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Neonatal Rhinovirus Infection Induces Mucous Metaplasia and Airways Hyperresponsiveness

Dina Schneider,* Jun Y. Hong,*† Antonia P. Popova,* Emily R. Bowman,* Marisa J. Linn,* Alan M. McLean,* Ying Zhao,* Joanne Sonstein,‡ J. Kelley Bentley,* Jason B. Weinberg,* Nicholas W. Lukacs,§ Jeffrey L. Curtis,§ Uma S. Sajjan,* and Marc B. Hershenson*†

Recent studies link early rhinovirus (RV) infections to later asthma development. We hypothesized that neonatal RV infection leads to an IL-13–driven asthma-like phenotype in mice. BALB/c mice were inoculated with RV1B or sham on day 7 of life. Viral RNA persisted in the neonatal lung up to 7 d postinfection. Within this time frame, IFN-α, -β, and -γ peaked 1 d postinfection, whereas IFN-λ levels persisted. Next, we examined mice on day 35 of life, 28 d after initial infection. Compared with sham-treated controls, virus-inoculated mice demonstrated airways hyperresponsiveness. Lungs from RV-infected mice showed increases in several immune cell populations, as well as the percentages of CD4-positive T cells expressing IFN-γ and of NKP46/CD335+, TCR-β+ cells expressing IL-13. Periodic acid-Schiff and immunohistochemical staining revealed mucous cell metaplasia and muc5AC expression in RV1B- but not sham-inoculated lungs. Mucous metaplasia was accompanied by induction of gob-5, MUC5AC, MUC5B, and IL-13 mRNA. By comparison, adult mice infected with RV1B showed no change in IL-13 expression, mucus production, or airways responsiveness 28 d postinfection. Intraperitoneal administration of anti–IL-13 neutralizing Ab attenuated RV-induced mucous metaplasia and methacholine responses, and IL-4R null mice failed to show RV-induced mucous metaplasia. Finally, neonatal RV increased the inflammatory response to subsequent allergic sensitization and challenge. We conclude that neonatal RV1B infection leads to persistent airways inflammation, mucous metaplasia, and hyperresponsiveness, which are mediated, at least in part, by IL-13.


Wheezy-associated acute respiratory viral infections in infancy, particularly those caused by respiratory syncytial virus (RSV) (1, 2), have been long considered risk factors for asthma. However, recent studies suggest a possible role for human rhinovirus (RV) in the pathogenesis of asthma. RV is the virus most commonly associated with acute respiratory hospitalizations in infants and young children (3). Results from the Childhood Origins of Asthma study suggest that, in patients with a family history of asthma, wheezing-associated illness with RV is the most important risk factor for subsequent asthma development (4, 5). In another study of infants hospitalized for respiratory infection-associated wheezing, RV was associated with the development of asthma (6), in contrast to RSV, which was negatively associated with later childhood asthma (7). In contrast, whereas it is conceivable that respiratory viral infection might lead to the development of asthma by damaging the developing airways or altering the immune response, viral infections may simply reveal a pre-existing tendency for asthma.

A number of rodent models aiming to elucidate the mechanisms linking early life viral respiratory infections to asthma have been developed. BALB/c mice infected with RSV at <1 wk of age show more mucus-producing cells in the airways, mild airway tissue eosinophilia, elevated IL-13 production, and airway cholinergic hyperresponsiveness (8). Mice infected with RSV at 1 wk of age also show airways hyperresponsiveness and IL-13 production that persists 69 d postinfection (9). One- to 2-d-old BALB/c mice infected with pneumonia virus of mice (PVM), a natural mouse pathogen that is closely related to human RSV (10), show airway lymphocytic inflammation, modest goblet cell metaplasia, and airways hyperresponsiveness at 7 wk of age, which is dependent on IL-4R signaling (10).

Chronic airways hyperresponsiveness and goblet cell hyperplasia following viral infection have also been shown in mature C57BL/6J mice infected with Sendai virus (murine parainfluenza virus type 1) (11). In these mice, IL-13 production by NK T cells and alternatively activated macrophages is responsible for persistent mucous metaplasia (12).

