Susceptibility to Acute Mouse Adenovirus Type 1 Respiratory Infection and Establishment of Protective Immunity in Neonatal Mice

Megan C. Procario,a Rachael E. Levine,a Mary K. McCarthy,b Eunnie Kim,a Lingqiao Zhu,b Cheong-Hee Chang,b Marc B. Hershenson,a,b and Jason B. Weinberg*a,b

Department of Pediatrics and Communicable Diseases, University of Michigan, Ann Arbor, Michigan, USAa; Department of Microbiology and Immunology, University of Michigan, Ann Arbor, Michigan, USAa; and Molecular and Integrative Physiology Graduate Program, University of Michigan, Ann Arbor, Michigan, USAb

There is an incomplete understanding of the differences between neonatal immune responses that contribute to the increased susceptibility of neonates to some viral infections. We tested the hypothesis that neonates are more susceptible than adults to mouse adenovirus type 1 (MAV-1) respiratory infection and are impaired in the ability to generate a protective immune response against a second infection. Following intranasal infection, lung viral loads were greater in neonates than in adults during the acute phase but the virus was cleared from the lungs of neonates as efficiently as it was from adult lungs. Lung gamma interferon (IFN-γ) responses were blunted and delayed in neonates, and lung viral loads were higher in adult IFN-γ−/− mice than in IFN-γ+/+ controls. However, administration of recombinant IFN-γ to neonates had no effect on lung viral loads. Recruitment of inflammatory cells to the airways was impaired in neonates. CD4 and CD8 T cell responses were similar in the lungs of neonates and adults, although a transient increase in regulatory T cells occurred only in the lungs of infected neonates. Infection of neonates led to protection against reinfection later in life that was associated with increased effector memory CD8 T cells in the lungs. We conclude that neonates are more susceptible than adults to acute MAV-1 respiratory infection but are capable of generating protective immune responses.

MATERIALS AND METHODS

Mice. All animal studies were approved by the University of Michigan Committee on Use and Care of Animals. Adult (4 to 6 weeks of age) BALB/cJ mice and IFN-γ-deficient mice backcrossed onto a BALB/c background [C.129S7(B6)-Ifngtm1Ts/J] were obtained from The Jackson Laboratory, BALB/cJ mothers with litters were also obtained from The Jackson Laboratory. All mice were maintained under specific-pathogen-free conditions.

Viruses and infections. MAV-1 was grown and passaged in NIH 3T6 fibroblasts, and titers of viral stocks were determined by plaque assay on 3T6 cells as previously described (10). Adult mice were anesthetized with ketamine and xylazine and infected intranasally (i.n.) with MAV-1 or controls. However, administration of recombinant IFN-γ to neonates had no effect on lung viral loads. Recruitment of inflammatory cells to the airways was impaired in neonates. CD4 and CD8 T cell responses were similar in the lungs of neonates and adults, although a transient increase in regulatory T cells occurred only in the lungs of infected neonates. Infection of neonates led to protection against reinfection later in life that was associated with increased effector memory CD8 T cells in the lungs. We conclude that neonates are more susceptible than adults to acute MAV-1 respiratory infection but are capable of generating protective immune responses.

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obtained by adding the values for each category, resulting in a total score that could range from 0 to 18.

In some experiments, neonates were treated with IFN-γ using a protocol similar to that described by Lee et al. (20). Recombinant mouse IFN-γ (R&D Systems) was resuspended in sterile PBS. Mice were treated with IFN-γ (10 ng in a total volume of 10 µl administered i.n.) at 1, 3, and 5 dpi. Control mice were treated with an equivalent volume of sterile PBS. At 7 dpi, mice were euthanized and organs were harvested as described above.

**Histology.** Lungs were harvested from a subset of mice and fixed in 10% formalin. Prior to fixation, lungs were gently inflated with PBS via the trachea to maintain lung architecture. After fixation, organs were embedded in paraffin and 5-µm sections were obtained for histopathology. Sections were stained with hematoxylin and eosin to evaluate cellular infiltrates. Periodic acid-Schiff (PAS) staining was performed to evaluate mucus production. All sectioning and staining was performed by the University of Michigan Comprehensive Cancer Center Research Histology and Immunoperoxidase Laboratory. Slides were viewed through a Laborlux 12 microscope (Leitz). Digital images were obtained with an EC3 digital imaging system (Leica Microsystems) using Leica Acquisition Suite software (Leica Microsystems). Final images were assembled using PowerPoint (Microsoft) and Adobe Illustrator (Adobe Systems). Adjustments to the color balance of digital images (10% reduction in the red channel) were applied in Adobe Illustrator equally to all experimental and control images.

