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The Migration of T Cells in Response to Influenza Virus Is Altered in Neonatal Mice

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Influenza virus is a significant cause of mortality and morbidity in children; however, little is known about the T cell response in infant lungs. Neonatal mice are highly vulnerable to influenza and only control very low doses of virus. We compared the T cell response to influenza virus infection between mice infected as adults or at 2 d old and observed defective migration into the lungs of the neonatal mice. In the adult mice, the numbers of T cells in the lung interstitia peaked at 10 d postinfection, whereas neonatal T cell infiltration, activation, and expression of TNF-α was delayed until 2 wk postinfection. Although T cell numbers ultimately reached adult levels in the interstitia, they were not detected in the alveoli of neonatal lungs. Instead, the alveoli contained eosinophils and neutrophils. This altered infiltrate was consistent with reduced or delayed expression of type 1 cytokines in the neonatal lung and differential chemokine expression. In influenza-infected neonates, CXCL2, CCL5, and CCL3 were expressed at adult levels, whereas the chemokines CXCL1, CXCL9, and CCL2 remained at baseline levels, and CCL11 was highly elevated. Intranasal administration of CCL2, IFN-γ, or CXCL9 was unable to draw the neonatal T cells into the airways. Together, these data suggest that the T cell response to influenza virus is qualitatively different in neonatal mice and may contribute to an increased morbidity. The Journal of Immunology, 2010, 185: 2980–2988.
coalesced together for 2 wk to synchronize estrus for timed pregnancies. The influenza A/Porto Rico/8/34 (PR8) strain of influenza virus was grown in the allantoic fluid of 10- to 12-d-old embryonated specific pathogen-free chicken eggs as previously described (19). Viral stocks were tested for common mouse pathogens and were shown to contain only influenza virus. Viral yield was quantified by titration in eggs to determine the 50% egg-infectious dose (EID50). Unless noted otherwise (Fig. 1), sublethal doses of approximate LD10 were used. Mice are considered neonates at less than 7 d of age and will be referred to as pups thereafter. At 2 d of age, neonates were infected intranasally (i.n.) under halothane or isoflurane anesthesia with an LD10 of PR8 virus (0.2 EID50/g body weight) in a 10-μl volume of HBSS. Adult mice were simultaneously infected with an LD10 of PR8 virus (2.0 EID50/g body weight) in a 50-μl volume. Following infection, mice were monitored daily for weight loss.

**Determination of lung virus titers**

Lungs were sterilely isolated from infected mice and frozen at −80°C until analysis. Viral burdens were determined by plaque assay on Madin Darby canine kidney (MDCK) cells (American Type Culture Collection, Manassas, VA). Cells were grown to confluency in six-well plates in DMEM (American Type Culture Collection) media supplemented with nonessential amino acids and 10% heat-inactivated FCS (Atlanta Biologicals, Lawrenceville, GA). Ten-fold dilutions of lung homogenate were incubated with the cells for 1 h at 37°C. The cells were then washed and overlayed with DMEM media containing 1% chicken embryo extract (CEE) and 0.1% CCE (Sigma-Aldrich) and 1 mg/ml collagenase A (Sigma-Aldrich) for 30 min at 37°C. The cells were then washed and overlaid with DMEM media containing 0.5% chicken embryo extract (CEE) and 0.1% CCE (Sigma-Aldrich). Alveolar spaces were highly defective in the neonates. Although the interstitium matched adult mice in both total numbers and percentage of alveolar cells. Blood was removed from the lungs by perfusion of HBSS into the right side of the heart. Lungs and tracheobronchial lymph nodes were excised and minced, and the lungs were incubated with 50 U/ml DNase (Sigma-Aldrich) and 1 mg/ml collagenase A (Sigma-Aldrich) in RPMI 1640 containing 3% FCS. Diced lung tissue and lymph nodes were pushed through cell strainers to obtain a single-cell suspension. Finally, RBCs were lysed by treatment with a hypotonic solution, and the remaining cells were washed and resuspended in HBSS.

