Neonatal Hyperoxia Enhances the Inflammatory Response in Adult Mice Infected with Influenza A Virus

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Rationale: Lungs of adult mice exposed to hyperoxia as newborns are simplified and exhibit reduced function much like that observed in people who had bronchopulmonary dysplasia (BPD) as infants. Because survivors of BPD also show increased risk for symptomatic respiratory infections, we investigated how neonatal hyperoxia affected the response of adult mice infected with influenza A virus infection.

Objectives: To determine whether neonatal hyperoxia increased the severity of influenza A virus infection in adult mice.

Methods: Adult female mice exposed to room air or hyperoxia between Postnatal Days 1 and 4 were infected with a sublethal dose of influenza A virus.

Measurements and Main Results: The number of macrophages, neutrophils, and lymphocytes observed in airways of infected mice that had been exposed to hyperoxia as neonates was significantly greater than in infected siblings that had been exposed to room air. Enhanced inflammation correlated with increased levels of monocyte chemoattractant protein-1 (CCL2) in lavage fluid, whereas infection-associated changes in IFN- γ , IL-1 β , IL-6, tumor necrosis factor- α , KC, granulocyte-macrophage colony-stimulating factor, and macrophage inflammatory protein-1 α , and production of virus-specific antibodies, were largely unaffected. Increased mortality of mice exposed to neonatal hyperoxia occurred by Day 14 of infection, and was associated with persistent inflammation and fibrosis.

Conclusions: These data suggest that the disruptive effect of hyperoxia on neonatal lung development also reprograms key innate immunoregulatory pathways in the lung, which may contribute to exacerbated pathology and poorer resistance to respiratory viral infections typically seen in people who had BPD.

Keywords: bronchopulmonary dysplasia; hyperoxia; infection; lung inflammation; virus

Bronchopulmonary dysplasia (BPD) is the most common form of chronic lung disease in the newborn and is frequently seen in premature infants with very low birth weight (1). Lungs of infants who died of BPD are less vascularized, with fewer and larger alveoli lined with type II epithelial cells. Fortunately, antenatal steroid administrations to mothers in preterm labor, and the application of exogenous surfactant and mild ventilation strategies to premature babies, have markedly reduced their mortality. Unfortunately, survivors continue to show de-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Despite improved therapies for treating bronchopulmonary dysplasia (BPD), it is unknown why survivors are at increased risk for symptomatic respiratory infections and are often rehospitalized.

What This Study Adds to the Field

This study shows that neonatal hyperoxia exacerbates the inflammatory response in adult mice infected with influenza A virus, implying that key innate immunoregulatory pathways may be disrupted in people who had BPD.

creased lung capacity at 5–10 years of age and even as young adolescents (2–4). Moreover, these children are at increased risk for asthma, infection, and other respiratory diseases, and are often rehospitalized after respiratory infection (5, 6). Thus, there is an urgent need to understand how oxidative stress permanently disrupts lung development and how this enhances susceptibility to future respiratory pathogens.

Like infants that died of BPD, chronic inflammation, alveolar dysplasia, fibrosis, and other pathologic signs of BPD are seen in newborn animals exposed to greater than 85% oxygen (7, 8). Intriguingly, some of these changes are permanent, as normal lung structure and function are not restored when newborn animals are returned to room air (9–11). Hyperactive airways with increased thickness of the underlying smooth muscle have also been reported in 21-day-old rats exposed to and recovered from greater than 95% oxygen (12, 13). We reported that exposure of newborn mice to greater than 85% oxygen between Postnatal Days 1 and 4 causes permanent alveolar simplification and altered lung compliance in adult mice (14, 15). As defined by expression of cell-specific markers, adult mice exposed to hyperoxia as newborns exhibit severe depletion of alveolar epithelial type II cells and an increased number of type I cells (15). Because significant alterations in airway structure, airway resistance, or vascular cells were not observed, these mice might model those humans who had mild BPD and survived, but present reduced lung function and enhanced susceptibility to infection later in life.

