Prenatal inflammation exacerbates hyperoxia-induced functional and structural changes in adult mice

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Prenatal inflammation exacerbates hyperoxia induced functional and structural changes in adult mice. Am J Physiol Regul Integr Comp Physiol 303: R279–R290, 2012. First published June 20, 2012; doi:10.1152/ajpregu.00029.2012.—Maternally derived inflammatory mediators, such as IL-6 and IL-8, contribute to preterm delivery, low birth weight, and respiratory insufficiency, which are routinely treated with oxygen. Premature infants are at risk for developing adult-onset cardiac, metabolic, and pulmonary diseases. Long-term pulmonary consequences of perinatal inflammation are unclear. We tested the hypothesis that a hostile perinatal environment induces profibrotic pathways resulting in pulmonary fibrosis, including persistently altered lung structure and function. Pregnant C3H/HeN mice injected with LPS or saline on embryonic day 16, Offspring were placed in room air (RA) or 85% O2 for 14 days and then returned to RA. Pulmonary function tests, microCTs, molecular and histological analyses were performed between embryonic day 18 and 8 wk. Alveolarization was most compromised in LPS/O2-exposed offspring. Collagen staining and protein levels were increased, and static compliance was decreased only in LPS/O2-exposed mice. Three-dimensional microCT reconstruction and quantification revealed increased tissue densities only in LPS/O2 mice. Diffuse interstitial fibrosis was associated with decreased micro-RNA-29, increased transforming growth factor-β expression, and phosphorylation of Smad2 during embryonic or early fetal lung development. Systemic maternal LPS administration in combination with neonatal hyperoxic exposure induces activation of profibrotic pathways, impaired alveolarization, and diminished lung function that are associated with prenatal and postnatal suppression of miR-29 expression.

pulmonary fibrosis; transforming growth factor-β; fetal origins; microcomputed tomography scans; pulmonary function tests

THE CAUSES AND CONSEQUENCES of preterm birth remain poorly understood and present a significant health burden. In the past three decades, advances in neonatal care, including use of antenatal corticosteroids, surfactant therapy, and high-frequency ventilation, have significantly improved survival rates of extremely preterm (<28 wk gestation) and low-birthweight infants (45). However, little is known about the long-term physiological consequences of a hostile perinatal environment. Data currently being collected indicate that preterm infants surviving to adulthood are at greater risk for the development of chronic health problems (9–11). Low birth weight, early gestational age, and respiratory support are highly associated with interrupted alveolarization and respiratory insufficiency. These pathologies can progress into the development of bronchopulmonary dysplasia (BPD) (29), a disease pathologically characterized by impaired alveolarization and diffuse interstitial fibrosis (2, 26). Furthermore, whether they develop BPD or not, extremely immature infants are at increased risk for developing adult pulmonary pathologies, including emphysema, chronic obstructive pulmonary disease, asthma, or pulmonary fibrosis (2, 14, 15, 26, 36, 57).

Maternal infections and/or inflammation and the subsequent inflammatory responses that contribute to preterm delivery can significantly impact fetal development (16, 18, 19, 22, 58). Research supporting the “fetal origins of adult disease” hypothesis has focused on cardiovascular and metabolic diseases (3, 4, 32, 43); however, disordered fetal development has profound effects on other organs, including the lung. Recently, Shi and colleagues (47, 48, 56) demonstrated that early exposure during periods of developmental plasticity contribute to the development of adult pulmonary diseases.

While the mechanisms responsible for the development of pulmonary pathologies are multifactorial, common diseases are often characterized by diminished lung function and interstitial fibrosis. Mechanistically, lung fibrosis is associated with dysregulated transforming growth factor-β (TGF-β) expression and Smad signaling in human patients and bleomycin-treated mice (6, 7). Previous studies have implicated a crucial temporal window for TGF-β signaling during lung development, that, if interrupted, leads to impaired alveolarization and pulmonary fibrosis (47, 49). TGF-β modulates the expression of profibrotic genes through suppression of micro-RNA (miR)-29 that, in turn, causes increases in TGF-β expression, in a feed-forward manner (12, 38). miR-29 has been demonstrated to target proteins regulated by TGF-β and Smad signaling, such as collagen and matrix-remodeling proteins (12). miRs regulate the expression of multiple genes by enhancement, suppression, or destabilization of target RNAs and are increasingly recognized as important contributors to developmental processes and disease pathogenesis. Furthermore, dysregulation of miRs has been linked to fibrosis in multiple organs, including the heart (52), kidney (8), and lung (12). The contributions of maternal influences on early disruption of fetal TGF-β pathways or miR expressions, in developing lungs are unknown.

