Interleukin-1 receptor antagonist prevents murine bronchopulmonary dysplasia induced by perinatal inflammation and hyperoxia

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Bronchopulmonary dysplasia (BPD) is a common lung disease of premature infants, with devastating short- and long-term consequences. The pathogenesis of BPD is multifactorial, but all triggers cause pulmonary inflammation. No therapy exists; therefore, we investigated whether the anti-inflammatory interleukin-1 receptor antagonist (IL-1Ra) prevents murine BPD. We precipitated BPD by perinatal inflammation (lipopolysaccharide injection to pregnant dams) and rearing pups in hyperoxia (65% or 85% O\textsubscript{2}). Pups were treated daily with IL-1Ra or vehicle for up to 28 d. Vehicle-injected animals in both levels of hyperoxia developed a severe BPD-like lung disease (alveolar number and gas exchange area decreased by up to 60%, alveolar size increased up to fourfold). IL-1Ra prevented this structural disintegration at 65%, but not 85% O\textsubscript{2}. Hyperoxia depleted pulmonary immune cells by 67%; however, extant macrophages and dendritic cells were hyperactivated, with CD11b and GR1 (Ly6G/C) highly expressed. IL-1Ra partially rescued the immune cell population in hyperoxia (doubling the viable cells), reduced the percentage that were activated by up to 63%, and abolished the unexpected persistence of IL-1α and IL-1β on day 28 in hyperoxia/vehicle-treated lungs. On day 3, perinatal inflammation and hyperoxia each triggered a distinct pulmonary immune response, with some proinflammatory mediators increasing up to 20-fold and some amenable to partial or complete reversal with IL-1Ra. In summary, our analysis reveals a pivotal role for IL-1αβ in murine BPD and an involvement for MIP (macrophage inflammatory protein)-1α and TREM (triggering receptor expressed on myeloid cells)-1. Because it effectively shields newborn mice from BPD, IL-1Ra emerges as a promising treatment for a currently irremediable disease that may potentially brighten the prognosis of the tiny preterm patients.

Modern perinatal medicine has substantially improved survival of extremely premature infants, but at the price of a steadily rising incidence of a severe lung disease known as bronchopulmonary dysplasia (BPD). BPD is now the most common chronic lung disease in infants and represents a major burden for babies, their families, and health services (1). The adverse impact of BPD on cardiovascular and respiratory health and neurodevelopmental outcome can persist into adulthood (2, 3). In addition, BPD exacts a considerable late mortality, with infants dying from common and usually harmless viral infections. According to National Institutes of Health estimates, treating infants with BPD in the United States costs $2.4 billion per year (4), making it the second most expensive childhood disease after asthma. Thus, BPD represents one of the greatest unmet therapeutic challenges in neonatology, yet efforts to find a safe and effective therapy have failed.

In the presurfactant era, BPD was largely characterized by ventilator-induced lung injury and consequent fibrosis, whereas the “new BPD” features severely reduced alveolarization and vascularization of the developing lung (5). The pathogenesis of BPD is multifactorial, resulting from pre- and postnatal infection and oxygen toxicity. Trauma caused by mechanical ventilation represents a third cause of BPD (Fig. S1), but its impact is on the decline as a result of modern, gentle ventilation strategies. These insults trigger pulmonary inflammation, now recognized as the common final pathway and main culprit in the pathogenesis of BPD. Inflammation leads to decreased alveolar septation, reduced maturation of epithelial type 1 and type 2 cells, parenchymal thickening, and reduced capillary density (6). The mediators of this process include chemokines, adhesion molecules, and proinflammatory cytokines such as IL-6, TNF, and particularly IL-1β, which have been associated with BPD and/or an increased risk for an adverse clinical course (6–11).

To date, the only anti-inflammatory agents tested in BPD are glucocorticoids, and as expected they reduce its incidence. However, glucocorticoids are used sparingly because their side effects are severe and include cerebral palsy and inhibition of alveolar growth, whereby they impede the very pathway essential for permanent healing of BPD (12).

Taking a fresh approach, we set out to test the hypothesis that IL-1 receptor antagonist (IL-1Ra) is effective in preventing BPD. Our rationale was that (i) IL-1α and IL-1β are already known to be important malefactors in BPD (6–9, 11, 13–15); (ii) tellingly, a low-bioactivity polymorphism of il1rn (odds ratio 11.7) with BPD (16); and (iii) IL-1Ra features a favorable safety profile that is well-established in a broad spectrum of diseases (17, 18), including neonatal cases (19, 20). We therefore embarked on the exploration of IL-1Ra as a candidate therapy for BPD using a clinically relevant mouse model.

