IL-1 β Disrupts Postnatal Lung Morphogenesis in the Mouse

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Pulmonary inflammation and increased production of the inflammatory cytokine IL-1 β are associated with the development of bronchopulmonary dysplasia (BPD) in premature infants. To study the actions of IL-1 β in the fetal and newborn lung *in vivo*, we developed a bitransgenic mouse in which IL-1 β is expressed under conditional control in airway epithelial cells. Perinatal pulmonary expression of IL-1 β caused respiratory insufficiency that was associated with increased postnatal mortality. While intrauterine growth of IL-1 β -expressing mice was normal, their postnatal growth was impaired. IL-1 β disrupted alveolar septation and caused abnormalities in α -smooth muscle actin and elastin deposition in the septa of distal airspaces. IL-1 β disturbed capillary development and inhibited the production of vascular endothelial growth factor in the lungs of infant mice. IL-1 β induced the expression of CXC chemokines KC (CXCL1) and macrophage inflammatory protein-2 (CXCL2) and of CC chemokines monocyte chemoattractant protein (MCP)-1 (CCL2) and MCP-3 (CCL7), consistent with neutrophilic and monocytic infiltration of the lungs. IL-1 β caused goblet cell metaplasia and bronchial smooth muscle hyperplasia. Perinatal expression of IL-1 β in epithelial cells of the lung caused a lung disease that was clinically and histologically similar to BPD.

Keywords: inflammation; bronchopulmonary dysplasia; cytokine

Bronchopulmonary dysplasia (BPD), defined as the need for supplemental oxygen at 36 wk postmenstrual age, occurs in $\sim$ 30% of infants with birth weight less than 1,000 g (1). BPD is a major cause of long-term hospitalization, poor growth, recurrent respiratory morbidity, and mortality in infants. Abnormal lung function and structure associated with BPD persist into adolescence (2, 3).

The physical examination of an infant with BPD typically reveals signs of respiratory insufficiency, including tachypnea and retractions. Lung pathology in BPD shows inflammation, enlarged airspaces, and abnormal capillary development (4). Airway changes include bronchial smooth muscle hyperplasia and increase in mucin secretion (4). These structural lesions also characterize animal models of BPD, induced by chronic ventilation of extremely immature baboons and lambs (5, 6).

Clinical studies indicate that pre- and postnatal inflammation are associated with the development of BPD (1). Maternal chorioamnionitis, a common antecedent of premature birth (7), increases the newborn's risk of developing BPD (8). Chorioamnionitis leads to increased levels of inflammatory cytokines in amniotic fluid (7, 9). The lower the gestational age at spontane-

CLINICAL RELEVANCE

The research shows for the first time that IL-1 production in the perinatal murine lung causes a disease that is similar to BPD. These results increase our understanding of the mechanisms by which BPD develops in infants with pulmonary inflammation.

ous preterm delivery, the higher the likelihood of intrauterine infection (7) and the greater the risk for the development of BPD in the newborn infant (1). In some pregnancies, amniotic fluid levels of inflammatory cytokines are elevated for weeks or months before preterm delivery (10), implying that some preterm infants are exposed to prolonged inflammation before birth. The association of chorioamnionitis with subsequent development of BPD suggests that the process leading to BPD may begin before birth, but the mechanisms by which inflammation modifies lung development are poorly understood. In addition to lung immaturity, oxygen injury and barotrauma/volutrauma from mechanical ventilation are important risk factors for BPD (1). Both oxygen therapy and mechanical ventilation cause pulmonary inflammation. Oxygen exposure increases the production of neutrophil chemoattractants, leading to infiltration of the lungs with neutrophils (11). Similarly, mechanical ventilation induces neutrophil and macrophage infiltration in the lungs of premature animals (12, 13). Inflammation thus emerges as a common feature of the major conditions associated with the development of BPD.

Corticosteroids have been widely used to treat BPD. Although this anti-inflammatory therapy makes it possible to extubate infants earlier, it does not improve the long-term pulmonary outcome or mortality of premature infants and may adversely affect neurodevelopment (14). Better and more selective therapies to prevent or treat BPD are therefore needed. Understanding of the pathogenetic mechanisms leading to BPD is a prerequisite for the development of such therapies.

IL-1 β , a central cytokine involved in the initiation and persistence of inflammation (15), is increased in amniotic fluid in chorioamnionitis and preterm labor (9). Increased amniotic fluid concentration of IL-1 β is associated with development of BPD (16). Levels of IL-1 β are also increased postnatally in tracheal aspirates of premature infants who subsequently develop BPD (17). In accordance with the clinical observation that chorioamnionitis lessens the risk of respiratory distress syndrome in preterm infants (8), we have previously shown that intra-amniotic administration of IL-1 β acutely enhances the maturation of the lung surfactant system and improves lung stability after premature birth (18). The effect of chronic perinatal exposure to IL-1 β on lung development has not been studied.

Macrophages and monocytes are a primary source of IL-1 β , but other cells, including fibroblasts, T cells, and neutrophils, as well as bronchial and alveolar epithelial cells, produce IL-1 β (15, 19, 20). IL-1 β is synthesized as a precursor, pro-IL-1 β , which essentially lacks biological effects. The active, mature IL-1 β is

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produced upon cleavage of pro-IL-1 β by a specific IL-1 β -converting enzyme (ICE or caspase-1) (15) or by proteases (21).

