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The Enhancement or Prevention of Airway Hyperresponsiveness during Reinfection with Respiratory Syncytial Virus Is Critically Dependent on the Age at First Infection and IL-13 Production[1]

Azzeddine Dakhama,[2,3] Jung-Won Park,[3] Christian Taube, Anthony Joetham, Annette Balhorn, Nobuaki Miyahara, Katsuyuki Takeda, and Erwin W. Gelfand

Respiratory syncytial virus (RSV) infection in early life is suspected to play a role in the development of postbronchiolitis wheezing and asthma. Reinfection is common at all ages, but factors that determine the development of altered airway function after reinfection are not well understood. This study was conducted in a mouse model to define the role of age in determining the consequences on airway function after reinfection. Mice were infected shortly after birth or at weaning and were reinfected 5 wk later, followed by assessment of airway function, airway inflammation, and lung histopathology. Infection of mice at weaning elicited a protective airway response upon reinfection. In this age group, reinfection resulted in increased airway inflammation, but without development of airway hyperresponsiveness (AHR) or eosinophilia and decreased IL-13 levels. By contrast, neonatal infection failed to protect the airways and resulted in enhanced AHR after reinfection. This secondary response was associated with the development of airway eosinophilia, increased IL-13 levels, and mucus hyperproduction. Both CD4- and CD8-positive T cells were a source of IL-13 in the lung, and inhibition of IL-13 abolished AHR and mucus production in these mice. Inoculation of UV-inactivated virus failed to elicit these divergent responses to reinfection, emphasizing the requirement for active lung infection during initial exposure. Thus, neonatal RSV infection predisposes to the development of airway eosinophilia and enhanced AHR via an IL-13-dependent mechanism during reinfection, whereas infection at a later age protects against the development of these altered airway responses after reinfection. ***The Journal of Immunology,*** **2005, 175: 1876–1883.**

Respiratory syncytial virus (RSV)[4] is the most common cause of acute bronchiolitis that primarily affects infants and young children worldwide (1). In the United States, RSV bronchiolitis was identified as the leading cause of infant hospitalization annually between 1997 and 2000, at estimated total costs of more than $2.6 billion (2). It has also been estimated that >200 deaths occur annually; ~80% of these deaths occur among infants (<1 year old) as a result of severe lower respiratory tract illness caused by RSV (3). Factors that predispose to severe RSV-related illness include premature birth and pre-existing conditions, such as bronchopulmonary dysplasia, congenital heart disease, and immune deficiency (4).

RSV infection can occur throughout life. However, unless immunocompromised either naturally or medically (5), adults develop mild symptoms, which are usually restricted to the upper airways (6). By contrast, individuals of the youngest ages are the most susceptible to develop severe symptoms, particularly involving the lower airways (7). Conceivably, because the size of the airways is smaller in young babies than in older children, they might be easily obstructed by inflammation, edema, and mucus, important physical factors that may contribute to the development of more severe lower respiratory tract illness. However, not all infants develop severe lower respiratory tract illness after RSV infection, suggesting that other factors, most likely host immune responses, are involved in determining and limiting the severity of the disease (8).

Almost all young children experience RSV infection at least once in the first few years of life, and >65% become infected during their first year, with the peak incidence for lower respiratory disease occurring between 2 and 7 mo of age (7, 9). Most intriguing, however, is the clinical and epidemiological associations that exist between RSV lower respiratory tract infections in early life and the subsequent development of persistent wheezing and asthma (10–13). Whether RSV infection in infancy causes subsequent alterations in airway function or whether underlying abnormalities predispose the young individual to develop persistent lower airway dysfunction after RSV infection are issues that remain to be resolved (14, 15). Nevertheless, recurrent RSV infection is frequent at all ages (9, 16), and it is possible that the type of host response that is elicited upon reinfection might distinguish which children will develop persistent wheezing and possibly asthma. We hypothesized that age at first RSV infection is a critical factor in determining the phenotype of airway response to subsequent RSV infection. The present study was conducted in a mouse model of RSV-induced AHR and inflammation. Mice were

Department of Pediatrics, Division of Cell Biology, National Jewish Medical and Research Center, Denver, CO 80206

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[2] Address correspondence and reprint requests to Dr. Azzeddine Dakhama, National Jewish Medical and Research Center, Denver, CO 80206. E-mail address: dakhamaa@njc.org

[3] A.D. and J.-W.P. contributed equally to this work.

[4] Abbreviations used in this paper: RSV, respiratory syncytial virus; AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; BM, basement membrane; cIg, control Ig; MBP, major basic protein; MCh, methacholine; PAS, periodic acid-Schiff; uvRSV, UV-inactivated RSV.

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first infected with purified RSV shortly after birth (at <1 wk of age) or at weaning (at 3 wk of age), then reinfected 5 wk later, after complete resolution of AHR and airway inflammation. Airway function, airway inflammation, and cytokine production were assessed after both primary and secondary infections. The results were compared with those obtained at the same time after primary infection of age-matched animals and demonstrated the important influence of age at first infection on the response to reinfection.

Materials and Methods

Animals

BALB/c mice were obtained from The Jackson Laboratory. Mice were bred and maintained under pathogen-free conditions at the Biological Resource Center, National Jewish Medical and Research Center. Mice were used under an experimental protocol approved by the institutional animal care and use committee.

