Lung development and the host response to influenza A virus are altered by different doses of neonatal oxygen in mice

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Buczynski BW, Yee M, Lawrence BP, O’Reilly MA. Lung development and the host response to influenza A virus are altered by different doses of neonatal oxygen in mice. Am J Physiol Lung Cell Mol Physiol 302: L1078–L1087, 2012. First published March 9, 2012; doi:10.1152/ajplung.00026.2012.—Oxygen exposure in preterm infants has been associated with altered lung development and increased risk for respiratory viral infections later in life. Although the dose of oxygen sufficient to exert these changes in humans remains unknown, adult mice exposed to 100% oxygen between postnatal days 1–4 exhibit alveolar simplification and increased sensitivity to influenza virus infection. Additionally, two nonlinear thresholds of neonatal oxygen exposures were previously identified that promote modest (between 40% and 60% oxygen) and severe (between 80% and 100% oxygen) changes in lung development. Here, we investigate whether these two thresholds correlate with the severity of lung disease following respiratory viral infection. Adult mice exposed to 100% oxygen at birth, and to a lesser extent 80% oxygen, demonstrated enhanced body weight loss, persistent inflammation, and fibrosis following infection compared with infected siblings exposed to room air at birth. In contrast, the host response to infection was indistinguishable between mice exposed to room air and 40% or 60% oxygen. Interestingly, levels of monococyte chemoattractant protein (MCP)-1 were equivalently elevated in infected mice that had been exposed to 80% or 100% oxygen as neonates. However, reducing levels of MCP-1 using heterozygous Mcp-1 mice did not affect oxygen-dependent changes in the response to infection. Thus lung development and the host response to viral infection are disrupted by different doses of oxygen. Our findings suggest that measuring lung function alone may not be sufficient to identify individuals born prematurely who have increased risk for respiratory viral infection.

fibrosis; inflammation; oxidative stress; premature; respiratory infections

PREMATURE INFANTS, especially those with very low birth weight, are at increased risk for developing neonatal lung disease, such as bronchopulmonary dysplasia (BPD) (17, 24). The lungs of infants who died from BPD are characterized pathologically by fewer, larger alveoli and abnormal pulmonary vasculature (23). Although milder ventilation strategies, administration of exogenous surfactant, and antenatal steroids have improved the survival of even the most premature infants, the prevalence of pulmonary sequelae among survivors of prematurity has unfortunately not decreased. Reduced pulmonary function and lung capacity have been observed in school-age children and beyond who have survived premature birth, suggesting that disrupted lung development may be permanent in these individuals (13–15, 40). Also, children born prematurely are at increased risk for respiratory viral infection and for developing asthma, retinopathy of prematurity, and neurodevelopmental delay (43, 51). These children often require rehospitalization or additional medical care to treat diseases now attributed to being born prematurely. Hence, there is an urgent need to understand how prematurity alters health later in life.

It is widely accepted that reactive oxygen species, created when the premature lung is exposed to the ambient oxygen in room air (RA) or therapeutically elevated levels of oxygen, cause cell and tissue injury via lipid peroxidation, DNA damage, and protein oxidation (50, 55). Because the maturation of antioxidant defenses does not occur until late in gestation, infants born prematurely are particularly at risk for sustaining lung injury attributable to oxidative stress. In fact, multiple studies have reported a significant correlation between oxygen supplementation in the neonatal period and decrements in lung function later in life (5, 26). Interestingly, premature infants treated with recombinant human copper-zinc superoxide dismutase had improved pulmonary function at 1 yr of corrected age, underscoring the influence of oxidant injury on the developing lung and perhaps other oxidant-sensitive organs like the brain (7). Nevertheless, supplemental oxygen continues to be used in the treatment of premature infants in respiratory distress, and several recent studies have focused on defining the optimal dose that maximizes efficacy and minimizes harm. The Surfactant, Positive Pressure, and Pulse Oximetry Randomized Trial (SUPPORT) evaluated the posttreatment effects of high (91% to 95%) and low (85% to 89%) oxygenation in premature infants (3, 19). Although the incidence of retinopathy was reduced in infants treated with low oxygenation, mortality was increased before discharge, and the investigators were unable to define an optimum target for oxyhemoglobin saturations. On the other hand, defining oxygen exposure as an area under the curve has been predictive for continued respiratory symptoms, use of medications, and general need for medical care during the first year of life (45). Despite the growing appreciation that lower levels of oxygen may still be beneficial, the appropriate exposure level of oxygen that is required for optimal target saturations without reprogramming organ development remains unknown.