**Flow cytometry**

For surface staining, 5 × 10^4 to 10^6 cells were stained with fluorochrome-conjugated Abs specific for murine CD4, CD8, CD44, CD62L, and CD69 (eBioscience, San Diego, CA) in PBS containing 0.1% BSA and 0.02% NaN3. Cells were then fixed in 5% formalin for 20 min at room temperature and resuspended in HBSS for multiparameter analysis using a FACSCalibur or LSR II cytometer (BD Biosciences, Mountain View, CA). Intracellular cytokine staining was performed on lungs from infected mice. Intracellular cytokine staining cells from digested lung tissue were stimulated for 4 h with 50 ng/ml PMA and 1 μg/ml ionomycin. Brefeldin A (10 μg/ml) was added for the final 2 h of incubation to inhibit secretion of cytokines. Cells were surface-stained with anti-CD8 as described above, fixed in 5% formalin, and permeabilized with PBS/BSA/azide containing 0.5% saponin. Nonspecific binding sites were blocked with anti-CD16/32 (eBioscience), and then cells were stained with anti–IFN-γ (BD Biosciences) and analyzed by flow cytometry.

**Histology and microscopy**

Ten days postinfection (dpi), mice were euthanized and lungs were inflated and fixed with 10% formalin. Lung lobes were then embedded in paraffin, cut in 5-μm-thick sections, and hydrated through graded alcohol washes. Slides were stained with H&E, dehydrated, and mounted.

For differential counts, an aliquot of 3 × 10^3 bronchoalveolar lavage (BAL) cells were spun onto glass slides, fixed in methanol, and stained with Diff-Quik (Dade International, Miami, FL). Images were obtained using a Spot digital camera attached to an Eclipse microscope (Nikon, Melville, NY).

**Ag presentation assay**

To make bone marrow dendritic cells (BMDCs), bone marrow progenitor cells from adult C57BL/6 mice were cultured with 90% complete media (RPMI 1640/5% heat-inactivated FBS/2-ME and antibiotics) and 10% supernatant from culture of GM-CSF–producing B16/F10/9GM cells for 14 d (20). BMDCs were infected with PR8 virus at 0.1 multiplicity of infection for 1 h or were left uninfected, then washed and cultured with lymphocytes isolated from 7 dpi lymph nodes at a 1:50 ratio in complete media. After 3 d, culture supernatants were harvested and assayed for IFN-γ by ELISA (eBioscience).

**Cytokine and chemokine analysis**

Lungs were lavaged with 1 ml HBSS/3 mMEDTA, and BAL fluid (BALF) was stored at −80°C until analysis. Lungs from separate mice were homogenized in HBSS containing a 100-fold dilution of Sigma protease inhibitor mixture (Sigma-Aldrich) with the Glus-Col Tissue Homogenizing System (Duigral, Vernon Hills, IL), ELISAs for TNF-α, IFN-γ, IL-4, IL-5, CCL5, and CXCL9 were performed on BALF and lung homogenates using ELISA kits according to manufacturer’s instructions (eBioscience). CCL11, CXCL1, CXCL2, and CCL3 levels in BALF were determined using Milliplex cytokine kits (Millipore, Billerica, MA) and KMAP technology (LumineX, Austin, TX), according to the manufacturers’ instructions. Cytokine levels in the lung homogenates were normalized using the RC DC Protein Assay (Bio-Rad, Hercules, CA).

For IL-4 mRNA analysis, lungs from infected mice were isolated and digested with MDM2 endonuclease and Minced in 1% Bacto Agar with 1% trypsin (Sigma-Aldrich, St. Louis, MO). Three days later, the cells were fixed with 20% acetic acid and the overlay removed. Plaques were visualized by staining with crystal violet and counted.

**Administration of i.n. cytokines and chemokines**

At 9 dpi with an LD10 dose of PR8 virus, 16 ng/g IFN-γ, 2 ng/g CXCL9, or 2 ng/g, 400 ng/g, or 2 μg/g CCL2 (PeproTech, Rocky Hill, NJ) was administered in a 10-μl volume of PBS or HBSS to pups i.n. under isoflurane anesthesia. Control mice were given 10 μl PBS or HBSS.

**Statistics**

Data were expressed as the mean ± SD of five mice per group, and each experiment was repeated at least twice. Statistics were performed using SigmaStat software (San Jose, CA) to run a two-way ANOVA followed by the Holm-Sidak post hoc test for pairwise comparisons. If variance or normality tests failed, then Kruskal-Wallis one-way ANOVA on ranks were performed at each individual time point followed by a Dunn’s pairwise post hoc test. Differences were considered statistically significant if p < 0.05.