Many animal models that include fetal inflammation or postnatal hypoxic or hyperoxic exposures have been developed to study newborn lung diseases. Hyperoxic exposure induces inflammation and disrupts cell proliferation, leading to alveolar...
dysplasia in newborn rodents (27, 60). However, the long-term pulmonary consequences of fetal exposures have not been extensively investigated (30). We have previously reported developmental alterations in alveolarization and pulmonary function 14 days after systemic maternal inflammation and neonatal hyperoxic exposure (53). This model was designed to mimic the hostile perinatal inflammatory environment often encountered by prematurely born human infants. In the current studies, we tested the hypothesis that the combination of LPS-induced systemic maternal inflammation and postnatal hyperoxic exposure would result in 1) persistently altered alveolarization, 2) lung fibrosis, and 3) impaired pulmonary function in adulthood.

MATERIALS AND METHODS

Animals and exposure. Animal study protocols were approved by the Institutional Animal Care and Use Committee at The Research Institute at Nationwide Children’s Hospital, Columbus, OH. All animals were handled in accordance with National Institutes of Health guidelines and housed in a “specified pathogen-free” facility. Mice were housed in our facility at least 7 days before breeding was started, and pregnancy was time dated by the presence of a vaginal plug. Pregnant C3H/HeN mice were injected on embryonic day 16 (E16) with LPS (80 μg/kg ip, serotype 0111:B4, no. 437627; Calbiochem, Gibbstown, NJ) or an equal volume of saline. The amount of LPS was chosen on the basis of preliminary studies to determine the highest dose that resulted in viable litter of equal size. Each litter of newborn mice was paired with a litter born to a dam receiving the same E16 treatment, and the pups were pooled and redistributed randomly, as previously described (53). One of the paired group of pups was exposed to 85% O₂ for 2 wk (saline/O₂, LPS/O₂) and subsequently returned to room air (RA), while the corresponding group was maintained in RA (saline/RA, LPS/RA). Nursing dams were rotated between their RA and O₂ litter every 24 h to prevent oxygen toxicity. Twenty-four hours of RA or oxygen exposure was designated as day 1. The mice were killed at E18, 7 or 14 days, or 8 wk of life, and only one pup per litter was used at each time point for analyses. One pup per litter was analyzed per experiment, and equal numbers of males and females were measured. For the pulmonary function tests, one male and one female were analyzed from each litter.

Histology. The left lung was inflation fixed with 10% buffered formalin at a pressure of 25 cm H₂O for 15 min. Following paraffin embedding, the tissue sections were cut, and 4-μm slides were stained with hematoxylin and eosin (H&E) for morphometric measurements, Mason’s trichrome, and Picrosirius red (PSR) stain to assess collagen deposition.

Immunohistochemistry. Inflation-fixed left lung tissue sections were cut and 4-μm slides stained for macrophages with Mac3 monoclonal antibody (catalog no. 550292, BD Pharmingen, San Diego, CA) as the primary antibody and rabbit anti-rat (catalog no. BA-4001; Vector, Burlingame, CA) as the secondary antibody. Macrophage counts were performed on five nonoverlapping fields per mouse lung tissues and were performed stepwise, increasing airway pressure to 30 cm H₂O and then reversing the process. For each parameter, three measurements were assessed and averaged. Measurements were excluded from analyses if they were disrupted by a spontaneous breath, and a coefficient of determination of 0.98 was used as the lower limit for each measurement.

MicroCT imaging. A General Electric Healthcare Xplore Locus microCT (General Electric, London, Canada) was used for pulmonary imaging. Mice were anesthetized with ketamine (20 mg/kg ip) and xylazine (2 mg/kg ip). Respiratory gated microCT images were acquired at inspiration, as previously described (17). Images were reconstructed with a nominal isotropic voxel spacing of 90 μm. Lung density data obtained from microCTs were normalized to density standards and converted to Hounsfield units (HU). Five representative regions of interest were measured, and the average was considered as the lung density of the individual mouse (40). After performing microCT in vivo, mice were killed by ketamine (150 mg/kg–)–xylazine (15 mg/kg) overdose; then they were tracheotomized, lungs were air inflated, and post-mortem high-resolution microCT were performed at a constant inflation of 30 cm H₂O. Three-dimensional images were reconstructed with a nominal isotropic voxel spacing of 20 μm and evaluated by a radiologist blinded to group assignment.