Analogous to premature infants who have died from complications of BPD, prolonged exposure of preterm baboons or term rodents to elevated levels of oxygen (hyperoxia) causes alveolar simplification, attributed in part to disrupted vascular development (21, 30, 31). Increased lung volumes and long-term changes in airway responsiveness have been observed in several species of newborn animals exposed to hyperoxia and recovered in RA, suggesting that altered lung development can...
be permanent (6, 10, 32, 42, 57). We have reported that the developing lungs of mice are responsive to multiple levels, or thresholds, of neonatal oxidative stress, leading to no (21–40% oxygen), modest (60–80% oxygen), or severe (100% oxygen) changes in lung structure and function that persist into adulthood (Fig. 1A) (56, 57). Additionally, adult mice exposed to 100% oxygen as neonates exhibit substantially reduced resistance to infection with a nonlethal dose of influenza virus compared with infected siblings exposed to RA at birth (35). Because children born prematurely also exhibit changes in lung function and are at increased risk for respiratory viral infection, our mouse model may clarify whether altered lung development can be used as a biomarker to reliably predict risk for respiratory infection. By infecting adult mice that had been exposed to RA, 80% oxygen and 100% oxygen (exposure paradigm 1) or RA, 40% oxygen and 60% oxygen (exposure paradigm 2) during postnatal days 1–4 (PND 1–4) (Fig. 1B), we investigate whether changes in metrics of the host response to influenza virus infection correlate with the thresholds of neonatal oxygen sufficient to cause modest or severe changes in lung structure and function.

MATERIALS AND METHODS

Exposure of mice to hyperoxia and influenza A virus. C57BL/6J mice and heterozygous monocyte chemoattractant protein (Mcp)-1 mice were used for this study. Mcp-1-deficient mice (Mcp-1<sup>−/−</sup>). The Jackson Laboratory, Bar Harbor, ME), on a C57BL/6J genetic background, were mated with C57BL/6j control mice to generate the Mcp-1<sup>−/−</sup> mice. Newborn mice were exposed to RA or 40%, 60%, 80%, or 100% oxygen between PND 1 and 4 (56, 57). On PND 4, oxygen-exposed mouse pups were returned to RA. Adult (8–10 wk of age) female mice exposed to RA or hyperoxia at birth were injected intranasally with 120 hemaglutinating units (250 g). Supernatant from the first lavage was retained as BAL fluid and was stored at −80°C. Cells recovered from all three lavages were pooled, and erythrocytes were removed by treatment with ammonium chloride lysing solution (0.15 M NH₄Cl, 10 mM NaHCO₃, 1 mM EDTA). The total number of cells was enumerated using a TC10 automated cell counter (Bio-Rad, Hercules, CA). Cells from individual mice were transferred to microscope slides using a cytological centrifuge and were stained with Diff-Quik. Macrophages, neutrophils, and lymphocytes were enumerated by differential cell counts of at least 200 cells on coded slides by two independent investigators.