Influenza A viruses are a common cause of illness in the general population, and children born prematurely are at increased risk of complications due to infection with influenza and other respiratory viruses (5, 6, 16). Influenza A viruses primarily infect cells of the respiratory tract because of the selective binding of viral coat proteins to specific terminal oligosaccharides on sialic acid residues, which in humans and mice are typically found on airway epithelial cells, alveolar type II epithelial cells, and antigen-presenting cells (17–20). It is generally considered that CD8⁺ cytotoxic T lymphocytes (CTLs) are the principal means for viral clearance during a primary

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infection, but both virus-specific antibodies and CTLs are important for host resistance on reinfection (21). Concomitant with the activation of the adaptive immune responses, leukocytes are recruited to the lung and numerous soluble mediators, such as surfactant proteins, cytokines, and chemokines, are produced by infiltrating immune cells and structural cells of the lung (22–25). Together, they help diminish viral replication, destroy infected cells, and promote tissue repair.

It is not clear why people who survive BPD are more susceptible to respiratory pathogens, in part because animal models for this disease have not been fully developed. Our finding that short-term neonatal hyperoxia causes permanent alveolar simplification in adult mice, much like the changes that are thought to occur in humans who had BPD, provides an opportunity to test whether adult mice exposed to hyperoxia as newborns were more susceptible to influenza A viral infection than adult mice exposed to room air at birth. We also examined whether neonatal exposure to hyperoxia causes long-lasting changes in the adaptive or innate immune response to infection and whether it altered alveolar remodeling after infection.

METHODS

See the online supplement for additional details on reagents and methods.

Exposure of Mice to Hyperoxia and Influenza A Virus

Newborn C57BL/6J mice were exposed to room air or 100% oxygen (hyperoxia) between Postnatal Days 1 and 4 (15). Oxygen-exposed pups were returned to room air on the morning of Postnatal Day 4 and allowed to grow to term. Adult (age, 8–9 wk) female mice that had been exposed to room air or hyperoxia at birth were infected intranasally with 120 hemagglutinating units of influenza A virus, strain HKx31 (x31; H3N2). All mice were housed in microisolator cages in a specified pathogen-free facility with the approval of the University Committee on Animal Resources (Johns Hopkins School of Medicine, Baltimore, MD).

Lung Histology

Paraffin-embedded sections of lungs fixed in 10% neutral buffered formalin were immunostained with mouse anti-human α -smooth muscle actin antibody followed by Texas red-conjugated secondary antibodies before counterstaining with 4',6-diamidino-2-phenylindole. Stained sections were visualized with a Nikon E800 fluorescence microscope (Nikon Instruments, Melville, NY). Images were captured with a SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI). The mean linear intercept was quantified on lung sections stained with hematoxylin and eosin as previously described (14).

Collection and Preparation of Cells

Leukocytes were obtained from lung airways by bronchoalveolar lavage with cold RPMI 1640 containing 1% bovine serum albumin and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid. Bronchoalveolar lavage fluid was centrifuged and the supernatant was stored at -80°C for quantifying levels of cytokine and chemokines in a fluorescent microsphere-based multiplex assay (Millipore, Billerica, MA). Erythrocytes were removed by treatment with ammonium chloride, and cells were enumerated with a Coulter Counter (Beckman Coulter, Fullerton, CA). Cells were centrifuged onto coated microscope slides, stained with hematoxylin and eosin, and identified by two independent investigators. For some experiments, mediastinal lymph node cells were prepared under aseptic conditions by pressing mediastinal lymph node cells from a single animal between the frosted ends of two sterile microscope slides. Cellular debris was removed by sedimentation, and cells were enumerated with a hemacytometer or a Coulter Counter.