**Results**

**Neonatal mice are more susceptible to influenza PR8 virus than adult mice**

To establish a dose of PR8 virus that was not lethal to neonatal mice, a dose response was performed as shown in Fig. 1. This study showed that neonatal C57BL/6 mice were more susceptible to lethal influenza infection than adults. Adult mice succumbed to 20 EID50/g of body weight, whereas 2 EID50/g was lethal for neonatal mice (Fig. 1A, 1B). This corresponded to an absolute dose of 500 EID50 in adults and 4 EID50 in pups. For both adults and pups, 10-fold lower doses of virus (2 EID50/g and 0.2 EID50/g, respectively) were lethal to only 10% of the mice (LD10, Fig. 1A, 1B). Morbidity was monitored by weight loss in adults and failure to gain weight in pups (Fig. 1C, 1D). Plaque assays performed on lungs from mice infected with an LD10 dose of PR8 showed that the neonates were productively infected, and viral burdens were as high in neonatal mice as in adults within a few dpi (Fig. 1E). Additionally, neonates cleared virus more slowly than adult mice (Fig. 1E).

**Neonatal T cell distribution differently within the lung**

We first examined the T cell response in the lungs of PR8-infected C57BL/6 mice. The development of the T cell response in the lung interstitium of the neonatal mice was delayed by ~4 d as compared with adults, peaking at 14 dpi (Fig. 2A, 2B). The CD8+ T cell response ultimately peaked at a lower magnitude than in adults (Fig. 2B). Beyond 14 dpi, CD4+ and CD8+ T cell responses in the lung interstitium matched adult mice in both total numbers and percentage of lung infiltrate (Fig. 2A, 2B and data not shown).

Interestingly, T cell migration from the interstitium into the alveolar spaces was highly defective in the neonates. Although the...
B

Mice were weighed daily (indicated dose of the PR8 strain of influenza virus based on body weight. Whereas adult alveoli remain thin-walled and open (Fig. 3), interstitium were considerably thickened with inflammatory cells, and neonatal alveoli are smaller in size and appear more "punctated." The absence of T cells in the lung airways of the neonatal mice (Fig. 2A, C) is suggestive of interstitial inflammation. To confirm this idea, we examined the activation status of the T cells in the lung interstitium and airways (Fig. 4). Early T cell activation is normal in the lungs of neonatal mice infected with influenza virus.

CD8+ T cells in the airways of influenza-infected adult mice are phenotypically different from those in the lung interstitium and tend to be uniformly Ag-specific and highly activated (18). When we examined the activation status of the T cells in the lung interstitia of neonatal mice, we found that the early activation markers CD69 and CD11a were expressed at similar levels on adult and pup CD8+ T cells throughout the course of infection (Fig. 4B, 4D). Additionally, lymph node cells that were isolated 7 dpi and cultured with PR8-infected BMDCs produced IFN-γ to levels comparable with adult lymph node cells (Fig. 4E), indicating that they were predominantly virus-specific T cells.

Cytokine expression is altered in influenza-infected neonates

Because the neonates consistently had eosinophilic airway infiltrates, we used PCR to assay for IL-4 by RNA expression. Both adult and neonatal T cells had the capacity to produce IFN-γ upon restimulation ex vivo (Fig. 5A), but IL-4 mRNA was detected at much higher levels in the neonatal lung than in adult lung (Fig. 5B). Although intracellular cytokine staining showed that the neonatal T cells had the potential to make IFN-γ upon ex vivo restimulation, we wanted to confirm whether IFN-γ and IL-4 were produced in the lungs of neonatal mice infected with influenza virus.

Early T cell activation is normal in the lungs of neonatal mice infected with influenza virus

Although T cells failed to enter the alveolar spaces, cytospin analysis showed that large numbers of polymorphonuclear leukocytes (PMNs) were able to enter the alveolar spaces (Fig. 3A, 3B). The neutrophil infiltration was slightly delayed in the neonates, but ultimately reached a similar magnitude as in adult mice (Fig. 2E). Interestingly, although eosinophils form a relatively minor fraction of the adult BALF, the numbers are highly elevated in neonates, peaking 14 dpi when they accounted for 30–40% of the total infiltrate (Fig. 2F). These data indicate that defective migration into the alveolar spaces does not extend to all leukocytes.