\textbf{Results}

\textbf{Perinatal Inflammation and 85% (vol/vol) \textit{O}_2 Produce a Profound and Irreversible BPD-Like Lung Injury.} The combination of perinatal inflammation and postnatal hyperoxia triggers a severe BPD-like lung disease in rodents (21, 22). We selected this double-hit model as the multifactorial etiology of BPD is taken into account.

Perinatal LPS and a fraction of inspired (Fi) \textit{O}_2 of 0.85 induced a severe lung disease in newborn mice. Histological analysis on day 28 demonstrated arrested alveolar development, with a decreased number of large, simplified alveoli and sparse secondary septation (Fig. L4). Quantitative analysis confirmed the histological appearance, revealing a more than 60% reduction


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O2 produced an irreparable BPD-like lung disease not amenable (gray bars), the increases in IL-1α (white bars) and by combined antenatal LPS and postnatal hyperoxia not they were treated with IL-1Ra (Fig. 1; lower magnification). Thus, perinatal inflammation and postnatal exposure to 85% O2 without antenatal exposure to LPS, but marked growth restriction, which may re...

Although treatment with IL-1Ra blunted the rise on day 3 in pulmonary IL-6 and macrophage inflammatory protein (MIP)-2 that is triggered by perinatal inflammation alone (Fig. 2, white bars) and by combined antenatal LPS and postnatal hyperoxia (gray bars), the increases in IL-1α and IL-1β were not affected. Dysalveolarization persisted in all hyperoxia groups, whether or not they were treated with IL-1Ra (Fig. 1; lower magnification). Thus, perinatal inflammation and postnatal exposure to 85% O2 produced an irreparable BPD-like lung disease not amenable to prophylaxis with IL-1Ra, although we observed inhibition of the proinflammatory cytokines IL-6 and MIP-2, but not IL-1α and IL-1β, in the lungs on day 3 of life. Murine BPD Induced by Perinatal Inflammation and Postnatal 65% O2 Is Prevented by IL-1Ra. The irreversible lung damage of 85% O2 prompted us to ask whether the hyperoxic insult overwhelmed the prophyllactic or treatment power of IL-1Ra. We thus repeated the experiments using 65% O2, a level that better reflects the modern clinical setting in which a high FiO2 is avoided whenever possible. Quantitative analysis of lung histology showed that the damage induced by 65% O2 in vehicle-treated pups was milder than that caused by 85% O2, with the alveolar number reduced by 44%, the alveolar size increased 2.3-fold, and the SVR reduced by 35% (Fig. 3 B–D; whole lung sections in Fig. S3).

The effects of IL-1Ra were remarkable at an FiO2 of 0.65. Whereas pups in the hyperoxia-vehicle group displayed a grossly abnormal, dysalveolarized lung histology (Fig. 3A, Lower Left) on day 28, treatment with IL-1Ra resulted in lungs almost indistinguishable from those of pups reared in room air (Fig. 3A, Lower Right). IL-1Ra completely prevented the hyperoxia/perinatal LPS-induced decrease in alveolar number (Fig. 3B) and increase in alveolar size (Fig. 3C). The SVR, corresponding to the surface area available for gas exchange, was also improved dramatically by IL-1Ra (Fig. 3D). The growth restriction observed with 85% O2 was present at 65%, but to a milder extent, and there was a trend toward improved growth with IL-1Ra treatment (Fig. 3E). When the lungs of pups reared in similar conditions for 14 d were compared structurally, the findings were similar to those in pups at 28 d, but the changes were less pronounced. Of note, there being no difference between them, data for the three room air groups were combined for calculating structural and molecular parameters; therefore, these groups are identical in Figs. 1–4. Hence, we demonstrated that IL-1Ra prevents murine BPD when the “second hit” of postnatal hyperoxia is milder.
of some of these mediators were further increased by the addition of 65% O₂, but others were unchanged or even lower. Comparing air vehicle with hyperoxia vehicle, we observed a marked increase in IL-1β (20-fold), BLC (sevenfold), and MIP-1α (sixfold); a moderate increase in KC (threefold) and TREM-1 (2.5-fold); a decrease in TNF (by 48%) and M-CSF (by 42%); and a minor increase (<twofold) or no change in the other mediators.