Quantitative real-time PCR. Total RNA was isolated from frozen lung tissue using an RNeasy mini kit (Qiagen, Valencia, CA). cDNA was synthesized using a Maxima first-strand cDNA synthesis kit for RT-qPCR (no. K1641; Thermo Scientific Fermentas, Glen Burnie, MD). Quantitative real-time PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (K0222; Thermo Scientific Fermentas) and the Mastercycler epgradient Realplex real-time PCR detection system (Eppendorf, Hamburg, Germany). ELISA. Frozen lungs were homogenized, and protein concentrations were determined by Bradford assay. TNF-α, IL-6, keratinocyte-derived chemokine (KC), and monocyte chemotactant protein-1 (MCP-1) levels were measured using ELISA (DuoSet ELISA kits; R&D Systems, Minneapolis, MN), according to the manufacturer’s protocols. Absorbances were determined spectrophotometrically using a Spectramax M2 Plate Reader ( Molecular Devices, Sunnyvale, CA).

MicroRNA analyses. miR fractions were isolated and enriched from lungs with Qiagen RNeasy Mini Kits (Qiagen, Valencia, CA). Subsequently, 100-ng enriched miR was reverse-transcribed with SA Biosciences miR first-strand cDNA synthesis kit for RT-qPCR (N. K1641; Thermo Scientific Fermentas, Glen Burnie, MD). Real-time PCR was performed on an Mastercycler epgradient Realplex real-time PCR detection system (Eppendorf, Hamburg, Germany) using RT² SYBR Green qPCR Mastermix with ROX and RT² miRNA qPCR assay primer sets (SA Biosciences).

Statistics. Analyses were performed using GraphPad PRISM 5 (GraphPad, La Jolla, CA). Data are expressed as means ± SE. Statistical analyses were performed using a two-way ANOVA with Bonferroni post hoc or Student’s t-test. P < 0.05 was considered statistically significant.
RESULTS

Lung and body weights. Body weights (BW) were assessed after birth and at 8 wk of age. BW was significantly lower in pups born to LPS-injected dams compared with pups born to saline-injected dams (means ± SE; 1.22 ± 0.01 vs. 1.32 ± 0.02 g, \( P < 0.001 \)). At 8 wk, BW was less in saline/O_2 and even lower in LPS/O_2-exposed mice compared with saline/RA controls. Absolute right lung weights were not different; however, ratios of right lung weight to BW were significantly greater in LPS/O_2-exposed mice than in saline/RA controls. Neither absolute nor relative liver weights were different and sexes were equally distributed (Table 1).

Table 1. Body, right lung, and liver weights from 8-wk-old mice after systemic maternal LPS and neonatal hyperoxia exposure

<table>
<thead>
<tr>
<th></th>
<th>Saline/RA</th>
<th>Saline/O_2</th>
<th>LPS/RA</th>
<th>LPS/O_2</th>
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<tr>
<td>BW, g</td>
<td>23.75 ± 0.58</td>
<td>21.82 ± 0.71*</td>
<td>22.77 ± 0.62</td>
<td>20.76 ± 0.62*</td>
</tr>
<tr>
<td>Right lung weight, mg</td>
<td>85.88 ± 0.40</td>
<td>86.66 ± 0.35</td>
<td>90.00 ± 0.31</td>
<td>88.82 ± 0.21</td>
</tr>
<tr>
<td>Ratio of right lung to BW, mg/g</td>
<td>0.36 ± 0.02</td>
<td>0.40 ± 0.02</td>
<td>0.40 ± 0.01</td>
<td>0.44 ± 0.02*</td>
</tr>
<tr>
<td>Liver weight, mg</td>
<td>141.29 ± 4.94</td>
<td>131.87 ± 5.07</td>
<td>130.38 ± 3.31</td>
<td>126.00 ± 3.92</td>
</tr>
<tr>
<td>Ratio of liver weight to BW, mg/g</td>
<td>6.00 ± 0.25</td>
<td>6.10 ± 0.24</td>
<td>5.81 ± 0.12</td>
<td>6.10 ± 0.15</td>
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Values are expressed as means ± SE. Data were analyzed by one-way ANOVA followed by Bonferroni post hoc test. \( n = 15–18 \) mice per group. *\( P < 0.05 \) compared to saline/RA-exposed mice.