To test the hypothesis that IL-1 β is an important mediator in BPD, we produced a bitransgenic mouse in which IL-1 β is conditionally expressed in epithelial cells of the lung, allowing the study of IL-1 β actions at specific times during antenatal or postnatal life. Perinatal pulmonary expression of IL-1 β causes a lung disease clinically and histologically similar to BPD.

MATERIALS AND METHODS

Conditional Expression of IL-1 β in the Mouse Lung

Transgenic mice in which tetracycline-responsive promoter (tet-O)₂CMV drives the expression of active, mature human IL-1 β were generated as previously described (22). To produce bitransgenic CCSP-rtTA-(tetO)₂CMV-IL-1 β fetuses and pups with regulatable IL-1 β production, heterozygous (tetO)₂CMV-IL-1 β ^{+/-} mice were mated with homozygous CCSP-rtTA^{+/+} activator transgenic mice (23) that express the reverse tetracycline transactivator (rtTA) under the control of the rat Clara cell secretory protein (rCCSP) promoter. Littermate single-transgenic CCSP-rtTA mice were used as controls. We chose to use these mice as controls to be able to study specifically the effects of IL-1 β expression on the lung phenotype (24). All mice were genotyped by PCR analysis of tail DNA using primers specific for transgene constructs as previously described (22).

Animal Care

The mice were housed in pathogen-free conditions and all experiments were conducted in accordance with local ethics committee guidelines. All animals were given access to water and laboratory chow *ad libitum*. For sample collection, pregnant dams or neonatal pups were anesthetized by intraperitoneal injection of a mixture of ketamine, xylazine, and acepromazine, the abdomen was opened, and the animal was exsanguinated by transection of abdominal vessels. For antenatal sample taking, vaginal plugs were observed and the plug date was counted as Embryonal Day zero (E 0). The date of birth was counted as Postnatal Day zero (PN 0).

Administration of Doxycycline

To induce IL-1 β transgene expression in the lungs of bitransgenic offspring, doxycycline (Sigma, St. Louis, MO) was administered in drinking water (0.5 mg/ml) to pregnant mice from the beginning of pregnancy and postnatally to lactating mice until the killing of fetuses or pups. The doxycycline solution was protected from light by covering cage bottles with aluminum foil, and the solution was changed three times per week.

RNA Isolation and Quantitative RT-PCR

Total RNA from fetal and postnatal lung tissue was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and treated with RNase-free DNase (DNA-free; Ambion, Austin, TX). One microgram of total RNA was reverse transcribed (Omniscript; Qiagen, Hilden, Germany), and cDNA was analyzed by real-time PCR using gene-specific, intron-spanning primers. For quantification of IL-1 β transgene, KC (the mouse homolog of human CXC chemokine gro- α [CXCL1]), macrophage inflammatory protein (MIP)-2, monocyte chemoattractant protein (MCP)-1, and MCP-3 mRNA levels, PCR was performed on cDNA equivalent of 20 ng of total RNA, using Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) and Mx3000P real-time PCR instrument (Stratagene). Specificity of polymerase chain reaction was confirmed by gel analysis during reaction optimization and melting curve analysis of each amplification product. Relative quantification of starting amounts of mRNA was performed with Mx3000P instrument software from amplification curves using standard curves obtained from dilution series of cDNA. The results were normalized to β -actin mRNA levels. Primer sequences used (forward and reverse, respectively, 5' to 3') were as follows: KC: GCA CCC AAA CCG AAG TCA TAG C, TTG TCA GAA GCC AGC GTT CAC C; MIP-2: CCC CCT GGT TCA GAA AAT CAT C, AAC TCT CAG ACA CGG AGC CAC ATC;

MCP-1: GCT CTC TCT TCC TCC ACC ACC AT, GCT CTC CAG CCT ACT CAT TGG GAT; MCP-3: TCT GCC ACG CTT CTG TGC CT, GCT CTT GAG ATT CCT CTT GGG GAT; (tetO)₂CMV-IL-1 β : CCA TCC ACG CTG TTT TGA CC, ACC AAG CTT TTT TGC TGT GAG TCC.

Lung Histology and Immunohistochemistry

Postnatal lungs were inflation-fixed, as described (22). The chest cavity was opened and the trachea exposed. A blunt cannula was inserted and tied to the trachea, and the lungs were inflated by instillation of PBS-buffered 4% paraformaldehyde fixative at a pressure of 25 cm H₂O. After overnight fixation at 4°C, the tissue was dehydrated and processed through conventional paraffin embedding. Five-micrometer tissue sections were stained with hematoxylin and eosin (H&E), Masson's trichrome, periodic acid Schiff (22), or Hart's elastin stain. For immunohistochemistry, the primary antibodies used were: rabbit anti-mouse KC (Abcam, Cambridge, UK), goat anti-mouse MIP-2 (Abcam), goat anti-mouse MCP-3 (R&D Systems, Abingdon, UK), monoclonal rat anti-mouse PECAM-1, clone MEC 13.3 (BD Pharmingen, San Diego, CA), monoclonal anti- α smooth muscle actin (α -SMA), clone 1A4 (Sigma), sheep anti-mouse FOXA2 (Upstate, Dundee, UK), monoclonal rat anti-mouse Mac-3, clone M3/84 (BD Pharmingen), and monoclonal rat anti-mouse neutrophils, clone 7/4 (Serotec, Oxford, UK). Biotinylated secondary antibodies, Mouse on Mouse immunodetection kit (used with α -SMA mouse monoclonal antibody), and avidin-biotin peroxidase with DAB-nickel chloride (Vectastain Elite ABC) were used according to manufacturer's instructions (Vector Laboratories, Burlingame, CA). Sections were lightly counterstained with nuclear fast red. After staining for FOXA2, lung sections were stained for acid mucins with Alcian Blue pH 2.5 method.