Virus preparation

Strain A2 of human RSV (catalogue no. VR-1302) was obtained from American Type Culture Collection. Stocks of purified RSV were prepared under endotoxin-free conditions and were assessed to be free of endotoxin contamination using a *Limulus* amebocyte lysate-based Pyrogent Plus Gel Clot assay with a sensitivity of 0.06 endotoxin units/ml (BioWhittaker; catalogue no. N283-06). The virus was propagated in monolayers of HEp-2 cells (American Type Culture Collection; catalogue no. CCL-23) grown in MEM (Invitrogen Life Technologies) supplemented with 5% FBS. At maximum cytopathic effect, the cells were harvested and disrupted by sonication in the same culture medium. The suspension was clarified by centrifugation at 10,000 × *g* for 15 min at 10°C, and the resulting supernatant was layered on top of a sucrose gradient (30% in 50 mM Tris-buffered normal saline solution containing 1 mM EDTA, pH 7.5) and additionally centrifuged at 100,000 × *g* for 2 h at 10°C. The pellet containing purified virus was resuspended in 10 mM PBS (pH 7.4) containing 15% sucrose and stored in aliquots at −70°C. Viral titers were determined by a standard plaque assay combined with immunostaining for RSV using a biotinylated goat anti-human RSV Ab (Accurate Chemical & Scientific) and an avidin-biotin peroxidase detection system (DakoCytomation). Uninfected HEp-2 cell cultures were similarly processed to obtain a control, virus-free, sham preparation.

Experimental design

At the indicated ages, mice were inoculated intranasally with 10^6 PFU of purified RSV, resuspended in 10 μl (for <1-wk-old mice) or 20 μl (for mice aged ≥3 wk of age) of endotoxin-free PBS. Age-matched control mice were inoculated with the virus-free, sham preparation or UV light (UV)-inactivated RSV. Full inactivation of RSV, as assessed by viral plaque assay, was achieved by exposing the viral suspension in a sterile glass vial to an UV light source (302 nm Chromato-Vue Lamp UVM-57) for 16 h at 4°C. The inactivated viral suspension was administered so that each mouse received a number of virions that would be equivalent to 10^6 PFU of active virus. Except for the youngest age (<1 wk old), all mice were inoculated under light anesthesia (2.5% avertin, 0.01 ml/g body weight). Airway function and airway inflammation were assessed at the peak of the response, as shown previously (17), on day 6 after infection. Immediately after measurement of airway function, the whole lung was lavaged with 1 ml of HBSS (twice with 0.5 ml after primary infection for the youngest animals), followed by fixation and processing of the right lobes into paraffin (whole lung for youngest animals, after primary infection). The left lobe (or whole lung from separate experiments with the youngest animals) was homogenized and used for determination of lung viral titers and IL-13 levels. The murine IL-13Rα2-human IgG fusion protein (IL-13i; provided by Dr. D. D. Donaldson, Wyeth Pharmaceuticals, Cambridge, MA), a potent IL-13 inhibitor of allergen-induced AHR and inflammation (18), was prepared as previously described (19). IL-13i was administered as previously reported (20) by i.p. injection (300 μg/mouse) 24 h before and on days 1, 3, and 5 after infection.

Assessment of airway function

Airway function was assessed in anesthetized, mechanically ventilated animals by measuring changes in lung resistance in response to increasing doses of inhaled methacholine (MCh) as previously described (21). Data are expressed as the percent change from baseline lung resistance obtained after inhalation of saline.

Measurement of cytokines

Levels of IFN-γ, IL-4, and IL-5 were measured in bronchoalveolar lavage fluid (BALF) using commercial ELISA kits according to the manufacturer's instruction (BD Pharmingen). The detection limits of the assays were as follows: IFN-γ, 62.5 pg/ml; IL-4, 7.8 pg/ml; and IL-5, 15.6 pg/ml. IL-13 was measured in lung tissue homogenate using a sensitive ELISA (limit of detection, 1.5 pg/ml; R&D Systems), as previously described (20).

Assessment of airway inflammation and mucus production

Airway inflammation was assessed by total and differential counting of cells recovered in BALF and by histopathological examination of lung tissue sections stained with H&E. Tissue-infiltrating eosinophils were detected by immunostaining of tissue sections using a polyclonal rabbit anti-eosinophil major basic protein (MBP; provided by Dr. J. J. Lee, Mayo Clinic, Scottsdale, AZ). Mucus-producing goblet cells were detected by staining of tissue sections using the periodic acid-Schiff (PAS) method. For quantitative analyses, the data were normalized to the perimeter of the basement membrane (BM) of the airway epithelium as previously described (22). The data are expressed as the mean ± SEM of MBP^+ and PAS^+ cells per millimeter of BM.

Detection of IL-13 in lung T cells

Intracellular IL-13 was detected by immunofluorescent staining of lung CD4 and CD8 T cells, as previously described (23). Briefly, lung T cells were isolated by collagenase digestion of lung tissue, followed by separation of mononuclear cells by density gradient centrifugation (Organon Teknika). Lung mononuclear cells were stimulated in culture for 4 h with PMA (5 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (10 μg/ml). After staining for cell surface markers with CyChrome-conjugated anti-CD3 (Caltag Laboratories) and FITC-conjugated anti-CD4 or anti-CD8 Abs (BD Pharmingen), cells were washed, fixed in paraformaldehyde (4% in PBS), and permeabilized with saponin (0.1% in PBS). After incubation with normal goat serum to block nonspecific background staining, the permeabilized cells were incubated for 30 min with a biotinylated goat anti-mouse IL-13 Ab or a similarly conjugated isotype-matching control Ab (R&D Systems). Cells were washed and stained for 30 min with PE-conjugated streptavidin (BD Pharmingen). Staining was analyzed by flow cytometry with gating on the CD3-positive cell population using CellQuest software on a FACSCalibur (BD Biosciences).

Statistical analysis

Data are expressed as the mean ± SEM of six to eight mice in each group. Statistical significance at a level of $p < 0.05$ was determined by ANOVA using the StatView 4.5 statistical analysis software package (Abacus Concepts). Differences between the groups were determined by multiple comparisons using Fisher's protected least significant difference test.

