Memory CD8⁺ T Cells Are Sufficient To Alleviate Impaired Host Resistance to Influenza A Virus Infection Caused by Neonatal Oxygen Supplementation

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Supplemental oxygen administered to preterm infants is an important clinical intervention, but it is associated with life-long changes in lung development and increased sensitivity to respiratory viral infections. The precise immunological changes caused by neonatal oxygen treatment remain poorly understood. We previously reported that adult mice exposed to supplemental oxygen as neonates display persistent pulmonary inflammation and enhanced mortality after a sublethal influenza A virus infection. These changes suggest that neonatal hyperoxia impairs the cytotoxic CD8⁺ T cell response required to clear the virus. In this study, we show that although host resistance to several different strains of influenza A virus is reduced by neonatal hyperoxia, this treatment does not impair viral clearance, nor does it alter the magnitude of the virus-specific CD8⁺ T cell response to primary infection. Moreover, memory T cells are sufficient to ameliorate the increased morbidity and mortality and alleviate the excessive lung damage observed in mice exposed to high oxygen levels as neonates, and we attribute this sufficiency principally to virus-specific memory CD8⁺ T cells. Thus, we show that neonatal hyperoxia reduces host resistance to influenza virus infection without diminishing the function of cytotoxic T lymphocytes or the generation of virus-specific memory T cells and that CD8⁺ memory T cells are sufficient to provide protection from negative consequences of this important life-saving intervention. Our findings suggest that vaccines that generate robust T cell memory may be efficacious at reducing the increased sensitivity to respiratory viral infections in people born prematurely.

Premature birth, as well as postnatal disorders associated with it, is the second leading cause of infant mortality in the United States (15). Premature infants often develop respiratory distress because their lungs are structurally immature, with reduced surfactant production and limited capacity for oxygen exchange. Treatment often involves prolonged early-life exposure to high concentrations of oxygen that can lead to bronchopulmonary dysplasia (BPD), a chronic form of lung disease often seen in preterm infants with very low birth weights (13, 18). Infants who die from complications attributed to BPD have simplified lungs with reduced vasculature (7). Although infant mortality has been diminished by the use of antenatal steroids, surfactant replacement, and milder ventilation strategies, long-term changes in lung function continue to be observed in children born prematurely (2, 10, 12). These children are also more likely to be rehospitalized following a respiratory viral infection and have an increased risk for asthma (14, 35, 42). The underlying cause of this increased disease susceptibility is not known, but early-life exposure to high oxygen levels is quite likely a contributor. Indeed, extensive studies in a variety of animal models demonstrate that exposure to high oxygen levels at birth permanently alters lung development, even in animals that recover in room air for many weeks after this early-life exposure (7, 9, 31, 46, 47). Neonatal hyperoxia has also been shown to increase the sensitivity of adult mice to a sublethal dose of influenza A virus infection, as defined by poorer survival, exacerbated weight loss, alterations in the number of leukocytes recruited to the lung, and parenchymal fibrosis (31). These changes suggest that neonatal hyperoxia may have disrupted the host response needed to clear the virus.

Respiratory infection by influenza virus triggers an integrated network of host responses that usually lead to the successful elimination of the virus 8 to 10 days later. Initially, this involves innate immune mediators, which strive to control viral replication until the adaptive response is fully engaged. Adaptive immune responses to influenza virus include the activation and differentiation of CD8⁺ T cells, CD4⁺ T cells, and B cells. The roles of CD4⁺ T cells during influenza virus infection are primarily to provide a helper function, to instruct isotype switching in B cells, and to regulate the generation of immunological memory (21, 23). Virus-specific antibodies provide critical defenses from repeated infections with homotypic virus strains, that is, viral subtypes that share homologous hemagglutinin and neuraminidase coat proteins (22). However, it is the creation of virus-specific CD8⁺ cytotoxic T lymphocytes (CTL) in lymphoid tissues, which traffic to the lung and kill infected cells, that is the principal means for viral clearance and survival during primary influenza virus infection (3, 21, 43). Virus-specific CD8⁺ T cells also play a role in host protective memory in repeated influenza virus infections. Although vaccines against influenza viruses are readily available, they can fail to protect due to frequent changes in the antigenic determinants of the virus coat proteins, which drift or shift from season to season. In the absence of vaccine-induced neutralizing antibodies,
CD8⁺ CTL are believed to contribute to cross-protective immunity in vaccinated individuals (38).

