IL-1β expression in the distal lung epithelium disrupts lung morphogenesis and epithelial cell differentiation in fetal mice

Anna Hogalm,1 Maija Bry,2 Birgitta Strandvik,3 and Kristina Bry1,4

1Department of Pediatrics, University of Gothenburg, Gothenburg, Sweden; 2Molecular/Cancer Biology Laboratory, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland; 3Department of Biosciences and Nutrition, Karolinska Institutet, NOVUM, Stockholm, Sweden; 4Division of Neonatology, Sahlgrenska University Hospital, Gothenburg, Sweden

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Hogalm A, Bry M, Strandvik B, Bry K. IL-1β expression in the distal lung epithelium disrupts lung morphogenesis and epithelial cell differentiation in fetal mice. Am J Physiol Lung Cell Mol Physiol 306: L23–L34, 2014. First published November 1, 2013; doi:10.1152/ajplung.00154.2013.—Perinatal inflammation and the inflammatory cytokine IL-1 can modify lung morphogenesis. To examine the effects of antenatal expression of IL-1β in the distal airway epithelium on fetal lung morphogenesis, we studied lung development and surfactant expression in fetal mice expressing human IL-1β under the control of the surfactant protein (SP)-C promoter. IL-1β-expressing pups suffered respiratory failure and died shortly after birth. IL-1β caused fetal lung inflammation and enhanced the expression of keratinocyte-derived chemokine (KC/CXCL1) and monocyte chemoattractant protein 3 (MCP-3/CCL7), the calgranulins S100A8 and S100A9, the acute-phase protein serum amyloid A3, the chitinase-like proteins Ym1 and Ym2, and pendrin. IL-1β decreased the percentage of the total distal lung area made up of air sacules and the number of air sacules in the lungs of fetal mice. IL-1β inhibited the expression of VEGF-A and its receptors VEGFR-1 and VEGFR-2. The percentage of the cellular area of the distal lung area made up of capillaries was decreased in IL-1β-expressing fetal mice. IL-1β suppressed the production of SP-B and pro-SP-C and decreased the amount of phosphatidylcholine and the percentage of palmitic acid in the phosphatidylcholine fraction of lung phospholipids, indicating that IL-1β prevented the differentiation of type II epithelial cells. The production of Clara cell secretory protein in the nonciliated bronchiolar (Clara) cells was likewise suppressed by IL-1β. In conclusion, expression of IL-1β in the epithelium of the distal airways disrupted the development of the airspaces and capillaries in the fetal lung and caused fatal respiratory failure at birth.

Surfactant; inflammation; microvascular development; alveolarization; bronchopulmonary dysplasia

Because the majority of early preterm deliveries are preceded by chorioamnionitis (32), premature infants are commonly exposed to inflammation already before birth. The duration, invasiveness, and severity of the intrauterine infection is generally unknown because the diagnosis of chorioamnionitis is basically based on histological examination or culture of the placenta at delivery. Intrauterine inflammation is present in the amniotic fluid already in midgestation in some pregnancies ending in preterm delivery (52). Postnatally, inflammation is induced in the lungs of premature infants by oxygen and respiratory therapy (49).

Several clinical studies have shown that chorioamnionitis suppresses the preterm infant’s risk of developing respiratory distress syndrome (RDS) (8), a disease caused by lack of production of pulmonary surfactant. However, a number of other studies have found no association between chorioamnionitis and oxygen requirement or RDS, and a large study associated chorioamnionitis with increased risk of RDS (8). The relationship of antenatal inflammation and the subsequent development of bronchopulmonary dysplasia (BPD), a disease characterized by decreased alveolar septation and decreased microvascular development, is also inconsistent. Some studies indicate that chorioamnionitis increases the risk for BPD, whereas others have shown no effect (8). Postnatal inflammation in the immature lung on the other hand may increase the risk of BPD (49). The discrepant pulmonary outcomes for preterm infants exposed to chorioamnionitis may be related to lack of information about the duration and intensity of the inflammation, to imprecision in making the diagnosis of RDS or BPD, to several confounding factors, and to various treatments that modify the clinical course of these pulmonary conditions (8).

IL-1 is a central inflammatory cytokine found in the amniotic fluid in chorioamnionitis (58) and in the lung effluent of intubated preterm infants after birth (11). Intra-amniotic administration of IL-1α or IL-1β enhances lung stability and the production of surfactant lipids and proteins in premature animals (13, 53). On the other hand, using transgenic mice overexpressing human IL-1β (hIL-1β) in the lung epithelium under the control of the Clara cell secretory protein (CCSP) promoter, we have shown that hIL-1β causes a lung disease similar to BPD in infant mice (14). In these mice, expression of hIL-1β during the saccular stage was sufficient to disrupt lung morphogenesis, whereas the lung was resistant to hIL-1β-induced injury during earlier and later stages of lung development (16).

The impact of inflammatory agents on lung morphogenesis may be dependent on their cellular location and levels in addition to the stage of lung development. In the present study, we used transgenic mice expressing hIL-1β under the control of the surfactant protein (SP)-C promoter to investigate how hIL-1β expression in the distal lung epithelium influences the development of the airspaces and the capillaries as well as the production of pulmonary surfactant in fetal lungs.