Lung morphometric. Alveolarization was assessed at 8 wk of age. Morphometric analyses of H&E-stained lung sections (Fig. 1A) revealed alveolar numbers that were 30%-45% lower in adult mice exposed to either LPS/RA or saline/O_2 and 60% lower in animals exposed to LPS/O_2 than the saline/RA controls (Fig. 1B). Mean alveolar areas were 72% greater in mice exposed to LPS/RA (9,002 ± 693 \( \mu \text{m}^2 \)), 110% greater in mice exposed to saline/O_2 (10,631 ± 1,465 \( \mu \text{m}^2 \)), and 123% greater

Fig. 1. Lung histology and morphometric analyses. Lung sections from saline/RA, saline/O_2, LPS/RA, and LPS/O_2-exposed mice were inflation fixed and hematoxylin-and-eosin stained at 8 wk of age. A: representative images, stained with hematoxylin and eosin, were taken at \( \times 100 \) magnification. Digital morphometric analyses were performed on five images per animal for number of alveoli (B) and septal thickness (C). Data were analyzed using two-way ANOVA and expressed as means ± SE; \( n = 8 \) pups in each treatment group, with no more than one animal from a given litter per treatment group. *Significant difference, \( P < 0.05 \) compared with saline/RA-exposed mice; #Significant difference, \( P < 0.05 \) compared with LPS/RA-exposed mice. +Significant difference \( P < 0.05 \) compared to saline/O_2. Scale bars = 100 \( \mu \text{M} \).
in mice exposed to LPS/O₂ (11,288 ± 1,659 μm²) than in saline/RA controls. Septal thickness was 12% greater in the saline/O₂, 25% greater in the LPS/RA, and 39% greater in the LPS/O₂ than in the saline/RA controls (Fig. 1C). In summary, alveolar simplification and increased septal thickness were evident in all treatment groups compared with saline/RA controls; however, deficits in alveolarization and increased septal thickness were most pronounced in mice that received the combination of prenatal LPS and neonatal hyperoxia.

Collagen deposition. Interstitial collagen deposition was assessed in Mason’s trichrome-stained lung tissue sections at 8 wk of age. The blue color indicates collagen fibers in the lung tissues. Saline/O₂ and LPS/RA-exposed mouse lungs showed traces of blue staining within the lung tissue. However, substantial increases in blue staining were observed within the tissues from LPS/O₂-treated mice compared with all other groups (Fig. 2A). Collagen deposition in blood vessels served as a positive control in saline/RA mice. Collagen protein levels were also quantified by Western blot analyses. No differences in collagen I or collagen III levels were detected in saline/O₂ or LPS/RA-exposed mice; however, our data indicate that collagen I and III levels were 4 and 20 times greater, respectively, in the lungs of LPS/O₂-exposed mice compared with saline/RA controls (Fig. 2B).

Collagen deposition was also quantified histologically. Additional tissue sections were stained with Picrosirius red and were observed under polarized light. Red (collagen I) or green (collagen III) fluorescence was quantified using digital analysis software. Significantly more red and green fluorescence was detected in the LPS/O₂ (red 6.0 ± 0.5; green 34.5 ± 2.0 μm²) mice than any other group. There were no differences in fluorescence intensities among the saline/O₂ (red 2.8 ± 0.3; green 21.5 ± 1.4 μm²), LPS/RA (red 2.1 ± 0.2; green 16.7 ± 0.9 μm²), or the saline/RA (red 3.6 ± 0.5; green 23.8 ± 2.3 μm²) groups. Both red and green fluorescence was significantly lower in LPS/RA-exposed mice compared with saline/RA controls (Fig. 2C).