Immunophenotypic Analyses

Cells were stained with previously determined optimal concentrations of fluorochrome-conjugated antibodies against the following cell surface antigens: NK1.1, CD4, or CD8 α , CD44, or CD62L. Data were

collected from 50,000 to 100,000 cells with a BD FACSCalibur (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Influenza-specific Antibody Measurements

The levels of influenza virus-specific antibodies IgG2a and IgA were analyzed by stacking enzyme-linked immunosorbent assay. Ninety-six-well plates were coated with 5- $\mu\text{g}/\text{ml}$ sucrose gradient-purified H3N2 X31 (Charles River/SPAFAS, North River, CT). Plasma was prepared in a series of fourfold dilutions and added to the plate. Plasma from naive mice was used as a negative control. For each isotype, a dilution of plasma that was in the linear range of absorbance was selected, and samples from room air- and hyperoxia-treated animals were compared at the same dilution.

Statistical Analysis

Values are expressed as means $\pm$ the standard error unless otherwise noted. Group means were compared by analysis of variance, followed by a Fisher least significant difference post hoc test, to compare the mean values from each treatment group at a specific point in time, or with an unpaired *t* test for paired analyses, using StatView software (SAS Institute, Cary, NC). Mouse mortality was evaluated by Kaplan-Meier test and analyzed for significance by Mantel-Cox test, using StatView software. All data were considered significant when $P < 0.05$.

RESULTS

Neonatal Oxygen Induces Alveolar Simplification in Adult Mice

Newborn mice were exposed to room air or 100% oxygen between Postnatal Days 1 and 4. Mice were then returned to room air until 8 weeks of age, at which time they were killed and their lungs were inflation fixed. Consistent with previous work showing that neonatal hyperoxia increases alveolar compliance (14, 15), alveolar simplification was clearly evident in adult mice that had been exposed to hyperoxia as neonates (Figures 1A and 1B). Quantitation of the mean linear intercept, a measurement of alveolar size, confirmed that alveolar size was significantly greater in adult mice that had been exposed to hyperoxia as neonates (Figure 1C).

Neonatal Hyperoxia Increases the Severity of Influenza A Virus Infection in Adult Mice

Adult mice exposed to room air or hyperoxia as neonates were infected with influenza A virus. Before infection (Day 0), the body weight of adult mice exposed to room air at birth was not different from that of siblings that had been exposed to hyperoxia as neonates (Figure 2A). Weights of infected mice exposed to room air at birth declined during the first 8 days of infection, returned to preinfection (naive) levels by Day 16, and then continued to increase. In contrast, weight loss was significantly greater in infected mice that had been exposed to hyperoxia as neonates. Although animal weight returned to preinfection levels by Day 20, it continued to lag behind that of infected mice that had been exposed to room air at birth. This suggests mice exposed to hyperoxia as neonates were having trouble recovering from influenza virus infection. Indeed, neonatal hyperoxia significantly reduced survival of infected adult mice (Figure 2B). By 28 days postinfection, 15 of 18 infected control mice versus only 9 of 21 oxygen-recovered and infected mice had survived. Thus, neonatal hyperoxia increased morbidity and mortality of adult mice infected with influenza A virus.

The Adaptive Immune Response to Infection with Influenza Virus Is Not Suppressed by Neonatal Hyperoxia

Although there was greater infection-induced mortality in the hyperoxia-exposed mice, we did not observe a corresponding

Figure 1. Neonatal hyperoxia promotes alveolar simplification in adult mice. (A and B) Representative images of adult mouse lung exposed to room air (A) at birth versus adult mouse lung exposed to hyperoxia (B) at birth. (C) The mean linear intercept of

newborn adult lung exposed to hyperoxia (O₂) is significantly greater than that of adult lung exposed to room air (RA) ($P < 0.02$). Each circle represents the mean linear intercept from a single lung and each horizontal line represents the group mean ($n = 4$).

impairment in the differentiation and expansion of T cells in the draining lymph nodes. Specifically, infection-associated expansion of CD8⁺ and CD4⁺ T cells and acquisition of an effector phenotype (i.e., up-regulation of CD44 and down-regulation of CD62L) were not suppressed in adult mice that had been exposed to hyperoxia at birth (data not shown). Similarly, levels of virus-specific antibodies in the blood were equivalent in infected mice, regardless of early life exposure to high oxygen (Figure 3).