MATERIALS AND METHODS

Transgenic mice. Bitransgenic human SP-C (hSP-C)-reverse tetracycline transactivator (rtTA)/tetracycline operator (tetO)-cytomegalovirus (CMV)-hIL-1β (abbreviated to SP-C-IL-1β) mice, which express the mature hIL-1β transgene in the presence of doxycycline, were generated as follows. Mice bearing the rtTA transgene driven by the hSP-C promoter were mated with mice bearing the mature hIL-1β
transgenic driven by the (tetO)–CMV (33) to produce litters of both bitransgenic hSP-C-rTA/(tetO)–CMV-hIL-1β mice and single-transgenic hSP-C-rTA mice. To specifically study the effects of hIL-1β, single-transgenic hSP-C-rTA littermates were used as controls (abbreviated to SP-C/control) (47).

Bitransgenic rat CCSP (rCCSP)-rTA/(tetO)–CMV-hIL-1β (abbreviated to CCSP/IL-1β) mice, which express the mature hIL-1β transgene in the presence of doxycycline, and single transgenic rCCSP-rTA control mice (abbreviated to CCSP/control) were generated as previously described (33).

The mice were genotyped by PCR analysis of tail DNA using primers specific for the (tetO)–CMV-hIL-1β transgenic construct and the rCCSP-rTA transgenic construct as described (33), and for the hSP-C-rTA transgene as follows: 5'-GAC ACA TAT AAG ACC CTG GTC A-3'; 5'-AAA ATC TTA TTG CCA GCT TTC CCC-3'.

Animal care. The experiments were approved by the Animal Research Ethics Committee at the University of Gothenburg. The mice were housed in pathogen-free conditions. They were given access to water and chow ad libitum. Before being sampled, the pregnant dams or infant mice were anesthetized by intraperitoneal injection of a mixture of ketamine, xylazine, and acepromazine.

Fetuses were removed by hysterectomy. For lung tissue sampling, the pregnant dams or infant mice were inflation-fixed with 4% PBS-buffered paraformaldehyde as described (14). After overnight fixation at +4°C, the tissue was rinsed in PBS and graded ethanol and processed through conventional paraffin embedding. Five-micrometer tissue sections were stained with hematoxylin and eosin. Macrophages, neutrophils, and proliferating cells were detected with immunohistochemistry as described (23). Apoptotic cells were detected by immunohistochemistry for cleaved caspase-3 as described (22). Cleaved caspase-3-positive cells were counted in the airspaces and parenchyma of the distal lung in at least five nonoverlapping high-power fields (HPFs) (×400 magnification) from five animals per group.

Administration of doxycycline. To induce hIL-1β expression in the lungs of bitransgenic offspring, doxycycline (0.5 mg/ml; Sigma, St. Louis, MO) was administered in drinking water to pregnant dams from the beginning of pregnancy until death as described (14).

Measurement of hIL-1β protein levels. Lung tissue from fetuses was homogenized for measuring the concentration of hIL-1β by ELISA and the total protein concentration using the bicinchoninic acid method, as described (23).

Lung histology and immunohistochemistry. At E18.5, the lungs were fixed in 4% PBS-buffered paraformaldehyde. Lungs from postnatal mice were inflation-fixed with 4% PBS-buffered paraformaldehyde as described (14). After overnight fixation at +4°C, the tissue was rinsed in PBS and graded ethanol and processed through conventional paraffin embedding. Five-micrometer tissue sections were stained with hematoxylin and eosin. Macrophages, neutrophils, and proliferating cells were detected with immunohistochemistry as described (23). Apoptotic cells were detected by immunohistochemistry for cleaved caspase-3 as described (22). Cleaved caspase-3-positive cells were counted in the airspaces and parenchyma of the distal lung in at least five nonoverlapping high-power fields (HPFs) (×400 magnification) from five animals per group.

Quantification of air saccule area fractions. Measurement of air saccule area fraction at E18.5 was performed from lung sections.
stained with hematoxylin and eosin. Five nonoverlapping fields (×20 objective lens) from lungs of five mice of each genotype were analyzed. The percentage of air sacccule area of the total distal lung area was analyzed on binarized lung micrographs using the public domain program ImageJ (available at http://rsb.info.nih.gov/ij/image). The number of air sacs was counted in the same images.

Analysis of airspace size at PN7. Measurement of distal airspace size at PN7 in the lungs of five to six mice of each genotype was performed as described (33).

Analysis of the ratio of the capillary area to the total parenchymal area of the lung sections. Lung sections at E18.5 were stained with rat anti-mouse endomucin (V.7C7: sc-65495; Santa Cruz Biotechnology, Santa Cruz, CA) and donkey anti-rat Alexa Fluor 594-conjugated secondary antibodies (Molecular Probes, Eugene, OR) and mounted with Vectashield with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories) for nuclear staining. Stainings were imaged using a Zeiss Axioplan2 fluorescence microscope with Zeiss AxioCam HRm 14-bit grayscale CCD camera. The total capillary vessel area (endomucin-positive area) and cellular area (DAPI-positive area) of the distal parenchyma were quantified using the ImageJ software (NIH) from four photomicrographs (1300 × 1030 pixels) of 1300/11001030 magnification (objective lens) per sample (22). Continuous endomucin-positive areas with a surface area larger than 50 pixels were excluded from the analysis to eliminate vessels larger than capillaries.