With regard to response to treatment with IL-1Ra, we found a more than 50% reduction in the abundance of IL-1β (94%), IL-6 (91%), IL-1α (90%), TREM-1 (85%), MIP-2 (75%), MIP-1α (72%), TNF (70%), KC (69%), MIP-1β (56%), and BLC (50%) in the hyperoxia-exposed animals. The only cytokines that were significantly changed by IL-1Ra in room air were IL-6, MIP-2, and TNF (reduced by 82%, 82%, and 58%, respectively).

To assess the abundance of IL-1α and IL-1β in the lungs on day 28, we performed IHC analysis. As expected, the lungs of the hyperoxia vehicle animals, but its abundance in the lungs on day 28. The overall number of immune cells was approximately three times lower in the hyperoxia groups at 65% and 85% than in the room air groups (e.g., 0.45 vs. 1.4 × 10⁶ cells at 65%; Fig. S4 and Fig. S5). IL-1Ra conferred a partial protection at 65% (hyperoxia vehicle 0.45 vs. hyperoxia IL-1Ra 0.85 × 10⁶ cells, P < 0.05). There was no difference in the percentage population of CD4⁺ and CD8⁺ T cells, B cells, conventional and plasmacytoid DC, or F4/80⁺ macrophages between any of the groups among 20,000 acquired CD45⁺ cells (Fig. 5 B and C); however, hyperoxia caused an increase in the activation of macrophages and DC (Fig. 5 D–G). This activation was demonstrated by assessing the surface expression of CD11b and GR1 (Ly6G/C), which were both increased in the hyperoxia vehicle animals compared with all pups reared in room air. Treatment with IL-1Ra reduced the percentage of CD11b⁺ and GR1⁺ cells. At 85% O₂, the inflammatory phenotype of the pulmonary monocytes and DC was more pronounced than than 65% (Fig. S5 B and C). In addition, we observed a marked increase in a population of CD11b⁺GR1hi granulocytes that was reduced by IL-1Ra (Fig. S5).

Taken together, these results demonstrate that perinatal inflammation plus postnatal hyperoxia induce a reduction in total immune cells in the lung and a marked activation of the remaining cells, particularly macrophages and DC. These effects can be ameliorated by IL-1Ra.

Discussion

The central role of perinatal inflammation and its possible consequence, the fetal inflammatory response syndrome, led the BPD Group of the American Academy of Pediatrics (AAP) to identify the development of anti-inflammatory agents as a research priority (23). By using a clinically relevant “double-hit” model of BPD that features the two threats commonly experienced by the neonatal lung in the age of gentle ventilation—perinatal inflammation and oxygen toxicity—we demonstrate that prophylactic administration of IL-1Ra can prevent this devastating lung disease in newborn mice. Importantly, we show the clear concentration-dependent nature of oxygen toxicity because IL-1Ra treatment effectively protected the lungs in 65% but not 85% O₂. This finding provides a strong basis for the ongoing efforts to limit the O₂ level to which babies are exposed (24). Beyond responding to the AAP’s call for action to reduce the burden of BPD, we also shed light on the neglected field of neonatal immunology.

We demonstrate that a moderate dose of maternal LPS substantially increases the abundance of a group of proinflammatory mediators in the lungs of newborn pups; nevertheless, the lungs develop normally without a second insult. In combination with hyperoxia, lung inflammation led to a severe BPD-like lung disease that was preventable by attenuating the immune response. These results emphasize that the etiology of BPD is indeed multifactorial; perinatal inflammation (e.g., deriving from chorioamnionitis) contributes to BPD provided other events occur. A recent review discussed this controversial concept (24), citing the finding of a decreased risk for BPD in infants born after chorioamnionitis isolated to the placenta and chorion, but an increased risk for BPD once the fetus itself was affected (25). The newborn mice in our model can be regarded as belonging to the latter category.