To quantify cellular inflammation in the lungs, slides were examined in a blinded fashion to determine a pathology index as previously described (5, 30), generating separate scores for the severity of cellular infiltrates around airway lumens and interstitial infiltrates (Table 1). Each score was multiplied by a number reflecting the extent of involvement of the specimen (5% to 25% = 1, >25% to 50% = 2, >50% = 3). The final pathology index score was obtained by adding the values for each category, resulting in a total score that could range from 0 to 18.

### Table 1: Quantification of cellular inflammation in histologic specimens.

<table>
<thead>
<tr>
<th>Score</th>
<th>Thickness (no. of cell diameters) of cellular infiltrates around airway lumens</th>
<th>Interstitial infiltrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>1–3</td>
<td>Increased cells visible only at high power</td>
</tr>
<tr>
<td>2</td>
<td>4–10</td>
<td>Easily seen cellular infiltrates</td>
</tr>
<tr>
<td>3</td>
<td>&gt;10</td>
<td>Extensive consolidation by inflammatory cells</td>
</tr>
</tbody>
</table>

* A score of 0 to 3 was given for each of the two categories. The score for each category was multiplied by a value reflecting the extent of involvement of the specimen (5% to 25% = 1, >25% to 50% = 2, >50% = 3). The final pathology index score was obtained by adding the values for each category, resulting in a total score that could range from 0 to 18.

**Analysis of inflammatory cells in BALF.** Mice were euthanized via pentobarbital overdose at the indicated time points. Lungs were lavaged three times with the same aliquot of 1 ml sterile PBS with protease inhibitor (complete, Mini, EDTA-free tablets; Roche Applied Science). For animals less than 21 days old, lungs were lavaged three times with the same 0.5-ml aliquot of lavage solution. Cells in bronchoalveolar lavage fluid (BALF) were counted using a hemocytometer.

**Analysis of cytokine protein in BALF.** The remaining cells in BALF were pelleted by centrifugation, and the supernatant was stored at −80°C. Measurements of cytokine protein concentrations in BALF were performed by enzyme-linked immunosorbent assay (ELISA [Duoset kits; R&D Systems]) according to the manufacturer’s protocol.

**Analysis of leukocytes in BALF.** Neutrophils, eosinophils, lymphocytes, and monocytes were counted using a hemocytometer.

**Analysis of mucus production.** Mucin production was determined using the alcian blue staining protocol.

**Susceptibility to MAV-1 Infection in Neonatal Mice**

MAV-1 viral loads in lungs were measured using real-time quantitative PCR (qPCR) as previously described (29). The primers and probe used to detect IFN-γ using a protocol similar to that described by Lee et al. (20). Recombinant mouse IFN-γ (R&D Systems) was resuspended in sterile PBS. Mice were treated with IFN-γ (10 ng in a total volume of 10 µl administered i.n.) at 1, 3, and 5 dpi. Control mice were treated with an equivalent volume of sterile PBS. At 7 dpi, mice were euthanized and organs were harvested as described above.

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### Table 2: Primers and probes used for real-time PCR analysis.

<table>
<thead>
<tr>
<th>Target</th>
<th>Oligonucleotide</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAV-1 E1A</td>
<td>Forward primer GCACTCCATGGCAGGATTCT</td>
<td>T(TGAPDH)</td>
</tr>
<tr>
<td></td>
<td>Reverse primer GGTGAAGGACGGTCTTC</td>
<td>TACTGCCACTTTCTGC</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Forward primer AAAGAGATAATTCTGGCTG</td>
<td>GCTCTGAGAATAAGCGCT</td>
</tr>
<tr>
<td></td>
<td>Reverse primer GCTCTGAGAATAAGCGCT</td>
<td>GCTCTGAGAATAAGCGCT</td>
</tr>
<tr>
<td>Musc5ac</td>
<td>Forward primer CGCAAGCCTCTGTTGGC</td>
<td>CTTGCAACACTCTGAGT</td>
</tr>
<tr>
<td></td>
<td>Reverse primer GCTCTGAGAATAAGCGCT</td>
<td>CTTGCAACACTCTGAGT</td>
</tr>
<tr>
<td>gob-5</td>
<td>Forward primer GAGTGGGCTCACTTCCGATG</td>
<td>GGAGTCGAGGATGATGCTC</td>
</tr>
<tr>
<td></td>
<td>Reverse primer GCTCTGAGAATAAGCGCT</td>
<td>GGAGTCGAGGATGATGCTC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward primer TGCACCCAACACTGCTTAG</td>
<td>GGAGTCGAGGATGATGCTC</td>
</tr>
<tr>
<td></td>
<td>Reverse primer GGAGTCGAGGATGATGCT</td>
<td>GGAGTCGAGGATGATGCTC</td>
</tr>
</tbody>
</table>