Results

Age-related differences in airway responsiveness to primary RSV infection

To examine whether the age at first infection determines the extent of airway responsiveness to a primary RSV infection, mice were first infected shortly after birth (<1 wk of age) or at weaning (3 wk of age). Airway function and airway inflammation were assessed on day 6 after infection. Without infection, baseline resistance was significantly higher in the youngest mice (mean ± SEM, 2.25 ± 0.08 cm $H_2O/ml \cdot s^{-1}$), reflecting smaller airway size, compared with weanling mice (mean ± SEM, 1.24 ± 0.05 cm $H_2O/ml \cdot s^{-1}$; $p < 0.01$). Similar to sham-inoculated mice, mice inoculated with UV-inactivated virus (uvRSV) exhibited normal airway responsiveness to inhaled MCh in both age groups (Fig. 1, *A* and *B*). After RSV infection, the baseline resistance was not altered in any of the age groups (2.36 ± 0.08 cm $H_2O/ml \cdot s^{-1}$ for the youngest mice and 1.36 ± 0.06 cm $H_2O/ml \cdot s^{-1}$ for weanling mice). However, RSV infection resulted in the development of AHR in response to inhaled MCh in both age groups.

After primary infection, the numbers of lymphocytes recovered in BALF were significantly increased for both age groups compared with either sham-inoculated or uvRSV-inoculated, age-matched control groups (Fig. 1, *C* and *D*). The extent of the lymphocytic response, however, was lower in the youngest group. In

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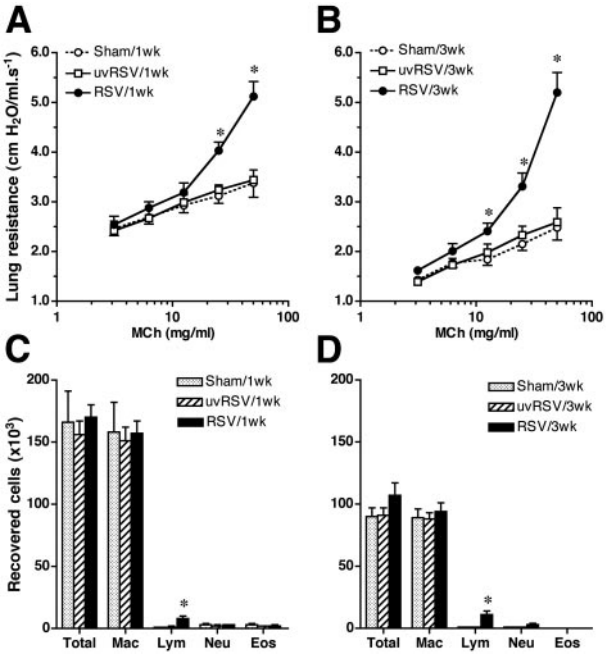

FIGURE 1. Primary RSV infection in newborn and weanling mice: airway function and BAL cellularity. Groups of mice were inoculated as newborns (<1 wk of age (1wk)) or at weaning (3 wk of age (3wk)) with RSV (n = 8/group), uvRSV (n = 4/group), or a sham preparation (n = 6/group). Airway responsiveness to inhaled MCh (*A* and *B*) and bronchoalveolar lavage cellularity (*C* and *D*) were assessed on day 6 after infection. In both age groups, primary RSV infection induced significant AHR and lymphocyte accumulation in the airways. *, Significant difference compared with sham or uvRSV group ($p < 0.05$).

tissue, RSV infection resulted in the development of a characteristic peribronchial and perivascular airway tissue infiltration predominantly by mononuclear cells (Fig. 2). Compared with mice infected at weaning, mice infected at <1 wk of age showed more mucus-producing cells in the airways (Fig. 2) and mild, but significant, airway tissue eosinophilia (Table I).

Cytokine levels were measured and compared between the two age groups. IFN-γ levels were higher in the BALF of RSV-infected weanling mice, but were barely detected in the BALF of RSV-infected newborn mice (Fig. 3*A*). By contrast, IL-13 levels were significantly higher in lung tissue homogenates of RSV-infected newborn mice compared with RSV-infected weanling mice (Fig. 3*B*). The levels of IL-4 and IL-5 were at the limit of the assay detection and were not different between the two age groups.

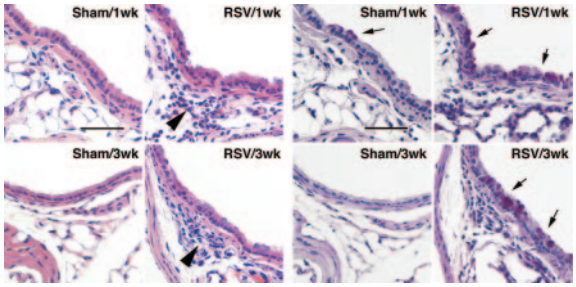

FIGURE 2. Primary RSV infection in newborn and weanling mice: lung histopathology. Lung tissue sections from RSV-infected or sham-inoculated mice (same groups as in Fig. 1) were stained with H&E (*left panels*) and PAS (*right panels*). In both age groups, RSV infection induced peribronchial airway tissue inflammation (arrowheads) and marked mucus production (arrows), which was higher in the youngest age group. Scale bars = 50 μm.

Virus replication and clearance were examined in the lungs of both age groups by measuring the amounts of replicating virus recovered from the lungs at different time points after inoculation. No replicating virus was recovered from the lungs of uvRSV-inoculated mice (data not shown), and no significant differences were detected between the two age groups in lung viral titers measured on day 1, 3, or 6 after RSV infection, indicating similar rates of viral replication and clearance for both age groups (Fig. 3*C*).

RSV infection at weaning protects mice against the development of AHR on reinfection

To define the pattern of airway response to a secondary RSV infection in mice initially infected at weaning and after full recovery, when no significant AHR or airway inflammation could be detected after the primary infection (data not shown), these mice were reinfected 5 wk later (at 8 wk of age). Airway function and airway inflammation were assessed in these animals on day 6 after the secondary infection, and the results were compared with those obtained in age-matched animals after primary infection with RSV (at 8 wk of age) with or without prior inoculation of uvRSV at weaning.