Despite the association of early-life RV infections with asthma development in children (4–6), the effects of neonatal RV infection have not been tested in an animal model. We have recently developed a mouse model of RV infection employing RV1B, a minor group virus that binds to low-density lipoprotein family receptors. In this model, RV1B replicates in the lungs of mature mice and induces a robust IFN response (13). In mice with pre-existing allergic airways disease, alternatively polarized macrophages are
in part responsible for RV-induced airway inflammation and hyperresponsiveness (14). In the current study, we tested the early and late effects of RV1B infection in neonatal mice, focusing on the role of IL-13. We also examined the effects of RV infection on NKT cell IL-13 expression and macrophage polarization.

Materials and Methods

Generation of RV

RV1B (American Type Culture Collection, Manassas, VA) were grown in HeLa cells, concentrated, partially purified, and titered, as described (15). Similarly concentrated and purified HeLa cell lysates were used for sham infection. Fifty percent tissue culture infectivity doses (TCID50) were determined by the Spearman-Karber method.

RV exposure

Experiments were approved by the Institutional Animal Care and Use Committee. Seven-day-old BALB/c or BALB/c-Il4ratm1Sz/J mice (The Jackson Laboratory, Bar Harbor, ME) were inoculated intranasally with 15 μl of 3 × 10⁸ TCID50/ml RV1B or an equal volume of sham. For selected experiments, 8-wk-old BALB/c mice were inoculated with 45 μl of 3 × 10⁸ TCID50/ml RV1B or an equal volume of sham (13).

Lung inflammation

To quantify inflammatory cells, lung digestes were minced, lysed in collagenase type IV (Life Technologies Invitrogen, Carlsbad, CA), and strained through 70-μm nylon mesh (BD Falcon, San Jose, CA) (16). The pellet was treated with RBC lysis buffer (BD Pharmingen, San Diego, CA), and leukocytes were enriched by spinning the cells through 20% Percoll (Sigma-Aldrich, St. Louis, MO). Cytospins were stained with Diff-Quick (Dade Behring, Newark, DE), and differential counts were determined from 200 cells.

Histology and immunohistochemistry

Lungs were fixed with 10% formaldehyde and paraffin embedded. Blocks were sectioned at 500-μm intervals at a thickness of 5 μm. Sections were stained with periodic acid-Schiff (PAS; Sigma-Aldrich) to visualize mucus accumulation. Additional sections were immunostained for goat anti-mouse muc5AC or its isotype control (Santa Cruz Biotechnology, Santa Cruz, CA), labeled with biotinylated anti-goat IgG (Vector Laboratories, Burlingame, CA), and developed using the Vectastain Elite ABC kit (Vector Laboratories) and diaminobenzidine (Sigma-Aldrich) as a substrate. The number of PAS-positive cells per basement membrane length and alveolar chord length was determined using NIH ImageJ analysis software, as described (17).

Quantitative real-time PCR

Whole-lung RNA was extracted with RNeasy (Qiagen, Valencia, CA) and analyzed by quantitative real-time PCR using specific primers and probes. Signals were normalized to GAPDH.

Measurement of lung cytokines

IL-4, IL-5, and IL-13 protein levels were measured by immune bioplex assay (Bio-Rad, Hercules, CA).

Flow cytometry

Staining and analysis by flow cytometry were performed, as described (18). Cells were analyzed on an LSR II flow cytometer (BD Biosciences, San Jose, Biotechnology, Santa Cruz, CA). Cells were analyzed on an LSR II flow cytometer (BD Biosciences, San Jose, CA) equipped with 488-nm blue, 405-nm violet, and 633-nm red lasers. Data were collected on an HP XW4300 Workstation (Hewlett-Packard, Palo Alto, CA) using FACSDiva software (BD Biosciences) with automatic compensation and were analyzed using FlowJo software (Tree Star, Ashland, OR). A range of cells (10,000–100,000) was analyzed per sample. Isotype-matched irrelevant control mAbs were tested simultaneously in all experiments. We used mAbs against the following Ags (clones and fluorochromes shown in parentheses): Ly6G (1A8; FITC or PE), Siglec-F (E50-2440; PE), and CD19 (1D3; allophycocyanin-cyanine 7) (each from BD Pharmingen, San Jose, CA); CD4 (GK1.5 PE-Cy7), CD11b (M1/70 Alexa Fluor 700), and TCR-b (H57-597 FITC or PE) (each from BD Biosciences, San Diego, CA); and CD206 (MR5D3 AF647) (AbD Serotec, Raleigh, NC). Appropriate isotype-matched controls were used in all experiments.

