

Age at First Viral Infection Determines the Pattern of T Cell-mediated Disease during Reinfection in Adulthood

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Abstract

Infants experiencing severe respiratory syncytial virus (RSV) bronchiolitis have an increased frequency of wheeze and asthma in later childhood. Since most severe RSV infections occur between the 8th and 24th postnatal week, we examined whether age at first infection determines the balance of cytokine production and lung pathology during subsequent rechallenge. Primary RSV infection in newborn mice followed the same viral kinetics as in adults but was associated with reduced and delayed IFN- γ responses. To study rechallenge, mice were infected at 1 day or 1, 4, or 8 weeks of age and reinfected at 12 weeks. Neonatal priming produced more severe weight loss and increased inflammatory cell recruitment (including T helper 2 cells and eosinophils) during reinfection, whereas delayed priming led to enhanced interferon γ production and less severe disease during reinfection. These results show the crucial importance of age at first infection in determining the outcome of reinfection and suggest that the environment of the neonatal lung is a major determinant of cytokine production and disease patterns in later life. Thus, simply delaying RSV infection beyond infancy might reduce subsequent respiratory morbidity in later childhood.

Key words: bronchiolitis • asthma • immunity • pneumovirinae • virus

Introduction

Although perinatal antigen exposure sometimes leads to T cell tolerance, neonates can generate immune responses to some vaccines and antigens, depending on the dose and the route of delivery (1–3). However, neonates tend to display Th2-biased responses with prolonged memory, whereas Th1 memory is unstable (4–6) and neonatal vaccination is often unsuccessful (7).

Respiratory syncytial virus (RSV) infects ~65% of children in the first year of life and causes most cases of viral bronchiolitis, the risk of which peaks at 2–6 mo of age (8). Severe infantile RSV infections are associated with recurrent wheezing and asthma diagnosis in later life (9), an effect which lasts 10–12 yr. Infants with bronchiolitis exhibit Th2-biased responses to nonspecific PBMC stimulation both during and after infection (10), and peripheral T cells from 7–8-yr-old children with a history of bronchiolitis frequently make IL-4 after stimulation with RSV in vitro (11).

We suspected that age at first infection might play a key role in shaping later immune responses. Since RSV regularly reinfects older children and adults (causing common colds, exacerbating preexisting lung disease and contributing to winter respiratory morbidity and mortality in the elderly), we studied the effects of age at first infection on reinfection in mice. We show that the timing of neonatal infection establishes the subsequent pattern of T cell responses and, consequently, the nature and severity of disease during of reinfection in adulthood.

Materials and Methods

Mice and Viral Stocks. BALB/c mice (Harlan Olac Ltd.) were maintained in individual filter cages. Pregnant mice were purchased at <14 d gestation and pups weaned at 3 wk. RSV A2 strain was grown in HEp-2 cells and the viral titre determined by plaque assay. Mice were infected intranasally at 1, 7, 28, or 56 d of age with 4×10^4 PFU of RSV per g body weight. In 1- and 7-d mice, virus was instilled without anesthesia in 5 or 10 μ l, and mice gently restrained until inhalation occurred. Older mice were lightly anaesthetized with isoflurane. All mice were reinfected at 12 wk of age. All work was approved and licensed appropriately.

Recovery of Cells. 7 d after rechallenge, mice were terminally anaesthetised and exsanguinated via femoral vessels. Lungs were

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inflated via the trachea five times with 1 ml EBSS with 12 mM lidocaine; 100 μ l of lavage was spun onto glass slides, air dried, and stained with H&E. Viable cells were counted with Trypan blue. For whole lung cell preparation, lungs were pressed through 100- μ m mesh, RBC lysed, and cells washed.

Flow Cytometry for Intracellular Cytokines. Lung cells were stimulated with PMA and ionomycin and stained with combinations of CD4-Tricolor (RM4-5), CD8-FITC (5H10) (Caltag), IL-5-PE (TRFK-5), IL-10-PE (JES5-16E3), IFN- γ -PE (XMG1.2), and IL-4-PE (11B11) (BD PharMingen) before analysis on a FACScalibur™ (Becton Dickinson) (12). Lymphocytes were gated for according to their forward and side scatter properties: the percentage that stained for CD4 or CD8 and the percentage of these groups that expressed particular cytokines was determined.