Fig. 2. Collagen deposition. Inflation-fixed lung sections from 8-wk-old mice were stained with Mason’s trichrome stain. A: representative images of saline/RA, saline/O₂, LPS/RA, and LPS/O₂-exposed mice were taken at ×100 magnification (scale bars represent 50 μM). Western blots were performed on lung tissue homogenates from 8-wk-old mice (n = 5). B: representative blots and quantification of collagen I and collagen III proteins are as indicated. C: inflation-fixed lung sections were stained with Picrosirius red and visualized under polarized light. Representative images were taken at ×100 magnification. D: additional tissue sections were stained Mac-3 antibody. Data were analyzed using two-way ANOVA and expressed as means ± SE; n = 5–7 pups in each treatment group, with no more than one animal from a given litter per treatment group; P < 0.05 compared with saline/RA (*), saline/O₂ (†), and LPS/RA (#) -exposed mice.
To assess the persistence of elevated pulmonary macrophage numbers, lung tissue sections from mice at 8 wk of age were stained with antimacrophage antibodies (Fig. 2D), and the number of immunoreactive cells was counted. Saline/RA-exposed mice showed only few [1.8 ± 0.3 cells/high-power field (HPF)], interstitial Mac-3-positive cells. While there was no significant increase in the number of Mac-3-positive cells in saline/O2 (2.7 ± 0.4 cells/HPF) or LPS/RA (4.2 ± 0.5 cells/HPF)-exposed mice, some positive stained cells were located in the intra-alveolar space. However, LPS/O2-exposed mice showed a significant increase (8.0 ± 1.0 cells/HPF) in the number of predominantly intra-alveolar Mac-3-positive stained cells compared with all other groups.

**Pulmonary function tests.** Physiological consequences of prenatal LPS and newborn hyperoxic exposure on adult pulmonary functions were evaluated at 8 wk of age. PV-loops were modestly upward shifted in LPS/RA-exposed mice compared with saline/RA controls; however, this shift was even more pronounced in LPS/O2 mice (Fig. 3A). Static compliance and inspiratory capacity from zero pressure were significantly lower in the LPS/O2 mice than in all other groups (Fig. 3B and C). Tissue resistance was greater in LPS/O2 than in saline/RA-exposed mice (Fig. 3D).

**MicroCT scans.** Thoracic microcomputed tomography (microCT) scans were performed on 8-wk-old mice and read by a clinical radiologist blinded to group assignment. Compared with saline/RA controls, the lungs of saline/O2, LPS/RA, and LPS/O2 mice were hyperexpanded, and the lung parenchyma exhibited patchy centrilobular areas of alveolar simplification. However, hyperexpansion was most exaggerated, alveolar simplification was most widespread, and interstitial/septal lung markings were coarsest in the LPS/O2 group compared with either saline/O2 or LPS/RA-exposed mice (Fig. 4A). Lung densities were quantified and are displayed as Hounsfield unit (HU) histograms (Fig. 4B). Compared with the saline/RA control histogram, the saline/O2 histogram was shifted left, indicating a less dense lung, while the LPS/O2 histogram was shifted to the right, indicating denser, fibrotic lung tissue. Three-dimensional microCT reconstructions revealed multiple dense spots that were only present in the lungs of LPS/O2-exposed mice (see Supplementary Movies, LPS CO2 vs. all others).

**Fetal inflammatory response.** mRNA levels of inflammatory genes were assessed in fetal lung tissues to determine the effects of maternal inflammation on the fetus. Fetal tissues were collected on E18, which was 48 h after intraperitoneal saline or LPS administration to the pregnant dam and the time that most of the inflammatory responses measured were highest in the fetus prior to birth (data not shown). TNF-α and KC mRNA levels were decreased (Figs. 5, A and D) and IL-1β mRNA was increased (Fig. 5B) in fetal lung tissues harvested from LPS-injected dams compared with saline injected dams. IL-6 and MCP-1 mRNA levels were not different between groups (Fig. 5, C and E). Interestingly, TGF-β and collagen I mRNA levels were increased (Fig. 5, F and G) in fetal lungs collected from LPS injected dams; however, collagen III mRNA levels were not different (Fig. 5H).
Neonatal inflammatory response during hyperoxia exposure. Inflammatory responses to hyperoxia were evaluated in neonatal lung tissues from mice born to saline or LPS-injected dams that were subsequently exposed to room air (RA) or 85% O₂. Tissues were harvested at 7 and 14 days, and mRNA and protein expressions were analyzed. Data were analyzed and compared with saline/RA controls at each corresponding time point. TNF-α mRNA levels were greater in LPS/O₂-exposed mice at 7 days and 14 days (Fig. 6A). IL-1β mRNA levels were significantly greater in LPS/RA and LPS/O₂-exposed mice at 7 days (Fig. 6A). IL-6 mRNA levels were greater in saline/O₂- and LPS/O₂-exposed mice at 14 days (Fig. 6A). KC mRNA levels...
were greater at 14 days in saline/O2-exposed mice and at 7 days and 14 days in LPS/O2-exposed mice (Fig. 6A). MCP-1 mRNA levels were higher in LPS/O2-exposed mice at 14 days (Fig. 6A).