Viruses titer measurements. Frozen lungs from individual mice (separate from those used for collection of BAL fluid) were resuspended in 1 ml of ice-cold 1× PBS, containing 100 U/ml of penicillin and 0.1 mg/ml of streptomycin, and mechanically homogenized (Tissuemiser; Fisher Scientific, Pittsburgh, PA). Following centrifugation of tissue homogenates (1,500 revolution/min for 5 min at 4°C), 15 μl of the supernatant was serially diluted (1:10<sup>9</sup> to 1:10<sup>3</sup>) in zero-serum refeed medium containing 4 μg/ml of 0.25% trypsin-EDTA. Virus titers were determined by immunocytochemistry on confluent Madin-Darby Canine Kidney cells in 96-well flat-bottom tissue culture plates in duplicate (2). Briefly, cells were incubated with 100 μl of serially diluted supernatant and centrifuged for 1.5 h at 700 g, after which wells were aspirated and replaced with fresh zero-serum refeed media. Following overnight incubation at 33°C, cells were fixed with 80% acetone/20% water for 30 min at −20°C, rinsed with washing buffer (SWB) (1X PBS, 2% FBS, 0.1% sodium azide), and then incubated with a biotinylated anti-influenza A monoclonal antibody (1:900 dilution in SWB; Millipore, Billerica, MA) for 1 h at 38°C. Cells were then incubated with Streptavidin-labeled alkaline phosphatase (1:500 dilution in PBS; Sigma-Aldrich, St. Louis, MO) for 1 h at room temperature, and viral foci, indicative of influenza infection in the cell monolayer, were detected upon addition of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma-Aldrich). Viral foci were counted, and viral foci units per milliliter were determined.

Lung histology and immunohistochemistry. Lungs from mice were inflation fixed with 10% neutral-buffered formalin, embedded in paraffin, and sectioned as previously described (56, 57). Tissue sections were stained with hematoxylin and eosin, and tissue sections were sampled from each lung for evaluation (Fig. 1). The amount of tissue sampled was based on the size of the lung. Tissues were prepared for immunohistochemistry and sections were stained with antibodies against MCP-1 (1:500 dilution in PBS; Sigma-Aldrich) for 1 h at 38°C. Cells were then incubated with Streptavidin-labeled alkaline phosphatase (1:900 dilution in SWB; Millipore, Billerica, MA) for 1 h at 38°C and then washed with washing buffer.

Fig. 1. Neonatal hyperoxia disrupts lung structure and the immune response to viral infection in adult mice. A: representative hematoxylin-eosin stained lung tissues from adult mice depicting the doses of oxygen that cause no (room air [RA] - 40% oxygen), modest (60–80% oxygen), or severe (100% oxygen) changes in lung structure and function. Scale bar, 100 μm. Exposure to 100% oxygen also causes increased sensitivity to influenza virus. B: cartoon model depicting the 2 experimental paradigms used to test whether the high (80% oxygen vs. 100% oxygen) and/or modest (40% oxygen vs. 60% oxygen) thresholds of oxygen exposure increase sensitivity to influenza A virus infection.
sections were stained with hematoxylin and eosin to determine changes in lung structure. Collagen deposition in tissue sections was detected with Gomori’s Trichrome stain according to the manufacturer’s instructions (Richard-Allan Scientific, Kalamazoo, MI). Separate tissue sections were rehydrated and incubated overnight at 4°C with anti-α-smooth muscle actin (α-SMA) primary antibody (35). Immune complexes were detected with fluorescently labeled secondary antibodies before tissue sections were counterstained with 4’,6-di-aminido-2-phenylindole (DAPI). Stained tissue sections were visualized with a Nikon E-800 fluorescence microscope (Nikon, Melville, NY), and images were captured with a SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Sircol assay. The Sircol collagen assay (Biocolor, Belfast, UK) was performed according to the manufacturer’s instructions. Briefly, lung homogenates were acid-pepsin digested at 4°C overnight. Test samples were then centrifuged at 10,000 g for 10 min, and the supernatant was collected. One milliliter of Sirius red reagent was then added to 100 μl of supernatant for each sample and mixed for 30 min at room temperature. The collagen-dye complex was precipitated by centrifugation at 10,000 g for 10 min, washed with an acid-salt solution, and dissolved in 500 μl of 0.5 M NaOH. The absorbance of each sample was determined at 555 nm, and the amount of collagen in each sample was quantified using a standard curve generated using collagen standards provided by the manufacturer.