**FIGURE 1.** RV1B infection of neonatal mice elicits a brisk IFN response. Seven-day-old BALB/c pups were intranasally infected with RV1B or sham control, and lungs were harvested for analysis 1–7 d postinfection. Positive-strand viral RNA (A) and mRNAs encoding IFN-α, -β, -γ, and -λ (B) were measured by quantitative PCR. IFN expression was normalized by expression of the housekeeping gene GAPDH. n = 3–5 animals per time point, *different from sham, p < 0.05, unpaired t test.
For intracellular staining, freshly isolated aliquots of the lung mince were stimulated for 5 h at 37°C in 5% CO₂ with PMA (50 ng/ml) and ionomycin (1 μg/ml) in the presence of brefeldin A. The lung minces were washed, and the cell pellets were resuspended in Dulbecco’s PBS. To eliminate potential artifacts due to dead cell contamination, the cells were first stained with Aqua Live/Dead Fixable Dead Cell Stain Kit (Invitrogen, Carlsbad, CA) and then stained for surface Abs, as above. The lung mince cells were fixed, permeabilized, and then incubated with anti-mouse IFN-γ, IL-13, and CD206, according to the eBioscience protocol for intracellular immunofluorescent staining of cells for flow cytometry.

**Airways responsiveness**

Airway cholinergic responsiveness was assessed by measuring changes in total respiratory system resistance in response to increasing doses of nebulized methacholine. Mice were anesthetized with pentobarbital. Resistance was measured plethysmographically (Buxco, Wilmington, NC).

**IL-13 neutralization**

For IL-13 neutralization, 30 μg goat anti-mouse IL-13 or normal goat IgG (R&D Systems, Minneapolis, MN) was administered i.p. on days 0, 2, 4,
and 6 postinfection. Mice were sacrificed for analysis 28 d after initial RV infection.

**OVA sensitization and challenge**

For OVA sensitization, mice were injected i.p. on days of life 17 and 24 with 100 μl PBS or a solution of alum and 50 μg endotoxin-free OVA (Sigma-Aldrich). Next, mice were challenged intranasally with 25 μl PBS or 50 μg OVA in PBS on days of life 32, 33, and 34. Mice were studied 1 d after the final challenge.

**Data analysis**

Data are represented as mean ± SEM. Statistical significance was determined by unpaired t test, rank sum test, or two-way ANOVA, as appropriate. Differences were pinpointed by Newman–Keuls’ multiple range test.

**Results**

**RV1B infection elicits IFN responses and inflammation in neonatal mice**

We initially characterized the response to viral inoculation in 7-d-old BALB/c pups. Pups were intranasally infected with RV1B or sham control, and lungs were harvested for analysis 1–7 d postinfection. Positive-strand viral RNA persisted in the mouse lung up to 7 d postinfection (Fig. 1A). The sustained level of viral copies 3 d postinfection strongly suggests that RV causes a replicative infection in neonatal mice. Virus was not detected >7 d following infection.

mRNAs encoding IFN-α, -β, and γ were each strongly induced at 1 d after RV1B infection, but not after sham inoculation (Fig. 1B). IFN-α, -β, and -γ levels decreased thereafter. In contrast, IFN-λ mRNA levels remained elevated through day 7. IFN-λ receptors are largely restricted to cells of epithelial origin (19), and IFN-λ receptor knockout mice show attenuated antiviral responses to dsRNA (20). It is therefore conceivable that dsRNA, released from RV-infected epithelial cells, stimulates further IFN-λ production.