Cell Counts

Lung sections were immunostained using primary antibodies specific to neutrophils and macrophages, at E 18.5, PN 0, and PN 7. The numbers of stained cells were counted in 10 random high-power fields ($\times 40$ lens) in each section section (four animals per treatment and age group), and average numbers of positive cells per square millimeter were calculated.

IL-1 β , Vascular Endothelial Growth Factor, Chemokine, and Total Protein Measurement in Lung Homogenates

Fetal and postnatal lung tissue was homogenized in PBS containing protease inhibitor (Complete protease inhibitor; Roche Diagnostics, Basel, Switzerland), and centrifuged to remove cell debris. Supernatant was used for analysis as described (22). Concentration of human IL-1 β in lung homogenate was measured using DuoSet hIL-1 β ELISA development kit (R&D Systems), specific for human IL-1 β , with no cross-reactivity with murine IL-1 β . DuoSet ELISA development kits (R&D Systems) were used to quantify mouse chemokines KC, MIP-3, and MCP-1. Mouse MCP-3 was quantified using Instant ELISA kit (Bender MedSystems, Vienna, Austria). Vascular endothelial growth factor (VEGF) levels were measured using Quantikine mouse VEGF kit (R&D Systems). Assay standard concentration ranges were 3.9–250 pg/ml (hIL-1 β , MCP-1), 7.8–500 pg/ml (VEGF), and 15.6–1000 pg/ml (all other assays). Total protein concentration was measured using the bicinchoninic acid method (Sigma).

Morphometric Analysis of Airspace Size and Septal Thickness

Quantification of distal airspace size at PN 7 was performed from H&E-stained lung tissue sections, using the mean chord length as a measure of alveolar size, as described (22). A minimum of 10 representative, nonoverlapping fields from lungs of four mice of each genotype and treatment group were acquired in 8-bit grayscale, using a $\times 20$ lens, at a final magnification of 1.89 pixels per micrometer using a Nikon Eclipse E800 microscope and DXM1200 digital camera (Nikon, Tokyo, Japan). Areas of bronchiolar airways and blood vessels, as well as atelectatic areas, were excluded from the analysis. Chord length analysis was performed using the public domain program NIH Image with a chord length macro (available from the U.S. National Institutes of Health at <http://rsb.info.nih.gov/nih-image>). To obtain consistent results, images were thresholded using equal threshold levels for all treatment groups. Binarized, inverted lung micrographs were subjected to a logical

"AND" operation with horizontal and vertical grids of straight lines. The lengths of lines overlying alveolar space were then averaged as the mean chord length. Chord lines touching the edges of the image fields were excluded from analysis, hence the calculated mean chord length is representative of line segments touching air space walls at both ends. The same binarized images at PN 7 and similarly acquired images at PN 0 were used to assess the thickness of saccular/alveolar septa, as described (25). Ten nonoverlapping image fields from lungs at PN 7 and five from smaller lungs at PN 0 per animal, from four animals in each genotype and age group, were analyzed. Straight lines (~ 30 –40 per field) were drawn at 90° angles across the narrowest segments (to minimize the number of tangential sections) of septa of distal airspaces. The mean length of lines crossing the septa was determined using NIH Image software.

Statistics

Student's *t* test was used for comparisons of group averages. Measurement values are expressed as mean $\pm$ SEM. Neonatal mortality data were analyzed using Kaplan-Meier survival analysis and logrank (Mantel-Cox) test. *P* values < 0.05 were considered statistically significant.

RESULTS

Conditional Expression of IL-1 β in Lungs of Fetal and Newborn Bitransgenic Mice

Doxycycline (0.5 mg/ml) was administered in drinking water to dams from the beginning of gestation until killing of the pups. Thus the fetal mice received doxycycline transplacentally, whereas newborn mice received doxycycline secreted into the milk of nursing dams. Lungs from fetuses were collected daily from E 13.5–18.5 and from newborn mice on PN 0 and 7. IL-1 β mRNA increased on E 14.5 and was maximal by E 16.5 (Figure 1A), consistent with the activity of the rat CCSP promoter used to express rtTA (26). Postnatally, IL-1 β expression

Figure 1. Conditional expression of IL-1 β in the respiratory epithelium. Pregnant and lactating mice were maintained on water containing doxycycline from the beginning of gestation until killing of the fetuses or newborns. hIL-1 β mRNA and protein were assessed in the lungs of bitransgenic offspring by quantitative RT-PCR and ELISA. *Panel A* shows the induction of hIL-1 β mRNA expression in the lungs of the offspring. IL-1 β mRNA increased rapidly after E 14.5 and decreased postnatally (PN 7 versus 0, **P* < 0.05). Five to eight mice were studied at each time point. *Panel B* shows the concentration of hIL-1 β protein in lung homogenate. IL-1 β increased at E 14.5, reached a plateau at E 16.5, and decreased postnatally (PN 7 versus 0, ***P* < 0.01). *n* = 4–8 mice were studied at each age. Data are mean $\pm$ SEM.