CTL Responses. CTL responses were measured from mice infected at 1 or 8 wk old as described previously (13). In brief, splenocytes were grown for 6 d with 1 μ M of RSV M2 82-90 peptide and IL-2; CTL activity was tested against peptide-pulsed P815 targets.

Quantification of Viral and Cytokine RNA. RNA was extracted from the lung with RNA-Stat 60 (Tel-Test Inc.) and cDNA generated with random hexamers using an Omniscript RT-kit (QIAGEN). PCR specific for RSV L gene was performed at 50°C for 2 min and 95°C for 10 min followed by 40 two-step cycles (15 s at 95°C, 1 min at 60°C) using Universal PCR Mastermix (Applied Biosystems) and 900 nM forward primer (5'-GAACTCAGTGTAGGTAGAATGTTTGCA-3'), 300 nM reverse primer (5'-TTCAGCTATCATTCTCTGCCAAT-3'), and 100 nM probe (5'-FAM-TTTGAACCTGTCTGAACAT-TCCCGTT-TAMRA-3'). Copy number was determined from standard curves of pCDNA3 plasmid vector containing a fragment of the RSV L-gene (gift of C. Ward). For IFN- γ mRNA, PCR was performed as described previously (14), but with Universal PCR Mastermix, quantified relative to a single positive control. Taqman GAPDH Control Reagents (Applied Biosystems) confirmed equal quantities of input cDNA, and PCR amplifications were measured in real time using the ABI 7700 (Applied Biosystems) and analyzed using Sequence Detections Systems (v1.6.3).

Statistical significances were assessed by ANOVA.

Results

Mice were infected with RSV as neonates (1 or 7 d old), as young mice (4 wk) or as young adults (8 wk) and rechallenged in adulthood (12 wk of age). Mice infected as neonates developed severe illness during rechallenge, weight loss peaking at 18% on day 6. By contrast, mice first infected in later life (or undergoing primary adult infection) exhibited only minor (<5%) weight loss ($P < 0.0001$; Fig. 1).

To see how primary infection was affected by age, 7-d pups and 8-wk mice were infected. Pups continued to thrive during primary infection, gaining weight at an equal rate to those that had received UV-inactivated RSV (Fig. 2 a), whereas young adult mice suffered slight weight loss (Fig. 2 b). Although the pup's lungs were smaller than those of adults, they contained similar numbers of cells and did not greatly increase in cellularity during primary infection (1.9 ± 0.7 in uninfected vs. $2.27 \pm 0.3 \times 10^6$ in 7-d-infected pups), whereas there was an increase in pulmonary

cellularity during primary infection in adults (1.7 ± 0.5 vs. $3.4 \pm 0.8 \times 10^6$).

Copy number of RSV L-gene RNA was lower in pups than adults, but peaked on day 4 and declined similarly regardless of age. (Fig. 2 c). However, IFN- γ mRNA levels were reduced and delayed in pups (Fig. 2 d), reflected also in CD4⁺ cells staining for intracellular IFN- γ (neonates, day 2: $0.9 \pm 1.0\%$; day 4: $0.5 \pm 0.3\%$; day 7: $5.4 \pm 0.9\%$; adults, day 2: $1.6 \pm 0.6\%$; day 4: $4.7 \pm 1.8\%$; day 7: $13.4 \pm 6.2\%$). To confirm CTL priming in neonates, mice infected at 1 or 8 wk were killed after 3 wk or at 11 wk of age and CTL isolated from splenocytes. In either case, RSV-specific CTL were found (but were absent from mice given UV-inactivated RSV), confirming immunological CTL priming dependent on virus infection.

During reinfection, mice first infected at 1 d recruited the most cells to the lung (Fig. 3 a), showing that increased weight loss reflected greater lung inflammation. In both the lung and the bronchoalveolar lavage (BAL), mice first infected at 1 d or 1 wk recruited relatively more CD8⁺ T cells and fewer CD4⁺ T cells to the lung during rechallenge (Fig. 3, b and c). Cytospin preparations of the BAL fluid showed striking pulmonary eosinophilia upon reinfection, which was greatest in neonates first infected at 1 d of age ($P < 0.05$; Fig. 4 a). All groups developed a neutrophilia after secondary challenge, but it was most marked in those first infected when very young (Fig. 4 b). Neutrophil numbers in mice first infected at 1 d or 1 wk of age were not significantly different, but were significantly higher than those first infected later in life ($P < 0.05$ at 4 wk and $P < 0.01$ at 8 wk).