Statistical analysis. Single comparisons of values were assessed by Student’s t-test, whereas group means were compared by ANOVA, followed by Fisher’s least-significant-difference post hoc test with StatView statistical software (Abacus Concepts, Piscataway, NJ). Values are expressed as means ± SE with P < 0.05 being considered significant.

RESULTS

Low levels of neonatal oxygen are not sufficient to disrupt the host response to influenza A virus infection. Because neonatal exposure to 100% oxygen increases the inflammatory response to influenza A virus infection in adult mice on postinfection days 5–9 and causes fibrosis by postinfection day 14 (35), the present study focused on how various levels of neonatal oxygen affected these metrics of the host response. Inflammation and fibrosis were evaluated on postinfection days 7 and 14, respectively, because this is when RA- and oxygen-exposed mice showed the greatest differences. Although there were no differences in mortality among the different exposure groups when examined, percent body weight loss was often significantly greatest in the infected mice exposed to 100% oxygen at birth (data not shown).

Viral titers were evaluated on postinfection day 7 in whole lung homogenates from adult mice that had been exposed to either a high (80% vs. 100% oxygen) or modest (40% vs. 60% oxygen) threshold of neonatal oxygen. Viral titers were significantly greater in infected adult mice exposed to 100% oxygen at birth compared with infected siblings exposed to RA at birth (Fig. 2A). In contrast, viral titers were not different between infected adult mice exposed to RA and 80% oxygen or the modest threshold of neonatal oxygen. Virus was cleared by postinfection day 14, regardless of whether the mice had been exposed to RA or hyperoxia as neonates (data not shown). Therefore, these findings demonstrate that exposure to low levels of neonatal oxygen (40% and 60%) is not sufficient to disrupt the ability of adult mice to limit viral replication during infection.

Consistent with prior observations, significantly more leukocytes were observed in the BAL fluid of infected adult mice exposed to the high threshold of neonatal oxygen (100% oxygen) compared with infected siblings exposed to RA at birth (Fig. 2B). The percentage and phenotype of leukocytes in the BAL fluid were also examined on postinfection day 7. There were no differences in the percentages of monocytes/macrophages, neutrophils, and lymphocytes between infected adult mice exposed to RA and 100% oxygen at birth (Table 1). This finding indicates a general increase in the numbers of those leukocytes examined, as opposed to a change in the number of a single cell type. Although total cell number was...
slightly increased in infected adult mice exposed to 80% oxygen at birth compared with infected siblings exposed to RA at birth, statistical significance was not reached. Additionally, there were no significant differences in total leukocyte numbers when we compared infected adult mice exposed to RA and the modest threshold of neonatal oxygen. These findings suggest that exposure to low levels of neonatal oxygen (40% and 60%) does not alter leukocyte recruitment in adult mice upon infection.

**High levels of neonatal oxygen increase fibrotic repair upon influenza A virus infection.** Fibrotic repair was evaluated on postinfection day 14 by staining lungs with Trichrome and antibodies against α-SMA. Trichrome staining revealed a striking increase in collagen deposition in infected adult mice exposed to 100% oxygen as neonates (Fig. 3A). Furthermore, fibrotic regions of lung tissue from these same mice also stained positive for α-SMA, a marker of activated myofibroblasts. This pathology was modestly observed in infected adult mice exposed to 80% oxygen at birth. Additionally, when total collagen deposition in the lungs of infected adult mice was measured by the Sircol assay on postinfection day 14, we observed a significant increase in the level of total collagen protein in infected adult mice exposed to 100% oxygen at birth compared with infected siblings exposed to RA at birth (Fig. 3B). Although the level of total collagen protein was increased in infected adult mice exposed to 80% oxygen at birth, the result was not statistically significant compared with infected adult mice exposed to RA at birth. Lastly, consistent with mice exposed to 100% oxygen at birth having a more difficult time resolving infection with influenza virus, the mean percentage of body weight lost among these mice through postinfection day 14 was often significantly greater than that of their infected siblings exposed to RA at birth (Fig. 3C). These findings suggest that exposure to high levels of neonatal oxygen (80% and 100%) can potentially disrupt alveolar repair and remodeling following infection in adult mice.