decreased by $\sim 30\%$ from PN 0 to PN 7. In accordance with the ontogenic changes in IL-1 β mRNA expression, hIL-1 β protein was increased in the lungs of bitransgenic fetuses on E 14.5, reached a plateau on E 16.5, and remained at this level until PN 0 (Figure 1B). IL-1 β content in the lungs decreased by $\sim 60\%$ from PN 0 to PN 7 (Figure 1B).

IL-1 β Caused Respiratory Insufficiency, Poor Postnatal Growth, and Increased Postnatal Mortality

Newborn mice expressing IL-1 β developed respiratory insufficiency (chest retractions) within several days after birth, whereas

Figure 2. IL-1 β decreased postnatal growth and survival. Doxycycline was administered to dams throughout pregnancy and after birth. (A) An IL-1 β -expressing pup and control littermate on PN 6. IL-1 β caused small size, lack of subcutaneous tissue, and poor hair development. (B) IL-1 β decreased the growth of newborn mice. Data represent 9–14 mice per group at each time point. ***P* < 0.01. (C) IL-1 β significantly increased mortality of newborn mice (logrank test, *P* < 0.0001). Ten litters containing 47 control and 48 IL-1 β -expressing pups were studied.

littermate controls (single-transgenic CCSP-rtTA-mice) were asymptomatic (Figure 2A). Although the birth weights of IL-1 β -expressing mice did not differ from those of the single-transgenic control littermates, their postnatal growth was decreased (Figure 2B). On PN 7, the bitransgenic mice weighed $\sim$ 36% less than their control littermates (Figure 2A). The lung disease in IL-1 β -expressing mice was associated with increased postnatal mortality (Figure 2C). A few mice in both groups died at or soon after birth (Figure 2C). No deaths beyond the immediate postpartum time occurred among the single-transgenic controls, whereas $\sim$ 50% of the bitransgenic newborn mice died before 7 d of age (Figure 2C).

IL-1 β Caused Inflammation and Enhanced the Production of Neutrophil- and Monocyte-Attractant Chemokines in Fetal and Neonatal Lungs

IL-1 β caused neutrophil and macrophage infiltration in fetal and neonatal lungs (Figures 3B and 5B, Table 1). Inflammatory cells were seen in airways and airspaces in the lungs of bitransgenic newborn mice on PN 0 (Figure 3B), whereas few inflammatory cells were present in control lungs (Figure 3A). In IL-1 β -expressing fetal lungs, numbers of both neutrophils and macrophages were increased (Table 1). The number of neutrophils increased rapidly from E 18.5 to PN 0 and remained stable thereafter, whereas the number of macrophages continued to increase during the first week of life (Table 1).

Since neutrophil and monocyte influx are regulated by CXC and CC chemokines, respectively, the expression of ELR+ CXC chemokines KC and MIP-2, and of the CC chemokines MCP-1 and MCP-3, were studied. IL-1 β increased the mRNA expression of these chemokines in fetal and postnatal lungs (Figure 4). On PN 7, KC, MIP-2, MCP-1, and MCP-3 mRNA expression were upregulated 23-, 16-, 19-, and 8.3-fold, respectively, in IL-1 β -expressing lungs compared with controls (Figure 4). The protein levels of the chemokines were likewise much higher in the lung homogenates of IL-1 β -expressing fetuses and newborns than in controls (Figure 4). Immunostaining confirmed the increased production of the chemokines in the lungs of bitransgenic animals (Figure 4). Neutrophils and macrophages in alveolar septa and in subepithelial cells in the airway stained for KC (Figure 4B). MIP-2-positive inflammatory cells were seen in airway walls and in the septa of distal airspaces of bitransgenic mice (Figure 4D). MCP-3 immunostaining was increased in epithelial cells and in subepithelial cells in airway walls and in airways of IL-1 β -expressing mice (Figure 4F).

IL-1 β Disrupted Alveolar Development in Newborn Mice

To study alveolarization in bitransgenic CCSP-rtTA-(tet-O)₂CMV-IL-1 β and single-transgenic CCSP-rtTA controls,

dams were given water with or without doxycycline during pregnancy and lactation until pups were killed on PN 7. Pulmonary histology showed normal septation in the lungs of control mice that had received doxycycline during pregnancy and postnatally (Figure 5A) as well as in bitransgenic mice that had not received doxycycline (Figure 5C). In contrast, septation was lacking in IL-1 β -expressing mice (Figure 5B). The mean alveolar chord length of IL-1 β -expressing mice was significantly greater than either in bitransgenic mice that had not received doxycycline or in control mice, independent of whether the latter had received doxycycline (Figure 5D).

On histological sections, the septa of distal airspaces appeared thicker in IL-1 β expressing mice than in controls (Figures 3A, 3B, 6A, and 6B). Morphometric analysis revealed that IL-1 β increased the thickness of saccular/alveolar walls by 23% on PN 0 and by 38% on PN 7 in the lungs of IL-1 β -expressing mice compared with controls (Table 2). In control lungs, the septa became thinner during alveolar development (Table 2), whereas this decrease in septal width did not occur in IL-1 β -expressing mice (Table 2).