Quantitative RT-PCR. Total RNA was isolated from lung tissue and reverse transcribed, and the cDNA was analyzed by quantitative real-time PCR, as described (14). Primer sequences (forward and reverse, respectively, 5’ to 3’) used were as follows: SP-A: GGA GCT TCA GAC TGT CCA GA, GAC TGA TGT CCC ATT GGT GGA AA; SP-B: CCA AAC CCC ACA CCT CTG AGA A, GCT TGT CCT CTG GAG CAG GCC TCT; SP-C: GAT ACT GTG TCC GAG TAT TCT, TTC TAC CGA CCC TGT GGA TGC T; SP-D: AGA GTG TGC CTT CTC CCA CTA TCA, GCC CAC ATC TGT CAT ACT CAG GAA; thyroid transcription factor 1 (TTF-1): GCT GCC GCC TTA CCA GGA, CGT GGG TGT CAG GTG AAT CAT; VEGF-B: AAG GAG AGT GCT GTG AAG CCA, GGA GTG GGA TGG ATG ATG TCA; VEGF-C: GGG AAA TGT GCC TGT GAA TGT A, TGG TGG ACT GAA AAC TGG TAT GA; VEGF-D: TCC ACT GCC TGG GAC AGA A, GCT TTA CAG ACG CAC TCA CAG C; VEGFR-3: TCT CCA GCA TCC TGA TCA C, TCC.

Fig. 2. Disrupted lung development in SP-C/IL-1β mice. A and B: lung histology at E18.5 of a SP-C/control mouse (A) and SP-C/IL-1β mouse (B). IL-1β disrupted lung development in fetuses. C: area of distal airspaces as percentage (%) of total distal lung area. A lower percentage of the lung tissue in SP-C/IL-1β mice was composed of air sacs than in SP-C-controls. D: number of distal air sacs. SP-C/IL-1β mice had fewer distal air sacs than SP-C-controls. E and F: lung histology at day of birth (PN0) of a SP-C/control mouse (E) and a SP-C/IL-1β mouse (F). Air sacs are large and the parenchyma thick in SP-C/IL-1β newborns. The images are representative of lung sections from 5 animals in each group. Scale bar = 200 μm. Open bars, SP-C/control mice; solid bars, SP-C/IL-1β-expressing mice. n = 5 per group. *P < 0.01 vs. littermate controls.
TGT GCT TTC CCG GAA CC. For primer sequences for β-actin, calgranulin A (S100A8), calgranulin B (S100A9), chitinase 3-like 3 (Ym1), chitinase 3-like 4 (Ym2), keratinocyte-derived chemokine (KC/CXCL1), monocyte chemoattractant protein 3 (MCP-3/CCL7), pyrophosphate (PP), and serum amyloid A3 (SAA3) at E15 and E18.5.

**Lipid analyses.** Lung phospholipids and fatty acids (FAs) were analyzed in whole lung samples from fetuses at E18.5. The lungs were immediately frozen and stored at −80°C until analysis. Samples were weighed, minced into pieces, and then homogenized with a Polytron PT 1200 CL (Kinematica, Luzern, Switzerland). Lipids were extracted by the procedure of Rose and Oklander (45) using chloroform and 2-propanol (7:1 vol/vol) and sonication. The phospholipid high-performance liquid chromatography method described by Silversand and Haux (46) was slightly modified for use of an internal standard and to collect lipid fractions from split postcolumn flow. The system consisted of two delivery pumps (Bischoff 2250, Bischoff Analysentechnik und -geräte, Leonberg, Germany), an injector of 20 μl, a gradient-mixing chamber 1.8 ml (SPARK), and a detector ELSD Varex MKIII (Alltech Associates, Deerfield, IL). The column was a LiChrospher 100 Diol 5 μm 250 × 4 mm with Si guard column. The column temperature was 55°C. Standards were composed of mixtures of phospholipids from L. rodan Fine Chemicals (Malmö, Sweden). Fractions corresponding to phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), diphasphatidylglycerol (DPG), and sphingomyeline (SM) were collected. Butylated hydroxytoluene was added (0.1 mg/ml chloroform). The fractions were dried under nitrogen and transmethylated in methanolic-HCl-3N at 80°C over 4 h. The FA methyl esters (FAME) were extracted with hexane, washed with water, dried over MgSO4, resolved in hexane (grade for spectroscopy), and separated by capillary gas-liquid chromatography in a Hewlett-Packard 6890 gas chromatograph equipped with a 30-m × 0.25-mm SP-2380 column; film thickness 20 μm. Helium at 1.4 ml/min was used as carrier gas. The injector and detector temperature was 250°C. The column oven temperature was programmed from 60–230°C at a heating rate of 8°C/min up to 155°C, 1.5°C/min up to 180°C and thereafter 6°C/min up to 230°C, where it was kept running for 10 min. The separation was recorded with HP GC Chem Station software (HP GC, Wilmington, DE). Heneicosanoic acid (21:0) was used as internal standard and the FAME identified by comparison with retention times of pure reference substances (Sigma Aldrich Sweden AB, Stockholm, Sweden). Total protein concentration was determined using ABX Pentra (Horiba Instruments, Irvine, CA).