For these measurements, 5-µl volumes of cDNA were added to reaction mixtures containing Power SYBR green PCR Mix (Applied Biosystems) and forward and reverse primers (each at a 200 nM final concentration) in a 25-µl reaction mixture volume. Analysis on an ABI Prism 7300 machine (Applied Biosystems) consisted of 40 cycles of 15 s at 90°C and 60 s at 60°C. Standard curves generated using known amounts of plasmid containing the MAV-1 E1A gene were used to convert cycle threshold (Cₜ) values for experimental samples to copy numbers of E1A DNA. Results were standardized to the nanogram amount of input DNA. Each sample was assayed in triplicate. The limit of detection of this assay is typically between 10³ and 10⁶ copies of MAV-1 genome per 100 ng of input DNA.

**Analysis of cytokine and mucus gene expression.** Cytokine and mucus gene expression was quantified using reverse transcription qPCR (RT-qPCR). First, 2.5 µg of RNA was reverse transcribed using Moloney murine leukemia virus RT (Invitrogen) in 20-μl reaction mixtures according to the manufacturer’s instructions. Water was added to the cDNA product to bring the total volume to 50 µl. cDNA was amplified using duplexed gene expression assays for mouse interleukin-4 (IL-4), IL-13, CCL5, CXCL1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Applied Biosystems). Five-microliter volumes of cDNA were added to reaction mixtures containing TaqMan Universal PCR Mix and 1.25 µl each of 20× gene expression assays for the target cytokine and GAPDH. The primers used to detect IFN-γ, Musc5ac, and gob-5 are described in Table 2. For these measurements, 5-µl volumes of cDNA were added to reaction mixtures containing Power SYBR green PCR Mix (Applied Biosystems) and forward and reverse primers (each at a 200 nM final concentration) in a 25-µl reaction mixture volume. Analysis on an ABI Prism 7300 machine (Applied Biosystems) consisted of 40 cycles of 15 s at 90°C and 60 s at 60°C. Quantification of target gene mRNA was normalized to GAPDH and expressed in arbitrary units as 2^(-ΔCₜ), where ΔCₜ = Cₜ(target) − Cₜ(GAPDH).

**Analysis of inflammatory cells in BALF.** Mice were euthanized via pentobarbital overdose at the indicated time points. Lungs were lavaged three times with the same aliquot of 1 ml sterile PBS with protease inhibitor (complete, Mini, EDTA-free tablets; Roche Applied Science). For animals less than 21 days old, lungs were lavaged three times with the same 0.5-ml aliquot of lavage solution. Cells in bronchoalveolar lavage fluid (BALF) were counted using a hemocytometer.

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**Analysis of lung lymphocytes.** In some experiments, left lungs were excised and cut into small pieces before digestion for 30 min at 37°C in a 1 mg/ml solution of collagenase A (Sigma). The digested tissue was then pushed through a syringe with a 1.5-in. 22-gauge needle and pelleted at...
3,000 rpm (402 × g) for 5 min. After lysis of red blood cells in 1× lysing buffer (BD PharMingen) for 3 min, tissue debris was removed by a brief spin (~5 to 10 s) at 1,000 rpm (45 × g). The remaining cells were pelleted at 1,200 rpm (64 × g) for 6 min prior to staining.

**Flow cytometry.** Cells were incubated with anti-FcγR monoclonal antibody 2.4G2 to block nonspecific antibody binding before they were stained with the following fluorescein isothiocyanate-, phycoerythrin (PE)-, peridinin-chlorophyll-protein complex-, PE-Cy7-, allophycocyanin (APC)-, or APC-Cy7-conjugated antibodies: CD4 (L3T4), CD8α (55-6.7), CD62L (MEL-14), CD25 (3C7), CD3ε (145-2C11), and Foxp3 (FJK-16S). All antibodies were purchased from BD PharMingen or eBioscience. For Foxp3 staining, cells were fixed and permeabilized with the Foxp3 Staining Buffer set (eBioscience). Events were acquired on a FACSCanto flow cytometer (BD PharMingen), and data were analyzed with the FlowJo software (Tree Star, Inc.).

**Statistics.** Analysis of data for statistical significance was conducted using Prism 3 for Macintosh (GraphPad Software, Inc.). For viral load data, differences between groups at a given time point in log-transformed viral loads were analyzed using Student’s t test. Otherwise, differences between groups were analyzed using one- or two-way analysis of variance (ANOVA), as appropriate, followed by Bonferroni’s multiple-comparison tests. P values of <0.05 were considered statistically significant.