**Reducing levels of the inflammatory chemokine MCP-1 does not attenuate the host response to influenza A virus in mice exposed to the high threshold of neonatal oxygen.** The altered response to influenza A virus infection in mice exposed to hyperoxia at birth was associated with persistent and elevated levels of MCP-1, but not IFN-γ, IL-1β, IL-6, TNF-α, keratinocyte cytokine, granulocyte-macrophage colony-stimulating factor, or macrophage inflammatory protein-1α (35). The selectively elevated expression of MCP-1 may contribute to the altered host response to infection because MCP-1 plays an important role in both inflammation and fibrosis. Interestingly, elevated levels of MCP-1 were observed in infected mice exposed to 80% and 100% oxygen at birth (Fig. 4). This finding gave us the opportunity to test the contribution of excess levels of MCP-1 to the altered host response to infection that we had primarily observed in mice exposed to 100% oxygen at birth. To test this, Mcp-1 wild-type (+/+) and heterozygous (+/-) mice exposed to 100% oxygen at birth were generated (Fig. 5A). Before infection, body weights and levels of MCP-1 in the BAL fluid were similar between both groups of mice (data not shown). Following influenza virus infection, the mean percentage of body weight lost was similar between Mcp-1+/− and Mcp-1−/− mice exposed to 100% oxygen at birth (Fig. 5B). Despite this reduction in the level of MCP-1 following infection in the hyperoxia-exposed heterozygous mice, viral titers and total leukocyte cell counts were similar compared with the hyperoxia-exposed wild-type mice (Figs. 6, A and B; Table 2). Note that the total leukocyte cell count reported in Table 2 for Mcp-1−/− mice exposed to 100% oxygen is lower than the count reported in Table 1 for wild-type mice exposed to 100% oxygen. Such differences in the host response to influenza virus are commonly observed, attributable to the variability of oxygen dosing and infection between experiments. To account for this variability, each experiment was internally controlled with either RA-exposed or wild-type mice and reproduced at least twice. Regardless of the values obtained in a given experiment, reducing excess levels of MCP-1 does not affect the host response of mice exposed to the high threshold of neonatal oxygen, as defined by differences in viral titers and total number of leukocytes recruited to the lung.

**Similar to the dose-response studies**, fibrotic repair was evaluated on postinfection day 14 by staining lungs with Trichrome and antibodies against α-SMA. Although Trichrome staining revealed considerable collagen deposition in lung tissues of both Mcp-1−/− and Mcp-1−/− mice, no differences were observed (Fig. 7A). Likewise, α-SMA staining was not noticeably different between Mcp-1−/− and Mcp-1−/− mice. Additionally, no differences in the level of total collagen were observed between Mcp-1−/− and Mcp-1−/− mice (Fig. 7B). Lastly, the mean percentage of body weight lost among the two groups of mice through postinfection day 14 was similar, indicating that the mice were likely responding to and resolving the infection similarly (Fig. 7C). Hence, excess levels of MCP-1 are not responsible for the oxygen-dependent changes in the host response to influenza virus infection because reducing levels using Mcp-1 heterozygous mice did not diminish lung disease. This genetic approach validates the finding that the persistent