Given the importance of antiviral CD8⁺ T cells to host resistance and the lack of any published experimental or clinical data investigating the potential for early-life oxygen supplementation to alter antiviral CD8⁺ T cell function, the present study investigated whether neonatal oxygen supplementation suppresses the virus-specific CD8⁺ T cell response in adult mice during primary influenza virus infection, resulting in enhanced mortality and impaired viral clearance. Due to the recurrent nature of influenza virus infections, we also examined the effects of neonatal hyperoxia on secondary influenza virus infection and determined whether the presence of virus-specific memory CD8⁺ T cells alters survival and postinfection lung inflammation. Knowing whether neonatal oxygen supplementation disrupts the normal function of CD8⁺ T cells is necessary for defining therapies to improve the long-term health and well-being of those born prematurely.

**MATERIALS AND METHODS**

**Exposure of mice to oxygen and viruses.** C57BL/6 and B6.SJL-Ptprcra Pepeb/BoyJ (CD45.1 transgenic) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Newborn C57BL/6 pups were exposed to room air or 100% oxygen (hyperoxia) within 12 h of birth until postnatal day 4, when the oxygen-exposed pups were returned to room air (31). Dams were rotated between litters exposed to room air and 100% oxygen every 24 h to minimize oxygen toxicity to the mothers. Humidity within the chamber was maintained between 30 and 70% during the exposure period. Adult female mice (7 to 9 weeks of age) were infected intranasally (i.n.) with influenza A virus strain HKx31 (H3N2), A/CA/04/09 (H1N1), or A/P8/83 (PR8; H1N1). For experiments investigating the memory response, adult mice that were exposed to room air or 100% oxygen as pups were challenged intraperitoneally (i.p.) with 200 hemagglutinating units (HAU) of HKx31. Sixty days later (at 15 to 19 weeks of age), HKx31–primed mice were infected (i.n.) with 0.005 HAU of PR8. To obtain high-mortality infections, we used 0.5 HAU of PR8. Mice were housed in microisolator cages within a specific-pathogen-free facility. In survival experiments, animals were monitored daily and were euthanized humanely when they were immobile and moribund. All experiments were conducted in accordance with protocols approved by the University of Rochester institutional animal care and use and institutional biosafety committees.

**Flow cytometry and cell sorting.** Isolated cells were stained in a phosphate-buffered saline (PBS) solution containing 1% bovine serum albumin (BSA) and 0.01% sodium azide. The following antibodies (clones) were used: CD3 (17A2), CD8 (53-6.7), gamma interferon (IFN-γ) (XMG1.2), CD45.1 (A20), CD45.2 (104), and CD44 (IM7) antibodies. Anti-CD16/32 (93) and rat IgG were used to block nonspecific binding. Isolated cells were stained in a phosphate-buffered saline (PBS) solution containing 1% bovine serum albumin (BSA) and 0.01% sodium azide. The following antibodies (clones) were used: CD3 (17A2), CD8 (53-6.7), gamma interferon (IFN-γ) (XMG1.2), CD45.1 (A20), CD45.2 (104), and CD44 (IM7) antibodies. Anti-CD16/32 (93) and rat IgG were used to block nonspecific binding. Antibodies were obtained from ebioscience. Major histocompatibility complex (MHC) class I tetramers for influenza A virus nucleoprotein (NP) and acid polymerase (PA) peptides were identified to CD8⁺ T cells specific for immunodominant viral epitopes (25). Live/Dead (Invitrogen) and 7-aminoactinomycin D (7-AAD; BD Biosciences) stains were used according to the manufacturers’ instructions and provided a means to exclude dead cells during analysis. To identify IFN-γ-producing cells, lung-derived immune cells were cocultured at a 2:1 ratio with DC2.4 (NP366–374 [ASNENMETM] and PA224–233 [SSLENFRAYV]) and 5 µg/ml brefeldin A. After staining for cell surface antigens, the cells were fixed with 2% formalin, permeabilized with 1% saponin, and incubated with an allophycocyanin (APC)-labeled anti-IFN-γ monoclonal antibody (MAb; ebioscience). Flow cytometry was performed on an LSR II flow cytometer, and cell sorting was performed using a FACS Aria instrument (BD Biosciences). Data were analyzed using FlowJo software (Treestar).

