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NF-κB Signaling in Fetal Lung Macrophages Disrupts Airway Morphogenesis

Timothy S. Blackwell,* Ashley N. Hipps,† Whitney J. Barham,‡ Michael C. Ostrowski,‖ Fiona E. Yull,‡ and Lawrence S. Prince*§

Bronchopulmonary dysplasia is a common pulmonary complication of extreme prematurity. Arrested lung development leads to bronchopulmonary dysplasia, but the molecular pathways that cause this arrest are unclear. Lung injury and inflammation increase disease risk, but the cellular site of the inflammatory response and the potential role of localized inflammatory signaling in inhibiting lung morphogenesis are not known. In this study, we show that tissue macrophages present in the fetal mouse lung mediate the inflammatory response to LPS and that macrophage activation inhibits airway morphogenesis. Macrophage depletion or targeted inactivation of the NF-κB signaling pathway protected airway branching in cultured lung explants from the effects of LPS. Macrophages also appear to be the primary cellular site of IL-1β production following LPS exposure. Conversely, targeted NF-κB activation in transgenic macrophages was sufficient to airway morphogenesis. Macrophage activation in vivo inhibited expression of multiple genes critical for normal lung development, leading to thickened lung interstitium, reduced airway branching, and perinatal death. We propose that fetal lung macrophage activation contributes to bronchopulmonary dysplasia by generating a localized inflammatory response that disrupts developmental signals critical for lung formation. The Journal of Immunology, 2011, 187: 2740–2747.

During fetal lung morphogenesis, simple epithelial tubes develop into a complex structure competent for gas exchange (1, 2). This process fails to occur normally in preterm infants with bronchopulmonary dysplasia (BPD), a chronic disease that affects ∼60% of preterm infants born before 28 wk (3). BPD results from arrested airway morphogenesis during the canalicular and saccular stages of lung development. Normally during these stages, small terminal airways branch, expand, and divide to form alveolar ducts. In patients with BPD, arrested development leads to fewer saccular stage airways, reduced numbers of alveoli, and lower capacity for gas exchange (4). Infants born after the early saccular stage of development rarely develop BPD, suggesting that the canalicular and early saccular stages represent a window of disease susceptibility. Various environmental factors have been implicated in development of BPD, involving either airway epithelial injury or lung inflammation (5, 6). Understanding how these factors lead to arrested airway morphogenesis will be critical for developing new therapeutic approaches.

Several lines of clinical and experimental evidence suggest infection or injury leads to an inflammatory response that causes or exacerbates BPD. First, chorioamnionitis (infection and inflammation of the amniotic membranes) is detected in up to 70% of preterm deliveries and is associated with increased BPD risk (7). Second, infants that develop BPD often have elevated levels of inflammatory mediators in their airway both at birth and during the later stages of disease progression (8, 9). Third, injecting Escherichia coli LPS into the amniotic fluid of pregnant animals inhibits airway branching and prevents subsequent alveolar development (10, 11). In developing an experimental mouse model for studying the mechanisms leading to BPD, we observed that LPS prevents saccular airway branching in both fetal mice and cultured fetal mouse lung explants. These data suggest innate immune activation and inflammatory signaling intersect with developmental pathways, interfering with processes required for branching morphogenesis.

LPS can inhibit airway branching in fetal lung explants in the absence of circulating inflammatory cells (10), suggesting that resident lung cells are competent to transduce signals that interrupt normal lung development. We have previously identified multiple gene targets of inflammatory signaling that are critical for normal airway morphogenesis (9, 12–14). However, the cellular site of the initial innate immune response critical for disrupting development is not known. Among the cell types in the fetal lung, airway epithelia and vascular endothelia appear at least somewhat capable of responding to microbial products (15, 16). To date, the potential role of macrophages in the fetal lung innate immune response has not been closely examined. Studies involving fetal macrophages have focused primarily on their ability to remove apoptotic cellular debris and remodel extracellular matrix (17–19). In this study, we report that macrophages are the primary cellular sites of the fetal lung innate immune response. Macrophage...
activation is required for the LPS-mediated production of inflammatory mediators that disrupt airway branching. In addition, targeted activation of fetal lung macrophages, both in cultured lung explants and developing mouse lungs in vivo, inhibits airway morphogenesis and produces a lung phenotype that closely resembles human BPD. Our findings indicate that macrophage activation and subsequent lung inflammation may play a key role in BPD pathogenesis.

