS	4.6 ± 0.6	1.9 ± 0.2	1.7 ± 0.7	453 ± 37
14d LPS	7.5 ± 2.6	2.4 ± 0.4	0.4 ± 0.1	362 ± 19
7d Beta	1.4 ± 0.2	1.1 ± 0.1	0.2 ± 0.1*	395 ± 28
14d Beta + 7d LPS	5.6 ± 3.3	1.8 ± 0.6	1.0 ± 0.3	295 ± 51
14d LPS + 7d Beta	10.5 ± 2.2*	2.7 ± 0.3*	0.6 ± 0.1	346 ± 26

Thyroid transcription factor-1 (TTF-1)-positive cells represent counts per high power microscopic field (HPF) corrected for the tissue space fraction (see METHODS for details). pulonary surfactant proteins A, B, and C (SP-A, SP-B, and SP-C) mRNA are expressed as fold increase relative to controls. Data are expressed as means ± SE *$P < 0.05$ vs. controls by 1-way ANOVA with Tukey's post hoc test.

methasone pretreatment prior to the LPS exposure suppressed this increase. The mRNA levels of *TLR2* (Fig. 4A) and *TLR4* (Fig. 4B) more than doubled after 7 days of LPS exposure but returned to control levels 14 days after exposure. Pretreatment with betamethasone 7 days prior decreased the LPS-induced increase in *TLR4* but not *TLR2* mRNA. Levels of *TLR9* mRNA did not change in experimental groups compared with controls (Fig. 4C).

Surfactant components. Exposure to LPS alone modestly and variably increased *SP-A* and *SP-B* mRNA levels (nonsignificant) (Table 4). However, the combined exposure to LPS followed by betamethasone resulted in a more consistent and significant increase in *SP-A* and *SP-B* mRNAs. SP-C mRNA expression did not change with either the betamethasone or LPS exposure. *SP-D* mRNA expression increased 14 days after the exposure to LPS alone or with betamethasone 7 days after LPS (Fig. 5A). Consistent with the mRNA data, combined exposure at 14 days to LPS followed by a 7-day exposure to betamethasone significantly increased SP-D protein in the BALF (Fig. 5B). To better assess the changes in the alveolar type II cells, we immunostained fetal lung sections using antibodies against TTF-1 and pro-SP-C (38). TTF-1, a transcription factor for

alveolar type II cells (38), did not change in any of the treatment groups, indicating no change in the alveolar type II cell numbers after exposure to either LPS or betamethasone (Table 4). However, compared with controls, the number of pro-SP-C-positive cells increased significantly 7 and 14 days after the exposure to LPS, and after 14 days LPS + 7 days betamethasone, indicating maturation of alveolar type-II cells (Fig. 6, A–E).

Sat PC is the major surfactant lipid (22). Exposure to intra-amniotic LPS for 7 or 14 days increased Sat PC in the fetal air spaces (Fig. 7A). Betamethasone alone did not significantly increase Sat PC expression. However, the 14-day LPS exposure followed by betamethasone for 7 days had the highest airway Sat PC levels (Fig. 7A). Consistent with increases in surfactant Sat PC, lung volumes, a measure of compliance, increased in all the LPS groups regardless of betamethasone exposure, with the highest lung volumes recorded in the 14-day LPS followed by 7-day betamethasone exposure group (Fig. 7B).