**Quantification of viral titers in the lung.** One lobe of frozen lung tissue was homogenized in 1 ml of PBS and added at 10-fold dilutions (1:10⁴ to 1:10⁹) to confluent monolayers of Madin-Darby canine kidney (MDCK) cells. Cells were cultured overnight, fixed, and stained with 1 µg/ml biotinylated anti-influenza virus NP antibody (Millipore). Free antibody was removed by washing, and bound antibodies were detected with streptavidin–alkaline phosphatase conjugates and 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium tablets (BCIP/NBT; Sigma Chemical Co.). Positively stained cells were identified and counted, and the number of viral focal units (VFU) per ml of lung homogenate was calculated.

**Cell isolation and adoptive transfer.** Leukocytes from the airways were collected by bronchoalveolar lavage (BAL) with cold RPMI 1640 containing 1% BSA as previously described (41). Leukocytes from the entire lung were obtained by digesting intact (i.e., not subjected to lavage) lungs with collagenase (RPMI 1640 medium containing 0.7 mg/ml collagenase A [Worthington Biochemical Corp.], 30 µg/ml DNase I, 2.5% fetal bovine serum [FBS], and 10 mM HEPES) (29). Prior to instillation of the collagenase solution via the trachea, circulating leukocytes were removed from the pulmonary vasculature by perfusing 5 ml of PBS containing 2 mM EDTA via the right ventricle. Collagenase-infused lungs were removed, mechanically minced, and incubated at 37°C and 5% CO₂ with gentle shaking for 25 min. Individual cells were collected by clarifying the disrupted tissue through 70-µm nylon filters, centrifuging the cells, and lysing erythrocytes with ammonium chloride. Splenocytes were obtained by mechanical disruption of the capsule followed by filtering through nylon mesh and lysing of erythrocytes. For adoptive transfer experiments, spleen cells were stained with fluorescein-conjugated antibodies, and cells with a CD8⁺ CD44hi phenotype were collected by cell sorting (FACS Aria cell sorters). A sample of the sorted cells was further stained with MHC class I tetramers specific for influenza virus NP366–374. Peptide to determine the frequency of this subset prior to adoptive transfer. Sorted cells were then concentrated to deliver 12,000 to 13,000 virus NP366–374-specific CD8⁺ T cells to each recipient (650,000 to 750,000 total CD8⁺ CD44hi cells), and cells were injected via the tail vein in a 200-µl volume.

**In vitro cytotoxicity assay.** The cytotoxicity assay was performed similarly to that described previously (20). Cells isolated by bronchoalveolar lavage were incubated at effector/target ratios of 8:1, 4:1, 2:1, and 1:1 with congenic (CD45.1⁺) splenocytes as target cells. Target cells consisted of a 50-50 mix of specific targets (pulsed with 1 µM influenza virus NP366–374 and PA224–233 peptides) and nonspecific targets (not pulsed with peptides). Specific targets were labeled with 4 µM carboxyfluorescein succinimidyl ester (CFSE), while unpulsed targets were labeled with 0.2 µM CFSE. Effector cells were incubated with CFSE-labeled target cells overnight and subsequently stained with 7-AAD and analyzed by flow cytometry. Specific lysis was determined by comparing the ratio of “live” (7-AAD negative) specific targets (CFSEhi) to nonspecific targets (CFSElo) by using the following formula: specific lysis = [1 – (ratio of CFSEhi to CFSElo) for live targets only/ratio of live targets in the presence of BAL fluid cells)] × 100.