**RESULTS**

**MAV-1 viral loads in lungs of neonates and adults.** To determine whether neonates were more susceptible than adults to acute MAV-1 respiratory infection, we infected neonates and adults i.n. with MAV-1. We then measured lung viral loads at various time points as a surrogate for susceptibility (Fig. 1). Neonates and adults had comparable lung viral loads at 4 dpi. We measured logarithmic increases in viral loads between 4 and 7 dpi in the lungs of both neonates and adults, consistent with productive MAV-1 replication in the lungs. Viral loads peaked at 7 dpi and decreased between 7 and 14 dpi in both neonates and adults, although viral loads were significantly higher in neonates at both time points. At 21 dpi, lung viral loads were detectable but were very low and did not significantly differ between neonates and adults. These data suggest that neonates are more susceptible than adults to acute MAV-1 respiratory infection but clearance of virus from the lungs is as efficient in neonates as in adults.

**MAV-1-induced lung cytokine production in neonates and adults.** Acute MAV-1 infection induces the expression of multiple cytokines and chemokines in the lungs of adult mice (40, 41). To determine whether virus-induced cytokine production differs between neonates and adults, we measured protein and mRNA levels of representative Th1 (IFN-γ) and Th2 (IL-4) cytokines along with those of chemokines (CCL5 and CXCL1) that are typically induced in the lung by acute MAV-1 respiratory infection. In both neonates and adults, concentrations of IFN-γ protein were greater in the BALF of infected mice than in that of mock-infected mice at 7 dpi but not at 14 or 21 dpi (Fig. 2A and B), although the difference did not achieve statistical significance in neonates. At 7 dpi, the concentration of IFN-γ protein in the BALF of adult mice was significantly greater (~10-fold) than in that of neonates. Similarly, MAV-1 infection increased concentrations of CXCL1 in the BALF of adults and neonates at 7 dpi (Fig. 2G and H), although peak CXCL1 concentrations were ~2-fold higher in adults than in neonates. There were no consistent changes in BALF IL-4 or CCL5 (Fig. 2E and F) concentrations in neonates or adults at any time point.

Increases in IFN-γ mRNA occurred later in neonates than in adults, with peak levels of IFN-γ expression occurring at 14 dpi in neonates and 7 dpi in adults (Fig. 2I and J). There were no statistically significant differences between the amounts of IL-4 mRNA detected in the lungs of mock-infected and infected neonates or adults at any time point (Fig. 2K and L). As with IFN-γ, MAV-1-induced increases in CCL5 mRNA occurred with delayed kinetics in neonates compared to those in adults (Fig. 2M and N). The kinetics of lung CXCL1 mRNA induction were similar in neonates and adults, although CXCL1 mRNA was more abundant in the lungs of adults than in those of neonates at 7 dpi (Fig. 2O and P). Collectively, these data indicate that lung cytokine responses to acute MAV-1 respiratory infection differ between neonates and adults. In particular, the amount of IFN-γ protein in BALF was less in neonates than in adults, and the induction of IFN-γ mRNA occurred later in neonates than in adults.

**Effects of IFN-γ deficiency on MAV-1 respiratory infection.** To determine whether the differences in IFN-γ induction described above contributed to the increased susceptibility of neonatal mice, we first infected adult IFN-γ-deficient (IFN-γ−/−) mice and wild-type BALB/cJ controls (IFN-γ+/+). All antibody-deficient (IFN-γ−/−) mice and IFN-γ+/+ mice, suggesting that the virus was cleared from the lungs with similar degrees of efficiency. Next, we treated neonatal mice i.n. with exogenous IFN-γ in order to determine whether supplementation of IFN-γ in the airways was sufficient to improve the control of viral replication in the lungs of neonates. At 7 dpi, the lung viral loads in mice treated with IFN-γ were not statistically significantly different from the lung viral loads of controls (Fig. 3B).
quantifying lung inflammation demonstrated small differences in overall lung inflammation between mock-infected and infected neonates that were not statistically significant (Fig. 4B). In contrast, total inflammation was more exuberantly increased in the lungs of infected adult mice than in those of uninfected controls at 7 dpi and then decreased by 14 and 21 dpi (Fig. 4C).

The distribution of inflammation differed between neonates and adult mice. Focal areas of inflammation surrounding airways were less common and less intense in the lungs of infected neonates (Fig. 4D) than in those of adult mice (Fig. 4E). Interstitial infiltrates (thickened and hypercellular alveolar walls) were greater in infected animals than in mock-infected controls regardless of age, although these differences were not statistically significant (Fig. 4F and G). While baseline scores for interstitial infiltrates in mock-infected neonates tended to be slightly greater than those in mock-infected adults at all time points, these differences were not statistically significant.