IL-1 β Decreased α -SMA Staining and Caused Abnormal Elastin Structure in the Distal Lung

Smooth muscle cells are located in vascular and bronchial walls and in alveolar septa of the lung. Septal smooth muscle cells (alveolar myofibroblasts) express α -SMA (27). α -SMA staining was decreased by IL-1 β (Figures 6A and 6B). While α -SMA-positive cells were seen in alveolar septa and at the tips of secondary septa in control mice (Figure 6A), few α -SMA-positive cells were detected in IL-1 β -expressing mice (Figure 6B). Elastin fibers were detected in walls of the distal airspaces and as dots at the tips of the developing secondary crests in control lungs on PN 7 (Figure 6C). In IL-1 β -expressing lungs, elastin fibers were poorly organized in the walls of airspaces, and elastin staining in septal crests was rare (Figure 6D). At 3 wk of age, elastin strands were smooth and narrow in the alveolar walls of control lungs and were visible as punctate staining on tips of secondary crests (Figure 6E). In contrast, elastin fibers were splayed and disorganized in the lungs of IL-1 β -expressing mice and were often perpendicular to the airspace wall (Figure 6F). Inflammatory cells were seen infiltrating the elastin bundles (Figure 6F). Abnormal elastin distribution was also notable in blunted secondary crests (Figure 6F).

IL-1 β Caused Abnormal Vascular Development of the Lung

PECAM immunostaining was performed to detect the vascular endothelium during the late saccular period and the alveolar phase of lung development (PN 3 and PN 14, respectively). In IL-1 β -expressing mice on PN 3, capillaries of varying sizes were

Figure 3. IL-1 β caused pulmonary inflammation. Pregnant and lactating mice were maintained on water containing doxycycline from the beginning of the pregnancy until killing of the newborn. Lungs were stained with H&E. Lung histology on PN 0 shows increased numbers of neutrophils and macrophages within the airspaces, airways, and blood vessels in IL-1 β -producing newborn pups (B) compared with control littermates (A). The saccular septa and the airway wall appear thicker in transgenic mice (B) than in controls (A). Figures are representative of $n = 4$ mice per group. Scale bar: 100 μ m.

Figure 4. IL-1 β stimulated the production of neutrophil- and monocyte-attractant chemokines in the lungs of fetal and newborn mice. Dams were given doxycycline from the beginning of pregnancy until killing of the fetal or newborn pups. IL-1 β increased the mRNA expression and protein levels of KC, MIP-2, and MCP-1, and MCP-3 at E 18.5, PN 0 and 7 (column diagrams). At least six animals in each group were studied by quantitative RT-PCR. Chemokine levels were determined by ELISA in lung homogenates from four to six animals in each group. * $P < 0.05$, ** $P < 0.01$. IL-1 β increased immunostaining for KC (B), MIP-2 (D), and MCP-3 (F), compared with control mice (A, C, and E, respectively). Data are from $n = 3$ mice per group. Scale bars: 100 μm (A–D), 50 μm (E, F).

Figure 5. IL-1 β disrupted alveolar morphogenesis. Water with or without doxycycline was administered to pregnant and lactating mice until killing of the pups on PN 7. Histologic sections showed that alveolarization was underway in the lungs of control pups that had received doxycycline (A) as well as in bitransgenic CCSP-rtTA-(tetO);CMV-IL-1 β mice that had not received doxycycline (C), whereas septation was lacking in the lungs of mice expressing IL-1 β (B). Tissue was stained with hematoxylin-eosin stain. Mean alveolar chord length was significantly increased in IL-1 β -expressing mice compared with control mice independent of whether the latter had received doxycycline or not, and to bitransgenic CCSP-rtTA-(tetO);CMV-IL-1 β mice that had not received doxycycline (D). Data represent the mean $\pm$ SEM with $n = 4$ mice in each group. *** $P < 0.005$. Scale bar = 200 μm .

TABLE 1. MACROPHAGE AND NEUTROPHIL COUNTS IN LUNG SECTIONS

	Neutrophils/mm ²	Macrophages/mm ²
E 18.5		
Control	8.9 ± 3.4	10.7 ± 2.2
Transgenic	28.3 ± 5.8*	25.1 ± 3.8*
PN 0		
Control	9.4 ± 2.1	9.5 ± 1.7
Transgenic	80.7 ± 11.7†	34.1 ± 6.1*
PN 7		
Control	15.4 ± 4.2	24.0 ± 3.3
Transgenic	55.1 ± 8.0†	109.5 ± 7.8†

Definition of abbreviations: E, embryonic day; PN, postnatal day.
Data are presented as average number of cells per square millimeter ± SEM; four animals were analyzed in each group.
* *P* < 0.05, IL-1β transgenic versus control.
† *P* < 0.01, IL-1β transgenic versus control.

scattered within the thick septa (Figure 7B), whereas in control lungs, capillaries lined the alveolar septa (Figure 7A). On PN 14, control lungs had an abundant capillary vasculature in the thin alveolar walls and in alveolar extensions (Figure 7C), whereas capillaries in IL-1β-expressing mice were dysmorphic (Figure 7D). IL-1β decreased the content of VEGF in the lung on PN 7 (Figure 7E).