Neonatal Hyperoxia Increases Leukocyte Recruitment to Lungs of Infected Adult Mice

The number and phenotype of leukocytes in airways were examined before and 1, 3, 5, and 9 days after infection. The total number of leukocytes was low and not different between naive mice that had been exposed to room air or hyperoxia as neonates (Figure 4A). Although the number of leukocytes had significantly increased by 3 days postinfection in both groups, they persisted and were significantly higher on Days 5 and 9 postinfection in the mice that had been exposed to hyperoxia at birth. The larger increase in leukocytes was principally due to increased numbers of macrophages and neutrophils at these times (Figures 4B and 4C, respectively). Although an increase in lymphocytes was observed earlier in infected mice that had been exposed to neonatal hyperoxia, there was no statistically

significant difference between infected mice exposed to room air versus hyperoxia at any time point (Figure 4D). Thus, influenza virus-infected adult mice recovered from neonatal hyperoxia exhibit elevated and persistent recruitment of macrophages and neutrophils compared with infected siblings that had been exposed to room air at birth.

Because differential cell counts cannot distinguish among lymphocyte subpopulations, we used flow cytometry to further examine the type of lymphocyte recruited to the lung during infection. On Day 9 of infection, mice exposed to hyperoxia as neonates had significantly more NK1.1⁺ cells than did room air-exposed littermates (Figure 5A). However, there was no significant difference in the number of CD4⁺ and CD8⁺ cells (Figures 5B and 5C, respectively). Thus, neonatal hyperoxia significantly enhances recruitment of macrophages, neutrophils, and lymphocytes during influenza virus infection. Although most of the increase in lymphocytes is represented by NK1.1⁺ cells, there appears to be a general increase in all subsets, suggesting that there may be a quantitative rather than a qualitative shift in the response to infection.

Neonatal Hyperoxia Augments Pulmonary Monocyte Chemotactic Protein-1 (CCL2) Levels in Infected Adult Mice

We next determined the effects of neonatal hyperoxia on the kinetics and levels of IFN- γ , KC, monocyte chemotactic protein (MCP)-1 (CCL2), IL-6, IL-1 β , granulocyte-macrophage colony-stimulating factor, macrophage inflammatory protein (MIP)-1 α ,

Figure 2. Neonatal hyperoxia increases the severity of influenza A virus infection in adult mice. Adult female mice exposed to room air (RA) or hyperoxia (O₂) at birth were infected intranasally with influenza A virus. (A) Mice were weighed before infection (Day 0) and every 2 days postinfection over the next 28 days. Mean average weight of infected adult mice exposed to hyperoxia at birth was significantly less than that of infected mice exposed to room air at birth (* $P < 0.05$ and † $P < 0.10$). (B) Survival of infected mice exposed to hyperoxia at birth (21 mice on Day 0)

was significantly lower than that of infected mice exposed to room air at birth (18 mice on Day 0) ($P < 0.01$).

Figure 3. Neonatal hyperoxia does not alter virus-specific antibody levels. Adult female mice exposed to room air (RA) or hyperoxia (O₂) at birth were infected intranasally with influenza A virus. Virus-specific IgA (A) and IgG2a (B) antibodies were determined by isotype-specific ELISA, using serial dilutions of serum collected on Day 9 postinfection. Graphs depict the mean absorbance (optical density) reading ($\pm$ SEM) for each dilution of plasma. The absorbance values of plasma samples taken from uninfected mice were typically less than or equal to 0.1 (data not shown).