The absence of T cells in the lung airways of the neonatal mice was suggestive of interstitial inflammation. To confirm this idea, sections of infected lung were stained with H&E at 10 dpi (Fig. 3). Because much of the lung alveolarization and septation in mouse lungs occurs postnatally (21), uninfected pups have larger alveoli and broader septa than adult mice (Fig. 3E, 3F). Our studies showed that during influenza infection, inflamed areas of the neonatal interstitium were considerably thickened with inflammatory cells, whereas adult alveoli remain thin-walled and open (Fig. 3C, 3D). By flow cytometry, we determined that the cells in the lung interstitium of infected pups are made up of ∼10% T cells, 30% monocytes, macrophages, and DCs, 8% NK cells, and 10% PMNs (data not shown).

FIGURE 1. Neonatal mice are more susceptible to PR8 virus than adult mice. Mice at 8 wk (A, C) or 2 d (B, D) of age were infected i.n. with the indicated dose of the PR8 strain of influenza virus. Viral burdens of mice infected with an LD10 dose of PR8 were determined by plaque assay of lung homogenates on MDCK cells (A). The dashed line indicates the limit of detection in this assay. E, Data represent the mean ± SD of at least 10 mice per group and are representative of eight separate experiments. *p < 0.05 compared with pups at the same time point.

FIGURE 2. Neonatal T cells are excluded from lung airways. Adult or 2-d-old mice were infected i.n. with an LD10 dose of the PR8 strain of influenza. BALF cells (C) and D) and cells in digested lung tissue (A, B) were stained with anti-CD4 (A, C) and anti-CD8 (B, D) and analyzed by flow cytometry. Aliquots of BALF cells were spun onto slides and stained with Diff-Quik (Dade International). Differential counts were performed to determine the percentage of eosinophils (E) and neutrophils (F). Data represent the mean ± SD of at least 5 mice per group and are representative of five separate experiments. *p < 0.05 compared with pups at the same time point.

Early T cell activation is normal in the lungs of neonatal mice infected with influenza virus

CD8+ T cells in the airways of influenza-infected adult mice are phenotypically different from those in the lung interstitium and tend to be uniformly Ag-specific and highly activated (18). When we examined the activation status of the T cells in the lung interstitium of neonatal mice, we found that the early activation markers CD69 and CD11a were expressed at similar levels on adult and pup CD4+ and CD8+ T cells throughout the course of infection (Fig. 4A, 4B and data not shown). However, early in infection, a significantly lower proportion of pup interstitial CD4+ and CD8+ T cells were CD44+CD62L+ Ag-experienced cells as compared with adults (Fig. 4C, 4D). Interestingly, by 14 dpi, the size of the lung interstitial infiltrate in the pups matched that of adults, and there were equivalent proportions of Ag-experienced T cells in the two groups of mice (Fig. 4C, 4D). Additionally, lymph node cells that were isolated 7 dpi and cultured with PR8-infected BMDCs produced IFN-γ to levels comparable with adult lymph node cells (Fig. 4E), indicating that they were predominantly virus-specific T cells.

Cytokine expression is altered in influenza-infected neonates

Because the neonates consistently had eosinophilic airway infiltrates, we used PCR to assay for IL-4 by RNA expression. Both adult and neonatal T cells had the capacity to produce IFN-γ upon restimulation ex vivo (Fig. 5A), but IL-4 mRNA was detected at much higher levels in the neonatal lung than in adult lung (Fig. 5B). Although intracellular cytokine staining showed that the neonatal T cells had the potential to make IFN-γ upon ex vivo restimulation, we wanted to confirm whether IFN-γ and IL-4 were produced in the lungs of neonatal mice infected with influenza virus.
lungs during infection. ELISA assay showed that upon infection, IFN-γ was upregulated in lung homogenates of both pups and adults; however, IFN-γ was slightly delayed and reached lower levels in pups (Fig. 5C). Additionally, no IFN-γ was detectable in the BALF from the neonates at any time point examined (Fig. 5E), corresponding with the lack of IFN-γ–producing T cells in the airways. Concomitant with the peak in IFN-γ, and a shift to a type 1-biased lung environment, the levels of IL-4 protein dropped in the lungs of adult mice (Fig. 5D). This drop was absent in neonatal mice, and instead, IL-4 protein levels doubled (Fig. 5D). In contrast, IL-5 levels in the lungs of adults and neonates did not increase significantly above baseline levels at any of the time points examined (data not shown).