Materials and Methods

Reagents

Gel-purified E. coli LPS (O55:B5) was purchased from Sigma-Aldrich. The following Abs were used for immunolabeling: rat anti-CD68 (Acris), rat anti-F4/80 (Acris), rat anti-E-cadherin (Zymed), rabbit anti-CD14 (Santa Cruz Biotechnology), and rabbit anti-anti-GFP (Abcam). DAPI, TO-PRO-3 iodide, ProLong Gold antifade reagent with DAPI, and Alexa-conjugated secondary Abs were purchased from Invitrogen. Anti-CD11b Microbeads, anti-CD68 (12G6) magnetic beads, and reagents for IC assay and analysis were obtained from Miltenyi Biotec. Reagents for preparing liposomal clodronate were obtained from Sigma-Aldrich. All cell culture media were purchased from Invitrogen. FBS was purchased from Thermo Fisher Scientific.

Mouse strains and lung explant culture

All animal experiments were thoroughly reviewed and approved by the Vanderbilt University Institutional Animal Care and Use Committee. BA-LysM-Cre and Rosa26-YFP mice were obtained from The Jackson Laboratory, NGL (20), LysM-Cre:IKKβ (21), and IFKβ (L. Connelly, W. Barham, H.M. Onishko, L. Chen, T. Sherrill, T. Zabuawala, M.C. Ostrowski, T.S. Blackwell, and F.E. Yull, submitted for publication). Mouse strains and lung explant culture. Mouse lung explants were isolated and cultured as previously described (16). For transgenic models, lungs of each mouse embryo were minced and cultured separately. Approximately 20 explants were isolated from each embryo. All explants studied were between 0.8 and 1.2 mm in diameter at initial time of culture. To quantify saccular airway branching in cultured lung explants, brightfield images of explants were acquired every 24 h of culture. Airway branching was expressed as the number of new branches formed between 24 and 72 h of culture in each explant. For macrophage depletion using clodronate, control or clodronate-containing liposomes were directly added to explants at the initial time of isolation. LPS was then added to the media 24 h later.

Fetal lung macrophage isolation

E15 fetal mouse lungs were dissected free of surrounding tissues and placed in ice-cold PBS. The lungs were homogenized and forced through 100-μm and 40-μm cell strainers. Cells were pelleted by centrifugation and resuspended in the presence of anti-CD11b–conjugated Microbeads (Miltenyi Biotec). The CD11b-labeled cells were collected using a magnetic separator, washed, and plated in RPMI 1640 with 10% FBS. Following overnight culture at 37˚C in 95% air/5% CO2, macrophages were washed, harvested, and treated for 4 h with LPS (250 ng/ml). NGL macrophages were similarly isolated, treated with LPS for various time points, and solubilized in Reporter Lysis Buffer (Promega). Luciferase activity was measured using SteadyGlo reagent (Promega) and a Synergy HT microplate reader (Bio-tek). Luciferase activity was normalized to total protein content as measured by Lowry’s method. Independent experiments were performed at least three separate times. Data between groups were compared by ANOVA or Student t test to test for significant differences. IL-1β concentrations were measured in homogenized fetal lung using the Quantikine Mouse IL-1β Immunoassay (R&D Systems). IL-1β concentrations were normalized to total protein as measured by BCA assay (Pierce).

Tissue processing and immunolabeling

Mouse lung tissue, fetal lung explants, and cultured macrophages were fixed, processed, and immunolabeled using standard techniques. For immunofluorescence, Alexa-conjugated secondary Abs were used for visualization, and nuclei were alternatively labeled with DAPI or TO-PRO-3. Immunohistochemical processing was performed using Vectastain Kits and 3,3’-diaminobenzidine visualization (Vector Laboratories) with Mayer’s H&E counterstaining (Sigma-Aldrich).