Figure 4. Neonatal hyperoxia increases leukocyte recruitment to lungs of adult mice infected with influenza virus. Adult female mice exposed to room air (RA) or hyperoxia (O_2) at birth were infected intranasally with influenza A virus. Total numbers of leukocytes, macrophages, neutrophils, and lymphocytes obtained in bronchoalveolar lavage (BAL) fluid on the indicated day after infection were quantified and graphed. Columns labeled D0 (Day 0) represent BAL cells obtained from uninfected adult mice. (A) The total number of leukocytes in infected mice significantly increased on Day 3 of infection ($^bP < 0.0005$). Although the number of leukocytes declined by Days 5 and 9, it remained significantly elevated in infected mice that had been exposed to hyperoxia as neonates ($^bP < 0.05$). (B) The total number of macrophages slightly declined on Day 1 of infection before significantly increasing on Day 5 ($^bP < 0.04$) and then on Day 9 ($^cP < 0.003$). Although similar changes were observed in infected mice that had been exposed to neonatal hyperoxia, the total number of macrophages was significantly augmented on Day 5 ($^dP < 0.04$) and Day 9 ($^dP < 0.03$). (C) The total number of neutrophils significantly increased on Day 3 ($^bP < 0.002$) and

declined to naive levels by Day 5. Although similar changes were seen in infected mice that had been exposed to neonatal hyperoxia, the total number of leukocytes remained significantly greater on Day 5 ($^bP < 0.005$) and Day 9 ($^cP < 0.05$). (D) The total number of lymphocytes significantly increased on Day 9 ($^bP < 0.002$) of infection. Although similar changes were seen in infected mice that had been exposed to neonatal hyperoxia, the number of lymphocytes was significantly increased on Day 5 ($^bP < 0.01$) and Day 9 ($^bP < 0.001$) of infection. Values were obtained from five mice per group at each point in time. Columns with the same letters are not significantly different from each other; columns with different letters are significantly different from each other.

and tumor necrosis factor- α . These cytokines and chemokines were chosen because they stimulate leukocyte recruitment and are known to be induced during influenza A virus infection (22, 26, 27). In room air-exposed mice, MCP-1 levels increased within 24 hours of infection and became significantly elevated on Day 3 before declining to naive levels by Day 9 (Figure 6A). MCP-1 levels were not different in naive mice that had been exposed to room air or hyperoxia as neonates. However, the infection-associated increase in MCP-1 in mice exposed neonatally to hyperoxia was considerably greater than observed in room air-exposed siblings, with twofold more MCP-1 detected on Days 5 and 9 of infection. Levels of MIP-1 α , tumor necrosis factor- α , and IL-6 also increased during infection, but were generally similar between mice that had been exposed to room air or hyperoxia as neonates (Figures 6B–6D, respectively). A close analysis of the data revealed that MIP-1 α was significantly elevated in oxygen-recovered mice 9 days postinfection, but these levels were quite low. Likewise, neonatal hyperoxia did not alter infection-induced increases in IL-1 β , KC, or granulocyte-macrophage

colony-stimulating factor (data not shown). Thus, neonatal hyperoxia does not appear to globally deregulate cytokine/chemokine levels in the infected lung. Instead, these data suggest a selective mechanism in which a subset of infection-associated cytokines, such as MCP-1, is altered.

Neonatal Hyperoxia Promotes Fibrosis in Infected Adult Mice

Although MCP-1 is chemotactic for monocytes and macrophages, studies have led to an appreciation that it also promotes fibrosis in response to bleomycin (28, 29) and affects the inflammatory response to influenza A virus infection (26). To determine whether elevated levels of MCP-1 were associated with fibrosis, lung histology was investigated during infection. Despite increased inflammation and cell injury on Postinfection Days 3, 5, and 9 (data not shown), infected mice that had been exposed to room air as neonates appeared remarkably normal, with minimal inflammation or interstitial thickening by Day 14 (Figures 7A and 7C). In contrast, persistent inflammation and fibrotic scarring was observed in infected mice that had

Figure 5. Neonatal hyperoxia enhances recruitment of NK1.1 $^+$ cells to airways. Adult female mice exposed to room air (RA) or hyperoxia (O_2) at birth were infected intranasally with influenza A virus. Lungs were lavaged on Day 9 postinfection and the proportion of NK1.1 $^+$ (A), CD4 $^+$ (B), and CD8 $^+$ (C) cells was determined by flow cytometry. Neonatal hyperoxia significantly increased the number of NK1.1 $^+$ BAL cells ($^*P < 0.03$). Values were obtained from five mice per group.