Lung differential cell counts revealed modest, but statistically significant increases in the numbers of neutrophils on days 3, 5, and 7, and in the number of macrophages on days 5 and 7 postinfection (Fig. 2A–D). In addition, lymphocytes and eosinophils were increased 7 d postinfection. Nevertheless, histology showed minimal cellular infiltration of the lungs after RV infection in neonatal animals (Fig. 2E, 2F).

Lungs were examined for selected mRNAs encoding proinflammatory cytokines and chemokines. Based on the early neutrophilic response to RV infection, we chose to examine three neutrophil chemotactic factors (CXCL1, CXCL2, and TNF-α), each of which are induced by RV1B infection in mature mice (21). mRNAs encoding CXCL1, CXCL2, and TNF-α were each increased compared with sham-inoculated mice (Fig. 3). mRNA expression of IL-13, which has previously been shown to be induced by neonatal RSV infection (8), was also increased.

**Neonatal RV1B infection induces airways hyperresponsiveness, inflammation, and mucus accumulation**

We examined the airway cholinergic responsiveness of sham- and RV-exposed mice on day 35 of life (28 d postinfection). We detected no viral RNA in either treatment group at 28 d postinfection (data not shown). There was no difference in weight between sham- and RV-infected mice at this time point (sham, 29.5 ± 1.9 g; RV, 29.4 ± 2.9 g). However, there was a significant increase in methacholine responsiveness in mice infected with RV as neonates (Fig. 4A). In contrast, mature mice infected with RV1B showed no increase in airways responsiveness 28 d postinfection.

To understand the change in responsiveness, we examined the lungs of 35-d-old mice infected as neonates. Most airways were histologically normal. Again, differences in inflammatory cells between sham- and RV-infected mice were slight. Selected airways from RV-infected mice showed a modest amount of peribronchial inflammation (Fig. 4B, 4C). In addition, RV-infected mice showed rare, localized collections of lymphocytes in the peribronchial, perivascular, and subpleural spaces (Fig. 4D, 4E). Compared with sham-infected mice, lung digests of RV-infected mice showed a small, but significant increase in the percentage of neutrophils from RV-infected epithelial cells, stimulating further IFN-λ responses and inflammation in neonatal mice. Virus was not detected >7 d following infection.

**FIGURE 4.** Neonatal RV infection induces persistent changes in airways responsiveness and inflammation. (A) Effects of sham and RV1B infection of neonatal and mature mice on airway cholinergic responsiveness, measured 4 wk after initial inoculation (day 35 of life for mice infected with RV as neonates, and day 84 of life for mice infected with RV at maturity). Airway cholinergic responsiveness was assessed by measuring changes in total respiratory system resistance in response to increasing doses of nebulized methacholine. n = 6–10 animals per group, *different from sham-exposed mice of the same age, p < 0.05, two-way ANOVA. (B–E) Representative images of lungs harvested 4 wk after neonatal sham (B) or RV1B infection (C–E). Lungs were stained with H&E. Original magnification ×200. These images are representative of three individual experiments of three mice per group. (F) Lung homogenate inflammatory cells from mice harvested 28 d after neonatal inoculation with sham or RV. Cytospins were stained with Diff-Quick. n = 6 animals per group, *different from sham-exposed neonatal mice, p < 0.05, unpaired t test.
Lungs of mice undergoing neonatal RV infection also demonstrated modest increases in total neutrophils, macrophages, lymphocytes, and eosinophils (Fig. 4F). There was no difference in mean alveolar chord length between sham- and RV-infected mice (sham, 25.3 ± 0.2 μm; RV, 24.9 ± 0.9 μm; p = 0.71). The minimal cellular infiltration of the lungs after RV infection in neonatal animals, particularly at later time points when airways responsiveness is increased, suggests that other mechanisms are responsible for the observed airways hyperresponsiveness.