**Lung histology.** Lungs were inflation fixed with 10% neutral buffered formalin (31). Fixed lungs were dehydrated in graded alcohol and embedded in paraffin, and 4-µm sections were prepared. Sections were stained with Gomori’s trichrome stain (Richard-Allan Scientific, Kalamazoo, MI) and visualized with a Nikon E-800 microscope (Nikon, Melville, NY). Images were captured with a Spot-RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

**Collagen assay.** Total lung collagen was measured using a Sircol collagen assay kit (Biocolor Ltd., Belfast, Northern Ireland, United Kingdom) according to the manufacturer’s instructions. Briefly, lung homogenates were hydrolyzed in acid-pepsin at 4°C overnight. Test samples were then centrifuged at 13,000 g × 10 min, and the supernatant was collected. One microliter 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 13,000 g × 10 min, washed with an acid-salt solution, and then dissolved in 500 µl of 0.5 M sodium dodecyl sulfate (SDS). The collagen-dye complex was precipitated by centrifugation at 13,000 g × 10 min, washed with an acid-salt solution, and then dissolved in 500 µl of 0.5 M sodium dodecyl sulfate (SDS). The collagen-dye complex was precipitated by centrifugation at 13,000 g × 10 min, washed with an acid-salt solution, and then dissolved in 500 µl of 0.5 M sodium dodecyl sulfate (SDS). The collagen-dye complex was precipitated by centrifugation at 13,000 g × 10 min, washed with an acid-salt solution, and then dissolved in 500 µl of 0.5 M.
sodium hydroxide. The absorbance of each sample at 550 nm was determined, and the amount of collagen in each sample was quantified using a standard curve generated with collagen standards provided by the manufacturer.

Statistical analysis. Statistical analyses were performed using StatView (SAS) or Prism (GraphPad Software). All data are expressed as means ± standard errors of the means (SEM), unless otherwise noted. Mean values for each treatment group were compared using Student’s t test. The Mann-Whitney U test was used to analyze changes in body weight over time. Survival was evaluated by the Kaplan-Meier test and analyzed for significance by the Mantel-Cox test. Fisher’s protected least significant difference (PLSD) test was used to determine significance for Sircol collagen assay data. Mean values were considered significantly different when P values were ≤0.05.

RESULTS
Neonatal hyperoxia does not impair viral clearance or reduce CD8+ T cell responses during primary influenza A virus infection. We previously reported that adult mice exposed to 100% oxygen at birth display increased morbidity and mortality after infection with a common laboratory strain of influenza A virus (HKx31; H3N2) (31). To test whether this phenotype is limited to a single strain of virus, adult mice that were exposed to either room air or 100% oxygen for 4 days at birth were infected with the recent pandemic influenza virus A/CA/04/09 (H1N1). Adult mice exposed to 100% oxygen as neonates lost more body weight during infection than their infected siblings exposed to room air only (Fig. 1A). Overall differences in body weight loss were significant between mice exposed to room air and those exposed to oxygen as neonates. Evidence of increased weight loss in the oxygen-exposed group was clearly evident between postinfection days 7 and 21, with significant changes observed on postinfection days 10 to 13. Mice exposed to 100% oxygen as neonates were also more likely than control animals to succumb to the infection (Fig. 1B). Similar findings were observed when mice were infected with influenza virus strain A/PR/8/34 (PR8; H1N1) (Fig. 1C and D). These data demonstrate that oxygen exposure at birth confers a defect in host defense against multiple subtypes of influenza A virus.