Figure 6. Neonatal hyperoxia selectively enhances levels of proinflammatory cytokines and chemokines in bronchoalveolar lavage (BAL) fluid of influenza-infected mice. Adult female mice exposed to room air (RA) or hyperoxia (O₂) at birth were infected with influenza virus and BAL fluid was obtained at the indicated times relative to infection. Protein levels of (A) monocyte chemoattractant protein (MCP)-1, (B) macrophage inflammatory protein (MIP)-1α, (C) tumor necrosis factor (TNF)-α, and (D) IL-6 in BAL fluid were analyzed in a fluorescent microsphere-based multiplex assay. The limit of detection was typically 8 pg/ml or more. Columns labeled D0 (Day 0) represent BAL fluid obtained from uninfected adult mice. Error bars indicate the SEM. (A) Levels of MCP-1 significantly increased on Day 3 of infection ($^{b}P < 0.03$). Although they declined to naive levels by Day 5, levels of MCP-1 remained significantly elevated on Day 5 ($^{b}P < 0.04$) and Day 9 ($^{d}P < 0.01$) in infected mice exposed to neonatal hyperoxia. (B) Levels of MIP-1α significantly increased on Day 1 of infection before declining to naive levels by Day 3 ($^{b}P < 0.001$). Although similar trends were seen in infected mice exposed to neonatal hyperoxia,

levels of MIP-1α were significantly elevated on Day 9 ($^{c}P < 0.001$). (C) Levels of TNF-α significantly increased on Day 1 and returned to naive levels by Day 3 ($^{b}P < 0.0001$). (D) Levels of IL-6 significantly increased on Day 1 and returned to naive levels by Day 3 ($^{b}P < 0.0001$). Values were obtained from five mice per group at each point in time. Columns with the same letters are not significantly different from each other; columns with different letters are significantly different from each other.

been exposed to hyperoxia as neonates (Figures 7B and 7D). Consistent with these regions containing active myofibroblasts, intense α -smooth muscle actin staining was observed in infected mice that had been exposed to neonatal hyperoxia. To assess whether these fibrotic regions contained active myofibroblasts, lungs were immunostained for α -smooth muscle actin. In infected mice that had been exposed to room air at birth, α -smooth muscle actin staining was detected underlying airway epithelium and surrounding large vessels, a pattern typically seen in naive mice (Figures 8A and 8B). In contrast, intense α -smooth muscle actin staining was observed in thickened fibrotic regions of infected mice that had been exposed to hyperoxia as neonates. Western blotting of whole lung homogenates confirmed increased α -smooth muscle actin expression in these mice (Figures 8C and 8D). Although α -smooth muscle actin levels were fairly uniform in infected mice exposed to room air at birth, it varied in infected mice that had been exposed to hyperoxia as neonates. These differences were consistent with the variable amount of fibrosis and associated mortality seen between room air- and hyperoxia-exposed mice at this time. To determine whether fibrosis resolves, tissue histology and expression of α -smooth muscle actin were investigated in mice that had survived 28 days after infection. Although some variability was seen between animals, persistent inflammation and fibrosis was clearly evident in all infected mice that had been exposed to hyperoxia at birth and had survived for 28 days. These pathologic findings were never observed in infected siblings that had been exposed to room air at birth. These observations suggest neonatal hyperoxia also disrupts alveolar remodeling after infection.