Compared with mice infected only once, at 8 wk of age (Fig. 4*A*) or at 3 wk of age (Fig. 2), mice that were initially infected at weaning and reinfected 5 wk later developed increased mononuclear airway tissue inflammation after secondary RSV infection (Fig. 4*C*). Mucus production and tissue-infiltrating eosinophil numbers, however, were not altered after secondary infection in these animals (Table I). In parallel, the numbers of lymphocytes recovered in BALF after reinfection at 8 wk were also increased compared with the numbers of lymphocytes recovered after a primary infection at either 3 or 8 wk of age (Fig. 4*D*). By contrast, inoculation of uvRSV at weaning did not alter the inflammatory response to subsequent infection with live RSV at 8 wk of age (Fig. 4, *B* and *D*). Most surprisingly, in contrast to age-matched control animals that developed significant AHR after a primary RSV infection at 8 wk of age, AHR was completely abolished in the reinfected animals despite the intense inflammatory response that developed in the lungs (Fig. 4*E*). In parallel, the peak viral titers were significantly reduced in the lungs of these animals (Fig. 4*F*).

After secondary RSV infection, the levels of IFN-γ detected in BALF were higher than those measured after a primary infection, but the peak response was shifted to an earlier time point after reinfection (Fig. 4*G*). By contrast, the amounts of IL-13 measured in lung tissue homogenates were significantly reduced after reinfection of mice that were initially infected at weaning (Fig. 4*H*).

Neonatal RSV infection predisposes mice to the development of eosinophilic airway inflammation and enhanced AHR after reinfection

To determine whether early age at infection predisposes to more severe alterations in airway responsiveness on RSV reinfection, mice were infected shortly after birth (<1 wk of age) and reinfected 5 wk later, when no significant AHR or airway inflammation could be detected after complete recovery from the primary infection (data not shown). Airway function and airway inflammation were assessed in these animals on day 6 after the secondary infection, and the results were compared with those obtained in age-matched animals that received a primary RSV infection (at 6 wk of age) with or without inoculation of uvRSV during the neonatal period (<1 wk of age).

Compared with mice infected only once, either at 6 wk of age (Fig. 5, *A* and *D*) or at <1 wk of age (Fig. 2), mice initially infected at <1 wk of age and reinfected 5 wk later developed an

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Table I. *Age-related differences in airway tissue eosinophilia and goblet cell hyperplasia after primary (1°) and secondary (2°) RSV infection*[a]

1° Infection 2° Infection	Sham	uvRSV	RSV	uvRSV RSV	RSV RSV
Tissue eosinophils (MBP-positive cells/mm BM)					
Age at 1° infection					
<1 wk	3.5 ± 0.8	3.3 ± 0.9	6.8 ± 1.0[b]	2.2 ± 0.8	15.6 ± 3.8[c]
3 wk	0.7 ± 0.3	0.5 ± 0.3	2.1 ± 0.9	1.5 ± 0.6	0.5 ± 0.1
6 wk	0.6 ± 0.3		1.6 ± 0.4		
8 wk	0.6 ± 0.2		1.3 ± 0.4		
Goblet cells (PAS-stained cells/mm BM)					
Age at 1° infection					
<1 wk	4.5 ± 1.2	5.0 ± 0.9	21.6 ± 3.0[b]	9.8 ± 3.3[b]	125.3 ± 15.8[c]
3 wk	1.3 ± 0.2	1.8 ± 0.5	13.3 ± 2.5[b]	12.3 ± 1.7[b]	10.0 ± 2.3[c]
6 wk	1.1 ± 0.2		11.0 ± 2.4[b]		
8 wk	1.0 ± 0.3		10.4 ± 2.3[b]		

[a] Data are expressed as the mean number of cells (±SEM) detected in central airways. Data are normalized to the length of airway epithelium BM.
[b] Significant difference compared with age-matched, sham-inoculated, control group ($p < 0.05$).
[c] Significant difference compared with 1° RSV in mice infected at <1 wk of age ($p < 0.05$).

additional increase in airway tissue inflammation (Fig. 5*C*) associated with markedly enhanced mucus production and pronounced airway tissue eosinophilia (Fig. 5*F* and Table I) after the secondary RSV infection. The numbers of lymphocytes recovered in the BALF of these animals were significantly increased after secondary RSV infection compared with those recovered after a primary infection in the age-matched group (Fig. 5*G*). Such altered responses, however, were not observed in animals that were first inoculated as neonates with uvRSV and subsequently infected with RSV 5 wk later (Fig. 5, *B*, *E*, and *G*). Unlike the primary infection, where only rare eosinophils were recovered in the BALF, the secondary infection induced significant airway eosinophilia, as shown by a markedly increased proportion and numbers of eosinophils in the BALF. Interestingly, in contrast to the absence of AHR after secondary infection in mice initially infected at weaning, AHR was not only observed, but was further increased after reinfection in mice that were initially infected early in postnatal life (Fig. 5*H*). However, despite the enhanced AHR and lung histopathology, the amounts of replicating virus recovered from the lungs of these mice were not different from those in age-matched control animals, which received a single RSV infection with or without previous inoculation with uvRSV (Fig. 5*I*).

Although IFN-γ was barely detected in BALF of mice that were infected only once, at <1 wk of age, the amounts of IFN-γ measured in BALF after reinfection at 6 wk of age were comparable to those measured after either a primary or a secondary infection in weanling mice (Fig. 5*J*). In parallel, the amounts of IL-13 detected in lung tissue homogenates were further increased after reinfection of mice that were initially infected at <1 wk of age (Fig. 5*K*).

Analysis of airway tissue-infiltrating T cells for intracellular IL-13 content by immunostaining and flow cytometry revealed that both CD4-positive and CD8-positive T cells expressed IL-13 (Fig. 6*A*), and the frequencies of these cells increased after reinfection of these animals (Fig. 6*B*). Of note, treatment of these mice during the secondary RSV infection with a recombinant murine IL-13Rα2-human IgG fusion protein (IL-13i) completely abolished AHR (Fig. 7*A*), indicating a central role for IL-13 in the altered airway response to secondary RSV infection in mice initially infected early in life. Inhibition of IL-13 also attenuated airway eosinophilia, without altering mononuclear cell infiltration, in both BALF (Fig. 7*B*) and airway tissue. The number (mean ± SEM) of airway tissue-infiltrating, MBP-positive cells was significantly lower in mice treated with IL13i (5.8 ± 1.4/mm BM) than in mice treated with control Ig (cIg; 18.3 ± 3.4/mm BM; $p < 0.05$). Furthermore, IL-13 inhibition completely abolished mucus hyperproduction in the airways of these animals (Fig. 7*D*). The number of PAS-positive goblet cells was dramatically reduced from 117 ± 13.2 cells/mm BM in cIg-treated animals to 16.4 ± 4.1 cells/mm BM in IL-13i-treated animals ($p < 0.05$).