DISCUSSION

Lung inflammation, increased airway surfactants, and increased lung volumes consistent with clinical lung maturation
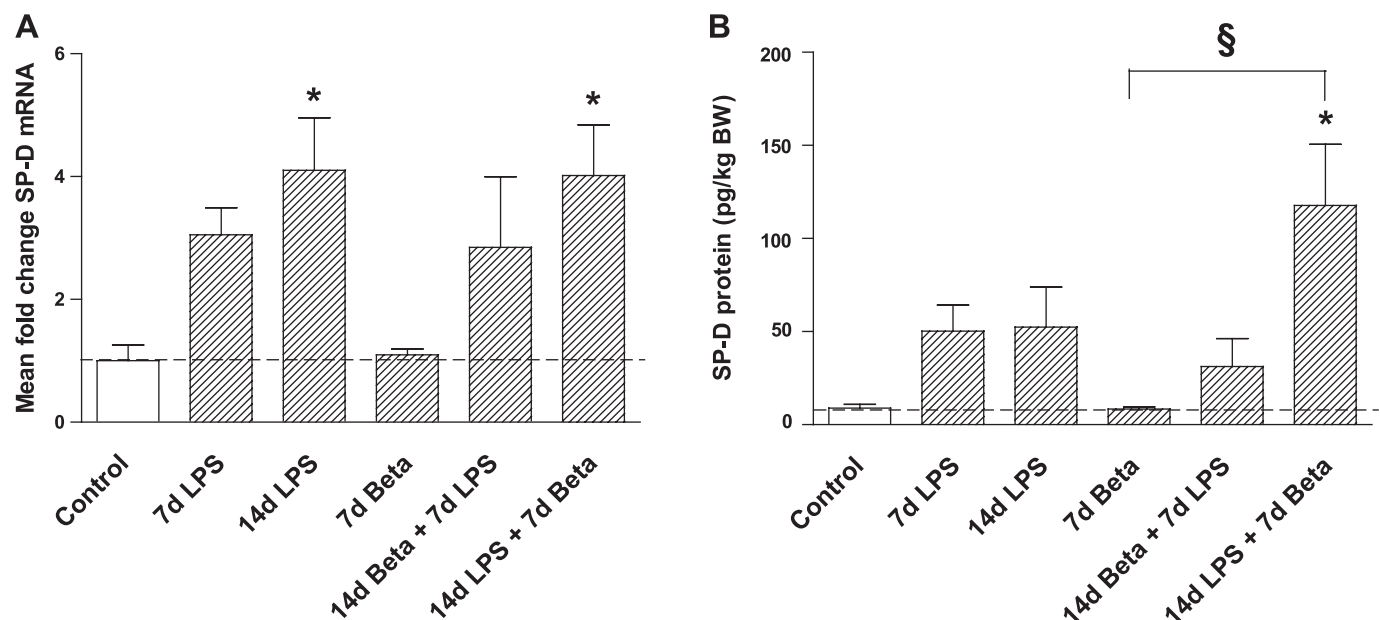
Fig. 5. Pulmonary surfactant protein D (SP-D) mRNA and protein expression. A: *SP-D* mRNA levels increased 14 days after the exposure to LPS irrespectively of the betamethasone posttreatment. B: only combined exposure to LPS for 14 days and betamethasone for 7 days increased SP-D protein expression in the bronchoalveolar lavage fluid. Exposure to LPS for 14 days did not increase SP-D protein expression. *$P < 0.05$ vs. controls; §$P < 0.05$ between experimental groups by 1-way ANOVA with Tukey's post hoc test.
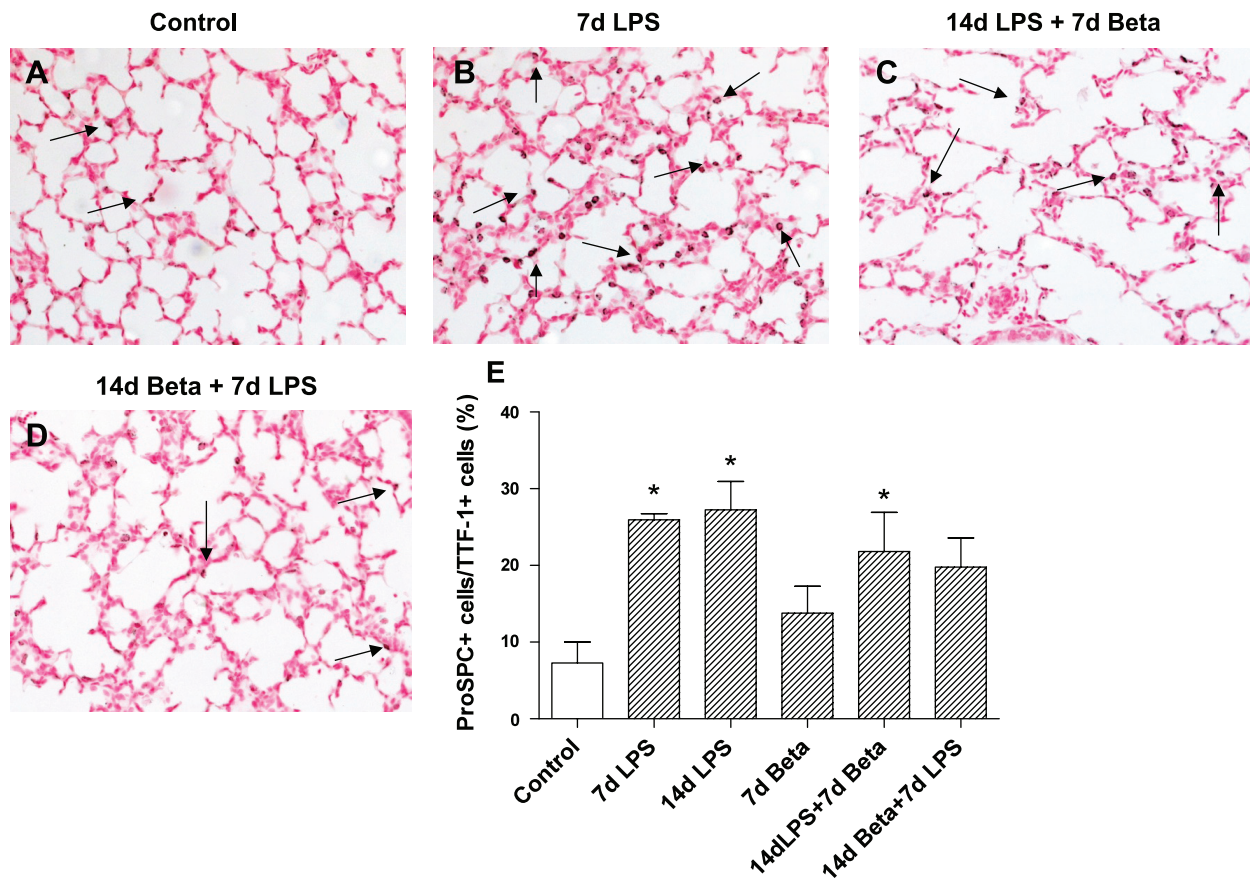
Fig. 6. Pro-pulmonary surfactant protein C (pro-SP-C) immunostaining in the lung. The number of pro-SP-C-positive cells was expressed as a percentage of TTF-1-positive cells (data in Table 4). Representative photomicrographs of immunostaining using lung sections from controls (*A*), 7d LPS (*B*), 14d LPS + 7d betamethasone (*C*), and 14d betamethasone + 7d LPS (*D*). *E*: quantitation of pro-SP-C-expressing cells in lung sections per microscopic field via a ×20 objective. LPS exposure increased pro-SP-C expression in the alveolar type II cells in the lung. (*$P < 0.05$ vs. controls, scale bar is 50 μm).