FIG 2 MAV-1-induced lung cytokine production in neonates and adults. Neonates and adults were infected i.n. with MAV-1 (black bars) or mock infected (white bars) with conditioned medium. (A to H) ELISA was used to quantify cytokine protein concentrations in BALF obtained at the indicated time points. (I to P) RNA was extracted from lungs harvested at the indicated time points, and RT-qPCR was used to quantify lung cytokine expression, which is expressed in arbitrary units. Combined data from 4 to 9 (for ELISA) and 5 to 16 (for RT-qPCR) mice per group are presented as means ± the standard errors of the means. Statistical comparisons were made using two-way ANOVA followed by Bonferroni’s multiple-comparison tests. *, P < 0.05; **, P < 0.01; †††, P < 0.001 (comparing uninfected mice to infected mice of the same age at a given time point); †††, P < 0.001 (comparing infected neonates to infected adults at a given time point).

FIG 3 Effects of IFN-γ on MAV-1 respiratory infection. (A) Adult IFN-γ−/− mice and IFN-γ+/+ controls were infected i.n. with MAV-1. DNA was extracted from lungs harvested at the indicated time points, qPCR was used to quantify the number of MAV-1 genome copies in lung DNA. (B) Exogenous IFN-γ or PBS was administered i.n. to neonates infected with MAV-1. At 7 dpi, qPCR was used to quantify the number of MAV-1 genome copies in lung DNA. DNA viral loads are expressed as numbers of copies of the MAV-1 genome per 100 ng of input DNA, and individual circles represent values for individual mice and horizontal bars represent means of groups. *, P < 0.05; **, P < 0.01 (comparing log-transformed values for two groups at a given time point using Student’s t test).
To determine whether recruitment of immune cells to the airways differed between neonates and adults, we counted the total inflammatory cells in the BALF of mock-infected and infected mice. Acute infection induced a significant increase in the number of inflammatory cells present in BALF of infected adult mice at 7 and 14 dpi (Fig. 4H and I). Cell numbers were higher in the BALF of infected mice than in that of mock-infected adult mice at 21 dpi, although this difference was not statistically significant. In contrast, no statistically significant differences in BALF cell numbers were noted between mock-infected and infected neonates at any time point. Although acute MAV-1 infection caused cellular inflammation in the lungs of both neonatal and adult mice, these data therefore suggest that recruitment of inflammatory cells in response to infection is impaired in neonatal mice.

FIG 4 MAV-1-induced lung inflammation in neonates and adults. Neonates and adults were infected i.n. with MAV-1 or mock infected with conditioned medium. Lungs were harvested at the indicated time points. (A) Hematoxylin-and-eosin-stained sections were prepared from paraffin-embedded sections. Scale bars, 100 μm. Pathology index scores were generated to quantify cellular inflammation in the lungs of mock-infected (white bars) and infected (black bars) neonates and adults. The data presented are total pathology index scores (B and C), scores of inflammation surrounding airways (D and E), and interstitial inflammation scores (F and G). Data from 2 to 9 mice per group are presented as means ± the standard errors of the means at each time point. (H and I) BALF was obtained from mice at the indicated time points, and inflammatory cells were counted using a hemocytometer. Combined data from 4 to 10 mice per group are presented as means ± the standard errors of the means. Statistical comparisons were made using two-way ANOVA followed by Bonferroni’s multiple-comparison tests. *, P < 0.05; **, P < 0.01; †, P < 0.05; ††, P < 0.01; †††, P < 0.001 (comparing uninfected mice to infected mice of the same age at a given time point); †, P < 0.05, ††, P < 0.01; †††, P < 0.001 (comparing infected neonates to infected adults at a given time point).
We used PAS staining to evaluate overall lung mucus production (data not shown). Scattered PAS-stained epithelial cells were occasionally observed in medium- to large-sized airways in the lungs of neonates. The number and distribution of PAS-stained cells were not qualitatively different between mock-infected and infected neonates at any time point. Likewise, although mRNA levels of Muc5ac and gob-5 tended to be higher in neonates than in adult mice at a given time postinfection, there were no statistically significant differences between mock-infected and infected mice at any time point.