In adult mice, TNF-α is produced rapidly postinfection and activates endothelial and epithelial cells to upregulate adhesion molecules and produce chemokines (22). Although high levels of TNF-α were detectable in BALF from adult mice 7 dpi, levels did not peak in the pups until 21 dpi (Fig. 5F). Notably, the increase in levels of TNF-α in the BALF correlated with the appearance of CD4⁺ T cells in the lung interstitium of pups (Figs. 2A, 5F).

**Chemokine expression is altered in influenza-infected neonates**

We hypothesized that delayed production of TNF-α and a lack of BALF IFN-γ would lead to a delay in TNF-α– and IFN-γ–induced chemokines, such as CXCL9 and CXCL10, which may in part explain the defect in CD4⁺ T cell infiltration into the airways of influenza virus–infected neonatal mice.

CCL5 (RANTES) and CCL3 (MIP-1α) are highly upregulated during influenza infection and are important for T cell migration to the lungs (23–25). CCL3 is also chemotactic for eosinophils and is associated with pulmonary eosinophilia (26, 27). Levels of CCL5 protein did not differ in either BALF or lung homogenate from infected adults or pups (Fig. 6A). CCL3 was expressed in the neonatal lung, but was delayed and ultimately peaked at a lower level than in adult mice (Fig. 6B). CCL3 and CCL5 may be important in drawing T cells and eosinophils into the lung.

We next examined the CXCR3 ligand CXCL9 (monokine induced by IFN-γ) and theCCR2 ligand CCL2 (MCP-1), both of which are important for T cell migration and function (25, 28). The adult mice expressed high levels of both CXCL9 and CCL2, which peaked in BAL 7 dpi. In contrast, CXCL9 and CCL2 were not detected in the neonatal lungs (Fig. 6C, 6D). This may be partly responsible for the defective migration of neonatal T cells into BALF and may be linked to reduced IFN-γ in the alveolar spaces.

In contrast to the T cells, neutrophils and eosinophils were able to enter the alveolar spaces of neonates (Fig. 3). We therefore examined the lungs for the neutrophil-attracting chemokines CXCL1 (keratinocyte chemoattractant) and CXCL2 (MIP-2) as well as the eosinophil chemokine CCL11 (eotaxin). Both CXCL1 and CXCL2 peaked in adult BALF prior to 10 dpi (Fig. 6F, 6G). Interestingly, CXCL1 concentrations were markedly reduced in pup BALF, whereas CXCL2 was expressed at similar levels as in adult BALF (Fig. 6F, 6G). CXCL2 expression corresponded with the neutrophil influx seen in Fig. 2E. Consistent with the low level of eosinophils in the lungs of adult mice, CCL11 was not expressed in adult lung homogenate at significant levels (Fig. 6E). In contrast, CCL11 was expressed highly in the lungs of influenza virus–infected neonates, consistent with the entry of eosinophils (Figs. 3F, 6E). This is also in agreement with the presence of CCL11 and eosinophils in the lungs of infants infected with influenza virus (7).

Overall, the neonatal lung expresses an array of chemokines distinct to those expressed in adults, consistent with differences in the lung infiltrate. Neonates express CCR5 and CCL3 that may draw
T cells into the lungs, but lack CCL2 and CXCL9, which may be linked to the absence of T cells in the alveolar spaces. Further, pups express CXCL2 for neutrophil migration and high levels of CCL11 (eotaxin) that likely are responsible for the elevation in eosinophils.

L.n. CCL2, IFN-γ, or CXCL9 treatment is insufficient to attract neonatal T cells into the alveolar spaces