Imaging and image analysis

Confocal images were acquired using either an Olympus FV1000 (Olympus) or Leica SPE (Leica Microsystems) laser scanning confocal microscope. Widefield fluorescence images and brightfield images of fetal mouse lung explants were obtained using an Olympus IX81 microscope equipped with a Hamamatsu Orca ER CCD monochrome camera and Slidebook software (Olympus). Color images of lung specimens were photographed using a Nikon TE800 and SPOT color CCD camera (Diagnostic Instruments). All microscopy images were saved in the Tagged Image File format and imported into Photoshop (Adobe Systems) for processing. Images for comparison were always identically processed. For live imaging of NF-κB activation, NGL explants were cultured in a stage-top incubator (Okolab) and imaged using an Olympus IX81 inverted microscope (Olympus) with a WeatherStation enclosure. Exact temperature, humidity, and CO2 concentration were maintained during the imaging experiment. Fluorescence images were acquired every 10 min for the first 2 h and then every 20 min for 2–24 h of culture. Time series images were processed in Slidebook (Olympus). Lung morphometry and cell counting were performed using the Image Processing Tool Kit (Reindeer Graphics) within Photoshop (Adobe Systems). Fractional lung volumes were measured by analyzing images from serial fetal lung sections using a counting grid function. The fractions of airspace, large airway epithelia, small or distal airway epithelia, and mesenchyme were measured on E-cadherin–labeled sections. Septal thickness in newborn lungs was measured using the global intercept function. To account for possible anisotropy, intercepts in each image were measured over multiple iterations with 10˚ of rotation between each measurement. The mean intercept length for each image was recorded.

Results

LPS activates NF-κB in fetal lung macrophages

Where LPS initially activates the innate immune system in fetal lungs has not been identified. We therefore investigated the localization and kinetics of LPS-induced NF-κB activation in E15 fetal lung explants from NF-κB transgenic reporter mouse (NGL), which expresses GFP and luciferase downstream of a promoter synthesized from the NF-κB–binding repeats within the HIV-1 long terminal repeat (20). Time-lapse imaging of NGL explants demonstrated NF-κB–dependent GFP expression by 90 min after LPS treatment, with continued GFP expression to 12 h (Fig. 1A–D, Supplemental Movies 1–4). GFP expression localized to cells throughout the lung mesenchyme. To visualize the cellular site of NF-κB–GFP expression at higher resolution, we imaged NGL explants using confocal microscopy. As seen in Fig. 1E–M, 2 h of LPS treatment stimulated GFP expression predominantly in cells that colabeled with the macrophage marker CD68. Following 12 h of LPS treatment, GFP reporter expression was detected both in CD68-positive macrophages and in adjacent mesenchymal cells (arrows in Fig. 1G, 1M). The close proximity of GFP-expressing mesenchymal cells to CD68-positive macrophages suggests release of secondary inflammatory mediators that could then activate NF-κB in nearby cells.

Because macrophages were present in E15 fetal lungs and positioned to regulate inflammatory responses, we examined the timing of macrophage appearance into the fetal mouse lung during development. Immunostaining identified CD68-positive cells in the lung mesenchyme as early as E10 during the initial steps of lung formation (Fig. 2A). Macrophages remained present in the mesenchyme throughout fetal development. Fetal lung macrophages also expressed F4/80 (Fig. 2B). To measure function, we tested if LPS could directly activate isolated fetal lung macrophages. We obtained macrophages from E15 lungs using anti-CD11b–coated magnetic beads (Fig. 2C), exposed them to E. coli LPS, and measured gene expression by RT-PCR. LPS stimulated macro-

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Phagocytosis is indicated by dotted lines. Scale bar, 25 μm.

Expression was also detected in CD68-negative cells (arrows, **G** for CD68 (red), **K** labeled with DAPI. In LPS-treated explants, NF-κB activation was required for LPS-dependent disruption of saccular airway branching (Fig. 3). We then measured the number of new airways that formed along the periphery of each explant between 24 h and 72 h. Although LPS inhibited formation of new saccular airways in control and empty liposome-treated explants (Fig. 3), clodronate depletion of macrophages protected saccular airway branching from the effects of LPS (Fig. 3).}