A dysfunctional CD8+ T cell response could be responsible for this increased sensitivity to influenza virus infection, as recruitment of functional CD8+ T cells to the lung has been linked directly to survival and severity of infection (3, 43). This hypothesis was tested by evaluating several parameters of CD8+ T cell function in adult mice that had been exposed to room air or 100% oxygen as neonates and infected with HKx31 (H3N2). Using flow cytometry and fluorescently labeled MHC class I tetramers (Fig. 2A) specific for influenza virus nucleoprotein (NP366–374) and acid polymerase (PA224–233), we found similar percentages (data not shown) and total numbers of virus-specific CD8+ T cells in the lungs of mice that were exposed to room air or 100% oxygen as neonates (Fig. 2B). Likewise, there was no difference in virus-specific cytotoxic activity (Fig. 2C) or in the frequency of IFN-γ+ CD8+ T cells (Fig. 2D) in lung-derived leukocytes cultured ex vivo with virus peptide-pulsed antigen-presenting cells. Since ex vivo stimulated cells may perform differently than they would function in vivo, viral clearance was investigated as a measure of in vivo CD8+ T cell function. Lung homogenates from adult mice that were exposed to room air or 100% oxygen as neonates were...
used to determine and compare lung viral burdens over the course of infection. Although viral titters were slightly elevated in infected mice exposed to 100% oxygen as neonates, they were not significantly different from those for infected mice exposed to room air at any given time point, and the virus was effectively cleared by postinfection day 9 (Fig. 2E). Collectively, these data suggest that the generation of virus-specific CD8+ effector T cells is not impaired by exposure to 100% oxygen as a neonate.

Memory T cells protect mice exposed to room air or 100% oxygen against influenza A virus infection. The consequences of neonatal oxygen supplementation on the generation and efficacy of immunological memory have not, to the best of our knowledge, been examined previously. Given that immunologic memory develops from primary infection or another initial encounter with antigen, it provides an indirect evaluation of whether neonatal oxygen supplementation impairs CD8+ T cell function during primary infection. To bypass the increased morbidity and mortality attributed to early-life oxygen supplementation followed by infection of the respiratory tract, a systemic immune response was elicited by administrating HKx31 by i.p. injection. This provides an antigen and stimulates a nonproductive infection that generates virus-specific effector and memory T cells (5, 6, 32). Sixty days later, the mice were given an intranasal challenge with a heterotypic influenza virus strain, PR8 (Fig. 3A). HKx31 and PR8 share the same internal viral proteins but have different surface proteins (H3N2 and H1N1, respectively). Therefore, while antibodies generated following primary HKx31 challenge will not recognize or provide protection against subsequent PR8 infection, the virus-specific CD8+ T memory cells created in response to HKx31 recognize and respond to viral epitopes from PR8 (22). Analogous to the case for intranasal infection with HKx31, the frequency of virus-specific CD8+ T cells after i.p. injection of HKx31 was not altered during the acute response (data not shown). Moreover, the number of virus-specific CD8+ T cells 60 days later was the same in mice exposed to room air or 100% oxygen as neonates (Fig. 3B). When mice were infected with PR8, those that were exposed to 100% oxygen as neonates and primed with HKx31 by i.p. injection exhibited changes in body weight and survival that were similar to those of HKx31–primed mice exposed to room air at birth (Fig. 3C and D). Similar data were observed during a more lethal PR8 infection (Fig. 3E and F), suggesting that this effect is relevant across a wide range of infection severities.

Despite the fact that mice exposed to 100% oxygen as neonates are fully capable of clearing influenza virus from the lungs, many are unable to survive infection, and those that survive eventually develop pulmonary fibrosis (31). It is interesting that most of the mortality occurs during or after the time frame in which the virus is generally cleared. A possible explanation for the poorer survival of mice exposed to 100% oxygen at birth is that impaired lung repair and/or postfixation fibrosis may lead to significantly reduced lung function and subsequent death. To evaluate whether the presence of memory T cells affects this, lung histopathology was compared between mice that were infected i.n. with HKx31 and those that were primed i.p. by HKx31 injection and then infected i.n. with PR8. As previously reported (31), fibrotic plaques were readily evident by postinfection day 28 in lungs of adult mice that were exposed to 100% oxygen as neonates and infected (i.n.) with HKx31 (Fig. 4A). Lungs of infected mice that had received high neonatal oxygen levels also stained extensively positive for smooth muscle α-actin, a marker of myofibroblasts, which was not observed in infected siblings that had been exposed to room air as neonates (data not shown). Interestingly, these fibrotic lesions were not readily observed when mice were primed by i.p. injection with HKx31 and then infected i.n. with a lethal dose of PR8 (Fig. 4B). Quantitative lung collagen measurements confirmed that priming of mice exposed to 100% oxygen as neonates with HKx31 i.p. resulted in a lack of collagen accumulation at 28 days postinfection (Fig. 4C). These observations suggest that the presence of memory T cells and the lack of postinfection fibrosis correlate with improved survival of mice exposed to 100% oxygen at birth.