To learn more about changes in leukocyte subsets following RV infection, we conducted flow cytometry of cells from minced lungs obtained from mice 3 wk after neonatal infection, focusing on NKT cells and macrophage polarization. We found increases in Gr1-positive neutrophils and Gr1-positive, SigF double-positive eosinophils, CD4-positive T cells, CD19-positive B cells, and CD11b-positive cells within monocyte/macrophage gates compared with sham controls (Fig. 5A–F). CD11b-positive cells from RV-infected mice showed a significant increase in CD206 expression (Fig. 5G), indicating alternative macrophage polarization. When cells were costained with Abs against IFN-γ and IL-13, lung cells from RV-infected mice showed increases in the percentage of CD4-positive T cells expressing IFN-γ (Fig. 5H), and the percentage of NKp46/CD335-positive TCR-β–positive cells (NKT cells) expressing IL-13 (Fig. 5I). Finally, CD11b-positive cells did not express IL-13 (data not shown).

We then examined PAS staining. Lungs from 35-d-old mice inoculated with sham on day 7 of life showed a range of PAS staining ranging from no signal to patches of dense staining in the large airways (Fig. 6A, 6B). In contrast, mice infected with RV at day 7 of life and studied at day 35 showed some large airways that were completely filled with PAS-positive epithelial cells, as well

![Graph A](https://example.com/graphA.png)

**Figure 5.** Neonatal RV infection induces prolonged changes in airway inflammation. Cells from the minced lungs of 35-d-old mice infected with sham or RV at 7 d of age were analyzed by flow cytometry. Compared with sham-exposed mice, RV-infected mice showed significant increases in Gr1-positive neutrophils (A); Gr1-positive, SigF-positive eosinophils (B); CD4-positive T cells (C); CD19-positive B cells (E); and CD11b-positive cells (F). There was no change in the number of CD8-positive cells (D). RV infection increased the CD206 expression of CD11b-positive cells (G; sham, gray line; RV, black line). Lung digests from RV-infected mice showed increases in the percentage of CD4-positive T cells expressing IFN-γ (H) and CD335-positive NK cells expressing IL-13 (I). n = 5–6 animals per group. *different from sham-exposed neonatal mice, p < 0.05, rank sum test.
as PAS staining in the medium-sized and small airways (Fig. 6C, 6D). Sham- and RV-infected mature mice showed only rare PAS-positive cells 28 d after treatment (data not shown). Mucus-secreting cells in the large airways were assessed 14, 21, and 28 d after neonatal infection by counting PAS-positive cells per basement membrane length. RV-infected mice showed significantly increased PAS staining (Fig. 6E). We also examined PAS staining 60 and 100 d after neonatal sham or RV treatment, on days 67 and 107 of life. At these time points, both groups showed only sparse PAS staining in the large airways, indicating that RV-induced mucous metaplasia had mostly resolved. However, some small airways of RV-infected 67-d-old mice continued to show PAS staining (Fig. 6F). Immunohistochemical staining for muc5AC confirmed increased mucus expression in the large and small airways of RV-treated mice (Fig. 6F–I).

We examined the time course of the mucus-related genes gob5, muc5AC, and muc5B. Given the sufficiency of pulmonary IL-13 expression for mucus hypersecretion in transgenic mice (22), we also examined the time course of this Th2 cytokine. The mRNA levels of genes related to mucus production were increased in mice infected as neonates, but not mice infected as adults (Fig. 7A–C). A similar pattern was observed for IL-13 mRNA, with significant increases on days 7, 14, and 21 (but not 28) postinfection (Fig. 7D). Lung levels of the Th2 cytokines IL-4, IL-5, and IL-13 were each significantly increased 3 wk after RV inoculation (Fig. 7E).