Discussion

The objective of this study was to define the role of age in the development of altered airway function after reinfection with RSV.

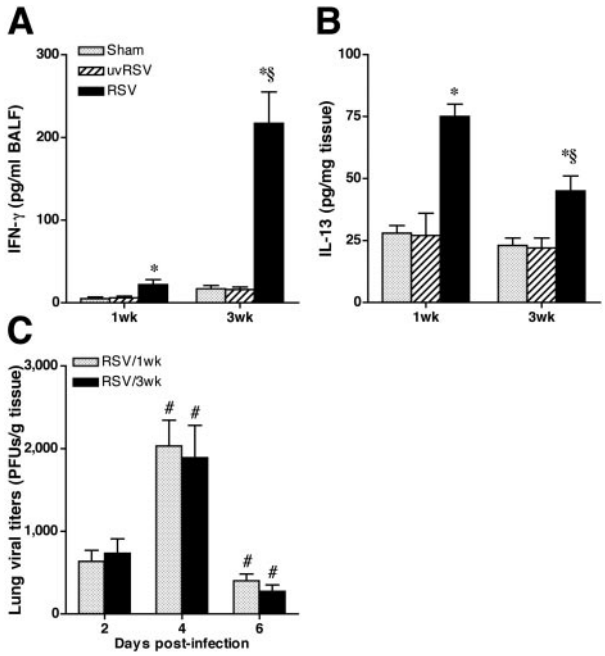

FIGURE 3. Primary RSV infection in newborn and weanling mice: cytokine levels and lung viral titers. BALF and lung tissue from RSV-infected, uvRSV-inoculated, and sham-inoculated mice (same groups as in Fig. 1) were examined for cytokine levels (*A* and *B*) and viral titers (*C*). Compared with weanling mice, newborn mice showed markedly lower levels of IFN-γ in BALF (*A*), but higher levels of IL-13 in lung tissue homogenate (*B*), after RSV infection. Lung viral titers were similar in both age groups (*C*). *, Significant difference compared with sham or uvRSV group ($p < 0.05$); §, significant difference compared with RSV-infected newborn mice ($p < 0.05$); #, significant difference compared with day 2 postinfection values ($p < 0.05$).

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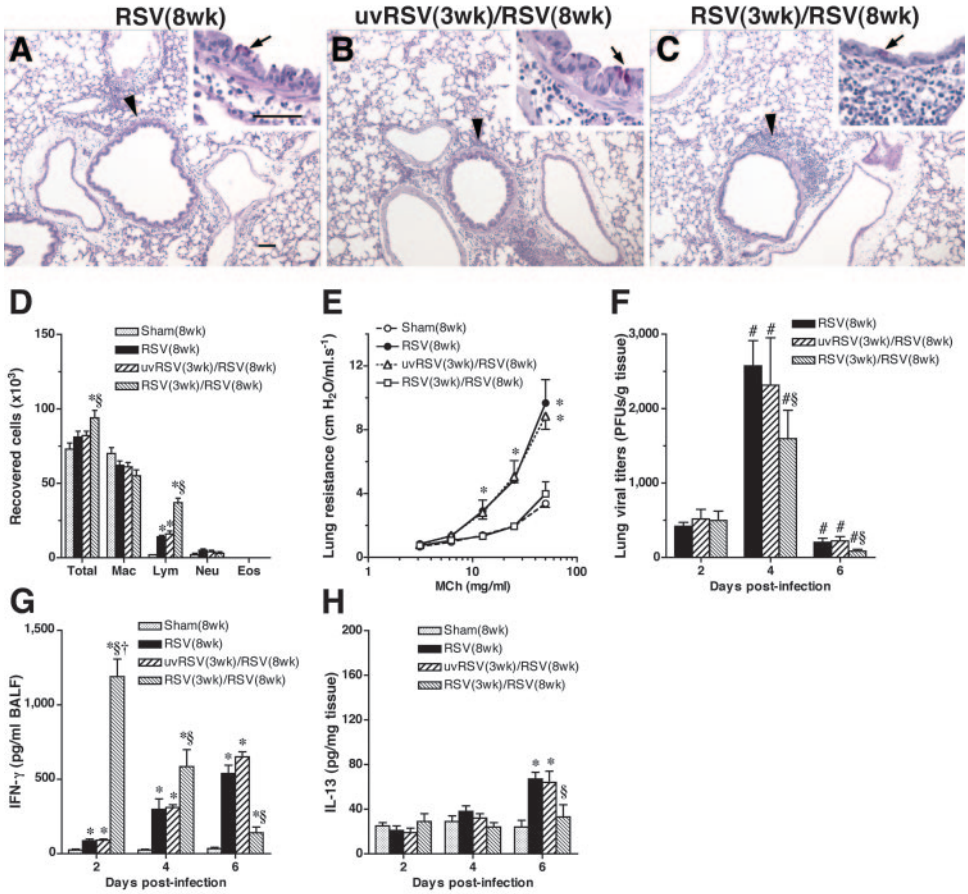