Neonatal immunology is a poorly researched field, with only a handful of studies devoted to the pulmonary immune response; thus, our report considerably expands knowledge in this field. A decreased proliferation of structural lung cells in response to hyperoxia has been described (14); we now report a significant loss of immune cells that was partially rescued by IL-1Ra. Another enlightening result was that activation of macrophages and DC emerged as a hallmark of murine BPD, a finding that accords with IL-1 receptor blockade. On day 28, IL-1Ra abolishes the persistence of IL-1 in lung tissue exposed to hyperoxia.

Effects of IL-1Ra on Cellular Aspects of the Immune Response to Perinatal LPS and 65% O₂.

Neutrophils, lymphocytes, monocytes/macrophages, natural killer (NK) cells, and dendritic cells (DC) were enumerated and evaluated for their level of activation on day 28. The overall number of immune cells was approximately three times lower in the hyperoxia groups at 65% and 85% than in the room air groups (e.g., 0.45 vs. 1.4 × 10⁶ cells at 65%; Fig. S4 and Fig. S5). IL-1Ra conferred a partial protection at 65% (hyperoxia vehicle 0.45 vs. hyperoxia IL-1Ra 0.85 × 10⁶ cells, P < 0.05). There was no difference in the percentage population of CD4⁺ and CD8⁺ T cells, B cells, conventional and plasmacytoid DC, or F4/80⁺ macrophages between any of the groups among 20,000 acquired CD45⁺ cells (Fig. 5 B and C); however, hyperoxia caused an increase in the activation of macrophages and DC (Fig. 5 D–G). This activation was demonstrated by assessing the surface expression of CD11b and GR1 (Ly6G/C), which were both increased in the hyperoxia vehicle animals compared with all pups reared in room air. Treatment with IL-1Ra reduced the percentage of CD11b⁺ and GR1⁺ cells. At 85% O₂, the inflammatory phenotype of the pulmonary monocytes and DC was more pronounced than than 65% (Fig. S5 B and C). In addition, we observed a marked increase in a population of CD11b⁺GR1hi granulocytes that was reduced by IL-1Ra (Fig. S5).

Similar studies were performed on day 14. Most of the findings shown in Fig. 5 and Fig. S5 for day 28 were consistent, but the changes were less pronounced and not statistically significant.

To assess the abundance of IL-1α in the lungs of newborn pups, we performed IHC analysis. As expected, the lungs of the hyperoxia vehicle animals, but its abundance in the lungs on day 28. The overall number of immune cells was approximately three times lower in the hyperoxia groups at 65% and 85% than in the room air groups (e.g., 0.45 vs. 1.4 × 10⁶ cells at 65%; Fig. S4 and Fig. S5). IL-1Ra conferred a partial protection at 65% (hyperoxia vehicle 0.45 vs. hyperoxia IL-1Ra 0.85 × 10⁶ cells, P < 0.05). There was no difference in the percentage population of CD4⁺ and CD8⁺ T cells, B cells, conventional and plasmacytoid DC, or F4/80⁺ macrophages between any of the groups among 20,000 acquired CD45⁺ cells (Fig. 5 B and C); however, hyperoxia caused an increase in the activation of macrophages and DC (Fig. 5 D–G). This activation was demonstrated by assessing the surface expression of CD11b and GR1 (Ly6G/C), which were both increased in the hyperoxia vehicle animals compared with all pups reared in room air. Treatment with IL-1Ra reduced the percentage of CD11b⁺ and GR1⁺ cells. At 85% O₂, the inflammatory phenotype of the pulmonary monocytes and DC was more pronounced than than 65% (Fig. S5 B and C). In addition, we observed a marked increase in a population of CD11b⁺GR1hi granulocytes that was reduced by IL-1Ra (Fig. S5).