**FIGURE 6.** Neonatal RV infection causes airway mucous metaplasia. Lungs from mice inoculated with sham or RV1B at 7 d of age were harvested on day 35 and stained with PAS. PAS staining in the large airways of sham-infected mice ranged from no signal to patches of dense staining (A). Sham-infected animals showed no staining in the small airways (B). In contrast, mice infected at day 7 of life showed some large airways that were completely filled with PAS-positive epithelial cells (C), as well as PAS staining in the medium-sized and small airways (D). These images are representative of four individual experiments of four to five mice per group. (E) PAS staining was quantified by using NIH ImageJ analysis software. RV-infected mice showed significantly increased PAS staining 14, 21, and 28 d postinfection. n = 3 animals per group, *different from sham-exposed mice, p < 0.05, unpaired t test. (F) We also examined PAS staining 60 and 100 d after neonatal sham or RV treatment, on days 67 and 107 of life. RV-infected 67-d-old mice continued to show mucus (arrow, inset). These images are representative of four individual experiments of four to five mice per group. (G–J) Lung tissues from sham-inoculated (G, H) and RV-infected mice (I, J) were also immunostained for MUC5AC. Signals were amplified and visualized using biotinylated anti-IgG and diaminobenzidine as a substrate. These images are representative of four individual experiments of four to five mice per group. Original magnifications ×50 (A, C), ×100 (G, I), and ×200 (B, D, F, H, J).
IL-13 neutralization attenuates RV-induced mucus accumulation and airways responsiveness

We hypothesized that IL-13 is required for mucus hypersecretion and airways hyperresponsiveness. We therefore administered neutralizing Ab to IL-13 or nonimmune serum days 0–6 post-RV infection. As noted previously, airways responsiveness was increased in BALB/c mice 28 d after neonatal RV infection. However, mice treated with anti–IL-13 showed a decreased level of methacholine responsiveness (Fig. 8A). Anti–IL-13 decreased the number of PAS-positive cells in the small airways, as defined by a circumference, 3 mm (Fig. 8B). Administration of anti–IL-13 had no effect on lung digest differential cell counts (data not shown).

IL-4R null mice show reduced mucous metaplasia in response to neonatal RV infection

BALB/c-IleIl4ratm1Sz/J mice (23) were inoculated with sham or RV1B, as described above. In contrast to wild-type mice, RV1B-treated IL-4R null mice showed no increase in PAS staining compared with sham-treated mice (Fig. 9A–C; compare with Fig. 6C, 6D). In addition, there was no significant increase in gob5, muc5AC, muc5B, or IL-13 mRNA expression (Fig. 9D; compare with Fig. 7).

Effect of early RV infection on the response to OVA sensitization and challenge

Mice were inoculated with sham or RV on day 7 of life, sensitized to PBS or OVA on days 17 and 24, and challenged with PBS or OVA on days 32 and 34. Mice were studied 1 d after the final challenge. Neonatal RV infection increased OVA-induced airway inflammation (Fig. 10A, 10B). Prior RV infection increased lung digest neutrophil and macrophage, but not lymphocyte or eosinophil counts (Fig. 10C). Finally, prior neonatal RV infection increased airways responsiveness following allergen sensitization and challenge (Fig. 10D).

Discussion

We demonstrate that, unlike mature mice, BALB/c mice inoculated with RV1B at 7 d of age display airways hyperresponsiveness, mucous metaplasia, and IL-13 production that last at least 1 mo postinfection. These data suggest that early life infection with RV can induce a prolonged state of airway pathology that recapitulates three hallmark features of allergic asthma.

It has previously been shown that infection of BALB/c mice shortly after birth with RSV and the closely related PVM causes persistent modest goblet cell metaplasia, airways hyperresponsiveness, and IL-13 production that last at least 1 mo postinfection. These data suggest that early life infection with RV can induce a prolonged state of airway pathology that recapitulates three hallmark features of allergic asthma.

In contrast to RSV, RV typically infects small clusters of cells in the airway epithelium (28) and elicits minimal, if any, cytotoxicity.
In mature mice, RV infection causes modest neutrophilic and lymphocytic inflammation (13) and, as shown in this work, no mucous metaplasia. In the current study, lung digests from BALB/c mice infected on day 7 of life showed modest infiltration of the airways with neutrophils, lymphocytes, macrophages, and eosinophils. Similarly, at age 35 d, the lungs of mice undergoing neonatal RV infection showed only a minor amount of peribronchial inflammation, with small but significant increases in total neutrophils, macrophages, and lymphocytes. Thus, the amount of cellular infiltration of the airways is unlikely to be the sole cause of the observed airways hyperresponsiveness.