Adaptively transferred influenza A virus-specific memory CD8+ T cells reduce oxygen-mediated sensitivity to influenza A virus infection. Heterosubtypic immunity involves virus-specific memory T cells that cross-react with related virus subtypes (27). Conversely, memory cells from an unrelated virus that do not cross-react with a subsequent virus will not contribute to a survival advantage (4). In addition to antigen specificity, the cell frequency and magnitude of the CTL response are also significant contributors to surviving a severe respiratory infection (8, 43). Since CD8+ T cells are associated with surviving an influenza virus infection, we wanted to determine whether virus-specific memory CD8+ T cells alone are sufficient to protect mice exposed to 100% oxygen as neonates. We began by injecting naïve C57BL/6 mice i.p. with HKx31 and sorted for splenic memory CD8+ T cells (CD44hi) 60 days later (Fig. 5A). A subset of the sorted cells were analyzed using MHC I tetramers to determine the frequency of influenza virus NP366–374+ specific CD8+ memory T cells and to ensure equal transfer of virus-specific cells across experiments. Memory CD8+ T cells were adaptively transferred into uninfected adult mice that had been exposed to room air or 100% oxygen as neonates. The following day, recipients were infected intranasally with PR8 and monitored for changes in body weight and mortality for 4 weeks. Survival and changes in body weight among mice exposed to 100% oxygen as neonates were not significantly different from those for mice exposed to room air (Fig. 5B and C). By postinfection day 28, interstitial thickening, collagen accumulation, and persistence of inflammatory cells were clearly evident and were markedly greater in mice that had been exposed to 100% oxygen as neonates (Fig. 6A and C). Administration of virus-specific memory CD8+ T cells prior to infection reduced the extent of collagen staining.
and the collagen levels in both groups of mice (Fig. 6B and C). The alveolar simplification seen in oxygen-treated mice was still evident in infected mice administered virus-specific CD8^+ T cells. Memory CD8^+ T cells are therefore sufficient to blunt the increased fibrosis, weight loss, and mortality seen in primary infection of adult mice that were exposed to 100% oxygen as neonates.

**DISCUSSION**

Conventional thinking is that premature infants are predisposed to more severe and frequent infections, such as influenza virus and respiratory syncytial virus (RSV) infections, due to the immaturity of their immune systems. However, recent data suggest that impaired responses to infection persist beyond infancy (19, 39), raising questions about whether and how early-life hyperoxia affects the immune system. To date, there have been no clinical or animal studies assessing antiviral CD8^+ T cell function in subjects that received neonatal oxygen supplementation. The findings in the current study indicate that the detrimental effects of neonatal oxygen supplementation on host responses to infection are unlikely due to intrinsic defects in the ability to mount an effective CD8^+ T cell response to the infection. This is evident from the equivalent numbers of virus-specific CD8^+ T cells, T cell functional readouts, and viral clearance kinetics for the two groups in the study. Combined with previously published data showing normal influenza virus-specific serum antibody titers in mice exposed to 100% oxygen as neonates (31) and with influenza immunization studies of premature infants demonstrating antibody responses comparable to those of full-term controls (11, 33), our findings support the idea that neonatal hyperoxia does not adversely affect adaptive immunity. In other words, premature infants are not predisposed to more frequent or severe respiratory infections simply due to the immaturity of the immune system.