FIGURE 4. Airway responsiveness to secondary RSV infection: effect of primary infection at weaning. Groups of mice were initially infected with RSV (RSV(3wk)/RSV(8wk); $n = 8$), inoculated with uvRSV (uvRSV(3wk)/RSV(8wk); $n = 4$) at 3 wk of age and reinfected 5 wk later, or infected with RSV (RSV(8wk); $n = 6$) or sham inoculated (Sham(8wk); $n = 6$) at 8 wk of age. Lung histopathology (*A–C*), bronchoalveolar lavage cellularity (*D*), and airway responsiveness to inhaled MCh (*E*) were assessed on day 6 after reinfection. Lung viral titers (*F*) and levels of IFN-γ (*G*) and IL-13 (*H*) were measured on days 2, 4, and 6 after reinfection. Infection at weaning elicited a protective airway response during reinfection. This was characterized by a more vigorous mononuclear cell inflammation (*C*, arrowhead, and *D*) with no eosinophilia and no increase in mucus (*C*, arrow) production, but a complete abolition of AHR (*E*). After the secondary infection, viral titers decreased (*F*), and IFN-γ levels increased with an early peak response (*G*), whereas IL-13 levels decreased (*H*). Scale bars = 50 μm. *, Significant difference compared with sham group ($p < 0.05$); §, significant difference compared with either RSV(8wk) or uvRSV(3wk)/RSV(8wk) ($p < 0.05$); #, significant difference compared with day 2 postinfection values ($p < 0.05$); †, significant difference compared with levels measured on day 6 in either RSV(8wk) or uvRSV(3wk)/RSV(8wk) ($p < 0.05$).

Because recurrent RSV infection is frequent at all ages in humans, but the peak incidence for primary and secondary RSV-related lower respiratory tract disease is in infancy (9, 16), we hypothesized that the development of altered airway function during reinfection with RSV is determined in an age-dependent manner, at the time of initial infection. As demonstrated in the present study, neonatal RSV infection predisposes mice to develop more severe airway disease on reinfection. This amplified and altered response to reinfection is characterized by the development of significant AHR associated with marked airway eosinophilia. By contrast, primary infection at weaning elicited a protective airway response to reinfection characterized by an increased inflammatory response, predominantly lymphocytic, but without development of AHR and airway eosinophilia. However, these divergent responses to reinfection were not elicited if the mice were initially inoculated with uvRSV, clearly demonstrating the requirement for active lung viral infection during initial exposures. Most remarkably, viral clearance was achieved at similar rates, and AHR developed to the same extent in both age groups, after the primary infection. The most distinctive differences between the two ages in terms of response to primary RSV infection were lower IFN-γ, but increased IL-13, levels in the lungs of neonatally infected animals. However, after reinfection, greater amounts of IFN-γ were detected in BALF and were comparable in both age groups. By contrast, after reinfection, the levels of IL-13 were further increased in mice initially infected as neonates, but were virtually undetectable in mice initially infected at weaning. From these data it is clear that the consequences of reinfection are established very early in life. Thus, if infected early in life, the host might not be able to mount the appropriate response that would protect against the consequences of reinfection.

In general, recurrent RSV infection results in milder symptoms, usually restricted to the upper respiratory tract in young children and adults (24, 25), but in some cases more severe symptoms involving the lower respiratory tract can develop after secondary RSV infection, resulting in recurrent wheezing in younger children. A recent prospective study evaluated RSV-specific T cell responses in the peripheral blood of 55 infants hospitalized for RSV bronchiolitis and found that the lymphoproliferative responses to RSV stimulation in vitro were similar during both acute and convalescent phases of bronchiolitis and were not enhanced after a subsequent episode of natural RSV infection during

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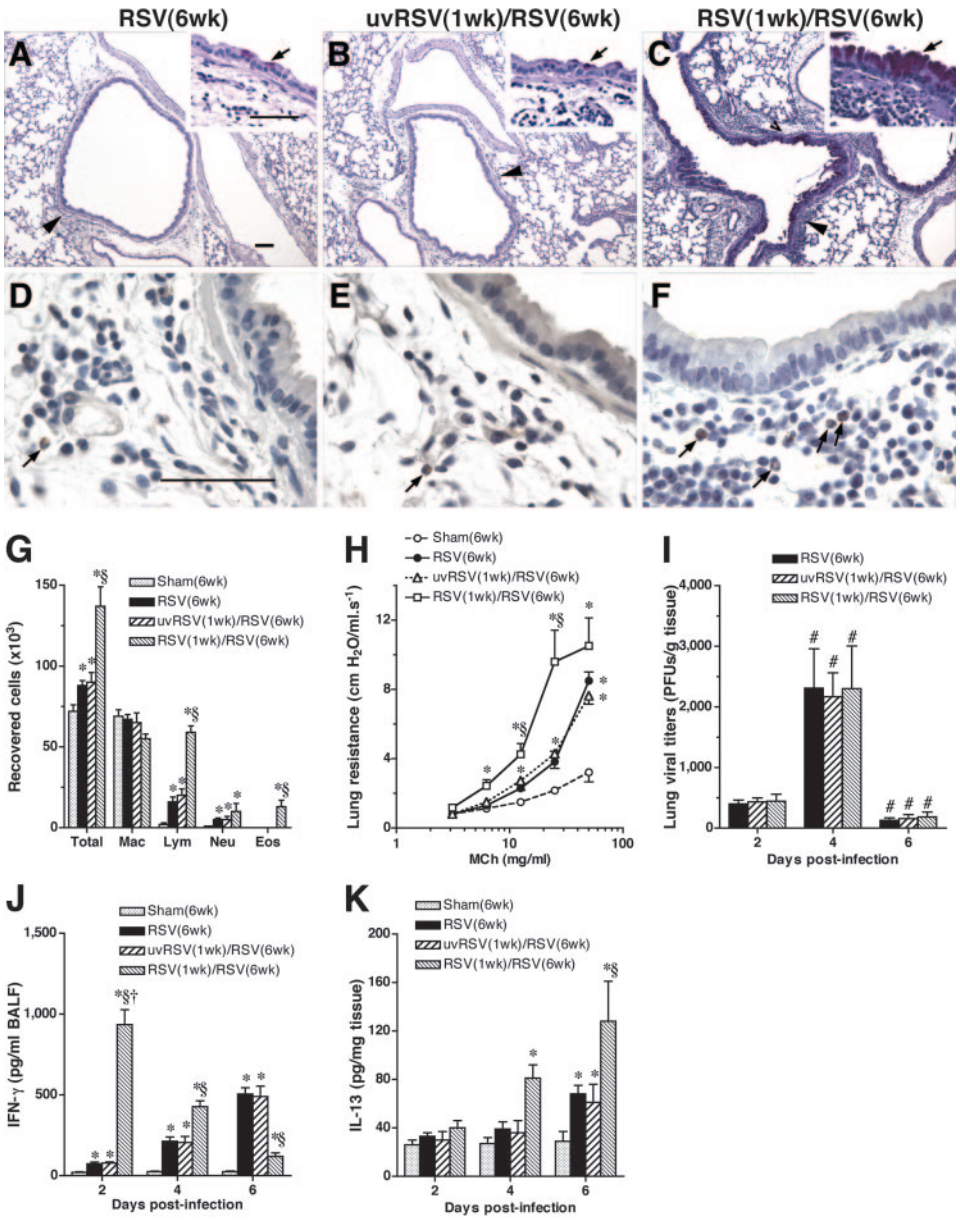