Taken together, these results demonstrate that perinatal inflammation plus postnatal hyperoxia induce a reduction in total immune cells in the lung and a marked activation of the remaining cells, particularly macrophages and DC. These effects can be ameliorated by IL-1Ra.
with a 1988 study that reported increased activation of alveolar macrophages in human infants with BPD compared with those without (26). In our mouse pups, this activation was so pronounced that despite the reduction in cell numbers, the abundance of most inflammatory mediators was greater in hyperoxia than in room air. IL-1Ra ameliorated this inflammatory response in macrophages and DC, as demonstrated by the reduction of CD11b and GR1. On the molecular level, protein array analysis of the lungs of three animals from each group. Data are plotted as OD normalized to the positive control spots on each membrane in arbitrary units ± SEM. (A–E) Semiquantitative protein analysis of cytokines (A), other mediators (B), and chemokines (D) was performed by multiplex immunoblotting on the lungs of three animals from each group. Data are plotted as OD normalized to the positive control spots on each membrane in arbitrary units ± SEM. (A–E) ▲ P < 0.05 and ▲▲ P < 0.01 for no antenatal LPS air vehicle vs. room air vehicle; * P < 0.05 for room air vehicle vs. room air IL-1Ra; * P < 0.05, ** P < 0.01, and *** P < 0.001 for no antenatal LPS air vehicle vs. hyperoxia vehicle; * P < 0.05 and ** P < 0.01 for room air vehicle vs. hyperoxia vehicle; P < 0.05 for hyperoxia vehicle vs. hyperoxia IL-1Ra. (F) Sections of the same day 28 lungs used to generate Fig. 3 were subjected to IHC assessment of the abundance of pulmonary IL-1β. One representative image for each group is shown; n = 4–7 per group. Scale bars: 100 μm. IL-1α was also determined and is shown in Fig. S4.

Although the involvement of IL-1β (6–9, 11, 16) and IL-6 (6, 7, 10) in human BPD is known, literature is sparse on BPD and MIP-2, and TREM-1. Besides other mononuclear cells, MIP-1α acts on monocytes and DC (27) and may therefore be involved in recruiting these cells into the lungs of the newborn mice. Consistently, antibodies to MIP-1α inhibited the harmful angiogenesis induced by intraamnionic injection of LPS in mice (28). Blockade of MIP-2, a neutrophil chemoattractant, reduces hyperoxia-induced alveolar septal thickening in a rat model of BPD (29). Increased serum levels of TREM-1, whose main function is to amplify inflammation, are present in preterm babies, although the association with BPD or death failed to reach significance (P = 0.15) (7). In view of evidence that TREM-1 facilitates neutrophil migration across airway epithelial cells in a model of pneumonia (30), our report on pulmonary abundance of TREM-1 may provide a more direct view of its involvement in BPD, albeit in mice.

The call by the AAP for research into anti-inflammatory strategies against BPD led to studies showing that sildenafil provides benefit, but less than we observed for IL-1Ra (31), and revealed some promise for bone marrow–derived mesenchymal
Fig. 5. Flow cytometric analysis of the lungs on day 28. The left lungs of the animals shown in Fig. 3 (perinatal LPS, 65% hyperoxia) were analyzed by flow cytometry. Among viable cells, all immune cells (A) or 20,000 CD45+ cells (B–G) were gated. The graphs show data from one experiment (n = 3–4 per group); a second experiment produced similar results (n = 3–4 per group). (A) The total number of cells isolated from the lungs is depicted. (B and C) Enumeration of CD45+ and CD8+ T cells, B220+CD11c− B cells, B220+CD11c+ conventional DC, and B220−CD11c+ plasmacytoid DC is plotted as percent of CD45+ cells. (D and E) CD11c+CD103− activated monocytes are shown as percent of CD45+ cells. (F) One exemplary histogram per group of the CD11b stain is depicted; red and blue line, room air groups; orange line, hyperoxia vehicle; green line, hyperoxia IL-1Ra. (F and G) Assessment of CD11b and GR1, markers of macrophage and granulocyte activation, on CD45+ cells. (F) Analysis of one experiment (n = 4 per group); (G) exemplary dot plots of one lung from each group. *P < 0.05, **P < 0.01, and ***P < 0.001 for all comparisons.

In addition to these promising prerequisites, IL-1α and IL-1β, both targets of IL-1Ra, have been shown to play an important and deleterious role in BPD pathogenesis. Several studies identified an association between elevated levels of IL-1β and development of BPD or an adverse clinical outcome (6, 9). An imbalance between IL-1β and IL-1Ra contributes to prolonged inflammation in BPD (11), and treatment with IL-1Ra protects from loss of function and structural damage in an ozone model of lung disease (35). Neonatal mouse lung inflammation is augmented in airway epithelial cells suffered from BPD-like disrupted alveolar septation and capillary growth and featured elevated levels of MIP-2 (8)—findings consistent with our data. This same study also reported that IL-1β reduced the production of VEGF, a mediator crucial for normal neonatal alveolarization (36). Hyperoxia-induced BPD has been associated with an increase in IL-1α and MIP-1α in mice (14), whereas in preterm lambs intratracheal IL-1α triggered severe lung inflammation (15). In another ovine model, IL-1Ra reduced the fetal pulmonary and systemic inflammatory response to intra-amniotic LPS (15). On a contradictory note, IL-1Ra has been tested unsuccessfully in a model of lung injury induced by high tidal volume ventilation in preterm sheep (37). However, the trigger for lung damage and the time points investigated were different from our study, which likely explains the absence of efficacy of IL-1Ra.