We also observed peribronchial, perivascular, and subpleural collections of lymphocytes in the lungs of RV- but not sham-infected mice. These collections, which resemble BALT, are only occasionally present in normal mice, but may be induced by sensitization and challenge with heat-killed *Pseudomonas aeruginosa* (30) or by heterologous viral infections (31, 32). BALT is also spontaneously increased in the peribronchial and perivascular lung tissue of mice deficient for the chemokine receptor CCR7 (33, 34). In humans, BALT is commonly present in infants, but not in normal adult lungs (35). The presence of BALT-like infiltrates is evidence of an adaptive immune response against RV infection.

RV infection of immature mice also induced significant mucous metaplasia that was present 14 d postinfection and persisted until 28 d postinfection. Consistent with this, RV-infected immature mice showed significant increases in mRNAs encoding the gel-forming glycoproteins MUC5AC and MUC5B. MUC5AC protein was also increased. Gob-5 (mCLCA3), a member of the calcium-activated chloride channel family that drives mucin release (36, 37), was also significantly increased. Neither sham-treated immature mice nor mature mice showed such increases. Like RV, RSV infection increases the number of mucus-producing cells in immature, but not mature mice (8). Together, these data suggest that the newborn airway is more susceptible to virus-induced mucous metaplasia.

Gob-5 expression and mucus secretion are positively regulated by the Th2 cytokines IL-13, IL-10, and IL-9 (22, 38, 39), and IL-10–induced mucous metaplasia is IL-13 dependent (38). Persistent mucous metaplasia and airways hyperresponsiveness do not occur in PVM-infected IL-4R knockout mice, implying a requirement for IL-13 in this process. We therefore examined the mRNA expression of IL-13 in RV-infected mice. Quantitative PCR demonstrated a biphasic increase in IL-13 mRNA expression (Fig. 3D), with an initial induction immediately after RV infection and

**FIGURE 8.** IL-13 neutralization partially attenuates RV-induced airways hyperresponsiveness and mucous cell metaplasia. (A) Sham- and RV1B-infected animals were treated with IgG or anti–IL-13. Airway cholinergic responsiveness was measured 4 wk after initial inoculation. n = 4–6 animals per group, *different from sham-exposed mice, p < 0.05, two-way ANOVA. (B) PAS staining in the large and small airways of RV-infected mice treated with anti–IL-13 or IgG. n = 6–8 animals per group, *different from sham-exposed mice, p < 0.05, unpaired t test.

**FIGURE 9.** Effect of neonatal RV infection on IL-4R null mice. BALB/c-Il4r<sup>−/−</sup> mice were inoculated with sham or RV at day 7 of life, and airway tissues were examined 28 d postinfection. Unlike RV-infected wild-type BALB/c mice (see Fig. 6), IL-4R null mice showed no PAS-positive cells in large, medium, or small airways (A–C). These images are representative of two individual experiments of three mice per group. Original magnifications ×50 (A), ×100 (B), ×200 (C). (D) Lung gob5, MUC5AC, MUC5B, and IL-13 mRNA expression was measured by quantitative PCR. Unlike wild-type mice (see Fig. 7), RV-infected IL-4R null mice failed to show an increase in mucus or IL-13 expression. n = 3–5 animals per group.
a second peak 21 d postinfection (Fig. 7D). Administration of anti–IL-13 neutralizing Ab significantly decreased PAS staining in the small airways and partially decreased airways responsiveness. Finally, IL-4R null mice failed to show RV-induced mucous metaplasia, implicating IL-13 in the pathogenesis of persistent airway changes in RV-infected neonatal mice.