CD8⁺ T cells that are biased toward type 2 cytokines show altered migration patterns in the lungs during influenza infection (29), whereas IFN-γ⁻/⁻ mice have reduced numbers of CD8 T cells in the BALF (14). To determine if IFN-γ played a role in migration of neonatal T cells to the lungs, murine IFN-γ (16 ng/g) or PBS was administered 7, 8, and 9 dpi. This resulted in concentrations of IFN-γ in BALF that were comparable to levels found in influenza virus-infected adult lungs at 7 dpi (Figs. 5E, 7B). Flow cytometry and differential counts were performed on BALF cells 10 dpi. This time period was selected because large numbers of T cells accumulated in the neonatal lung interstitium, but did not enter the alveolar spaces (Fig. 2). The cytokine treatment led to increased total cellularity in both BALF and lung homogenate (data not shown), but did not increase the proportion of lymphocytes in the BALF of neonatal mice (Fig. 7A). The percent of CD4⁺ and CD8⁺ T cells remained in the range observed in the BALF of uninfected neonates. Adult or 2-d-old mice were infected i.n. with an LD₁₀₀ dose of the PR8 strain of influenza virus-infected neonates. Adult or 2-d-old mice were infected with PR8 at LD₁₀₀ doses. Lung homogenate (A, E) and BALF (B–D, F, G) were analyzed for CXCL15 (A), CXCL3 (B), CXCL9 (C), CCL2 (D), CCL11 (E), CXC11 (F), or CXCL2 (G) by ELISA or luminex multiplex assay. Data for uninfected adults are shown at day 0, and data for uninfected pups are shown at time points corresponding to when infected littermates were analyzed. Data for uninfected controls is not shown for CCL11 (E). These data represent the mean ± SD of five mice per group and are representative of at least two separate experiments. *p < 0.05 compared with pups.
numbers of PMNs were not able to enhance viral clearance in influenza-virus-infected neonatal mice. Together, these data indicate that the cytokine response to influenza in neonatal mice is a mixture of type 1 and type 2 cytokines rather than the typical type 1 response of adult mice. This is associated with infiltration of eosinophils but not T cells into the BAL and increased susceptibility to influenza virus infection. Moreover, treatment with IFN-γ or IFN-γ–induced chemokines did not alter the neonatal response to influenza virus.

**Discussion**

Although neonatal mice are highly susceptible to the lethal affects of influenza virus infection, they were able to clear an LD_{10} albeit with some delay, compared with adults (Fig. 1). Importantly, although activation and entry of neonatal T cells into the lungs was delayed, pups were capable of generating robust interstitial T cell responses (Fig. 2). The delay in the T cell response corresponded with delayed and/or reduced expression of the proinflammatory cytokines TNF-α and IFN-γ (Fig. 5D, 5F). Although neutrophil migration was normal, and eosinophil numbers were elevated, very few T cells entered the alveolar spaces of the neonatal mice (Fig. 2). Our data suggest that the altered composition of the BALF infiltrate was linked to altered chemokine expression. Although CCL5, CCL3, and CXCL2 were upregulated normally in the neonatal lung, the chemokines CXCL9 and CCL2 were markedly reduced (Fig. 6).

The data presented in this study are complicated by the fact that neonates are more susceptible to lethal influenza infection than adults and only tolerate small doses of virus. In this study, adult and neonatal C57BL/6 mice were infected with a LD_{10} dose, corresponding to a 2-fold difference per gram of body weight and a large difference in total virus. Titration experiments that were performed in adult mice suggested that the kinetic differences in the T cell response were unlikely to be only dose related (33). Others have also found that adult mice that are infected with a 100-fold range of influenza virus do not exhibit significant differences in kinetics of T cell infiltration into the lungs (33). Moreover, our own data show a similar lung burden of virus in pups and adults through 7 dpi and even higher levels through 10 dpi without a concomitant infiltration of T cells into the alveoli. These data indicate that there is an intrinsic defect in migration of T cells into the alveoli of neonatal mice that is unrelated to the level of Ag in the lungs.

In earlier studies, we showed delayed entry of T cells into the lungs of neonatal mice that were infected with *Pneumocystis carinii* (34). In this model, the delay in the T cell response was also independent of the dose of the inoculum and corresponded with delayed TNF-α expression as well as slow upregulation of VCAM-1 and ICAM-1 (35). These studies were consistent with the influenza model, which showed delayed expression of TNF-α in the BAL of neonatal mice (Fig. 5F). Others have shown that cord blood cells have a markedly reduced capacity to produce TNF-α in response to TLR ligands (36, 37), which may be partly explained by reduced MyD88 expression on monocytes (37). Defects in TNF-α production by adult blood monocytes have also been attributed to factors that are found in cord blood plasma (36). The importance of TNF-α in T cell entry of the lungs is illustrated by the fact that neutralization of TNF-α with a TNF-α–specific Ab reduces cell recruitment to the lungs of adult mice in response to influenza (38). However, even though TNF-α production was delayed in our model of neonatal influenza virus infection, the appearance of this cytokine after 7 dpi did not significantly influence the migration of T cells from the lung interstitium into the alveolar spaces, leaving us to surmise that the lack of IFN-γ may have been responsible for the failure of T cells to migrate across the epithelial barrier into the alveoli.