### Table 1. High-threshold and modest-threshold oxygen exposure

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<th></th>
<th>RA</th>
<th>80% oxygen</th>
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<th>RA</th>
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<td>Cell Number, ×10⁵ ml⁻¹</td>
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<td>Neutrophils, %</td>
<td>43.8 ± 6.9</td>
<td>67.2 ± 9.9</td>
<td>98.4 ± 27.9*</td>
<td>47.8 ± 5.3</td>
<td>23.8 ± 6.4</td>
<td>30.8 ± 10.7</td>
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<td>Macrophages, %</td>
<td>13.0 ± 1.4</td>
<td>13.1 ± 1.0</td>
<td>13.2 ± 1.4</td>
<td>11.1 ± 0.5</td>
<td>8.4 ± 1.6</td>
<td>9.8 ± 2.4</td>
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<td>Lymphocytes, %</td>
<td>80.9 ± 1.7</td>
<td>81.7 ± 1.3</td>
<td>79.7 ± 1.0</td>
<td>84.2 ± 2.1</td>
<td>86.5 ± 1.4</td>
<td>83.7 ± 2.1</td>
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Values are means ± SE. Exposure to neonatal hyperoxia does not change the percentages of leucocytes recruited to the lungs of adult mice following influenza virus infection. Adult female mice exposed to room air (RA), 80% oxygen, and 100% oxygen (high threshold) or RA, 40% oxygen, and 60% oxygen (modest threshold) at birth were intranasally infected with a nonlethal dose of influenza A virus. Leukocytes in the bronchoalveolar lavage fluid were collected on postinfection day 7, and differential leukocyte counts were performed; n ≥ 4 mice per group, *P < 0.05.
DISCUSSION

Although supplemental oxygen is often used to treat premature infants in respiratory distress, defining an acceptable level that does not adversely affect the developing lung and other organs has been difficult because clinical treatment regimens are often variable. Excess levels of oxygen clearly increase the risk for disrupted vasculogenesis and retinopathy, neurodevelopmental delay, and chronic lung disease, or BPD, among infants born prematurely (1, 18, 41). Although tracking survivors of prematurity into childhood and beyond has also been difficult, several studies have reported a reduction in lung function and capacity, as well as an increased incidence of viral infection, in individuals prematurely exposed to oxygen at birth (15, 40, 51). Lung function has been used as a biomarker to identify and monitor the progression of certain respiratory diseases, such as chronic obstructive pulmonary disease and asthma (4, 44). Although increased sensitivity to respiratory viral infection may manifest in children before pulmonary function can be accurately assessed, it is unknown whether lung function can also be used to identify at risk individuals. Here, using a mouse model of neonatal oxidative stress, we provide evidence that the dose of neonatal oxygen sufficient to disrupt lung development (60% oxygen) is not associated with an altered host response to influenza virus infection in adult mice. Hence, altered pulmonary function parameters alone may not be a sufficient biomarker for determining whether children born prematurely are at risk for respiratory viral infections.

Although respiratory viruses are a significant cause of illness in the general population, children born prematurely are at increased risk for complications attributable to infection with influenza A virus, as well as other respiratory viruses, such as...
respiratory syncytial virus (RSV). During a primary influenza virus infection, epithelial and innate immune cells are the initial responders, secreting soluble mediators, such as chemokines, cytokines, and surfactant proteins, into the local environment and precede the induction of adaptive immune responses (28). Ultimately, it is the generation of virus-specific CD8+ T lymphocytes and antibodies that are responsible for viral clearance from the lung and host resistance upon reinfection (34). Although a tightly coordinated innate immune response functions to initially control viral replication, the present study shows that this response is unchanged in mice exposed to low levels of neonatal oxygen (40% and 60%). On the other hand, exposure to 100% oxygen at birth significantly increases viral titers in adult mice following infection, whereas negligible changes in viral titers were observed in mice exposed to 80% oxygen at birth. Increased viral titers were associated with a concomitant increase in the total number of inflammatory leukocytes recruited to the lung in mice exposed to 100% oxygen at birth. Whereas total cell number was modestly, but not significantly, increased in mice exposed to 80% oxygen at birth, no remarkable differences were noted in mice exposed to low levels of neonatal oxygen (40% and 60%). The increase in numbers of inflammatory leukocytes and viral titers following infection in mice exposed to high levels of

![Graph showing MCP-1 levels](image-url)
neonatal oxygen (80% and 100%) may be contributing to the morbidity observed in these mice. Although influenza virus-associated fatalities are rare and often limited to the very young or elderly, excessive inflammation and increased viral titers have been associated with disease pathogenesis and severe lung pathology (8, 25, 27).