To determine whether the interval between priming and challenge accounted for the results, mice were in-

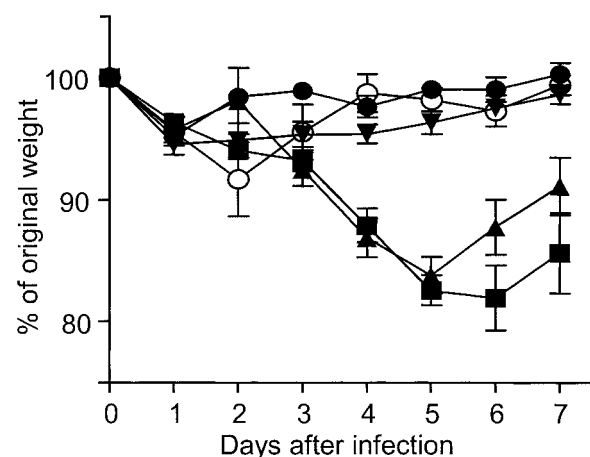


Figure 1. Age at primary infection determines weight loss after secondary challenge. Mice were weighed daily after RSV rechallenge at 12 wk of age. Groups had been previously infected at 1 d (black squares), 1 (black triangles), 4 (inverted black triangles), or 8 wk (black circles) of age or had not previously been challenged (white circles). Data is normalized to group weight at challenge and shown as mean \pm SEM. Weight loss in groups first infected as pups were significantly lower on day 3 ($P < 0.01$) and days 4-7 ($P < 0.0001$). There was no significant difference between groups first infected after weaning (4 wk, 8 wk, or no primary infection) and no difference between groups first infected as pups (1 d or 1 wk primary) ($P > 0.05$).

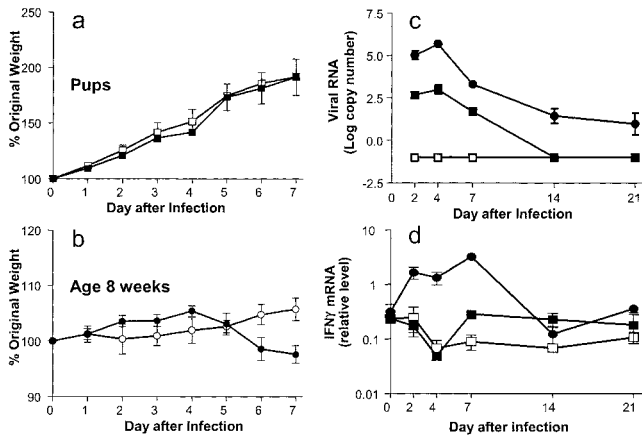


Figure 2. Primary RSV infection. Mice were infected at 1 or 8 wk old and weighed daily for 7 d. In neonates, each litter was weighed and the average weight per pup in each litter calculated (a). Data shows the average of three litters, either RSV (black squares) or UV-RSV (white squares) challenged. Average weight of adult mice (b) either RSV (black circles) or UV-RSV (white circles) challenged. Copy number of RSV L-gene RNA (c) or relative IFN- γ mRNA production (d) was determined by RT-PCR on whole lung from mice infected at 1 (black squares) or 8 wk of age (black circles) or in 1-wk-old mice challenged with UV-inactivated RSV (white squares).

infected at 1 wk and then reinfected at 4 wk, comparison being made with mice first infected at 8 wk then reinfected at 11 wk (i.e., the interval between primary and secondary infection was maintained at 3 wk). Mice first infected as pups developed a severe weight loss after rechallenge, losing $20.7 \pm 10.9\%$ weight and developing pulmonary eosinophilia ($5.8 \pm 4.3\%$ of BAL cells), which was absent in mice first infected at 8 wk or undergoing primary infection as adults. Therefore, the critical factor is age of primary infection rather than interval between primary infection and rechallenge. Mice receiving UV-inactivated RSV as neonates did not develop severe weight loss during secondary challenge (unpublished data), show-

ing that live viral infection was necessary for priming the augmented responses.

Intracellular staining of lung lymphocytes showed that the proportion of CD4⁺ lymphocytes expressing IFN- γ during reinfection increased with age at first infection. Intracellular IL-4 showed a reverse trend (Figs. 5 and 4, c and d). IL-10 followed a similar pattern to IFN- γ , but the difference between groups did not reach significance (unpublished data). By contrast, the proportion of IFN- γ ⁺ CD8⁺ cells was unaffected by the age of primary infection (Fig. 4 e) and very few (<1%) stained for IL-4 (Fig. 4 f) or IL-10 (unpublished data) in any situation.