In summary, we demonstrate that prophylactic administration of IL-1Ra affords nearly complete protection against BPD and allows almost normal lung development in a clinically relevant mouse model. We also provide intriguing insights into neonatal pulmonary immunology, including the identification of macrophages and DC as well as IL-1α, IL-1β, MIP-1α, and TREM-1 as the major cellular and molecular perpetrators of pulmonary inflammation. Earlier work has shown that IL-1Ra is safe and efficacious in the treatment of human diseases, including in the pediatric setting. We therefore propose that clinicians and researchers would be justified in proceeding with a clinical trial of a nonsteroidal anti-inflammatory drug to fundamentally change the prognosis of premature infants by preventing the currently irreducible BPD, a disease with devastating short- and long-term consequences, including significant mortality.

Materials and Methods

Murine Model of BPD. Based on previous publications (21, 22, 38), pregnant C57BL/6J dams received an i.p. injection of 150 μg/kg of LPS or volume-matched vehicle (normal saline) at 14 d gestation. Within 24 h after birth, pups and dams were randomized to a chamber through which we passed gas at 10 L/min with an FiO2 of 0.21 (room air), 0.65, or 0.85 for a total of 3, 14, or 28 d. Pups were also randomly allocated to receive daily s.c. injections of IL-1Ra (10 mg/kg) [a moderate dose considering (i) the Food and Drug Administration recommendation to use 12-fold higher drug doses in murine models of human disease (32) and (ii) the dose of 1–5 mg/kg in human infants with NOMID (19)] or a similar volume of vehicle (5%). Maternal temperature (22°C) and humidity (50%) were kept constant. Light was cycled in a 12-h day/night rhythm. Dams were rotated between the groups in a 3-d cycle to limit dam-effects on study outcome and to protect dams from pronounced hyperoxia. At 3, 14, or 28 d, mice were...
anesthetized with isoflurane, then humanely killed by cervical dislocation. Lungs were processed for histology, flow cytometry, or cytokine assay.

**Lung Preparation, Histology, and IHC.** After cervical dislocation, 14- and 28-d-old mice were intubated via the trachea and the left lung lobe was tied off at the main bronchus and removed for flow cytometry or cytokine analysis. The right lung was fixed with 4% paraformaldehyde (PFA) (pH 7.4, adjusted at a pressure of 20 cmH2O) removed from the thorax, kept in 4% PFA for a minimum of 2 h, then immediately processed for paraffin embedding and sectioning. Lung tissue was cut into 3-μm sections at three levels from the apex to base of the lung and H&E-stained for histology. For IHC, 4-μm sections were cut and deparaffinized. Antigen retrieval was performed in 10 mM citrate buffer, pH 6.0, in a pressure cooker for 10 min. Endogenous peroxidase activity was inhibited using a 0.5% H2O2/methanol solution applied to the slides for 15 min followed by a 30-min blocking step using 1.5% rabbit serum in PBS (part of the Vectastain Elite ABC kit, Vector Laboratories). Slides were then incubated with goat anti-mouse IL-1α or IL-1β antibodies (both 1:100, R&D Systems) for 1 h at room temperature. Secondary antibody incubation and diaminobenzidine (DAB) staining were performed according to the instructions of the Vectastain kit. Sections were counterstained with Harris hematoxylin (Scientific) and scanned on an Aperio Scanscope (ePathology Solutions).

**Cytokine Analysis.** At day 3, the entire lung was removed, washed in ice-cold sterile saline, snap frozen in liquid nitrogen, and stored at −80 °C. For analysis, the lungs were homogenized in lysis buffer (39) using an Ultra Turrax. The homogenate was centrifuged for 10 min at 14,000 × g and the supernatant was assayed for protein and cytokines. Changes in cytokine abundance resulting from tissue rarefaction, reduced growth, or other confounding factors were corrected by normalization to total protein content. ELISAs (R&D Systems, MIP-2, IL-1β) were performed according to the manufacturers’ instructions. ProteomeProfiler Arrays (R&D Systems) were used as described previously (40).