**Macrophage activation inhibits saccular airway branching**

We have previously developed an E15 fetal mouse lung explant model to study the mechanisms regulating canalicular and saccular stage airway branching (10, 16). Following 72 h of culture, the structures along the periphery of these explants resemble early saccular lungs by undergoing type I/type II cell differentiation, thinning of the mesenchyme, and branching of small airways. This model parallels the period of lung development occurring postnatally in extremely preterm infants born between 23 and 28 wk gestation, making it relevant for investigating BPD pathogenesis. Airway branching in E15 explants requires fibronectin, mesenchymal α5β1-integrin expression, and fibroblast growth factor-10 (FGF-10) (10, 12, 13). We have also previously shown that TLR agonists, hyperoxia exposure, and tracheal aspirate fluid from preterm infants exposed to antenatal inflammation can each inhibit airway branching in cultured explants (10, 13, 22). To initially test if the inhibitory effects of LPS on branching required macrophage activation, we depleted macrophages from E15 fetal lung explants using clodronate-containing liposomes (Fig. 3). We then measured the number of new airways that formed along the periphery of each explant between 24 h and 72 h. Although LPS inhibited formation of new saccular airways in control and empty liposometreated explants (Fig. 3E, 3G), clodronate depletion of macrophages protected saccular airway branching from the effects of LPS (Fig. 3F, 3G). Macrophage depletion also substantially reduced the amount of LPS-induced IL-1β within lung explants (Fig. 3H), suggesting macrophages produce most of the IL-1β within LPS-treated fetal lung explants. Macrophages, therefore, are important for both production of inflammatory mediators within the fetal lung and inhibiting airway branching following exposure to LPS.

As clodronate could have off-target toxic effects, we next used a molecular approach to test if NF-κB activation in macrophages was required for LPS-dependent disruption of saccular airway
branching. For these experiments, we used mice with macrophage-specific IkB kinase β (IKKβ) deletion. LysM-Cre:IKKβΔ/Δ mice (IKKβΔ) lack functional IKKβ in myeloid cells, including macrophages and monocytes (21). LysM-Cre recombinase activity was confirmed in fetal lung explant macrophages using Rosa26-YFP reporter mice (Fig. 4A, 4B). We next measured the effects of LPS on explants from IKKβΔ and littermate controls. LPS failed to inhibit saccular airway branching in IKKβΔ explants (Fig. 4C, 4D), supporting the critical role of macrophages in inflammation-dependent disruption of airway morphogenesis and identifying NF-kB as the operative signaling pathway in mediating this effect. The total amount of IL-1β produced in LPS-treated IKKβΔ explants was much lower than in explants from littermate controls, again demonstrating that macrophages are responsible for a majority of the IL-1β produced in the LPS-treated fetal lung (Fig. 4E).

Having observed that activation of the NF-κB pathway in macrophages appears to mediate the effects of LPS on saccular airway morphogenesis, we next tested if NF-κB activation in macrophages is sufficient to disrupt airway morphogenesis and whether macrophages can impact airway formation in vivo. For these experiments, we used IKFM transgenic mice that express a macrophage-specific doxycycline-inducible c-fms transactivator and a constitutively active IKKβ mutant (cIKKβ) gene downstream of a tet-responsive promoter (L. Connelly et al., submitted for publication). We cultured saccular stage fetal lung explants from IKFM mice and control littermates in the absence or presence of doxycycline (Fig. 5A, 5B). Doxycycline had no effect on branching in control explants, but caused airway dilation and reduced branch formation in IKFM explants (Fig. 5B). Excessive LPS treatment (*p < 0.05 LPS compared with untreated [n = 4], #p < 0.01 LPS compared with LPS + clodronate [n = 4]).

FIGURE 3. Macrophage depletion protects fetal lung explants from the effects of LPS. A and B, E15 fetal mouse lung explants were cultured in the absence or presence of liposomal clodronate (50 mg/ml). Following 72 h of culture, explants were immunostained with Abs against the macrophage marker CD68 to verify macrophage depletion (B). C–F, Brightfield images show that LPS inhibits formation of new saccular airway branches (E), but this effect is prevented in clodronate-treated explants (F). Scale bar, 50 μm (A–F). G, LPS inhibited formation of new saccular airways in control and empty liposome-treated explants (*p < 0.001; n = 24). However, LPS did not inhibit branching in clodronate-treated explants. H, Clodronate depletion of macrophages reduced the amount of IL-1β released following LPS treatment (*p < 0.05 LPS compared with untreated [n = 4], #p < 0.01 LPS compared with LPS + clodronate [n = 4]).