**Statistics.** Measurement values are presented as means ± SE. Groups of normally distributed data were compared by unpaired t-test followed by Bonferroni correction. Otherwise, the Mann-Whitney test was used. Statistical analysis was conducted using GraphPad Prism 5.01 software (GraphPad Software, San Diego, CA). P values < 0.05 were considered statistically significant.

**Fig. 3.** Lung inflammation in fetal SP-C/IL-1β mice. A: neutrophil and macrophage count in lung parenchyma at E18.5. B: neutrophil and macrophage count in the air sacules at E18.5. Expression of hIL-1β increased the number of neutrophils and macrophages in the parenchyma and in the airspaces. C: Messenger RNA expression of keratinocyte-derived chemokine (KC/CXCL1), calgranulin A (S100A8), calgranulin B (S100A9), monocyte chemoattractant protein 3 (MCP-3/CCL7), and serum amyloid A3 (SAA3) at E15 and E18.5. IL-1β increased the mRNA expression of KC/CXCL1, S100A8, S100A9, MCP-3/CCL7, and SAA3. Open bars, SP-C/control mice; solid bars, SP-C/IL-1β mice. n = 4–8 per group. *P < 0.05, **P < 0.01, or ***P < 0.001 vs. littermate controls.
RESULTS

Levels of hIL-1β in the lungs of fetal SP-C/IL-1β mice. Doxycycline was administered to pregnant dams from the beginning of pregnancy. The mRNA expression of hIL-1β was analyzed in the lungs of SP-C/IL-1β mice at E12, E15, and E18.5, and hIL-1β protein levels were measured at E15 and E18.5. Levels of hIL-1β mRNA increased strikingly from E12 to E15 but not thereafter (hIL-1β/β-actin mRNA: E12, 0.037 ± 0.018, n = 5; E15, 54.22 ± 3.66, n = 9; E18.5, 65.30 ± 8.33, n = 8; E12 vs. E15 or E18.5, P < 0.0001; E15 vs. E18.5, P = 0.2). However, hIL-1β protein levels increased from E15 to E18.5 (hIL-1β protein/total protein: E15, 1.526 ± 231 ng/mg, n = 4; E18.5, 3.014 ± 370 ng/mg, n = 5; P < 0.05). We have previously shown that the levels of hIL-1β in the lungs of CCSP/IL-1β fetuses given doxycycline are much lower (hIL-1β protein/total protein: E15, ~10 ng/mg; E18.5 ~140 ng/mg) (14).

Differences in localization of hIL-1β production in SP-C/IL-1β and CCSP/IL-1β mice. Immunohistochemistry demonstrated that hIL-1β was localized to the epithelium of terminal bronchioles and air sacs at E18.5 and PN0 in SP-C/IL-1β mice (Fig. 1, A and C). In these mice, hIL-1β was not detected in proximal bronchioles or proximal airways (Fig. 1, A and C). In contrast, CCSP/IL-1β mice produced hIL-1β in the epithelium of bronchi and proximal bronchioles at E18.5 and PN0 but not in terminal bronchioles or in air sacs (Fig. 1, B and D).

Fetal growth and postnatal mortality of SP-C/IL-1β mice. The Mendelian ratios of genotypes were as expected at E15 and E18.5, and litter sizes were similar at E15, E18.5, and at birth (data not shown), suggesting that hIL-1β expression did not cause antenatal deaths.

After birth, SP-C/IL-1β pups were cyanotic and had respiratory distress (chest retractions). All SP-C/IL-1β mice (n = 40) died at or shortly (within the first few hours) after birth. SP-C/control pups had normal breathing and color. Four SP-C/control pups of 40 (10%) died before 2 days of life, all the others (90%) survived until death at PN7.

The body weights of SP-C/control and SP-C/IL-1β mice were similar at E15 (SP-C/control, 0.371 ± 0.024 g, n = 21; SP-C/IL-1β, 0.347 ± 0.018 g, n = 21; P = 0.4); E18.5 (SP-C/control, 1.183 ± 0.018 g, n = 28; SP-C/IL-1β, 1.142 ± 0.020 g, n = 24; P = 0.1), and PN0 (SP-C/control, 1.312 ± 0.048 g, n = 5; SP-C/IL-1β, 1.254 ± 0.045 g, n = 5; P = 0.4). All SP-C/control mice had normal postnatal growth and weighed 4.8 ± 0.1 g (n = 18) at PN7. In the absence of doxycycline, all SP-C/IL-1β (n = 26) and SP-C/control (n = 21) pups survived until death at PN7 and gained weight postnatally as expected (data not shown).

Abnormal lung morphogenesis in SP-C/IL-1β fetal mice. The area of peripheral airspaces as a percentage of total distal lung area was 41% in SP-C/control mice but only 27% in SP-C/IL-1β mice at E18.5 (Fig. 2, A–C). In addition, the number of air sacs was lower in SP-C/IL-1β than in SP-C/control mice (Fig. 2, A, B, and D). At PN0, the lungs of SP-C/IL-1β pups had much larger distal airspaces and thicker lung parenchyma than controls (Fig. 2, E and F).