Consistent with prior observations, we observed in the present study an increase in the level of MCP-1 within the BAL fluid of infected adult mice exposed to 80% and 100% oxygen at birth. To date, MCP-1 is the only chemokine or cytokine that we have identified that is selectively different in infected mice exposed to hyperoxia as neonates (35). In other words, the abundance of other chemokines or cytokines is equivalently changed in infected mice, regardless of their prior exposure to RA or hyperoxia as neonates. MCP-1 is a chemotactic cytokine expressed primarily by epithelial cells, endothelial cells, fibroblasts, and monocytic cells during infection and inflammatory processes. When bound to its receptor, CCR2, MCP-1 regulates the migration and infiltration of monocytes, memory T lymphocytes, and natural killer (NK) cells to sites of tissue injury and inflammation, thus playing an important role in the immune response to pathogens (11). Studies have shown that transgenic mice overexpressing MCP-1 are protected against viral or bacterial infection (12, 37). Other studies have suggested that MCP-1 contributes to significant pulmonary inflammation and fibrosis when aberrantly expressed. For example, increased levels of MCP-1 have been detected in the serum or BAL fluid obtained from children and adults with interstitial lung disease (22, 46). Despite these conflicting conclusions, the present studies found that reducing MCP-1 levels using MtCP-1 -1 mice exposed to 100% oxygen at birth did not attenuate the host response to influenza virus infection. Although MCP-1 signaling certainly plays an important role in mediating a proper immune response to infection, our data suggest that excess levels of MCP-1 are not sufficient to change metrics of the host response to influenza virus in mice exposed to hyperoxia at birth. Moreover, it suggests some other mechanism inherently related to early-life exposures with high levels of oxygen that is altering the way adult mice respond to and resolve an influenza virus infection.

Our findings also show that infected adult mice exposed to 100% oxygen, and to a lesser extent 80% oxygen, exhibit pathological markers of fibrosis, possibly attributable to unbalanced epithelial repair. Fibrosis is rarely a pathological outcome of influenza virus infection, and the role of infection in the genesis of pulmonary fibrosis has often been speculated upon (20). Nevertheless, there have been documented cases, albeit rare, of influenza pneumonia being associated with the development of pulmonary fibrosis (29, 38). Although influenza infection by itself does not lead to the development of fibrosis in our model, it does when mice are also exposed to high levels of neonatal oxygen. This implies that neonatal

Table 2. Leukocyte counts 5 and 7 days postinfection

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<td>Cell Number,</td>
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<tr>
<td>×10⁶ ml⁻¹</td>
<td>34.6 ± 7.1</td>
<td>27.5 ± 4.6</td>
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<td>Neutrophils, %</td>
<td>12.0 ± 1.5</td>
<td>14.7 ± 3.2</td>
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<tr>
<td>Macrophages, %</td>
<td>82.5 ± 1.9</td>
<td>80.4 ± 3.5</td>
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<td>Lymphocytes, %</td>
<td>5.1 ± 0.9</td>
<td>4.8 ± 0.5</td>
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Values are means ± SE. Reducing excess levels of monocyte chemoattractant protein (MCP)-1 does not change differential leukocyte counts in adult mice exposed to hyperoxia at birth upon influenza virus infection. Adult female MtCP-1 wild-type (+/+I) and heterozygous (+/−) mice exposed to 100% oxygen at birth were intranasally infected with a nonlethal dose of influenza A virus. Leukocytes in the bronchoalveolar lavage fluid were collected on postinfection days 5 and 7, and differential leukocyte counts were performed; n = 5 mice per group.
hyperoxia reprograms how the respiratory epithelium repairs following viral infection. Interestingly, neonatal oxygen disrupts the balance of alveolar epithelial cells, as defined by reduced expression of the alveolar type II epithelial cell-specific marker proSP-C and increased expression of the alveolar type I epithelial cell-specific marker T1/H251 (56, 57). However, oxygen-dependent changes in lung structure and function were not entirely associated with changes in expression of these genes. For example, whereas levels of T1/H251 are increased in adult mice exposed to 40% oxygen at birth, measurable changes in lung structure and function are not observed until mice are exposed neonatally to at least 60% oxygen (modest threshold). Whether changes in the balance of epithelial cells contribute to the increased sensitivity to respiratory viral infection remains to be determined.