IL-1β Caused Airway Remodeling and Goblet Cell Hyperplasia
In the airway walls, the epithelium and the subepithelial smooth muscle were thickened in IL-1β-expressing mice (Figure 8B)

TABLE 2. SACCULAR/ALVEOLAR SEPTAL THICKNESS IN NEWBORN LUNGS

	Septal wall thickness (μm)	
	PN 0	PN 7
Control	4.24 ± 0.12*	3.86 ± 0.08*
Transgenic	5.20 ± 0.29†	5.32 ± 0.28‡

Definition of abbreviation: PN, postnatal day.
Measurement was performed as described in MATERIALS AND METHODS. Data are presented as mean ± SEM. Four animals were studied in each group.
* *P* < 0.05, control PN 0 versus PN 7.
† *P* < 0.05, IL-1β transgenic versus control.
‡ *P* < 0.01, IL-1β transgenic versus control.

compared with controls (Figure 8A). Periodic Acid Schiff and Alcian blue staining was increased in airway epithelial cells, consistent with goblet cell hyperplasia (Figures 8D and 8E). Immunostaining for FOXA2 was absent or decreased in the goblet cells (Figure 8E).

DISCUSSION
IL-1β-Induced Lung Disease as a Model of BPD

While clinical studies have shown that increased levels of IL-1β in amniotic fluid or tracheal aspirate fluid are associated with the development of BPD (16, 17), the role of IL-1β in the pathogenesis of BPD has not been elucidated. The present results demonstrate for the first time that perinatal pulmonary expression of IL-1β

Figure 6. IL-1β decreased α-SMA staining and caused abnormal elastin deposition in alveolar septa. Pregnant and lactating mice were given water containing doxycycline from the beginning of gestation until killing of the pups. A and B show immunostaining for α-SMA in the lung of infant mice on PN 7. α-SMA immunostaining was seen in secondary crests (arrows) and in alveolar septa in control mice (A), whereas few α-SMA-positive cells were seen in the lungs of mice expressing IL-1β (arrow) (B). C and D show Hart's stain for elastin on PN 7. In control lungs (C), elastin fibers were detected in the walls of distal airspaces (arrowheads) and as punctate dots at the tips of the developing secondary crests (arrows). In IL-1β-expressing mice (D), elastin fibers of variable length were scattered in the walls of airspaces (arrowheads); few secondary crests were seen (arrows). In control animals at 3 wk of age (E), smooth and tight elastin bundles were observed in alveolar septa (arrowheads) and as well-defined dots at the tips of secondary crests (arrows) (E). In contrast, in IL-1β-expressing mice (F), elastin bundles were disorganized and splayed in the walls of distal airspaces (arrowhead) and elastin was blunted and thick at the tips of secondary crests (arrows). Inflammatory cells infiltrated the disordered elastin fibers (F, double arrow). Micrographs are representative of at least *n* = 3 animals in each group. Scale bar = 100 μm.

Figure 7. IL-1 β disrupted pulmonary vascular development and decreased the production of VEGF. Pregnant and lactating mice were maintained on water supplemented with doxycycline until killing of the newborn on PN 3 (A and B) and PN 14 (C and D). Lung sections were immunostained for PECAM-1. On PN 3, abundant capillaries lined the septa in the control lung (A). In IL-1 β -expressing lung on PN 3, capillaries were of varying sizes and were randomly located in the thickened septa (B). In control lungs on PN 14, abundant capillaries in the thin alveolar septa were in close proximity to the alveolar space and formed outsproutings into the secondary crests (C). In contrast, in IL-1 β -expressing lungs on PN 14, the walls of the distal airspaces were of variable thickness, with capillaries arranged in a dual parallel pattern or in the interior of the septa; some septa and capillaries appeared disrupted (D). Representative results for $n = 3$ mice in each group are shown. Scale bar: 100 μ m. Lung VEGF content (pg/mg protein) was significantly decreased in IL-1 β -expressing mice on PN 7. Data are mean $\pm$ SEM, $n = 6-8$ mice in each group; $*P < 0.05$.

Figure 8. IL-1 β caused airway remodeling and goblet cell hyperplasia. Dams were maintained on water supplemented with doxycycline from the beginning of pregnancy until killing of the pups on PN 7. Airway structure was evaluated using Masson's trichrome stain (A, B). IL-1 β caused thickening of airway epithelium and hyperplasia of the bronchial smooth muscle (B) compared with controls (A). PAS staining revealed goblet cell hyperplasia in the lungs of IL-1 β -expressing mice (D) compared with controls (C). FOXA2 staining was absent or decreased in nuclei of Alcian Blue-positive goblet cells in mice expressing IL-1 β , whereas adjacent non-mucus-producing cells stained for FOXA2 (E). Panels represent $n = 3$ mice per group, scale bar = 100 μ m.

causes a lung disease that clinically presents with respiratory distress and is histologically characterized by lung inflammation, alveolar hypoplasia, abnormal capillary development, airway remodeling, and goblet cell hyperplasia in the newborn murine lung. These clinical and pathologic features resemble those seen in infants with BPD (4). Pulmonary disease caused by IL-1 β was associated with poor postnatal growth and decreased survival. Similarly, infants with BPD often fail to thrive (28) and have increased mortality (29).