**T-cell responses to MAV-1 respiratory infection.** We hypothesized that differences between neonates and adults in susceptibility and in the kinetics of IFN-γ induction could be due to differences in lymphocyte recruitment or activation following infection. To test this hypothesis, we analyzed cells recruited to the lungs of infected mice at various time points. Total CD3⁺ lymphocyte numbers were quantified from the left lungs of mock-infected and infected mice at multiple time points. CD3⁺ lymphocyte numbers were not significantly different between mock-infected and infected mice at any time point (Fig. 5A and B). There were slightly more CD3⁺ lymphocytes in the lungs of infected mice than in those of mock-infected mice by 14 dpi. At 7 dpi, there was a very small but statistically significantly lower percentage of CD8 T cells present in the lungs of infected neonates than in those of mock-infected controls, whereas infection induced a higher percentage of CD8 T cells in both neonates and adults at 14 and 21 dpi. The magnitude of this increase was somewhat greater in neonates than in adults, with up to 60% ± 2% CD8 T cells in the lungs of infected neonates compared to 47% ± 2% CD8 T cells in those of infected adults at 14 dpi. By 7 dpi, effector memory (CD62Llow) CD4 (Fig. 5G and H) and (CD62Llow) CD8 (Fig. 5I and J) T cells were present at a significantly higher percentage in the lungs of infected neonates and adults than in those of mock-infected controls. The appearance of effector memory T cells occurred with similar magnitudes and kinetics in neonates and adults. Interestingly, the percentage of CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells (Tregs) was higher in the lungs of infected neonates than in those of mock-infected controls at 7 dpi and then was similar to levels seen in mock-infected controls at later time points (Fig. 5K). In contrast, the percentage of Tregs was not higher in infected adults than in mock-infected controls at any time point. Instead, the percentage of Tregs was lower in infected adults at 14 and 21 dpi (Fig. 5L).

**Protective immunity following primary infection of neonates.** Although lung viral loads were greater in acutely infected neonates than in adults at 7 and 14 dpi, the virus was cleared from the lungs with similar efficiency. In addition, effector memory CD4 and CD8 T cells were generated following neonatal infection.
We therefore hypothesized that adaptive immune responses that developed following neonatal infection would provide protection against reinfection with MAV-1 at a later time. To test this hypothesis, we either infected neonates i.n. with $10^5$ PFU of MAV-1 or mock infected them and then rechallenged them with the same amount of virus or conditioned medium at 28 dpi (35 days of age). At 7 days after the second challenge, we measured lung viral loads and lung cytokine production and assessed cellular responses in the airways and lungs. MAV-1 DNA was readily detectable in the lungs of mice that were mock infected as neonates and then infected for the first time as adults (Mock/MAV-1), and lung viral loads in this group were comparable to those in the adult mice in previous experiments (Fig. 1). In mice that were originally infected as neonates and then mock infected as adults (MAV-1/Mock), lung viral loads were much lower, essentially at the limit of detection. Lung viral loads were significantly lower in mice that were infected as neonates and then reinfected as adults (MAV-1/MAV-1) than in mice infected for the first time as adults.

Compared to mock-infected controls (Mock/Mock), IFN-γ protein in BALF (Fig. 6B) and mRNA (Fig. 6D) in whole lung were upregulated in mice that were infected for the first time as adults. In contrast, there were no significant differences in IFN-γ protein or mRNA measured in the airways and lungs of uninfected control mice, mice infected as neonates and then mock infected as adults, and mice infected as neonates and then reinfected as adults. There were no statistically significant differences between groups in the amount of IL-4 protein in BALF (Fig. 6C) or IL-4 mRNA (Fig. 6E) in whole lung. Although there were no differences between groups in total CD3+ lymphocytes and the indicated lymphocyte populations in the lungs (Fig. 6G), we detected a slightly lower percentage of lung CD3+ lymphocytes (Fig. 6F) and a higher percentage of CD8 T cells (Fig. 6H) in mice that were infected as neonates than in mice that were mock infected as neonates. No differences were detected between groups in the percentage of effector memory (CD62Llow) CD4 T cells in the lungs (Fig. 6I). However, we detected a higher percentage of CD62Llow CD8 T cells in the lungs of mice that were infected as neonates, regardless of the nature of the secondary challenge (Fig. 6J). Taken together, these data suggest that neonates are capable of generat-
ing a protective immune response following MAV-1 respiratory infection and that effector memory CD8 T cells may contribute to this protection.

**DISCUSSION**

Infants are at an increased risk of developing infections and disease caused by respiratory viruses. This is likely due to many factors, including, but not limited to, Th2-skewed immune responses in neonates (1, 2, 4, 22), impaired B and T cell function or recruitment (6, 21, 33), and defects in dendritic cell maturation and recruitment to the lung (13, 28). In this study, viral loads were significantly higher in the lungs of neonates than in those of adult mice at 7 and 14 dpi (Fig. 1), suggesting that neonates were more susceptible than adults to acute MAV-1 respiratory infection. This was not simply an effect of an increased relative inoculum size (putting the same amount of virus used for infection of adult mice into the smaller lungs of neonatal mice), since lung viral loads were similar in neonates and adults at 4 dpi. It is possible that differences in the breathing patterns of unanesthetized neonates and anesthetized adults affected the distribution of virus in the lungs and indirectly influenced viral replication. Both unanesthetized infants and anesthetized adults inhaled the inoculum completely (data not shown), suggesting that there was not an overt difference in the amount of virus with which the groups were infected. The equivalent lung viral loads at 4 dpi provide additional support for this supposition.