are the major effects of experimental chorioamnionitis (32, 35). In this study we evaluated whether the exposure to the commonly used anti-inflammatory drug betamethasone altered the responses to LPS in the fetal lung. The major conclusion was that betamethasone when administered 7 days after intra-amniotic LPS did not inhibit lung inflammation but resulted in a striking lung maturation. However, when betamethasone was given 7 days before intra-amniotic LPS, lung inflammation was suppressed and lung maturation was more modest.

Intra-amniotic LPS caused lung inflammation at 7 and 14 days after exposure. Both neutrophils and T lymphocytes were recruited to the lung. Furthermore, the neutrophils were activated as demonstrated by the MPO expression (10). The influx of proinflammatory cells was also accompanied by an increase in Foxp3-positive cells, a prototypic marker of the anti-inflammatory T-regulatory cells (13). The number of cells expressing PU.1 also increased, which suggests maturation of lung monocytes, a characteristic for LPS-mediated pulmonary inflammation (34). Both the *TLR2* and *TLR4* mRNA levels increased in the lung tissue after LPS. Consistent with our previous results demonstrating an early rapid induction profile for cytokine expression (32), the mRNAs for proinflammatory cytokines were only modestly increased 7 days after exposure in the present study. Interestingly, betamethasone treatment 7 days prior to LPS significantly decreased LPS-induced lung inflammation. However, when LPS exposure preceded betametha-

sone treatment, lung inflammation was not altered. We previously reported that a concomitant administration of maternal betamethasone and LPS resulted in an early suppression but later amplification of lung inflammation (28, 33). Thus the timing of exposure to betamethasone in relation to the proinflammatory stimulus is a major determinant of modulation of lung inflammation.