In the absence of doxycycline, the mean alveolar chord length, a measure of alveolar size, was similar in SP-C/IL-1β (39.8 ± 1.75 μm, n = 5) and SP-C/control (35.7 ± 0.99 μm, n = 5; P > 0.05) pups at PN7.
E18.5 (SP-B/β-actin mRNA: CCSP/control, 1.120 ± 0.083, n = 10; CCSP/IL-1β, 0.762 ± 0.044, n = 9; CCSP/IL-1β vs. CCSP/control P = 0.0018). However, the SP-B mRNA levels in CCSP/IL-1β mice were 2.7-fold higher than in SP-C/IL-1β mice (CCSP/IL-1β vs. SP-C/IL-1β, P < 0.0001; CCSP/control vs. SP-C/control, P = 0.11). In contrast to SP-C/IL-1β mice, CCSP/IL-1β mice did not have lower levels of SP-C mRNA than littermate controls at E18.5 (SP-C/β-actin mRNA: CCSP/control, 1.121 ± 0.099, n = 10; CCSP/IL-1β, 0.991 ± 0.044, n = 9; CCSP/IL-1β vs. CCSP/control P = 0.26). CCSP/IL-1β mice had higher SP-C mRNA levels than SP-C/IL-1β mice at E18.5 (CCSP/IL-1β vs. SP-C/IL-1β, P < 0.0001; CCSP/control and SP-C/control mice, P = 0.15).

Expression of hIL-1β modified the lipid composition of the lungs of fetal SP-C/IL-1β mice. Most (80%) of the lipid content in pulmonary surfactant is PC (1). Analysis of the amounts of different phospholipids in the lungs of fetal mice showed that hIL-1β suppressed the amount of PC, whereas no differences in the amount of DPG, PE, PI, SM, or PS were found between SP-C/IL-1β and SP-C/control lungs at E18.5 (Fig. 6).

A large percentage of the PC in lung surfactant is dipalmitoylphosphatidylcholine (1). In SP-C/IL-1β fetal lungs at E18.5, the levels of palmitic acid (16:0) as molar percentage of total FAs in the PC fraction of lung phospholipids were lower than in SP-C/controls (Table 1). The decrease in PC and palmitic acid content in SP-C/IL-1β mice suggests that the production of surfactant lipids was suppressed in these mice.

Inhibition of the expression of VEGF-A, VEGFR-1, and VEGFR-2 and suppressed microvessel formation in fetal SP-C/IL-1β lungs. VEGF-A mRNA expression increased with gestational age in both SP-C/control and SP-C/IL-1β fetuses given doxycycline (Fig. 7A). VEGF-D mRNA levels were higher at E18.5 than at E15 in SP-C/control mice (Fig. 7A). IL-1β inhibited the expression of VEGF-A at E18.5 and VEGF-D at E15 and E18.5 (Fig. 7A). No differences in the expression of VEGF-B and VEGF-C were detected between SP-C/IL-1β and SP-C/control mice at either time point (Fig. 7A).

In SP-C/control mice, the expression of VEGFR-1, VEGFR-2, and VEGFR-3 increased with advancing gestation (Fig. 7B). This increase in VEGF receptor expression was
prevented by hIL-1β (Fig. 7B). At E18.5, the expression of VEGFR-1 and VEGFR-2 was lower in SP-C/IL-1β mice than in SP-C/controls (Fig. 7B). The mRNA expression of VEGFR-3 did not differ significantly between SP-C/IL-1β mice and SP-C/control mice (Fig. 7B).

To assess the vessel area, lung sections were stained for endomucin, a marker of endothelial cells. Vessels larger than 10 μm were measured from whole sections of the lungs of SP-C/IL-1β mice at E18.5 in SP-C/control and SP-C/IL-1β mice (Fig. 7). DAPI staining was used to identify epithelial cells. Vessels larger than 10 μm were excluded from the analysis. DAPI staining was used to identify epithelial cells. Vessels larger than 10 μm were excluded from the analysis.

Apoptosis but not proliferation was decreased in SP-C/IL-1β mice. Because apoptosis and proliferation are important processes during fetal lung development (30, 55), the numbers of apoptotic cells and proliferating cells in the fetal lungs were assessed. The number of apoptotic cells (cleaved caspase-3-positive cells) was lower in the parenchyma, but not in the airspaces, in SP-C/IL-1β mice compared with SP-C/controls mice at E18.5 (Fig. 8A). In both SP-C/IL-1β and SP-C/controls lungs, the percentage of nonciliated columnar epithelial cells was 26% of the cellular area, indicating that hIL-1β inhibited the development of capillaries in these mice (Fig. 7, C–E) (P < 0.05 vs. SP-C/controls).

IL-1β decreased the production of CCSP in SP-C/IL-1β fetuses. The mRNA expression of CCSP increased from E15 to E18.5 in SP-C/control and SP-C/IL-1β mice but was suppressed by hIL-1β at E18.5 (Fig. 8A). In both SP-C/IL-1β and SP-C/controls lungs, the percentage of nonciliated columnar cells in the bronchioles stained positive for CCSP in SP-C/IL-1β mice but in SP-C/controls (Fig. 8, B–D). These results suggest that hIL-1β suppressed the production of CCSP but not the number of Clara cells.