FIGURE 4. Targeted deletion of IKKβ in macrophages protects fetal lung explants from the effects of LPS. A, LysM-Cre recombinase activity in E15 fetal mouse lung explants. Fluorescence images of cultured LysM-Cre:Rosa26-YFP reporter explants. YFP expression is seen in macrophages throughout the explant. Cell nuclei visualized with DAPI. Scale bar, 10 μm. B, LysM-Cre:Rosa26-YFP macrophages immunostained with Ab against CD68 (red) to show overlapping expression. Scale bar, 50 μm. C, LPS does not inhibit saccular airway branching in IKKβΔ explants. Scale bar, 50 μm. D, LPS inhibited the number of new saccular airways that formed between 24 and 72 h of culture in IKKβΔ/littermate controls, again demonstrating that macrophages are responsible for a majority of the IL-1β produced in the LPS-treated fetal lung. E, Macrophage expression of IKKβ is required for normal IL-1β expression following LPS treatment. LPS increased IL-1β production in littermate control IKKβΔ explants (*p < 0.05; n = 6), but did not significantly increase IL-1β in IKKβΔ explants (*p < 0.05 compared with LPS in IKKβΔ/F+ explants; n = 6).

IKKβ activity in IKFM macrophages inhibited branching, as did adding LPS (Fig. 5C). Inducing cIKKβ expression in macrophages increased IL-1β expression in fetal lung explants, demonstrating activity of the transgene (Fig. 5D). These results suggest that NF-κB activation specifically in macrophages is sufficient to disrupt normal fetal lung morphogenesis.

Targeted NF-κB activation in mouse macrophages disrupts lung morphogenesis

We next tested if macrophage-specific overexpression of cIKKβ in IKFM mice could alter lung morphogenesis in intact fetal mice.
Pregnant IKFM mice were treated with doxycycline to activate macrophages during either the late pseudoglandular stage (E12–E16) or from the late pseudoglandular stage through the canalicular and early saccular stages (E14–E18, Fig. 5). Doxycycline administration from E12–E16 did not cause apparent changes in airway formation (Fig. 5E,5F). However, transgene activation from E14–E18 caused significant changes with less airspace and increased mesenchyme in IKFM lungs compared with littermate controls (Fig. 5E, 5G). These differences were consistent with defects in saccular airway branching morphogenesis. The lack of effect in mice treated E12–E16 suggests that airway branching in the E14–E18 lung may have a window of vulnerability to macrophage activation. These data support the idea that macrophage activation and subsequent lung inflammation disrupt the later stages of airway formation.

To investigate how the morphological changes observed in IKFM mice affect respiratory function, mice treated with doxycycline from E14–E18 were allowed to deliver at E19 and E20. FIGURE 5. IKKβ activity in macrophages is sufficient to inhibit saccular airway branching. Brightfield images of E15 fetal lung explants from IKFM mice (B) and littermate controls (A) cultured under control conditions (top panels), in the presence of doxycycline (middle panels), and in the presence of LPS (bottom panels). Scale bar, 50 μm (A, B). C. Doxycycline induction if cIKKβ in macrophages inhibited saccular airway branching. Doxycycline had no effect in littermate control explants, whereas LPS inhibited formation of new saccular airway branches in both control and IKFM explants (*p < 0.05 compared with branches formed in the absence of doxycycline and without LPS [n = 9]). D. Doxycycline induction of cIKKβ in macrophages increased IL-1β expression in IKFM explants (*p < 0.05; n = 9). Dotted line indicates baseline expression level. E. Overexpression of cIKKβ in macrophages inhibits saccular stage lung morphogenesis in vivo. IKFM mice were given doxycycline (Dox) from either E12–E16 (top panels) or E14–E18 (bottom panels). Sections were immunostained using anti-E-cadherin to label epithelia. Scale bars, 100 μm. Lung morphogenesis did not appear altered in mice given Dox from E12–E16, either by histology or morphometric measurement of large and small airway epithelia, airspace (air), and mesenchymal (mes.) volumes (F). Dox administration from E14–E18 disrupted normal lung development, with IKFM lungs having smaller airways with reduced branching and expansion (E, G). Morphometry measurements confirmed decreased airspace and increased mesenchymal volumes in IKFM mice treated with Dox (G; *p < 0.001; n = 27).
Disruption of normal lung development in IKFM pups led to reduced survival in the first 24 h. Newborn IKFM mice appeared cyanotic, and many died soon after birth, whereas control littersmates appeared healthy (Fig. 6A, 6B). Histological examination of newborn IKFM lungs again showed a thickened lung interstitium compared with control littersmates (Fig. 6C, 6D). As with mice harvested at E18, the microscopic appearance of newborn IKFM mouse lungs, with thickened interstitium and simplified airways, showed similarities to the lungs of patients with BPD. Macrophages were present both lining the airway lumen and within the interstitium in both control and IKFM newborn lungs (Fig. 6E). IKFM mice had increased numbers of F4/80-positive cells, which may represent increased macrophage recruitment, maturation, or proliferation (Fig. 6F). IKFM and control lungs contained similar numbers of lymphocytes and neutrophils (Fig. 6G), without signs of consolidated infiltrates or edema. These data further demonstrate that macrophage NF-κB activation, which could occur secondary to infection or other insults, can inhibit saccular airway morphogenesis.