**FIG 3** Virus-specific memory T cells reduce oxygen-mediated morbidity and mortality of mice infected with influenza A virus. (A) Experimental method. Adult mice exposed to room air (RA) or 100% oxygen (O2) as neonates were primed (i.p.) with 200 HAU of influenza virus HKx31 and subsequently infected (i.n.) 60 days later with a low-mortality (C and D) or high-mortality (E and F) dose of influenza virus PR8. (B) Average numbers of CD8^+ T cells specific for the influenza virus peptide NP366–374 in the lung and spleen prior to PR8 infection (i.e., 60 days after HKx31 injection). Body weight (C and E) and survival (D and F) were monitored for 28 days after PR8 infection, and results are shown as percent survival and percent changes in body weight. Data are representative of two independent experiments, and error bars indicate the SEM (n ≥ 15 mice per group for morbidity and mortality experiments [C to F] and n ≥ 6 mice per group for the experiment depicted in panel B). Analyses of differences between body weight and survival over time were determined by Mann-Whitney and Mantel-Cox tests, respectively (*, P < 0.05).
In the absence of intrinsic deficits in key adaptive responses to infection, it is unclear which mechanisms are responsible for the poorer survival of adult animals that received supplemental oxygen for a few days after birth. One possibility is that early-life exposure to a high oxygen level reprograms innate host responses to infection. This could include lung epithelial and endothelial cells. Indeed, it has long been known that exposure of newborn rodents to high oxygen levels causes alveolar simplification and defects in vascular development, which are attributed in part to loss of vascular endothelial growth factor signaling and circulating endothelial cell precursors (7). Furthermore, studies of mice suggest that supplemental oxygen disrupts the frequency of type I and type II epithelial cells within the developed lung (47). These changes in lung cell programming and frequency may contribute to the increased inflammation observed (31) or adversely influence tissue repair mechanisms.

A surprising finding from the current study was the contribution of memory CD8\(^+\) T cells to the development of infection-induced fibrosis in mice exposed to 100% oxygen as neonates. Very few investigations have evaluated how memory CD8\(^+\) T cells affect fibrosis development in infectious models. Previous studies have suggested that fibrosis associated with chronic hepatitis is promoted by nonspecific activation of memory CD8\(^+\) T cells (28, 40). For a bleomycin-induced fibrosis model, it was hypothesized that recently activated memory T cells may contribute to lung pathology via interaction with inflammatory dendritic cells (DCs) (1). The potential role of CD8\(^+\) T cells in these models is believed to be as IFN-γ-mediated activators of inflammatory macrophages/dendritic cells, which generate fibrosis-inducing molecules such as tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β), interleukin-1β (IL-1β), and IL-13 (44, 45). Whether immune activation of these molecules alone or in the context of severe epithelial injury drives fibrosis remains to be determined. While the issue of antigen-specific activation versus nonspecific activation may explain why pathology was improved in our infectious model compared to the aforementioned studies, it is possible that the altered kinetics of immune activation is responsible for the improved outcome. It is well known that the presence and early recruitment of memory T cells to the lung confer accelerated pathogen clearance and improved survival (16, 21, 37). In addition to reducing the duration of infection, recent studies are starting to address how the presence of memory T cells affects the nature and magnitude of the innate immune response (24, 36). The altered kinetics of proinflammatory cytokines may alter the activation state or reduce the recruitment of innate immune cells that contribute to the development of fibrosis in mice exposed to 100% oxygen as neonates.

In addition to reduced lung pathology, the presence of memory T cells also improved the survival of adult mice that had received supplemental oxygen as neonates. While it was expected that having virus-specific memory would improve host resistance, the fact that generating memory T cells restored survival to that observed in controls exposed to room air, even at virus doses that induce 70% lethality, was surprising. This suggests that whatever benefit is conferred by the memory T cells is independent of infection severity. As with the effects on fibrosis, the presence of T cell memory likely influenced the kinetics of the innate immune response and accelerated viral clearance. A similar phenotype has been observed for influenza virus-infected obese mice, where pathology in obese mice was enhanced independent of viral clear-

**FIG 4** Virus-specific memory T cells reduce oxygen-associated pulmonary fibrosis in mice infected with influenza A virus. Adult mice exposed to room air or 100% oxygen as neonates were infected (i.n.) with HKx31 (A) or primed (i.p.) with HKx31 and then infected (i.n.) with PR8 (B). Lungs were collected on day 28 postinfection and stained with Gomori’s trichrome stain to visualize collagen deposition. Images are representative of observations for 8 to 14 mice in each treatment group. (C) Soluble collagen in lung homogenates was measured in the day 28 postinfection samples described above (n = 5 for each group). Significance was determined by Fisher’s PLSD test (***, P < 0.0001).
ance (30). Despite the ability of obese mice to effectively clear the infection, survival was improved in these mice with the addition of antiviral medication that reduced the duration of infection. These data support the idea that reducing the duration of infection may keep neonates exposed to 100% oxygen from developing persistent, pathological inflammation and may improve survival.