**Flow Cytometry.** For lung cell isolation on days 14 and 28, the left lung was minced and digested with collagenase D (1 mg/mL) at 37 °C for 30 min. After digestion, lung homogenates were macerated through a 70-μm cell strainer, red blood cells were lysed (BD Pharm Lyse) for 2 × 10 min, then centrifuged at 400 × g for 10 min at 18 °C. Pellets were washed twice with PBS and cells were stained and acquired using a FACS Canto II flow cytometer (BD). Fc receptors were blocked using anti-CD16/CD32 (eBioscience). Cells were determined by surface expression of CD11c, CD11b, and GR1 for monocytic and macrophages; B220/CD11c for plasmacytoid DC; B220/CD11c for conventional DC; F4/80/CD11b for macrophages; B220/CD11c for B cells; DX5/CD3 for NK cells; and CD4 or CD8 for T cells. A total of 20,000 CD45+ cells were analyzed per sample. A forward scatter gate was used to eliminate cell debris and gates were set using fluorescein minus one staining gate. Data were analyzed using FlowJo software. All antibodies were obtained from BD Pharmingen, except F4/80 (Serotec).

**Statistical Analysis, Ethics, and Analysis of Lung Structure.** Please see Supporting Information.

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Supporting Information
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SI Materials and Methods

Ethics. All experimental procedures conformed with the guidelines established by the National Health and Medical Research Council, had the approval of Monash University’s Ethics Committee, and complied with the Declaration of Helsinki.

Analysis of Lung Structure. Each slide was scanned at 200-fold magnification using an Aperio Scanscope. Images of whole lungs were analyzed using ImageJ software (National Institutes of Health) by measuring the alveolar number and size and by calculating the SVR.

Statistical Analysis. Sigma Plot (Systat Inc) was used to test groups for normality and equal variance (P to reject 0.05), then one-way ANOVA or one-way ANOVA on ranks were applied to test for significant differences. Where ANOVA revealed significance, post hoc Student–Newman–Keuls or Dunn’s comparisons were performed (threshold for significance P < 0.05). Flow cytometry data were analyzed by Student t test.

Fig. S1. An overview of the pathogenesis of bronchopulmonary dysplasia (BPD). PA, pulmonary artery; PDA, patent ductus arteriosus.
Fig. S2. Whole lung histology in 28-d-old pups exposed to antenatal LPS and postnatal hyperoxia at 85%. Each image depicts a right lung of one 28-d-old pup in its entirety that is representative for the group. Each higher magnification image in Fig. 1 is a 500 × 500 μm detail from the corresponding slides. n = 10–27 per group. IL-1Ra, IL-1 receptor antagonist.

Fig. S3. Whole lung histology at 65% O₂. Shown are the whole right lungs from which the 500 × 500 μm details in Fig. 3 were excerpted. n = 5–27 per group.

Fig. S4. Immunohistochemical analysis of the abundance of pulmonary IL-1α on day 28. Additional sections were cut from the lungs of the same animals shown in Fig. 3 and Fig. S3 and were subjected to immunohistochemical assessment of IL-1α. One exemplary image for each group is shown; n = 4–8. (Scale bar: 100 μm.)
Fig. S5. Flow cytometry of pulmonary cells from the right lung on day 28 of 85% hyperoxia or room air and treatment with IL-1Ra or vehicle as well as antenatal LPS. n = 4–7 per group. (A) The total number of cells obtained from the isolations is plotted. (B and C) Abundance of cell surface CD11b and GR1 (Ly6G/C) is shown. (B) One representative dot plot from each group is depicted and illustrates the gating that was used to differentiate between the groups shown in C. (C) CD11b\textsuperscript{hi}GR1\textsuperscript{hi} inflammatory macrophages, CD11b\textsuperscript{−}GR1\textsuperscript{hi} granulocytes, CD11b\textsuperscript{+}GR1\textsuperscript{−} monocytes, and CD11b\textsuperscript{+}GR1\textsuperscript{int} macrophages evolving into the inflammatory phenotype were enumerated and are shown as percent of total lung population. *P < 0.05, **P < 0.01, and ***P < 0.001 for all comparisons.