Recent studies have led to a growing appreciation that genes controlling lung development and repair are also involved in host defense (54). For example, thyroid transcription factor-1 (TTF-1/NKx2.1) is required for normal alveolar lung development (9, 33, 58). However, overexpression of TTF-1 in transgenic mice causes pulmonary fibrosis and eosinophilia, associated with increased expression of eotaxin and IL-6 (52). Similarly, overexpression of a mutant form of TTF-1 that cannot be phosphorylated in the lung causes severe lung hypoplasia at birth and altered expression of genes involved in innate immunity (9). Moreover, the Forkhead box A transcription factor Foxa2, a known transcriptional regulator of lung morphogenesis and perinatal lung maturation, plays a critical role in controlling the expression of genes involved in innate host defense, such as lysozyme and surfactant proteins A and D (53). Deletion of Foxa2 leads to air-space enlargement, neutrophilic pulmonary infiltrates, the induction of severe Th2-mediated inflammation, and goblet cell metaplasia (36, 49). Additionally, cell signaling pathways known to influence lung development, such as Ras, Myc, and B-catenin, are also present during repair processes within the lung to maintain a normal lung structure (47). Interestingly, the signaling proteins fibroblast growth factor-10 and bone morphogenetic protein-4 are expressed during hyperoxia injury repair in the mouse lung, as well as during development as part of a mechanism that putatively controls epithelial growth (39). Based on these examples, it is reasonable to speculate that neonatal hyperoxia disrupts a common pathway or pathways that control both postnatal lung development and host defense against respiratory infections.

It is difficult to extrapolate the dose-dependent effects of oxygen in this study to outcomes in children born prematurely because the clinical use of oxygen in premature infants is based on need and therefore is highly variable. However, the findings we report here demonstrate for the first time that exposure to high levels of neonatal oxygen (80% and 100%) results in a multifactorial response to influenza virus infection, with changes in individual outcomes being dependent on the dose of oxygen exposure at birth. In other words, the outcome of a
given measurement cannot always be predicted by the dose of oxygen exposure. Although several studies have shown that neonatal exposure to greater than 40% oxygen is sufficient to disrupt lung development and function in mice (6, 56), our findings demonstrate that exposure to low levels of oxygen (40% and 60%) at birth are not sufficient to alter the host response to influenza virus infection in mice. Therefore, these data suggest that the level of neonatal oxygen required to disrupt lung development is different than the level required to alter the host response to influenza virus infection in adult mice. In fact, it has been suggested that infants born prematurely are not predisposed by diminished premorbid lung function to respiratory viral infections. Ultimately, such interventions may not be sufficient to restore normal lung development and function. Precedence for this hypothesis derives from a single study showing that treatment of preterm infants with recombinant human copper-zinc superoxide dismutase alleviated rehospitalization rates in the first year of life, but not mortality in the neonatal intensive care unit (7). Because changes in lung development are observed in adult mice exposed to >40% oxygen at birth, our findings suggest that lung structure and function may not be suitable markers for identifying those individuals at increased risk for respiratory viral infections. Ultimately, understanding how exposure to different levels of oxygen in the immediate postnatal period reprograms lung development and the way the lung responds to respiratory pathogens through childhood and beyond could provide insight into biomarkers for identifying at risk individuals or mechanisms of disease.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: B.W.B., B.P.L., and M.A.O. conception and design of experiments; B.W.B. prepared figures; B.W.B. drafted manuscript; B.W.B., M.P.L., B.P.L., and M.A.O. approved final version of manuscript.

REFERENCES


EFFECT OF NEONATAL HYPEROXIA ON RESPIRATORY VIRAL INFECTIONS