Table 1. Distribution (molar percentage) of fatty acids within phosphatidylcholine in lungs of SP-C/ control and SP-C/IL-1β mice at E18.5

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>SP-C/Control</th>
<th>SP-C/IL-1β</th>
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<tr>
<td>12:0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>14:0</td>
<td>2.584 ± 0.157</td>
<td>2.512 ± 0.112</td>
</tr>
<tr>
<td>14:1α5</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>16:0</td>
<td>56.36 ± 0.27</td>
<td>53.07 ± 0.39†</td>
</tr>
<tr>
<td>16:1α7</td>
<td>6.153 ± 0.156</td>
<td>5.271 ± 0.268*</td>
</tr>
<tr>
<td>18:0</td>
<td>6.138 ± 0.157</td>
<td>6.822 ± 0.187</td>
</tr>
<tr>
<td>18:1ω9</td>
<td>15.05 ± 0.24</td>
<td>15.86 ± 0.38</td>
</tr>
<tr>
<td>18:2ω6</td>
<td>3.983 ± 0.274</td>
<td>4.861 ± 0.244*</td>
</tr>
<tr>
<td>18:3ω3</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>18:5ω6</td>
<td>0 ± 0</td>
<td>0.061 ± 0.033</td>
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<tr>
<td>20:0</td>
<td>0.020 ± 0.001</td>
<td>0.024 ± 0.002</td>
</tr>
<tr>
<td>20:2ω6</td>
<td>0.231 ± 0.014</td>
<td>0.312 ± 0.017*</td>
</tr>
<tr>
<td>20:3ω6</td>
<td>0.468 ± 0.027</td>
<td>0.566 ± 0.032*</td>
</tr>
<tr>
<td>20:4ω9</td>
<td>0.071 ± 0.006</td>
<td>0.069 ± 0.007</td>
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<tr>
<td>20:5ω3</td>
<td>6.556 ± 0.266</td>
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<td>20:4ω6</td>
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<td>0.036 ± 0.004</td>
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<td>22:0</td>
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<td>0 ± 0</td>
</tr>
<tr>
<td>22:4ω6</td>
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<td>22:5ω3</td>
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<td>0.181 ± 0.007</td>
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<td>22:6ω3</td>
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<td>0.197 ± 0.012</td>
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<td>22:6ω3</td>
<td>1.188 ± 0.020</td>
<td>1.454 ± 0.043†</td>
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<td>24:0</td>
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<td>0 ± 0</td>
</tr>
<tr>
<td>24:1ω9</td>
<td>0.064 ± 0.003</td>
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Values are means ± SE, n = 5–6 per group. †P < 0.05 surfactant protein C (SP-C/IL-1β) vs. SP-C/control. †P < 0.01 SP-C/IL-1β vs. SP-C/control, embryonal day.

DISCUSSION

The present study shows that antenatal expression of hIL-1β in the distal lung epithelium caused lung inflammation, disrupted lung morphology and microvascularization, inhibited the expression of VEGF-A and its receptors, decreased apoptosis, inhibited surfactant production, and caused fatal respiratory failure at birth.

IL-1β, inflammation, and disrupted lung development. During the pseudoglandular stage of lung development (E9.5–E16.5 in the mouse), the primary buds develop into a complex tree-like structure ending in thousands of terminal tubules that later narrow during the canalicular stage (E16.5–E17.5 in the mouse). In the saccul ar stage (E18.5 to P5), the terminal buds develop numerous small sacs that later become the alveoli (36). The formation of sacs was prevented in SP-C/IL-1β fetuses. Lung morphology in these fetuses at E18.5 appeared similar to that found in wild-type mice earlier in gestation (36).

IL-1β, a central cytokine that regulates inflammatory and immune responses (20), may have disrupted lung development both directly and through its multiple downstream mediators. IL-1 signaling activates NF-kB via the IκB kinase complex. NF-κB activation reduces airway branching in fetal mouse lung explants (10). In addition, both IL-1β and direct NF-κB activation reduce the expression of fibroblast growth factor-10, an important growth factor in branching morphogenesis (9). Intra-amniotic administration of endotoxin, an activator of NF-κB, to fetal lambs causes abnormalities in alveolar development (37). Repeated maternal exposure to endotoxin during pregnancy also delays postnatal alveolarization in rats (17).

IL-1β expression caused infiltration of the lungs with neutrophils and macrophages and induced the expression of the neutrophil-attractant CC chemokine KC/CXCL1 and of the monocyte-attractant CC chemokine MCP-3/CCL7. Treatment with antibodies to KC/CXCL1 preserves alveolar development in hyperoxia-exposed newborn rats (6). Using newborn mice...
expressing hIL-1β under the control of the CCSP promoter, we have previously shown that blocking the action of CXC chemokines abolishes the migration of neutrophils into the airspaces, but the effects of inhibition of the CXCR2 pathway on lung morphogenesis were dependent on the developmental stage of the lung at the time of hIL-1β expression (22).

Antimacrophage chemokine treatment has been shown to suppress neutrophil and macrophage influx and to reduce protein oxidation in the lungs of hyperoxia-exposed newborn rats (56). hIL-1β also stimulated the production of S100A8 and S100A9. These proteins are highly expressed in neutrophils, but they are also found in monocytes, early differentiation stages of macrophages, and under inflammatory conditions in epithelial cells (31). High levels of S100A8/A9 proteins induce...