Despite the greater viral loads in neonates at 7 and 14 dpi, lung viral loads were equivalent in neonates and adults at 21 dpi. Thus, our data suggest that while neonates were unable to control viral replication in the lungs early during the acute phase of infection as well as adult mice were, adaptive immune responses that developed later in infection were sufficient for clearance of the virus from the lungs. These responses are apparently intact in neonates, or they are at least capable of developing by 28 days of life following neonatal infection. This increased susceptibility of neonates to acute MAV-1 respiratory infection is similar to that seen in mouse models of influenza virus infection, in which neonates have greater lung viral loads and mortality rates than adults do (23). This is not a universal feature of neonatal infection with respiratory viruses. For example, in mice infected with pneumonia virus of mice (a mouse paramyxovirus), lung viral loads at 4 and 7 dpi do not differ based on the age of inoculation, although virus-induced inflammatory responses are more robust when infection occurs in older mice (8). Lung viral loads during the acute phase of infection do not differ between neonates and adults infected with murine gammaherpesvirus 68, but long-term persistence in the lungs and dissemination from the lungs were greater following neonatal infection than following adult infection (34).

While there were several differences between neonates and adults in the induction of lung cytokine production by acute MAV-1 respiratory infection, the differences in IFN-γ production seemed particularly relevant and consistent with previous reports, suggesting an impaired Th1 immune response in neonates (1, 2, 4, 22). IFN-γ contributes to the immune control of many viruses (7). MAV-1 is relatively resistant to the effects of type I and type II IFNs in vitro (19). However, mice deficient in IFN responses show increased susceptibility to systemic MAV-1 infection following intraperitoneal (i.p.) inoculation (Kathy Spindler, personal communication), suggesting that IFNs do contribute to the control of MAV-1 infection in vivo. We have previously demonstrated the upregulation of lung IFN-γ production following i.n. inoculation of adult mice (5). We therefore measured MAV-1-induced lung IFN-γ production to determine whether increased susceptibility to infection in neonates was correlated with differing IFN-γ production. We observed substantially lower levels and delayed kinetics of MAV-1-induced IFN-γ responses in both the airways and lungs of neonates compared to those of adults.

The differences in IFN-γ production were consistent with impaired Th1 responses in neonates and are similar to delayed IFN-γ induction in neonatal mice infected with RSV (12). Our results showing higher viral loads in adult IFN-γ−/− mice than in IFN-γ+/− controls indicate that IFN-γ is likely to play at least some role in the control of MAV-1 in the lungs. The differences in lung viral loads between adult IFN-γ−/− and IFN-γ+/− mice were fairly small, and IFN-γ supplementation in neonates did not lead to a decrease in lung viral loads at 7 dpi, suggesting that IFN-γ is not the only factor contributing to control of MAV-1 in the lung. Although we did not observe a significant increase in BALF IFN-γ concentrations in the IFN-γ-treated neonates (data not shown), the protocol that we used led to biologically relevant effects in a separate model (20). It is possible that increased doses of recombinant IFN-γ and/or an increased dosing frequency would have an effect on virus control in neonates. It remains possible that differences in the induction of cytokines that we did not measure in this study, such as type I or type III IFNs, could also contribute to the observed differences in susceptibility between neonates and adults.

It is unclear whether measurements of IFN-γ in the airways (BALF) or in whole lung are most relevant in the context of MAV-1 respiratory infection. There were some discrepancies between the kinetics of IFN-γ protein levels in BALF and mRNA levels in whole lung. This was also the case for CCL5. It is possible that these discrepancies relate to differences between airways and lungs in the recruitment of specific cell types that produce these and other cytokines. Although the different BALF volumes used in neonates versus adults may complicate a direct comparison of cytokine concentrations between the two age groups, the most pronounced difference between neonates and adults in IFN-γ concentrations at 7 dpi (Fig. 2A and B) is large enough that it is unlikely to be due exclusively to the differences in BALF volumes. In addition, the RT-qPCR data (Fig. 3I and J), which are normalized to GAPDH and would not be affected in a similar way, demonstrate similar differences in IFN-γ induction.