Considering the fragility of infants who require supplemental oxygen and their sensitivity to respiratory infections, they are prime candidates for receiving the seasonal influenza vaccine. The recent emergence of the 2009 pandemic influenza (H1N1) and avian influenza (H5N1) viruses has triggered a renewed interest in elements that confer immune protection across multiple strains of influenza virus. Multiple groups have reported that seasonal influenza vaccination can generate CD8\(^+\) T cells that cross-react against pandemic and avian influenza virus antigens (26,34, 38). In a mouse model, adoptive transfer of influenza virus H3N2-specific T cells can confer protection against the 2009 H1N1 influenza virus (17). These studies predict that cross-protective memory T cells generated by the seasonal influenza vaccine may reduce the susceptibility of infants treated with supplemental oxygen and/or diagnosed with BPD to related influenza virus infections. Considering the relatively small number of influenza virus-specific cells that were adoptively transferred into the mice in our study—equivalent to the number of cells found in the lungs of i.p. challenged mice (Fig. 3B)—protection may not require a large expansion of memory T cells by the vaccine. If this proves true for humans, it will mean that many current vaccine formulations might be adequate to prime the immune response of infants requiring supplemental oxygen at birth, better protecting them from respiratory infection later in life. Since there is no current RSV vaccine and many infants become infected with viruses before vaccination, the use of antiviral drugs may be beneficial to shorten the duration of infection and reduce the risk of developing chronic inflammation. Further studies with children who received the seasonal influenza vaccine are needed to ascertain whether the incidences of influenza virus infection and/or hospitalization are different between children born at term and premature infants requiring supplemental oxygenation.

In conclusion, the current findings demonstrate for the first time that neonatal hyperoxia confers susceptibility to various subtypes of influenza virus but does not impair the CD8\(^+\) T cell response. Conversely, the presence of memory CD8\(^+\) T cells protects oxygen-exposed neonates from enhanced pathology and mortality associated with infection. These results suggest that premature infants who require supplemental oxygen therapy would benefit from vaccination strategies that promote T cell immunity against respiratory viral infections as well as from early antiviral treatments that reduce the duration of infection. A better understandi

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**FIG 5** Prophylactic administration of virus-specific memory CD8\(^+\) T cells reduces oxygen-mediated morbidity and mortality of mice infected with influenza A virus. (A) Experimental design. Untreated adult mice were primed (i.p.) with 200 HAU HKx31 to generate virus-specific memory T cells. Sixty days later, CD8\(^+\) T cells were enriched from spleens, and then CD8\(^+\)CD44\(^{hi}\) T cells (80% purity) were obtained by sorting. Sorted memory CD8\(^+\) T cells were adoptively transferred (intravenously [i.v.]) into naive, adult mice exposed to room air (RA) or 100% oxygen (O\(_2\)) as neonates (6.5 \(\times\) 10\(^5\) to 7.5 \(\times\) 10\(^5\) total CD8\(^+\) T cells, per recipient). Control mice received PBS (i.v.). One day after adoptive transfer, mice were infected (i.n.) with influenza virus strain PR8. Mean body weight changes (B) and survival (C) were monitored for 28 days after PR8 infection, and results are shown as percent survival and percent changes in body weight (n \(\geq\) 15 mice per group). Data are representative of two independent experiments. Significance of differences between body weight and survival curves was determined by Mann-Whitney and Mantel-Cox tests, respectively.
standing of how oxygen supplementation of neonates contributes to postinfection pathology will be helpful in developing appropriate vaccines and/or drugs to protect this fragile population.

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