Discussion

Our results show that the age of primary infection with RSV has a profound effect on the outcome of RSV rechallenge and that weight loss during rechallenge is driven by inappropriate immune responses primed by infection in early life. The stronger inflammatory response during reinfection in mice first infected as pups was not due to a stronger response during primary infection, nor to higher viral replication and delayed clearance, but to immune-driven disease augmentation.

The strongest Th2 responses were seen in mice primed at the youngest age, suggesting that the eosinophilic lung disease seen in adults primed as neonates is likely to be caused by CD4 T cells making type 2 cytokines. Others have suggested a role for IL-5-producing CD8⁺ (Tc2) cells in driving Th2 responses to RSV (15), but we found no evidence for Tc2 cells in our model. Had neonates failed to generate a CTL response, this might have explained the subsequent eosinophilia, since we and others have previously shown that CD8⁺ T cells and NK cells making IFN- γ play a role in preventing it (16, 17). However, neonatal and adult mice both developed good CTL responses after primary infection and the proportion of CD8⁺ cells during

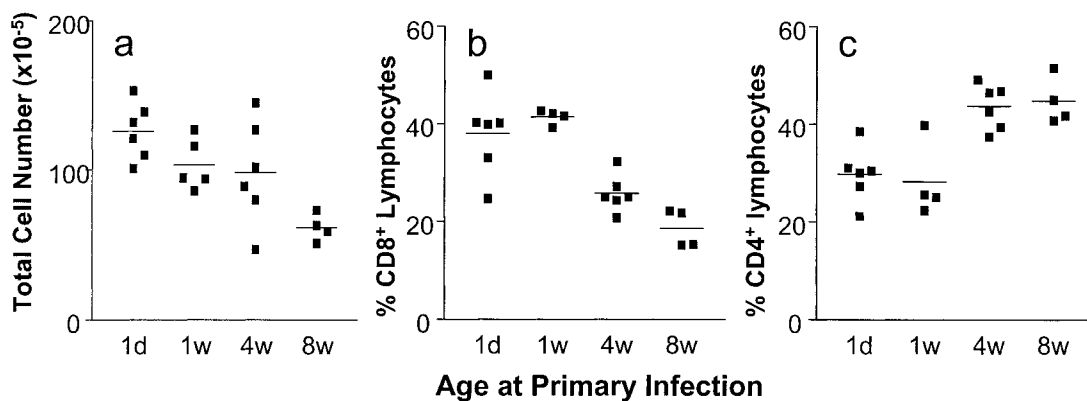


Figure 3. Age at primary infection determines inflammatory cell recruitment after secondary challenge. Mice were infected with RSV at either 1 d, 1, 4, or 8 wk of age and rechallenged at 12 wk of age. On day 7 after secondary challenge, viable cell numbers in bronchoalveolar lavage were significantly higher in mice first infected at 1 day of age than in mice first infected as adults ($P < 0.01$). Primary infection as neonates (1 d of age or 1 wk of age) resulted in a significantly higher proportion of CD8⁺ cells ($P < 0.01$) recruited to the lung after secondary infection (b), and a lower proportion of CD4⁺ cells ($P < 0.001$) (c). There was no significant difference between groups first infected after weaning (4 wk or 8 wk) and no difference between groups first infected as pups (1 d or 1 wk primary) ($P > 0.05$).