Inflammation, Chemokines, and Neonatal Lung Injury

IL-1 β caused infiltration of the newborn lung with neutrophils and macrophages, probably mediated, at least in part, by enhanced production of ELR+ CXC chemokines KC and MIP-2 and of CC chemokines MCP-1 and MCP-3. The production of KC and MIP-2 can be induced by inflammatory stimuli in macrophages, neutrophils, and in nonmyeloid cells, including endothelial cells and fibroblasts (30). Besides being neutrophil attractants, ELR+ CXC chemokines are potent promoters of angiogenesis (31). MCP-1 is essential for monocyte chemotaxis (32), whereas MCP-3 is a pluripotent chemokine, acting on multiple cell types including monocytes, lymphocytes, eosinophils, basophils, dendritic cells, and natural killer cells (33). MCP-1 and MCP-3 are produced by a variety of cells, including airway epithelial cells and airway smooth muscle cells, on stimulation with cytokines such as IL-1, bacterial and viral products, or mitogens (34). MCP-1 is also produced by alveolar type II cells in response to inflammatory agents (35).

Neutrophils and macrophages infiltrate soon after birth the lungs of infants developing BPD (36). The levels of IL-8, the functional homolog of which are the mouse chemokines KC and MIP-2, and of MCP-1 and MCP-3, are increased in the lungs of these infants (37, 38). While the role of neutrophils or neutrophil-attractant chemokines in BPD is not well defined, the CXC chemokines are important in the pathogenesis of lung injury caused by mechanical ventilation (39) and hyperoxia (40) in adult animals. Similarly, in neonatal animals exposed to hyperoxia, preventing neutrophil accumulation by treatment with an antagonist to the mouse CXC chemokine receptor CXCR2 or with antibodies to neutrophil chemokines (41, 42) preserves alveolar development. Likewise, treatment with anti-MCP-1 antibody decreases neutrophil and macrophage influx into hyperoxia-exposed lungs (43).

Alveolar Hypoplasia and Abnormal Vascular Development in BPD

IL-1 β impaired alveolarization and caused abnormal vascular development of the lung in neonatal mice. These pathologic lesions are common in human infants with BPD in whom similar large, simplified alveolar structures and dysmorphic capillary configuration are present (4). Animal models of BPD caused by chronic ventilation of preterm lambs and baboons are also characterized by alveolar hypoplasia and abnormal pulmonary vascular development (6, 44). Saccular/alveolar septa were thicker in IL-1 β -expressing lungs compared with controls. While saccular/alveolar septal thickness decreased during alveolar development in control mice, as expected (6), the septa of IL-1 β -expressing mice failed to become thinner. Increased septal thickness is also a feature of other models of BPD (25, 45, 46). Alveolar septation occurs in rats and mice from PN 4 through PN 14. Alveoli are formed by the outgrowth of secondary septa from the primary septa present in the newborn lung. During lung morphogenesis, expression of α -SMA in subsets of stromal cells in the alveoli is closely associated with elastogenesis (47). Consistent with previous results (47), we detected α -SMA-

containing septal cells, likely representing myofibroblasts, and elastin deposition in the alveolar septa and at the tips of the developing secondary septa in the lungs of control mice during the alveolarization phase. In contrast, IL-1 β -expressing mice had few α -SMA-containing cells and abnormal elastin structure in these locations. Abnormalities in elastin caused by IL-1 β were remarkably similar to the disordered elastin deposition in chronically ventilated lambs and in human infants with BPD (4, 48). The essential role of elastogenesis in alveolar septation has been demonstrated by studies in elastin-null mice and in mice lacking platelet-derived growth factor A (49, 50). Abnormal elastin structure in the lungs of IL-1 β -expressing mice may be the result of a combination of abnormal elastin deposition and of elastin degradation by elastolytic enzymes, such as neutrophil elastase and matrix metalloproteases MMP-9 and MMP-12, produced by neutrophils and macrophages (51, 52).

Decreased production of VEGF in the lungs of IL-1 β -expressing mice may contribute to their abnormal pulmonary development. Inhibition of VEGF signaling disrupts pulmonary vascular development and impairs alveolarization (53). VEGF expression is inhibited in the lungs after pulmonary injury caused by long-term ventilation in premature baboons (44) and in infants dying from BPD (54). In addition to VEGF, angiogenesis is regulated by multiple pro- and antiangiogenic factors. Chronic inflammation in the lung (55, 56) and other organs, such as in joints in rheumatoid arthritis (57), is associated with intense but aberrant angiogenesis. Many inflammatory mediators, including ELR+ CXC chemokines (31), have both direct and indirect angiogenic activities. The increased production of ELR+ CXC chemokines may promote angiogenesis in the lungs of IL-1 β -expressing mice. In addition, IL-1 β itself is a potent inducer of angiogenesis (58). The dysmorphic pulmonary vasculature in IL-1 β -expressing mice may thus result from abnormalities in the production of angiogenic factors in the lung. De Paepe and coworkers recently showed that vascular growth may not be inhibited in BPD, although the vasculature is structurally abnormal (59).