Expressions of inflammatory proteins were analyzed in lung tissue homogenates using ELISA or Western blot. TNF-α protein levels were increased only in LPS/O2-exposed mice at 14 days compared with saline/RA controls (Fig. 6B). IL-6 protein levels were increased in LPS/O2-exposed mice at 14 days (Fig. 6B). KC protein levels were increased at 7 days and 14 days in both saline/O2 and LPS/O2 groups compared with saline/RA controls (Fig. 6B). MCP-1 protein levels were higher in LPS/O2-exposed mice at 14 days compared with saline/RA controls (Fig. 6B).

Neonatal markers of fibrosis. mRNA levels of fibrotic markers were also assessed. TGF-β1 levels were higher in all treatment groups at 7 days and correlated with increases in IL-1β; however, increased levels were sustained only in LPS/O2-exposed mice at 14 days (Fig. 7A). Collagen I levels were...
higher at 14 days in LPS/O2-exposed mice (Fig. 7A). Collagen III levels were higher in all treatment groups at 7 days, but increased levels were sustained only in LPS/O2-exposed mice at 14 days (Fig. 7A). As an indicator of TGF-β activation, we assessed protein levels of phospho-Smad 2 (p-Smad 2) by Western blot analysis. p-Smad 2 was not different at 7 days (Fig. 7B); however, levels were significantly increased at 14 days in saline/O2 and greater in LPS/O2-exposed mice compared with saline/RA controls (Fig. 7B).

Levels of miR-29b were assessed in fetal lung tissues at embryonic day 18 (E18) and at 14 days, the end point of the hyperoxia exposure. miR-29b levels were significantly decreased in fetal lung tissue harvested from LPS-injected dams compared with tissues from saline-injected dams (Fig. 7C). At 14 days,
miR-29b expression was lower only in the LPS/O2-exposed mice, while miR-29b expression was greater in saline/O2 and LPS/RA groups compared with saline/RA controls (Fig. 7C).

**DISCUSSION**

The findings of the present study indicate that mouse lungs are particularly vulnerable to perinatal inflammation. Using our previously established mouse model (53), we have identified pathways modulated by TGF-β and miR29b expression. Additionally, we observed deficits in alveolarization, diffuse interstitial fibrosis, and impaired pulmonary function in exposed male and female offspring as adults. Our data indicate that, in mice, activation of proinflammatory and profibrotic pathways during the fetal and neonatal period contributes to the development of an adult phenotype characteristic of pulmonary fibrosis in humans.

There is increasing recognition that hostile perinatal environments influence fetal organogenesis and contribute to adult diseases (47, 48, 53). Specifically, maternal infection and

Fig. 7. Neonatal fibrotic response during hyperoxia. Pups born to E16 saline or LPS-injected dams were RA or 85% O2 exposed. Mice were euthanized at 7 and 14 days, and pulmonary mRNA and protein were isolated. A: expression of TGF-β, collagen I and collagen III was analyzed by quantitative real-time PCR. B: protein expression of p-Smad2 and total Smad2/3 were assessed by Western blot. C: pulmonary miRs were isolated, and expression of miR29b was analyzed on embryonic day 18 or at day 14. Expression was normalized to Rnu6. Data were analyzed using unpaired t-test or two-way ANOVA and Bonferroni post hoc and expressed as means ± SE; n = 5 pups in each treatment group, with no more than one animal from a given litter per treatment group. *P < 0.05 compared with saline/RA at the corresponding time point.
inflammation are common during pregnancy and has adverse repercussions on the developing fetus (25). The consequences of intrauterine infections and inflammation have been extensively studied in the context of early lung development (28, 30). However, recent evidence indicates that subtle systemic maternal inflammatory responses likely influence both the developing fetus and timing of parturition (5, 23, 44, 55). In rodent models, low-dose systemic LPS administration induces inflammatory responses in the pregnant dam (54). Maternally derived inflammatory mediators rapidly cross the placenta and are transmitted to the fetus, while only traces of maternally administered LPS are detectable in the fetus (34, 46). Recent advances in medical therapies have led to steadily increasing survival rates in prematurely born infants that comprise a unique population of children and young adults, many of which have been exposed to both inflammation in utero and proinflammatory medical interventions after birth.