Fig. 7. Expression of hIL-1β inhibited microvascularization in the fetal SP-C/IL-1β lung. A: messenger RNA expression of VEGF-A, -B, -C, and -D at E15 and E18.5. IL-1β decreased the expression of VEGF-A and VEGF-D at E18.5. B: messenger RNA expression of VEGF receptor (VEGFR)-1, -2, and -3 at E15 and E18.5. IL-1β decreased the expression of VEGFR-1 and VEGFR-2 at E18.5. C and D: endomucin staining for endothelial cells (red) in the distal lung at E18.5 in a SP-C/control (C) and a SP-C/IL-1β mouse (D). Blue, DAPI staining. Scale bar = 100 μm. E: total capillary area as percentage of parenchymal area. Expression of hIL-1β decreased capillary development in the lung. Open bars, SP-C/control mice; solid bars, SP-C/IL-1β mice. n = 4–8 per group. *P < 0.05, or **P < 0.01 vs. littermate controls. †P < 0.05, ††P < 0.01, or †††P < 0.001 vs. same genotype at E15.

Fig. 8. Decreased mRNA expression and production of CCSP in SP-C/IL-1β fetuses. A: messenger RNA expression of CCSP at E15 and E18.5. IL-1β decreased CCSP mRNA at E18.5. B and C: CCSP-positive cells (red) within airways of a SP-C/control mouse (B) and a SP-C/IL-1β-expressing mouse (C). Scale bar = 50 μm. D: percentage of CCSP-positive cells in proximal airway epithelium at E18.5. The percentage of CCSP-positive cells in airway epithelium was decreased in SP-C/IL-1β fetuses. Open bars, SP-C/control mice; solid bars, SP-C/IL-1β mice. n = 5–8 per group. *P < 0.01 vs. littermate controls. †P < 0.01 vs. same genotype at E15.
neutrophil influx, aggravate ventilator-induced inflammation, and are associated with lung injury (31).

The expression of the acute-phase protein SAA3 that is induced by ventilation and endotoxin in the airway epithelium of preterm animals (54) was enhanced in hIL-1β-expressing lungs. On the other hand, SAA3 has been shown to enhance the production of IL-1α and IL-1β and to elicit IL-1-dependent neutrophilic pulmonary inflammation (5). SAA3 also promotes allergic asthma in mice in a IL-1-dependent manner (5).

IL-1β also upregulated the expression of the epithelial anion transporter pendrin and of the chitinase-like proteins Ym1 and Ym2. Pendrin regulates airway surface liquid, increases airway transporter pendrin and of the chitinase-like proteins Ym1 and Ym2 are associated with alternatively activated macrophages (15), allergic lung inflammation (51), and murine models of asthma (35, 59).

Lack of capillary development in fetal SP-C/IL-1β lungs. For normal lung development, alveolar microvasculature and airspace development must be coordinated. The walls of the sacculles, the primary septae, are tightly associated with the vascular plexus (36). VEGF-A is a member of a family of angiogenic and lymphangiogenic growth factors, which also includes VEGF-B, VEGF-C, and VEGF-D. The binding of VEGF-A, the main angiogenic factor, to VEGFR-2 induces proliferation and migration of endothelial cells (41, 57). Expression of hIL-1β inhibited the expression of VEGF-A and of its receptors VEGFR-1 and VEGFR-2 in SP-C/IL-1β fetuses. Because VEGF-A and VEGFR-2 have been shown to play critical roles in pulmonary vascular and alveolar development (24, 50), inhibition of VEGF action probably impaired microvascularization of the lung and may also have reduced formation of the distal airspaces in the present model. In addition, hIL-1β decreased the expression of VEGF-D, which stimulates both angiogenesis and lymphangiogenesis (41). Intra-amniotic exposure of fetal lambs to endotoxin also decreases the expression of VEGF and VEGFR-2 (28). On the other hand, IL-1β plays a role in ischemia-induced neovascularization by mobilizing endothelial precursor cells and by upregulating expression of VEGF and VEGFR-2 (3). We have shown that IL-1β induces persistent lymphangiogenesis but not angiogenesis in adult mouse airways (7).

Apoptosis in hIL-1β-expressing mice. Apoptosis during fetal lung development is needed for normal branching morphogenesis (55). Decreased prenatal apoptosis in the parenchyme may have contributed to the abnormal lung development in SP-C/IL-1β mice.

Inhibition of surfactant production in SP-C/IL-1β fetuses. The number of SP-B- or pro-SP-C-positive epithelial cells was decreased in the distal lung of SP-C/IL-1β fetuses, indicating that the differentiation of type II epithelial cells (34, 60) was prevented by hIL-1β. The mRNA expression of SP-B and SP-C in whole lungs was also decreased by hIL-1β. Absence of SP-B causes fatal respiratory failure (18, 40). The content of PC, the major lipid component of surfactant (1), was also decreased by hIL-1β in the fetal lung. In SP-C/IL-1β lungs, the molar percentage of palmitic acid was lower in the PC fraction than in SP-C/control lungs, suggesting that hIL-1β suppressed the production of dipalmitoyl PC, the major surfactant phospholipid. Lack of SP-B and of surfactant lipids may have contributed to the mortality of hIL-1β-expressing pups soon after birth.

Compernolle et al. (19) showed that loss of hypoxia-inducible factor-2α and reduction in VEGF resulted in fatal RDS in neonatal mice (19). Administration of VEGF-A intra-amniotically to fetuses or intratracheally to newborn mice prevented the development of RDS (19). VEGF-A also stimulated the production of SP-B and SP-C by type II cells in vitro (19). It is therefore possible that suppression of VEGF activity may have contributed to the lack of surfactant production in the present model.