DISCUSSION

Epidemiologic studies indicate children who had been exposed to elevated oxygen at birth for treatment of BPD are more

likely to have viral infections, asthma, increased sensitivity to second-hand cigarette smoke, and more out-of-school sick days than children who were not exposed to oxygen (5, 6, 30). Whereas it is well known that hyperoxia permanently disrupts postnatal lung development, little is known about how that might affect the response to respiratory pathogens. Here, we provide evidence that short-term hyperoxia during postnatal lung development significantly increases the sensitivity of adult mice to influenza A virus infection. Unlike infected siblings that developed disease and recovered, oxygen-exposed and infected mice showed enhanced recruitment of macrophages, neutrophils, and NK1.1⁺ lymphocytes; and increased MCP-1 levels, alveolar fibrosis, and mortality. This implies that neonatal hyperoxia disturbs key innate immunoregulatory pathways in lung, which may contribute to the increased susceptibility to respiratory viral infections typically seen in people who had BPD.

Influenza A viruses are a common cause of illness in humans and are a significant health threat for children who were born prematurely (6). Influenza A virus primarily infects airway epithelial and antigen-presenting cells by binding to cell surface terminal oligosaccharides on sialic acid residues. Studies have shown that type II cells also express influenza virus receptors (17–20). In fact, avian influenza H5N1 virus has also been detected in type II cells of humans who died during infection (31). The adaptive immune system becomes activated during infection and responds by creating virus-specific CD8⁺ CTLs and antibodies that help clear virus and provide protection against subsequent infection (21). Because neither the expansion and differentiation of CD8⁺ cells nor virus-specific antibody titers were suppressed by neonatal hyperoxia, the decreased survival observed among these mice is probably not attributable to their inability to mobilize an adaptive immune response or to clear the virus.

Concomitant with the activation of the adaptive immune responses, inflammatory cells are recruited to the lung and nu-

Figure 7. Persistent inflammation and fibrosis in infected adult mice that had been exposed to hyperoxia as newborns. Adult mice that had been exposed to room air (A and C) or hyperoxia (B and D) as neonates were infected with influenza A virus. Lungs were harvested on Day 14 postinfection and stained with hematoxylin and eosin. Scale bars: 100 μ m.

merous soluble mediators of host defense are produced, including surfactant proteins, cytokines, and chemokines (22–25). The expression of surfactant protein (SP)-A and SP-D is one mechanism by which pulmonary epithelial cells protect against influenza virus infection. Airway and alveolar type II cells express SP-A whereas basal cells of the trachea and alveolar type II cells express SP-D (32, 33). SP-A and SP-D are members of a larger family of proteins (called collectins) that includes the acute-phase reactant of serum (called mannose-binding protein) (34). Collectins form larger oligomeric structures via collagen-like domains. Like mannose-binding protein, the pulmonary collectins SP-A and SP-D can bind and aggregate pathogens (bacteria, fungi, and viruses), thereby enhancing phagocytosis and killing by immune cells. Consistent with their ability to opsonize pathogens, clearance of bacteria, fungi, and viruses is defective in *SP-A*-deficient mice, and is associated with enhanced inflammation (25, 35, 36). *SP-D*-deficient mice also show

defects in fungal and viral clearance, but they still have some capacity to clear bacteria (24, 37). Thus, a reduction in SP-A and SP-D caused by the loss of type II cells in adult mice exposed to hyperoxia could affect viral clearance, and therefore be partially responsible for increasing sensitivity to influenza A virus infection.