The type 1-biased cytokine IFN-γ was reduced in the neonatal BALF (Fig. 5E). We found that the IFN-γ–induced chemokine CXCL9 was also markedly reduced in neonatal BALF (Fig. 6C). A number of studies have demonstrated that T cells from both human and murine newborns are defective in IFN-γ production (39–41) and that this may be due to defects in IL-12 and IL-18 expression (42). IFN-γ signaling is not required for clearance of influenza, and IFN-γ−/− mice do not show significantly increased mortality (14, 43). However, IFN-γ has an important role in ameliorating immunopathology, and IFN-γ–deficient responses are associated with delayed T cell recruitment and increased PMN influx (44). Additionally, it appears that IFN-γ receptor 1–deficient mice show defects in migration of CD8+ T cells into the
Although neonatal T cells can produce IFN-γ during influenza virus infection, the lungs also contain IL-4 mRNA and increased IL-4 protein, suggesting that the response is less strongly type 1 biased (Fig. 5B). The bias of cytokines that T cells produce is known to correspond with their migratory properties. For example, the chemokine receptors CCR2, CCR5, and CXCR3 are expressed on type 1-biased T cells, whereas type 2-biased T cells tend to express CCR3 and CCR4 (45). Additionally, type 2-biased T cells also show defects in migration and cluster in the lung parenchyma at locations distinct from influenza virus-infected epithelial cells (29). The interstitial inflammation observed in neonates infected with influenza virus may be linked to the less type 1-biased T cell response. In support of such an idea, interstitial pneumonia has been associated with type 2-biased cytokines in humans (46). Further, studies in adult mice have shown that type 2-biased T cells are ineffective against influenza virus infection and are associated with eosinophilia, increased morbidity, and delayed clearance (6). We observed these features in influenza-infected neonatal mice (Figs. 1–3). The lack of a clear type 1 bias in neonates is consistent with data demonstrating that neonatal T cells have more of an intrinsic type 2 bias to a number of Ags (47–49), even upon adoptive transfer into adult hosts (12). Additionally, because the strength of TCR interactions influence Th1 differentiation (50), and neonates have a more restricted TCR repertoire (51–53), this may hinder full Th1 biasing.

In addition to T cell-intrinsic factors, the neonatal lung environment and/or APCs may influence the cytokine profile of the T cells. For example, a lack of DC maturation is associated with failure to induce good Th1-biased responses (54, 55). It has also been shown that CD8αCD4+ DCs that produce IL-12 are significantly reduced in number prior to 6 d of age (56). In this vein, the respiratory tract of rats lacks MHC class II+ DCs until days 2 to 3 after birth, and mature DCs do not reach adult levels until after weaning (57). DC recruitment to the lung and DC maturation are deficient in neonates as compared with adult rodents during infection with aerosolized Moraxella catarrhalis in rats and Pneumocystis carinii in mice (34, 58). During influenza infection, lung DCs are critical for T cell activation and function, so DC deficiency could potentially impact T cell function during neonatal influenza virus infection. This could account for the delayed migration of T cells into the lungs of pups compared with adults (Fig. 2), although early activation marker expression and robust IFN-γ production ex vivo (Fig. 4) argue that initial activation events may be normal in neonates in response to influenza virus.

A study by You et al. (15) described infection of 7-d-old mice with influenza virus. In this model, they saw infiltration of only small numbers of neutrophils and no eosinophils. Additionally, they saw little IFN-γ or IL-4 production by neonatal T cells in the lung (15). These differences are likely due to the older age of the pups at infection and differences in experimental methods. We did not see peaks in eosinophils or T cell responses until after 10 dpi (Fig. 2). You et al. (15) did not examine time points between 10 dpi and 109 dpi and generally did not compare pup to adult responses. The authors indicated that both adults and pups cleared virus by 7 dpi, whereas at this time point, both adults and pups were still infected in our study (Fig. 1), suggesting that they may have used lower infecting doses. The lack of a clear type 1-biased response in our model is consistent with the existence of type 2 cytokines and eosinophils in the lungs of children infected with influenza virus (7). Additionally, the interstitial pneumonia that we observed is consistent with lung specimens of children that have died of influenza virus infection (59). Our model may be more comparable with severe childhood infections, whereas the You et al. (15) study may recapitulate subclinical infections.