Macrophage activation from E14–E18 in IKFM mice caused a persistent inhibition of expression of multiple genes critical for branching morphogenesis in newborn lungs (12, 23, 24), including reduced expression of the α8 integrin subunit, BMP4, and Wnt7b (Fig. 6H). Expression of BMPR1A and FGF-10 also trended lower in IKFM lungs. Macrophage activation and release of inflammatory cytokines can therefore inhibit gene expression in the developing lung, leading to altered airway morphogenesis. These findings are consistent with our findings in lung explants, where macrophage activation can inhibit airway branching in the absence of systemic circulation. Activation of NF-κB in fetal lung macrophages could therefore be a common feature of insults that disrupt normal airway morphogenesis. As such, our model of a BPD-like phenotype in IKFM mice may be useful in further defining the pathogenesis and course of BPD.

Discussion

In this study, we demonstrate that NF-κB activation in macrophages is a key initial step in the fetal lung inflammatory response and sufficient to disrupt fetal lung morphogenesis. Macrophages in the canalicular or saccular stage fetal lung reside in the interstitium and respond to endotoxin. The NF-κB–dependent release of inflammatory mediators from these activated macrophages may then cause a second wave of signaling in neighboring cells, including the mesenchymal cells critical for airway morphogenesis. Even when the entire fetal lung is exposed to LPS, as is the case when explants are treated with LPS, macrophages appear to be the major cellular site of NF-κB activation and cytokine production. Macrophage depletion or targeted IKKβ deletion dramatically reduced the amount of IL-1β produced in response to LPS treatment and protected airway branching from the inhibitory effects of LPS. In addition, targeted activation of fetal lung macrophages was sufficient to disrupt lung morphogenesis, causing perinatal lethality and airway pathology that resembles human BPD.

These findings fill an important gap in our knowledge of how the immature fetal lung responds to inflammatory stimuli. When preterm infants are exposed to chorioamnionitis in utero, inflammatory cytokines accumulate in their airways (9). The infants with high levels of inflammatory mediators in their lungs at birth are more likely to develop BPD (25). Not only are macrophages the major source of inflammatory mediators in the lung, but also their location in the fetal lung interstitium may increase the likelihood that macrophage-derived cytokines can affect immediately adjacent cells. Recent studies have demonstrated increased macrophage recruitment to fetal or newborn lungs following either mechanical or endotoxin-mediated injury (26, 27). However, our current study is the first to connect macrophage activation and altered lung morphogenesis, to our knowledge. We propose a two-wave mechanism for NF-κB activation leading to arrested lung development in BPD. In this model, microbial products initially activate NF-κB in lung macrophages. The release of inflammatory mediators, particularly IL-1β and/or TNF-α, then causes NF-κB activation in the adjacent mesenchymal cells. NF-κB activation in the mesenchyme disrupts expression of genes important for the precise, controlled epithelial–mesenchymal interactions that regulate airway branching. This mechanism is supported by our previous findings that NF-κB activation in fetal lung mesenchymal cells interferes with normal expression of important developmental genes, including FGF-10 and integrin αβ1 (9, 12–14). The decreased BMP4 expression in IKFM lungs may be due