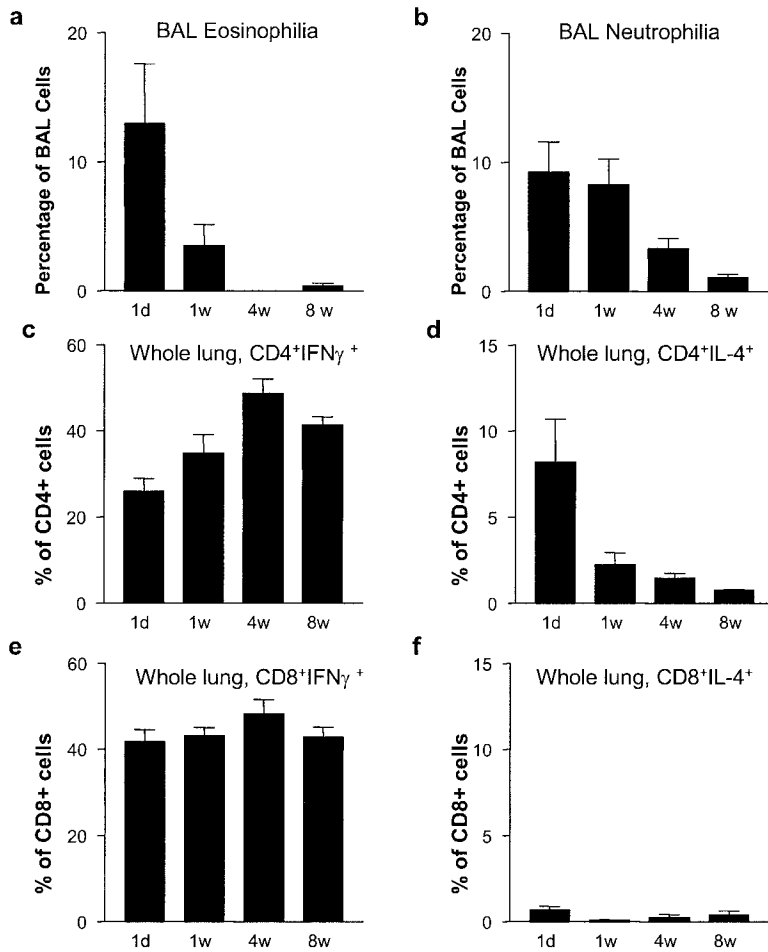


Figure 4. Cell recruitment to the lung after secondary infection. Eosinophil numbers in the BAL were greatest in neonates first infected at 1 d old ($P < 0.05$) and eosinophilia was absent in mice first infected at 4 or 8 wk of age (a). Neutrophil numbers also declined with age at primary infection (b). Lung cell suspensions were stained for CD4 or CD8 and the cytokines IFN- γ and IL-4. Data is expressed as the mean proportion of CD4⁺ and CD8⁺ cells staining for each cytokine \pm SD. There were significantly fewer CD4⁺ IFN- γ ⁺ cells in mice first infected as neonates than in those first infected at 4 or 8 wk of age (c; $P < 0.001$ and $P < 0.01$, respectively). Conversely, intracellular IL-4 staining decreased with increasing age at primary infection: Mice first infected at 1-d-old exhibited the greatest proportion of IL-4⁺ CD4⁺ cells (d; $P < 0.05$). The proportion of CD8⁺ cells staining for each of the cytokines did not differ significantly with the age at primary infection (e and f).