Maternal betamethasone and intra-amniotic LPS have different pharmacokinetic profiles in the fetus. In a human study, peak fetal serum betamethasone levels were measured 1–2 h after maternal betamethasone treatment with a return to baseline within 2 days of treatment (4). In fetal sheep, maternal betamethasone treatment resulted in peak fetal betamethasone levels 3 h after treatment with a decrease to 50% of the peak levels at 6 h (9). In contrast, the half-life of endotoxin was measured as ∼30 h in the amniotic fluid of preterm lambs after an intra-amniotic administration and exhibited first order kinetics of elimination (39). Neither antenatal betamethasone nor intra-amniotic LPS significantly change the levels of endogenous cortisol in the preterm fetus (21, 25). These results suggest that fetal betamethasone levels 7 days after maternal treatment must be negligible. Therefore, the inhibition of LPS-induced fetal lung inflammation 7 days after maternal betamethasone suggests priming or conditioning of the inflammatory response cells rather than a straight forward drug-drug interaction. This interpretation is also consistent with our
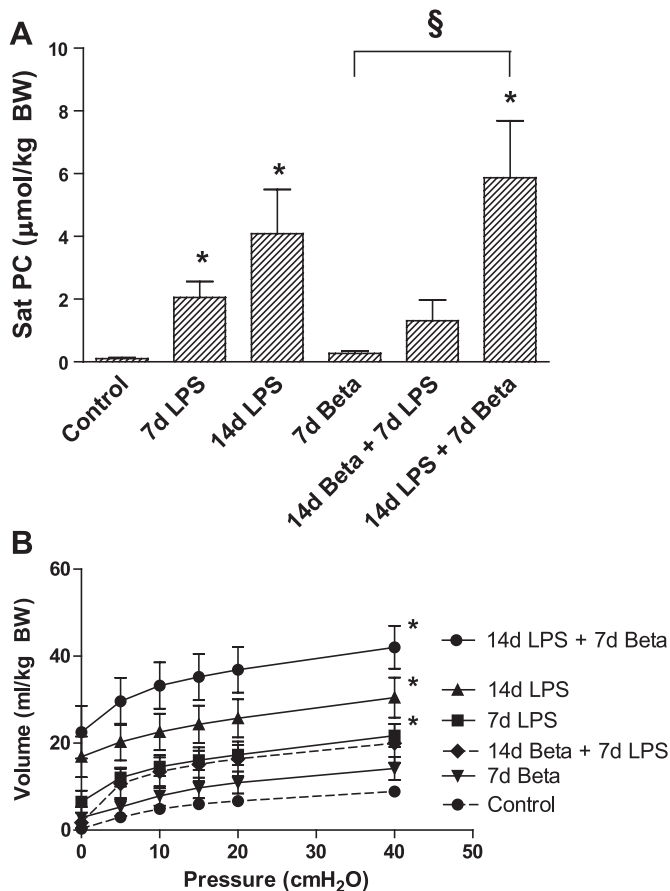
Fig. 7. Saturated phosphatidylcholine (Sat PC) and pressure-volume curves. *A*: Sat PC levels in the bronchoalveolar lavage fluid were increased 7 and 14 days after exposure to intra-amniotic LPS. Betamethasone posttreatment 7 days after the exposure to LPS increased Sat PC levels further. *B*: pressure-volume curve of animals after 14 days of LPS exposure was significantly higher compared with controls irrespectively of treatment with betamethasone. *$P < 0.05$ vs. controls; §$P < 0.05$ between experimental groups by 1-way ANOVA with Tukey's post hoc test.

mRNAs are differentially regulated by betamethasone (3). The more pronounced effect of LPS compared with betamethasone in increasing mRNA levels of surfactant proteins in this study is consistent with previous reports showing a rapid but reversible effect of betamethasone, but a more lasting effect of intra-amniotic LPS on surfactant protein mRNA expression (2, 44). Previous reports suggest that the effects of simultaneous exposure to explants of fetal rabbits to IL-1α and glucocorticoids could stimulate or inhibit surfactant protein mRNA depending on the gestational age (45). Taken together, the combined effects of a proinflammatory exposure and glucocorticoids on the fetal lung are variable and dependent on context including gestational age and the order of exposure.