CCSP in SP-C/IL-1β mice. The expression of CCSP was reduced in the proximal bronchiolar epithelium of hIL-1β-expressing mice. The 3.7-kb human SP-C promoter drives the expression of rtTA in the transgenic mouse model used in our study. Human 3.7-kb SP-C promoter studies using neutral reporter transgenes demonstrated strong expression along the entire embryonic respiratory epithelium that over time is silenced in the proximal airways but sustained in progressively more distal bronchiolar cells and in cells that have become

![Fig. 9. Apoptosis in fetal lungs.](http://ajplung.physiology.org/Downloadedfrom/howtoaccess)
alveolar cells (43). It is possible that the CCSP downregulation seen in the current model reflects a temporal transient early transgenic hIL-1β expression in those early proximal cells that was extinguished by the later time points collected for this study. On the other hand, pulmonary inflammation, known to inhibit CCSP expression (4), may also have suppressed the production of CCSP in SP-C/hIL-1β mice.

Effects of IL-1β on the fetal lung: differences between different models. A single intra-amniotic injection of IL-1 or of endotoxin enhances the production of SP-B and other surfactant components and improves lung stability after preterm birth (12, 13, 26, 53). The maturational effects of endotoxin are at least partially mediated by IL-1 (29). Administration of IL-1 intratracheally to fetal lungs has also been shown to promote the production of surfactant phospholipids (48). The beneficial effects on lung maturation of acute fetal exposure to inflammation are consistent with epidemiological studies suggesting that premature infants exposed to chorioamnionitis have a decreased risk of developing RDS (8).

We have previously shown that hIL-1β expression in CCSP/hIL-1β mice causes a BPD-like illness in the infant mouse (14). Expression of hIL-1β during the saccular stage was sufficient to disrupt alveolar development in the mouse (16). In contrast to SP-C/hIL-1β mice, CCSP/hIL-1β mice did not have abnormal lung structure prenatally, respiratory distress at birth, or early neonatal mortality (14, 22). When doxycycline is administered to pregnant dams, expression of rtTA, which drives the expression of hIL-1β in SP-C/hIL-1β and CCSP/hIL-1β mice, is initiated at ~E14 in CCSP-rtTA mice (14) but at ~E11 in SP-C-rtTA mice (42). Production of hIL-1β is therefore initiated earlier in SP-C/hIL-1β than in CCSP/hIL-1β mice. In addition, the levels of hIL-1β were higher in SP-C/hIL-1β than in CCSP/hIL-1β mice. When doxycycline was administered, hIL-1β was detected in the epithelium of the terminal bronchioles and air sacs/alveoli in SP-C/hIL-1β mice, whereas hIL-1β was only present in the proximal bronchioles and bronchi of CCSP/hIL-1β mice. Different phenotypes have previously been observed in mice overexpressing VEGF_164 under the control of the CCSP and SP-C promoters (2).

The levels of IL-1β are elevated in amniotic fluid in chorioamnionitis and spontaneous preterm labor (44) as well as in the lungs of preterm infants developing BPD (27). The prevalence of chorioamnionitis is inversely related to gestational age at birth, and its duration before delivery is generally unknown. Intrauterine infection leading to preterm labor may occur quite early in pregnancy and remain undetected for months (21). In some pregnancies subsequently ending in preterm delivery, elevated levels of the inflammatory marker IL-6 are found in amniotic fluid samples already at midgestation, corresponding to the late pseudoglandular stage of lung development (52).

Epidemiological studies on the role of chorioamnionitis in the pathogenesis of diseases such as RDS or BPD are hampered by lack of information about the duration and intensity of antenatal inflammation before birth, by clinical variation in the presentation and severity of diseases such as RDS or BPD, by imprecision in making these diagnoses, and by the impact of various therapies (such as oxygen exposure or mechanical ventilation) on these illnesses (25). In the present murine model, the duration of inflammation can be timed and lung development can be studied in animals that have not been exposed to oxygen or mechanical ventilation. However, the relevance of mouse studies as models of human lung disease is limited. A limitation of the present model is that the levels of hIL-1β production in the lungs of the bitransgenic mice are much higher than those found in amniotic fluid in chorioamnionitis or in lung effluent in ventilated preterm infants (27, 44). In the present study, hIL-1β was expressed in the lungs from E11 to PNO, i.e., from the pseudoglandular to the saccular stage of lung development (36). It is possible that induction of hIL-1β for a limited time during specific stages of lung development would have had different effects on the pulmonary development of these mice (16).

In summary, expression of hIL-1β in the distal lung epithelium disrupted fetal lung morphogenesis and surfactant production in the murine lung. The impact of IL-1β on fetal lung development is dependent on cellular location, time, and levels of production of the cytokine.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: A.H. and K.B. conception and design of research; A.H. and M.B. performed experiments; A.H. and M.B. analyzed data; A.H., M.B., B.S., and K.B. interpreted results of experiments; A.H. and M.B. prepared figures; A.H. and K.B. drafted manuscript; A.H., M.B., B.S., and K.B. edited and revised manuscript; A.H., M.B., B.S., and K.B. approved final version of manuscript.

REFERENCES