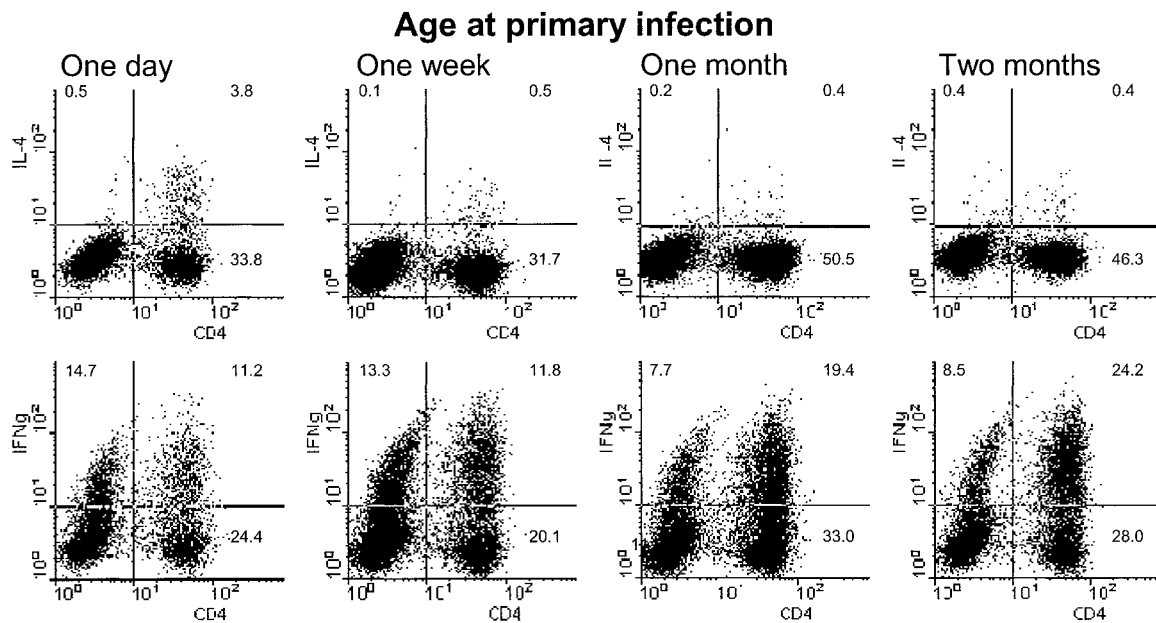


Figure 5. FACS[®] analysis of intracellular cytokine production during secondary infection. Lung cells were surface stained for CD4 and intracellular cytokine staining performed for IFN- γ and IL-4. The proportion of total lymphocytes staining for IL-4 production is greatest in pups first infected at 1 d of age, and the majority of this cytokine is within the CD4⁺ population. Conversely, the proportion of lymphocytes staining for IFN- γ increases with increasing age at primary infection. In pups infected as neonates the IFN- γ production is approximately equally distributed between CD4⁺ and CD4⁻ cells. However, in mice first infected at 4 or 8 wk, CD4⁺ cells account for a higher proportion of the IFN- γ -producing cells.

adult reinfection decreased as age at first infection increased. Moreover, the proportion of IFN- γ ⁺ CD8⁺ cells was unaffected by the age of primary infection.

In both mouse and man, primary RSV infection rarely leads to pulmonary eosinophilia. However, adult BALB/c mice develop a Th2 response to RSV after dermal scarification with recombinant vaccinia virus expressing the RSV G-protein (vvG) (18) which primes a dominant population of V β 14⁺ CD4⁺ cells specific for the 183–197 epitope (19, 20). Heightened T cell responses are believed to underlie the cellular infiltrate seen in younger children who received formalin-inactivated RSV vaccine (21, 22), although heightened bronchial reactivity may have been related to immune complex deposition and complement activation (23).

Bronchiolitis may be the first manifestation of a predisposition to recurrent respiratory disorders or severe RSV disease may lead directly to chronic, persistent, or delayed disease. Our current studies show that disease enhancing responses can be induced by normal exposure to live RSV if infection occurs in the early postnatal period. This finding supports the proposition that the delayed effects of infantile bronchiolitis are acquired, and that neonatal RSV infection could itself cause subsequent recurrent wheeze.

Infections in early life seem to play an important role in shaping immunological development. According to the hygiene hypothesis, lack of exposure to Th1-promoting pathogens (or their components) contributes to the rise in the incidence of allergy and asthma (24). Existing T cell memory to one respiratory viral infection has been shown to profoundly alter the nature of the Th1/Th2 balance and dynamics of subsequent infection with an antigenically unrelated virus (25, 26). Therefore, it is possible that Th2-biased memory responses to RSV may direct responses to other antigens in the lung toward a more 'allergic' phenotype. Others have reported reduced and delayed IFN- γ production after neonatal challenge with *Pneumocystis carinii* and speculated that this was due to suppression of T cell responses in the environment of the neonatal lung (27). Our data suggest that such differences in neonatal immune responses can have long-lasting consequences for the host. We suggest that a weaker Th1 response during primary infection may allow the development of stronger Th2 responses to rechallenge later in life, so explaining the enhanced disease, lymphocyte, and granulocyte recruitment in adult reinfection.

It is currently unclear whether the Th2 bias in the neonatal immune system is due to the T cells, their environment, or both. For example, neonatal dendritic cells are deficient in IL-12 production, an important factor in the differentiation of Th1 cells (28) and respiratory tract dendritic cells are less numerous and mature in the neonatal than in the adult lung (29). The intrinsic properties of the T cell may differ in neonates. For example, reduced IFN- γ production by neonatal CD4⁺ T cells may be explained by differential patterns of methylation of the IFN- γ promoter (30).

In conclusion, our results show that the timing of RSV infection in newborn mice is a crucial factor in determining the outcome of reinfection in adulthood and that early neonatal infection is associated with long lasting bias toward Th2 responses to rechallenge. Therefore, delaying

RSV infection might avoid the delayed consequences of bronchiolitis, possibly reducing the frequency of respiratory symptoms in later childhood. These novel findings highlight the importance of early life infections in determining subsequent patterns of disease and suggest that efforts should be directed toward delaying RSV disease until children are more immunologically mature.

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