**FIGURE 6.** Targeted expression of a cIKKβ in fetal macrophages causes arrested lung development and perinatal lethality. A and B, Pregnant mice were treated with doxycycline from E14–E18 and then allowed to deliver. Newborn IKFM mice were born alive but developed respiratory distress and became cyanotic and lethargic soon after delivery. Littermate controls appeared healthy and vigorous. C and D, Newborn IKFM lungs have abnormal structure, with wider interstitium between airways and increased mean septal thickness (p < 0.01; n = 15). Scale bar, 100 μm (C, D). E and F, Immunostaining for F4/80 shows increased numbers of macrophages in newborn IKFM lungs compared with control littermates. Scale bar, 100 μm (E, F). G, The numbers of lymphocytes and neutrophils (PMN) in the lungs of newborn IKFM mice and littermate controls were similar. H, Reduced expression of genes required for normal branching morphogenesis in newborn IKFM lungs treated from E14–E18 with doxycycline. Expression measured by real-time PCR (p < 0.05; n = 7). Dotted line indicates control expression level.
to the direct effect of inflammatory cytokines on the airway epithelium (28) or secondary to reduced FGF-10 expression in the mesenchyme (23). Although inflammatory mediators that signal via other pathways may also affect lung development, particularly if the phenotype can be modulated so that affected pups can survive the neonatal period.

The inducible cIKKβ transgene allows macrophage activation at distinct stages of lung development, as compared with postnatal rodent models that are restricted to studying the late saccular and alveolar stages of lung development (29). Human infants born at a comparable stage of lung development to that of newborn mice very rarely develop BPD, making these experimental models less useful for investigating the early mechanisms of BPD pathogenesis (3). IKFM mice may also allow us to test if the effects of macrophage activation are reversible or persistent, how repetitive activations affect lung development, and if macrophage activation alters the susceptibility to other types of injury including hyperoxia, mechanical trauma, and viral infection. These future studies may help us better understand why some infants develop only mild BPD and others have more severe or progressive disease. The IKFM experimental model will potentially permit studies that target different phases of the inflammatory response in the fetal and newborn lung to better identify potential strategies for treatment.

Although fetal lung macrophages clearly respond to innate immune stimuli, their functional role in normal embryogenesis remains uncertain. In developing tissues, macrophages may remove cellular debris, remodel extracellular matrix, or express growth factors (30). Removal of apoptotic cells may be critical for lung morphogenesis, as at least one study has demonstrated that mice lacking the phosphoserin receptor are unable to phagocytose apoptotic cells and die following birth with abnormal lung structure (31). In the developing mammary gland, macrophages localize to collagen fibrils along elongating ductal branches, possibly playing a role in extracellular matrix remodeling (32).

Macrophages present in the fetal kidney may contribute to development by expressing specific Wnt ligands (33). We do not yet know if fetal lung macrophages play a similar trophic role in normal airway morphogenesis. In addition to the downstream effects of macrophage-derived cytokines on mesenchymal cell gene expression, inflammatory activation could divert macrophages from their yet uncharacterized developmental or trophic roles. This intersection of inflammatory and developmental pathways could therefore be a focal point for disease pathogenesis.

The two-wave model for propagation of inflammatory signals that disrupt branching morphogenesis suggests several potential therapeutic strategies for preventing BPD. Targeting macrophage activation could prevent the initial wave of fetal lung inflammation. The feasibility of this approach is supported by our experimental data using macrophage depletion and targeted deletion of IKKβ. However, many women clinically present already in preterm labor and with evidence of chorioamnionitis (34). In these situations, fetal lung macrophage activation may have occurred prior to delivery. Therefore, alternative strategies could target inflammatory mediators such as IL-1β and TNF-α to prevent the effects of inflammation on mesenchymal gene expression and altered epithelial–mesenchymal interactions. Finally, approaches that restore the mesenchymal cell phenotype to a normal fetal lung gene expression pattern and cell behavior could promote ongoing lung morphogenesis even in preterm infants with previous inflammatory exposures.

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