It is known that central memory T cells are only found in the parenchyma of the lung and not within the airways. This has been suggested to indicate the existence of further selection criteria for airway entry, in which chemokines and chemokine receptors may be important (18). Recently, it was demonstrated that CCR5 is critical for the early recruitment of memory CD8+ T cells into the alveolar spaces but is dispensable for interstitial recruitment (17). In contrast, CCR5 was not necessary for entry of T cells into alveolar spaces during acute infections. In vitro, migration across epithelial cells is dependent on CCL2 but is independent of CCL5 (28). Other possible candidates are the CXCR3 ligands, which are expressed at the epithelial cell surface (60). CXC3+/− mice have decreased T cell infiltration of the BAL (25), suggesting that CXCR3 ligands, such as CXCL9, may be important for alveolar T cell responses. A role for CCL2 and CXCL9 in alveolar trafficking is consistent with the lack of these chemokines and T cells in neonatal BALF (Figs. 2, 6C, 6D). However, these chemokines appeared not to be the only defect in alveolar trafficking, because the alveolar administration of these chemokines was unable to induce T cell migration into the airways (Fig. 7). We previously reported that neonatal T cells failed to migrate across an endothelial cell line in vitro in response to either CCL2 or CCL5 (36) indicative of an intrinsic defect in migration of neonatal T cells to the alveoli. Notably, endothelial cells produce chemokines, such as CCL2, and the differential migration of T cells to the neonatal lung interstitium but not to the alveolar space may be due to differential expression of chemokines by endothelial cells and not epithelial cells or alveolar macrophages. We are currently examining chemokine production by endothelial versus epithelial cells as well as chemokine receptor expression in these mice.

The differential chemokine expression in the lungs of influenza virus-infected neonates appeared to lead to a different assembly of leukocytes. The main chemokines we observed in the neonatal lungs were CCL5 and CCL3 (Fig. 6A, 6B). These chemokines are also detected in nasal secretions of children infected with respiratory viruses, including respiratory syncytial virus, adenovirus, para-influenza, and influenza virus (61). Both of these chemokines are produced by macrophages and epithelial cells and are potent chemotactants for eosinophils (28, 62), which we observed at high levels in the BALF of neonates (Fig. 2F) along with eotaxin (CCL11; Fig. 6E). CCL3 and CCL5 are also able to activate eosinophils and induce degranulation and adhesion molecule expression (63). Neutrophil influx into the lungs of neonates may be a result of CXCL2 in the BALF (Fig. 6G), a known chemoattractant for neutrophils. We also have preliminary data that IL-17 is elevated in the alveoli of influenza-infected neonates, which may also contribute to neutrophil attraction to the lungs of neonates.

In addition to chemokines, adhesion molecules both on T cells and epithelial cells may be important selection criteria for migration of leukocytes into the alveolar spaces. We have previously reported that expression levels of both ICAM-1 and VCAM-1 on Pneumocystis-infected lungs is significantly reduced in neonates compared with adults, and exogenous TNF-α can upregulate these adhesion molecules (36). We have observed that ICAM-1 expression is also reduced in neonatal lungs infected with influenza virus despite the
presence of TNF-α in the BALF late during influenza virus infection (data not shown). Crossing the endothelial barrier requires expression of the integrins CD18 and CD11a (LFA-1), whereas for the epithelial barrier, VLA-4 may be more important (64). However, during Pneumocystis infection, the levels of VLA-4 and LFA-1 were comparable between adult and pup T cells (35). Despite this, Pneumocystis-specific neonatal T cells were less responsive to in vitro migration across an endothelial cell monolayer than adult cells, indicating that there may be some T cell-intrinsic defects in chemokine and/or integrin signaling. We are currently examining T cell integrins during influenza infection in neonates.

Overall, our data indicate that there are fundamental differences in the migration pattern of neonatal T cells into the lungs in response to influenza virus. Similar results have recently been reported in autopsies specimens from young children that died of influenza virus infection (59). As in human children (7), the lungs of neonatal mice contained elevated levels of eosinophils and neutrophils and were less strongly Th1 biased. We postulate that failure of T cells to appropriately bias to type 1 responses in infected infants may contribute to increased susceptibility and poor outcome.

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Disclosures

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References