We also considered the possibility that differences in cellular responses to infection could account for the observed susceptibility differences between neonates and adults. IFN-γ can be produced by a variety of immune cells, including lymphocytes and NK cells (35). We focused on T cells because they are major contributors to IFN-γ production and because of demonstrated defects in the production of IFN-γ by neonatal T cells (3, 9, 38). Previous reports demonstrated a role for T cells in MAV-1 pathogenesis. MAV-1-specific cytotoxic T cells are detectable by 4 days after i.p. inoculation (17). Despite viral titers equivalent to or greater than those of wild-type mice, typical signs of disease associated with MAV-1-induced encephalomyelitis do not occur in T cell receptor alpha-deficient (TCRα−/−) and TCRβ6−/− mice or in mice deficient in perforin or major histocompatibility complex class I (25). TCRα−/− and TCRβ6−/− mice succumb to infection between 9 and 16 weeks postinfection, but mice lacking CD4 or CD8 T cells do not (25). T cells are therefore critical both for acute
immunopathology and for long-term host survival following i.p. MAV-1 infection.

No published data are available regarding specific roles of T cells in the host response to primary MAV-1 respiratory infection of infants or adults or in the subsequent formation of protective immunity. Interestingly, we observed little difference in overall CD3⁺ lymphocyte numbers in either mock-infected or infected lungs from neonates or adults. Because neonatal lungs are smaller than adult lungs, it may therefore be that there are proportionately more CD3⁺ lymphocytes in neonatal lungs (both mock infected and infected) than in adult lungs. It also seems possible that the lack of an age-based difference in CD3⁺ lymphocyte numbers could be due to an increase in lung parenchyma with age that is not accompanied by a proportionate increase in overall lymphocyte numbers.

Although it is possible that relevant differences in MAV-1-specific T cell responses would be revealed by more detailed T cell functional assays, we demonstrate in this study that there is little difference between neonates and adults in the recruitment of CD4 and CD8 T cells to the lungs or in the generation of effector memory (CD62Llow) T cell populations in response to MAV-1 infection (Fig. 5). Human fetal stem cells possess a greater potential to differentiate to Tregs than do adult stem cells (24). Consistent with these human data, MAV-1 infection induced a transient increase in the percentage of Tregs in the lungs of MAV-1-infected neonates, while lower percentages of Tregs were detected in the lungs of infected adult mice (Fig. 5K and L). It is possible that this relative increase in a lymphocyte population with suppressive capability contributed to increased viral replication in the lungs of neonates. It is also possible that the exclusion of lymphocytes or other immune cells from the airways of neonates, as described during influenza virus infection (23), facilitates increased viral replication in the neonatal lung. This may be the case in our experiments, in which immune cells were recruited to the airways of infected adults but not neonates (Fig. 4). The difference in recruitment of cells to the airways and other differences in the distribution of inflammatory responses in the lungs (Fig. 4) could also be explained if replicating virus were localized differently in the lungs of neonates than in adults.

Despite greater MAV-1 replication in the lungs of neonates than in those of adults during the acute phase of infection, clearance of virus from the lungs by 21 dpi occurred with equal efficiency. In addition, we demonstrated that primary infection of neonates provided protection against a second infection 28 days later (Fig. 6). Reinfection of mice that were previously infected as neonates did not induce lung production of IFN-γ or other measured cytokines, although it is possible that we missed responses that occurred earlier after reinfection by making measurements only at 7 dpi. The higher percentage of effector memory CD8 T cells that was noted during the acute phase of infection of neonates was also seen at this later time point (Fig. 6, MAV-1/mock and MAV-1/MAV-1 groups), suggesting the possibility that this lymphocyte population contributes to protective responses induced by primary infection of neonates. A similar protective CD8 T cell response has been described following neonatal RSV infection (37). Additional components of adaptive immunity are likely to contribute to this protective effect. For instance, B cell deficiency and Btk deficiency increase susceptibility to MAV-1, and antiserum from immune adult mice protects Btk⁻/⁻ mice (26). We plan to investigate the role of these and other adaptive immune responses in neonates with ongoing work in our laboratory.

In conclusion, we present data that provide evidence for increased susceptibility of neonates to MAV-1 respiratory infection. Increased lung viral loads were associated with impaired IFN-γ responses, impaired recruitment of inflammatory cells to the airways, and a transient increase in Tregs in the lungs of neonates. Despite these differences between neonates and adults in virus-induced immune responses during the acute phase of infection, our results suggest that adaptive responses to MAV-1 respiratory infection are relatively intact in neonates. Lung T cell populations developed in infected neonates and adults in similar manners, and primary infection of neonates provided protection against reinfection. Continued work with this model will provide valuable insight into neonatal immune function, potentially aiding in the design of vaccines to prevent respiratory viral infection.

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REFERENCES