We reported previously that intra-amniotic LPS but not maternal betamethasone improved lung compliance, ventilatory efficiency index, and the alveolar wash Sat PC pool sizes 15 days after treatment (2, 22). We did not study the effects of betamethasone alone 14 days after exposure, because of high rate of abortions in this group. Interestingly, the highest expression of surfactant components and the consequent increase in lung volumes was in the group exposed to LPS for 14 days along with betamethasone 7 days before delivery. This group of animals also had the most pronounced lung inflammation. These results are consistent with our previous studies demonstrating that lung inflammation was required for LPS-induced lung maturation (30, 31).

Clinical studies indicate that maternal glucocorticoids can decrease fetal weight and head size (14). We reported previously that simultaneous exposures of fetal sheep to maternal corticosteroid and intra-amniotic LPS protected the fetus from the growth restriction caused by the antenatal corticosteroids (40). In this experiment, the maternal corticosteroid exposure did not decrease fetal weight, a result that probably represents animal variability. The new observation is that the combination of 14-day LPS + 7-day betamethasone caused fetal growth restriction. Since exposure to intrauterine inflammation is the major cause for preterm labor and cervical dilation leading to preterm birth, pretreatment with betamethasone will rarely be an option to diminish or counteract inflammation in the clinical setting. An inference from the study is that prenatal betamethasone in conjunction with inflammation promotes the maturity of the surfactant system resulting in reduced respiratory distress syndrome, but does not prevent bronchopulmonary dysplasia as noted in clinical studies (8, 47). However, the interactions of LPS and betamethasone on fetal growth are complex and not fully understood, and this observation needs verification by replication.

Antenatal corticosteroids are routinely administered to mothers who are at risk of preterm birth to mature the fetal organs, suppress inflammation, and thus reduce perinatal morbidity and mortality (12a). Antenatal steroids also reduce adverse neonatal outcome after preterm birth associated with chorioamnionitis (15). Our experiments do not identify new concerns for the use of antenatal corticosteroids. It remains unknown whether these antenatal corticosteroids also prevent or amplify the developmental and structural complications in the fetal lung, which are induced by infection/inflammation (11, 48). In summary, this study provides further molecular evidence into the protective effects of antenatal corticosteroids on the fetal lung and its interactions with proinflammatory stimuli in the setting of chorioamnionitis.

previous observation of an early suppression with a late amplification of lung inflammation with concomitant administration of maternal betamethasone and intra-amniotic LPS (28, 33). Our results also demonstrate that modulation of LPS-induced fetal inflammation does not occur when betamethasone is administered after LPS.

Intra-amniotic exposure to LPS followed by betamethasone 7 days before delivery strikingly increased lung maturation as seen by increased Sat PC, SP-D, and the increase in the pressure-volume curve. In contrast, betamethasone pretreatment followed by exposure to LPS 7 days before delivery induced less lung maturation. The large effect of LPS on SP-D expression is consistent with recently reported upregulation of LPS-induced SP-D expression in fetal mice (43). SP-D is an innate host-defense molecule protecting the host against a number of pulmonary pathogens (12). Alveolar type II cell maturation was demonstrated by increased pro-SPC expression. The expression of surfactant protein mRNAs for *SP-B* increased but SP-C was unchanged after LPS. Betamethasone alone did not increase expression of any of the surfactant protein mRNAs. We previously reported a differential effect of LPS on *SP-B* and SP-C mRNA levels (2). In vitro, these two

DISCLOSURES

None of the authors have a commercial interest in any entity related to subject of the manuscript or have a conflict of interest relative to the manuscript.

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