On the other hand, adult mice exposed to hyperoxia as newborns express more T1 α , suggesting that they have more type I cells (15). Although type I cells comprise 95% of the alveolar surface and are often thought to be inactive bystanders, they may in fact be quite active because they express caveolin-1 and contain numerous calveolae. Calveolae are small, flask-shaped vesicles enriched in cholesterol, glycosphingolipids, and ceramide that function to transport receptors, heterotrimeric G proteins, and enzymes within the cell. As such, type I cells might actively respond to and be high producers of cytokines and other inflammatory signaling molecules. Consistent with this concept, type I

Figure 8. Expression of α -smooth muscle actin (α -SMA) is increased in infected adult mice that had been exposed to hyperoxia as newborns. Lungs prepared from postinfection Day 14 mice exposed to room air (A) or hyperoxia (B) as neonates were stained with antibodies against α -SMA (red) and counterstained with 4',6-diamidino-2-phenylindole (blue). (C) Lung homogenates prepared from mice 14 days after infection were immunoblotted for α -SMA and β -actin. (D) Band intensity of α -SMA was quantified, normalized to β -actin, and graphed. Values represent means $\pm$ SEM ($n = 3$; $*P < 0.05$).

cells express intercellular adhesion molecule (ICAM)-1 (38, 39). Whereas ICAM-1 on endothelial cells serves as a ligand for trafficking leukocytes, its expression by type I cells is believed to promote alveolar macrophage activation and motility (40). Thus, increased numbers of type I cells may also be partially responsible for increased inflammation seen in infected mice that had been exposed to hyperoxia.

The current study also found that MCP-1 was selectively elevated in lavage fluid of infected mice that had been exposed to hyperoxia as newborns. Studies have led to the appreciation that MCP-1 plays an important role in lung inflammation and fibrosis. MCP-1 expressed by epithelial and endothelial cells, fibroblasts, and macrophages is the major ligand for the C-C chemokine receptor-2 (CCR2) found on leukocytes. As shown by overexpressing MCP-1 in transgenic mice, MCP-1 recruits monocytes and natural killer cells to the lung and protects against bacterial or viral infection (26, 41–43). Although protective, overexpression of MCP-1 also causes bronchiolitis obliterans on pneumococcal challenge. MCP-1 may also promote the fibrotic phenotype. For instance, mice lacking CCR2 or overexpressing mutant MCP-1 are protected against bleomycin-induced lung fibrosis (28, 29). Intriguingly, elevated levels of MCP-1 have been detected in lavage fluid obtained from children with interstitial lung disease (44). Thus, the elevated expression of MCP-1 may be responsible for the enhanced inflammatory response, fibrosis, and mortality seen in infected mice exposed to hyperoxia.

In addition to MCP-1, neonatal hyperoxia likely adversely affects other signaling pathways involved in protection against infection. For instance, newborn mice exposed to cigarette smoke for 2 weeks show attenuation of type I and II IFN pathway genes, increased oxidative stress and transforming growth factor- β signaling, and mild alveolar simplification (45). These mice also showed reduced expression of Toll-like receptor (TLR)-3, retinoic acid-inducible gene (RIG)-1, melanoma differentiation-associated gene (MDA)-5, and myxovirus resistance-1 (MX1), which are genes that respond to double-stranded RNA. Hypothetically, such mice would respond more poorly to infection, much like the mice exposed to neonatal hyperoxia. Indeed, ozone, particulates, and house dust mites can also disrupt postnatal lung development and have been associated with increasing the incidence of asthma (46, 47). Because these pollutants target different regions of the lung, they are likely to cause different types of defects in lung development, and hence may selectively alter how the lung responds to various respiratory pathogens. Importantly, it is unlikely that neonatal hyperoxia affects only how the adult lung responds to influenza A virus infection. In fact, these mice also develop fibrosis when exposed to and allowed to recover from 100% oxygen (data not shown).

In summary, the current findings show for the first time that neonatal hyperoxia adversely impacts how adult mice respond to influenza A virus infection. Our results suggest that critical aspects of neonatal lung development are affected by exposure to high oxygen, leading to long-lasting changes in the innate response to respiratory viral infection. Understanding how hyperoxia reprograms key innate signaling and cellular pathways involved in host protection and remodeling of the injured lung could provide new opportunities for treating people who had BPD.

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

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