FIGURE 5. Airway responsiveness to secondary RSV infection: effect of primary neonatal infection. Groups of mice were initially infected with RSV (RSV(1wk)/RSV(6wk); $n = 8$), inoculated with uvRSV (uvRSV(1wk)/RSV(6wk); $n = 4$) at <1 wk of age and reinfected 5 wk later; or infected with RSV (RSV(6wk); $n = 6$) or sham inoculated (Sham(6wk); $n = 6$) at 6 wk of age. Lung histopathology (*A–F*), bronchoalveolar lavage cellularity (*G*), and airway responsiveness to inhaled MCh (*H*) were assessed on day 6 after reinfection. Lung viral titers (*I*) and levels of IFN-γ (*J*) and IL-13 (*K*) were measured on days 2, 4, and 6 after reinfection. After neonatal infection, secondary RSV infection resulted in the development of an enhanced lung histopathology (*C*), characterized by marked increases in airway tissue inflammation (arrowhead), mucus hyperproduction (arrow), and the appearance of eosinophilia in airway tissue (*F*, arrows) and BALF (*G*). Reinfection of this age group also resulted in enhanced AHR (*H*). Despite these changes, the rates of viral replication or clearance were unaffected in this group (*I*). However, IFN-γ levels increased with an early peak response (*J*), whereas IL-13 levels further increased on day 6 after the secondary infection (*K*). Scale bars = 50 μm. *, Significant difference compared with sham group ($p < 0.05$); §, significant difference compared with either RSV(6wk) or uvRSV(1wk)/RSV(6wk) ($p < 0.05$); #, significant difference compared with day 2 postinfection values ($p < 0.05$); †, significant difference compared with levels measured on day 6 in either RSV(6wk) or uvRSV(1wk)/RSV(6wk) ($p < 0.05$).

the second winter season (26). These observations led the investigators to conclude that RSV-specific T cell responses do not provide protection against reinfection and to speculate that RSV-specific T cells may fail to expand in vivo after reinfection. However, it is possible that these cells could be compartmentalized to specific locations in the lung and respond more vigorously in situ upon secondary RSV infection, as demonstrated in previous experimental animal studies (27, 28). A recent study conducted in mice suggested that the extent of the BALF inflammatory response to reinfection in adulthood is determined by the age at first infection (29). They showed that the pattern of inflammatory (lymphocytic) response to reinfection in adulthood differs depending on the age at first infection with RSV, and that neonatal infection primes the host to develop a Th2-biased response. Our data not only extend these findings, but additionally demonstrate that the enhancement of AHR during reinfection requires IL-13, is associated with airway eosinophilia and mucus hyperproduction, and is determined in an age-dependent manner at the time of initial infection with RSV. This is contrasted with the protective response against development of AHR and the absence of airway eosinophilia and mucus production despite the marked BAL and lung lymphocytic accumulation elicited on reinfection of mice initially infected at weaning.

In both humans and animal models, the Th2 cytokine IL-13 has been identified as a central mediator of AHR (18, 30). IL-13 is also a major regulator of mucus production in the airways (31), and to some degree it contributes to the development of airway eosinophilia (32). The role of IL-13 in RSV-mediated airway disease is to some extent controversial. IL-13 production is increased along with that of other Th2 cytokines in the lungs of mice previously primed with formalin-inactivated RSV and subsequently infected with live virus (33). Unlike formalin-inactivated RSV, the secreted form of the attachment G glycoprotein of RSV appears to preferentially prime the animals to develop high levels of IL-5 and IL-13 and pulmonary eosinophilia, even in the complete absence of IL-4 (34). Given the marked increases in IL-13 production detected in the present study, especially in mice reinfected after primary infection as neonates, we determined the cellular source of this cytokine and defined its role in the development of mucus production, airway eosinophilia, and AHR in this group. Intracellular staining of isolated

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lung T cells revealed that both CD4 and CD8 T cells were a source of IL-13, and the frequency of these cells was significantly increased after reinfection of the youngest mice. Furthermore, treatment of these animals with murine IL-13Rα2-human IgG fusion protein during secondary RSV infection completely abolished the development of AHR and mucus production and significantly inhibited airway eosinophilia. In contrast to our earlier studies of the role of IL-13 in primary RSV infection (20), these data clearly identified IL-13 as a central mediator involved in the development of AHR after reinfection of mice initially infected as neonates.