Chronic airways hyperresponsiveness and goblet cell hyperplasia following viral infection were first shown in C57BL/6J strain infected with Sendai virus (murine parainfluenza virus type 1) (11), a negative sense, ssRNA virus. In these mice, IL-13 production by NK T cells and alternatively activated macrophages is responsible for persistent mucous metaplasia (12). We have recently shown that alternatively polarized macrophages are responsible for RV-induced airway inflammation and hyperresponsiveness in mature mice with allergic airways disease (14). In the current study, we found increased expression of CD206, also known as macrophage mannose receptor 1 and C-type lectin domain family 13 member D, in CD11b-positive macrophages, indicative of a switch in macrophage activation state. Alternative macrophage activation has been demonstrated to be IL-13 dependent in Cryptococcus neoformans-infected mice (40). We also found an increase in the percentage of CD335-positive, TCR-β–positive cells expressing IL-13. Together, these results are consistent with the notion that IL-13, produced by NK T cells, plays a significant role in chronic mucous metaplasia and hyperresponsiveness following viral infection. Other mechanisms are possible, however. We have not excluded the possibility that IL-13–producing CD4-positive Th2 lymphocytes play a role. It is therefore conceivable that RV infection has direct effects on airway epithelial cell mucus production. RV infection of NCI-H292 airway epithelial cells induces TLR3- and epidermal growth factor receptor-dependent MUC5AC expression (41). We have also noticed that RV infection of primary airway epithelial cells grown at air-liquid interface induces mucous metaplasia (U. Sajjan, M. Hershenson, unpublished observations). In addition, the proinflammatory cytokine IL-17A has been shown to regulate airway epithelial cell MUC5AC and MUC5B expression via an IL-6 paracrine loop (42). Finally, whereas it is conceivable that early RV infection increases airway responsiveness by inducing subtle alterations in airway and parenchymal development (43), importantly, neonatal RV infection had no effect on lung alveolarization.

As noted above, infection of mature mice failed to induce mucous metaplasia or long-lasting airways hyperresponsiveness as it did in neonatal mice. The mechanisms underlying this age-dependent effect are unclear. First, it is conceivable that neonatal mice are more susceptible to viral infection, leading to exaggerated airway effects. It has previously been shown that the activation and entry of neonatal T cells into the lungs are delayed after influenza infection (44). In the current study, RV copy number was sustained for 3 d in neonatal mice, and lung lymphocyte infiltration did not increase until 7 d postinfection. This contrasts with mature mice in which viral copy number peaks 24 h postinfection and lymphocyte infiltration increased 1 d postinfection. In addition, neutrophilic inflammation and IFN-γ expression by CD4+ T cells persisted for weeks after neonatal infection. Together, these results suggest a delayed and prolonged response to acute viral infection in neonatal
animals. Second, the neonatal airway epithelium may be more susceptible to rhinovirus infection than the mature epithelium. Mucous metaplasia has been shown to increase susceptibility of the airway epithelium to RV infection (45). In rhesus monkeys, goblet cells in the trachea decrease in abundance with increasing age (46). We noted that sham-infected 35-d-old mice show a range of PAS staining, extending from no signal to patches of dense staining in the large airways, whereas sham-infected 8 wk-old mice show only rare PAS-positive cells. Third, it is possible that early life viral infection causes mucous metaplasia and airway hyperresponsiveness by augmenting or maintaining the neonatal immature response, which is skewed toward a Th2 phenotype (47–49). The relatively high IL-13 levels in both sham- and RV-treated immature mice are consistent with Th2 polarization.

Neonatal RV infection also enhanced the inflammatory response to allergen sensitization and challenge. Prior infection with RV has been shown to increase subsequent allergenic airways responses in both immature (9) and mature mice (50). Interestingly, in contrast to the previous study of neonatal RV infection, prior infection with RV significantly increased OVA-induced neutrophilic inflammation. The mechanisms by which allergen exposure induces neutrophilic airway inflammation have not been well studied, but may involve the activation of the IL-17/IL-23 axis (51, 52).

Finally, we note that the effects of neonatal RV infection appeared to fade with time, with mucous metaplasia substantially reduced by 60 d postinfection and absent by 100 d postinfection. Because babies are exposed to RV numerous times during infancy, it is conceivable that there could be additive or synergistic effects of infection over time.

We conclude that RV1B infection of neonatal BALB/c mice leads to airways inflammation, mucous metaplasia, and hyperresponsiveness, which are mediated, at least in part, by IL-13.

Disclosures

The authors have no financial conflicts of interest.

References