Airway Pathology in BPD and in IL-1 β -Expressing Newborn Mice

IL-1 β caused airway inflammation, goblet cell hyperplasia, and bronchial smooth muscle hyperplasia. Hyperplasia or metaplasia of the bronchial epithelium, goblet cell hyperplasia, and increased mucus production are frequent findings in infants with BPD (29). In infants with severe BPD, lobar or segmental atelectasis resulting from retained secretions and airway obstruction is common (29). A pathologic increase in airway smooth muscle in preterm infants with chronic lung disease provides a structural basis for the pathophysiologic increase in airway resistance that accompanies BPD (60). Immunostaining for the winged helix transcription factor FOXA2 was decreased or absent in goblet cells in the airways of IL-1 β -expressing mice, whereas FOXA2 staining was maintained in nongoblet bronchiolar epithelial cells. Lack of FOXA2 is sufficient to cause goblet cell hyperplasia in the bronchial epithelium in the mouse (61). Decreased FOXA2 is also associated with goblet cell hyperplasia in human lung tissue with bronchiectasis or BPD (61).

Growth in IL-1 β -Expressing Mice and in Infants with BPD

Several factors may contribute to the poor postnatal growth of IL-1 β -expressing pups. The severe respiratory illness of IL-1 β -expressing newborn mice is likely to limit their milk intake and thereby compromise postnatal growth. In addition, increased energy loss due to greater work of breathing may limit the growth of IL-1 β -expressing pups. Poor nutrition may directly contribute

to alveolar hypoplasia in IL-1 β -expressing pups, since malnutrition in adult animals (62) and humans (63) causes emphysema.

Inadequate growth is a well-known complication of BPD (28). A series of studies has demonstrated both increased resting energy expenditure and inadequate nutrient and energy intake in infants with BPD (28). It is likely that in addition to lack of vitamin A, known to be associated with the development of BPD (64), lack of calories and of protein and other nutrients is important in the etiology of poor alveolar development in infants with BPD.

Stage of Lung Development in IL-1 β -Expressing Mice and in Infants Developing BPD

When doxycycline was administered during pregnancy, IL-1 β production began in bitransgenic fetuses on E 14, consistent with previous results demonstrating that the rat CCSP promoter driving the expression of the rtTA gene becomes active at that time (26). The levels of IL-1 β were similar in fetal and adult lungs of bitransgenic mice (22), but lower in bitransgenic pups. This difference in IL-1 β production probably results from less efficient doxycycline administration to the newborn than the fetus or adult. Doxycycline is excreted in milk, but chelation of the drug with calcium in milk limits its absorption from the gastrointestinal tract (65).

IL-1 β expression in the lungs in the present study started in the late pseudoglandular–early canalicular phase of lung development and continued until mice were killed in the saccular (fetuses killed E 18.5 and newborns killed PN 1–3) or alveolar phase (PN 4 onwards). BPD primarily affects newborns whose gestational age at birth is between 23 and 27 wk. The lungs of these infants at birth are in the late canalicular or early saccular period. However, elevated levels of inflammatory cytokines are already present in amniotic fluid in the early second trimester in pregnancies that lead to spontaneous preterm birth (10), implying that the inflammatory process leading to premature labor may start weeks or even months before birth, and that fetuses may already be exposed to inflammation during the late pseudoglandular–early canalicular phase of lung development. Since chorioamnionitis and elevated IL-1 β levels in amniotic fluid are associated with the development of BPD in newborn infants, it is conceivable that BPD results from a chronic pulmonary injury that is initiated well before the infant manifests signs of respiratory illness.

The present results suggest that BPD may result from a chronic inflammatory process in the immature lung. This process is likely worsened and accelerated by postnatal insults such as exposure to mechanical ventilation and oxygen therapy. However, many immature infants who develop BPD have only mild respiratory disease at birth and are therefore not exposed to mechanical ventilation and high oxygen concentrations (1), suggesting that mechanical ventilation or exposure to high concentrations of oxygen is not necessary for the development of this illness.

IL-1 β -Induced Lung Injury in Newborn versus Adult Mice

We have previously shown that adult mice expressing IL-1 β develop histologic abnormalities consistent with the pathologic changes in COPD or asthma, including lung inflammation and emphysema, degradation of elastin structure, goblet cell hyperplasia, and airway thickening and fibrosis, but the mice are asymptomatic and maintain normal weight and activity (22). This contrasts with the poor weight gain, increased mortality, and clinically evident lung disease resulting from perinatal pulmonary IL-1 β expression. While the severity of the adult phenotype thus differs from the neonatal phenotype, the phenotypes

share common features, such as decreased alveolar size, goblet cell hyperplasia, and airway thickening. The present results suggest that similar inflammatory pathways may lead to BPD in infants and to COPD in adults and that these two illnesses may result from developmentally dependent responses of the lung to similar insults.

In summary, expression of IL-1 β in the lungs of fetal and newborn transgenic mice caused a pulmonary disease with characteristics that closely resemble BPD. These results suggest that IL-1 β -induced inflammation in the lung is sufficient to cause BPD, without additional insults such as prematurity, hyperoxia, or trauma caused by mechanical ventilation. The present animal model may be useful in identifying the mechanisms by which inflammation disrupts lung morphogenesis and causes lung remodeling, and in testing the efficacy of therapies to prevent or treat BPD.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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