The role of IFN-γ in RSV-mediated airway disease is not clear. Several clinical studies have described an imbalance in Th1/Th2 cytokine expression with a predominant Th2-like response. This has been generally attributed to deficient IFN-γ production, associated with lower respiratory tract disease in infants and young children with documented RSV infection (35–38). More recently, a prospective study examined type 1 and type 2 cytokine responses in high risk infants (at least one atopic parent with asthma) to their first natural RSV infection and related the response to the onset of disease (39). In particular, the levels of IFN-γ measured in nasal lavage samples taken at the first signs of symptoms were significantly lower in infants who developed lower respiratory tract disease (bronchiolitis) than in those who developed upper respiratory symptoms alone. When PBMC were stimulated in vitro, deficient IFN-γ production could also be demonstrated in infants with more severe symptoms, corroborating the results obtained in vivo. IFN-γ production is restricted to T cells and NK cells. Although produced in large amounts in the lungs of RSV-infected adult mice, IFN-γ did not appear to be involved in the development of AHR (40). The present study provides additional evidence that IFN-γ is not involved in the development of AHR. In particular, AHR was completely abolished after reinfection despite high levels of IFN-γ in the lungs of mice that were initially infected at weaning. Similarly, IFN-γ was not apparently protective, because mice infected at <1 wk of age and reinfected 5 wk later developed enhanced AHR despite high levels of IFN-γ. As demonstrated by the inhibition studies, IL-13 appears to be a major regulator of AHR and mucus hyperproduction after reinfection.

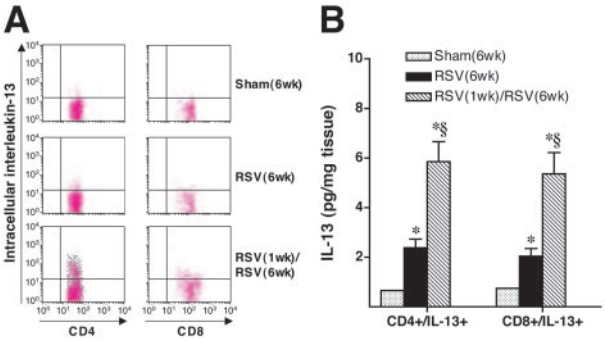

FIGURE 6. Cellular source of IL-13 production in the lung after the secondary infection in mice initially infected as neonates (same groups as described in Fig. 5). T cells were isolated from collagenase-digested lung tissue and simultaneously stained for surface expression of CD3 and CD4 or CD8, followed by intracellular staining for IL-13 and analysis of expression by flow cytometry. *A*, Representative flow cytometry panels showing IL-13-expressing lung T cells in the three groups of mice. *B*, Proportion (mean ± SEM) of IL-13-expressing lung T cells. Both CD4-positive and CD8-positive lung T cells expressed IL-13, and the proportions of these cells were further increased after the secondary infection compared with primary infection. *, Significant difference compared with sham group ($p < 0.05$); §, significant difference compared with RSV(6wk) ($p < 0.05$).

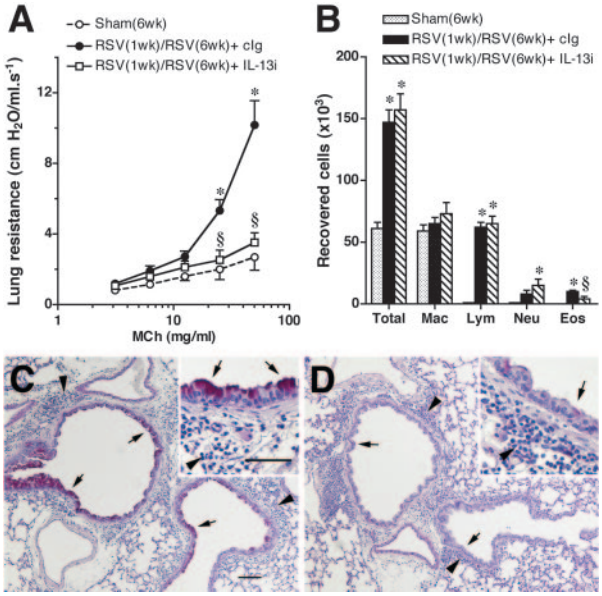

FIGURE 7. Role of IL-13 in the development of enhanced AHR, airway inflammation, and mucus production during secondary RSV infection. Mice were initially infected as neonates and treated with IL-13i (RSV(1wk)/RSV(6wk) + IL-13i; $n = 6$) or control Ig (RSV(1wk)/RSV(6wk) + cIg; $n = 6$) during reinfection. Airway responsiveness to inhaled MCh (*A*), bronchoalveolar lavage cellularity (*B*), and lung histopathology (*C* and *D*) were assessed on day 6 after the secondary infection. Neutralization of IL-13 resulted in significant inhibition of AHR (*A*) and airway eosinophilia in both bronchoalveolar lavage (*B*) and tissue (see text), and abolished mucus hyperproduction in the airways (*D*, arrows) without altering mononuclear airway inflammation in bronchoalveolar lavage (*B*) or tissue (*C* and *D*, arrowheads). Scale bars = 50 μm. *, Significant difference compared with sham group ($p < 0.05$); §, significant difference compared with cIg-treated group ($p < 0.05$).

It is well known that immune responses change with advancing age. In general, the responses are compared at widely different ages; in the mouse aged is defined as older than 6 mo in some studies. In contrast, it appears that significant differences in terms of the consequences of re-exposure are established very early in life. Thus, in an allergen challenge model, the age at initial sensitization was critical in dictating the degree of airway (eosinophilic) inflammation, the profile of cytokine production, and AHR on subsequent allergen challenge (41). In an analogous fashion, the present study demonstrates that the age at first RSV infection, even within the narrow window of primary infection at 1 wk vs primary infection at 3 wk, is sufficient to establish the consequences of reinfection. It is not entirely clear what the underlying mechanisms are. It is possible, at least in part, that a neonatal deficiency in IFN-γ production during primary infection, perhaps due to immaturity of the host immune response rather than the result of an aberrant response to viral infection, allows for the development of an unopposed, Th2-biased response that persists and can be restimulated during subsequent infection by RSV. However, it is unlikely that simple differences in the levels of IFN-γ dictate or account for this narrow window of susceptibility. IL-13 does emerge as a major regulator, at least of the enhanced response to reinfection of young mice. When extrapolated to the human situation and exposures in the neonate/young infant, these studies highlight the importance of developing preventative or delaying strategies, so that the consequences of initial RSV infection may be mitigated by the age at which they occur.

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Disclosures

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