At 8 wk of age, mice exposed to LPS-induced maternal inflammation and postnatal hyperoxia are slightly smaller in total body weight but have relatively heavier lungs (Table 1), despite dramatic decreases in alveolarization (Fig. 1). Deficits in alveolarization are commonly observed with neonatal hyperoxic exposure in a dose-dependent manner (60) and are evident in Fig. 1. Interestingly, maternal LPS exposure also decreases alveolarization, and the decreases are even more profound in the animals exposed to both insults. Given that a large number of preterm infants are born to mothers with inflammation (from a variety of sources), the additive effects of maternal inflammation and neonatal hyperoxia could have profound effects of the pulmonary function of the offspring in later life.

The greater lung weights are most likely due to increased collagen deposition detected in histological sections and in lung homogenates (Fig. 2). While the position and temporal expression of collagen are essential for normal lung growth; just as important is the remodeling of the collagen network as the lung grows and becomes more complex. Additionally, macrophages can contribute to tissue remodeling and collagen deposition. The persistent presence of increased numbers of macrophages at 8 wk of age in the LPS/O2-treated mice supports their role in the phenotype we have observed (Fig. 2). Altered expression, cross-linking, or changes in the relative amounts of collagen I and III are all associated with fibrotic lung diseases and may be linked to dysregulation of cross-linking enzymes (35, 47, 49). In the developing human lung, collagen I and collagen III are weakly to moderately expressed in the alveoli, arteries, veins, and adventitia (31). Redistribution of collagen protein precursors in the alveolar walls below the alveolar epithelium or changes in expression of elastin/collagen cross-linking enzymes are reported in infants diagnosed with BPD and in patients with idiopathic pulmonary fibrosis (31, 41). Our Mason’s trichrome staining in tissues (Fig. 2A) and the quantification of Picrosirius red staining (Fig. 2B) as well as protein levels indicate expression of collagen I and III in the alveolar walls is much like those observed in infants with BPD and in animal models of pulmonary fibrosis (31, 41, 59). Consequently, we speculated that the increases in collagen observed in our model are more likely a deficiency in matrix remodeling rather than a dysregulation in deposition.

Pulmonary function tests in the present studies revealed stiffer lung tissues in LPS/O2-exposed, mice as indicated by reduced static compliance, reduced inspiratory capacity from zero pressure, and increased tissue resistance (Fig. 3). A similar reduction in static compliance was reported in a mouse model of bleomycin-induced pulmonary fibrosis (13, 42). The fact that the LPS/O2-exposed mice show alterations in pulmonary mechanics similar to animals in an acute model of pulmonary fibrosis highlights the significant physiological consequences of perinatal exposures on lung development.

Lung structure was evaluated radiologically in adult mice using microCT imaging. While our data revealed alveolar simplification in all treatment groups, this was most severe in LPS/O2-exposed mice, which is also indicated by increased alveolar areas in histological sections. Consistent with previous reports, saline/RA and LPS/RA-exposed mice had lung densities in the range of −450 to −550 HU (24, 40). Interestingly, the saline/O2-exposed group had lower lung densities (leftward shift) that are consistent with our histological findings of fewer and larger alveoli and are similar to that seen clinically in adult patients with emphysema (39). Conversely, LPS/O2-exposed mice had lung densities that were greater than controls (rightward shift), which is indicative of more dense lung tissues. Increases in lung densities measured by microCT have been reported in animal models of pulmonary fibrosis (24, 40), as well as in human patients (50). Greater densities in the present studies are supported histologically and biochemically by the observed increases in collagen deposition in the LPS/O2 group (Fig. 2).

Inflammation can disrupt developmental pathways, and our data indicate alterations in mRNA expression levels of proinflammatory and profibrotic genes in fetal lung tissues obtained from LPS-injected dams. Surprisingly, TNF-α and KC mRNA levels were lower in fetal lung tissues harvested from LPS-injected dams (Fig. 6). We speculate that E18, which was 48 h postinjection, was too late to detect transient increases in the mRNAs expression of these molecules. However, IL-1β was substantially elevated at E18 and correlated with increased levels of TGF-β and collagen I in fetal lung tissues from LPS-injected dams. Our current findings indicate that maternal LPS injection induced fetal inflammatory response, making the organism more vulnerable to subsequently occurring insults (6).

mRNA expression and protein levels of mediators that regulate leukocyte infiltration were increased during neonatal hyperoxic exposure. KC, TNF-α, IL-6, and MCP-1 mRNA expression and protein levels were increased in saline/O2 and LPS/O2-exposed mice throughout the hyperoxic exposure period compared with RA controls (Fig. 6). While MCP-1, IL-6, and TNF-α protein levels were lower in saline/O2 mice by 14 days, they remained elevated in LPS/O2 mice. Increases in IL-6 mRNA expression and protein levels have been previously reported in the offspring of rats exposed to intra-amniotic LPS and neonatal hyperoxia (33) and in human infants born to mothers with diagnosed chorioamnionitis (44). However, our model differs from these studies in that elevations in IL-6 levels are more closely linked to hyperoxia exposure than to the combination of LPS/O2 (Fig. 6).

Collagen expression and fibrosis are regulated by TGF-β and/or Smad-related signaling (21), potentially through modulation of miR-29b expression (12). The TGF-β pathway has been implicated in development of BPD, as well as other fibrotic lung diseases (21). In addition to its role in matrix remodeling and fibrosis, TGF-β plays a key role in alveolar
development and maintenance of alveolar structure (1). Developmental overexpression of TGF-β results in impaired alveolarization and Smad-dependent interstitial fibrosis in monkeys (51) and rats (20, 21). Increases in IL-1β, TGF-β, and collagen I mRNA levels were persistent after birth in hyperoxia-exposed mice. There were additional increases in collagen III in hyperoxia-exposed mice, particularly at 7 days. By day 14, IL-1β levels were similar to controls but TGF-β and collagen I and III mRNA expression remained elevated solely in the LPS/O2-exposed mice (Figs. 6 and 7). Additionally, elevated levels in TGF-β and collagen I and III mRNA were associated with increases in p-Smad2 protein levels in only the LPS/O2-exposed at day 14.

Previous investigations have linked macrophage-induced increased IL-1β levels with altered TGF-β and Smad expressions, and subsequent collagen deposition and fibrosis, suggesting that this phenotype more closely resembles impaired tissue repair rather than acute injury (6, 37). One explanation of our current findings could be that the LPS exposure in utero initiates a lung injury that is either unable to resolve or interrupted during the healing process by the inflammatory responses to hyperoxia exposure. Regardless, the sustained increases in MCP-1, IL-6, TNF-α, IL-1β, TGF-β, and collagen I and III, distinguish the LPS/O2 from the other treatment groups and are, in part, responsible for the physiological deficits in lung structure and function observed at 8 wk.

Our data provide the first evidence that TGF-β mRNA levels in fetal lungs are increased by LPS-induced maternal inflammation and that subsequent hyperoxia exposure caused the induction to persist. TGF-β regulation of downstream profibrotic genes has been linked to suppression of miR-29b expression (12). Our data indicate that miR-29b is decreased at E18 in fetal lung tissues from LPS-treated dams (Fig. 7) and is inversely correlated with TGF-β and collagen I and III expressions. On day 14, miR-29b expression was further suppressed in LPS/O2 mice; however, miR-29b expression was elevated in saline/O2 compared with saline/RA mice probably as a compensatory response. miR-29b reduces expression of proteins that are regulated by TGF-β stimulation, such as collagen I and III (12). We hypothesize that elevated mRNA levels of collagen I and III in LPS/O2 on E18 and 14 days are due to suppression of miR-29b levels. Whether directly or indirectly through miR-29b, our data suggest that TGF-β expression is fundamentally involved in the fibrotic phenotype observed in the LPS/O2-exposed mice.

In summary, the present study demonstrates that systemic maternal LPS administration induces a profibrotic response in fetal lung tissue that begins prior to birth and that results in an ongoing activation of profibrotic pathways when combined with neonatal hyperoxic exposure. These dual inflammatory exposures lead to the development of diffuse interstitial fibrosis, impaired alveolarization, and compromised pulmonary mechanics in both adult male and female offspring with no differences between sexes. Our data suggest that these changes are mediated through reduction in miR-29b expression, resulting in activation of profibrotic pathways, including, but not exclusive to, TGF-β-mediated signaling. The finding that perinatal exposures result in persistently impaired pulmonary physiology in adulthood provides a novel model to investigate the influence of the perinatal environment on the development of adult pulmonary diseases.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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