Early-life heterologous rhinovirus infections induce an exaggerated asthma-like phenotype

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GRAPHICAL ABSTRACT

Early life heterologous rhinovirus infections induce an exaggerated asthma-like phenotype

Background: Early-life wheezing-associated respiratory tract infection by rhinovirus (RV) is a risk factor for asthma development. Infants are infected with many different RV strains per year.

Objective: We previously showed that RV infection of 6-day-old BALB/c mice induces a mucous metaplasia phenotype that is dependent on type 2 innate lymphoid cells (ILC2s). We hypothesized that early-life RV infection alters the response to subsequent heterologous infection, inducing an exaggerated asthma-like phenotype.

Methods: Wild-type BALB/c mice and Rora\textsuperscript{fl/fl} Il7r\textsuperscript{cre} mice lacking ILC2s were treated as follows: (1) sham on day 6 of life plus sham on day 13, (2) RV-A1B on day 6 plus sham on day 13, (3) sham on day 6 plus RV-A2 on day 13, and (4) RV-A1B on day 6 plus RV-A2 on day 13.

Results: Mice infected with RV-A1B at day 6 and sham at day 13 showed an increased number of bronchoalveolar lavage eosinophils and increased expression of IL-13 mRNA but not expression of IFN-\gamma mRNA (which is indicative of a type 2 immune response), whereas mice infected with sham on day 6 and RV-A2 on day 13 showed increased IFN-\gamma expression (which is a mature antiviral response). In contrast, mice infected with RV-A1B on day 6 before RV-A2 infection on day 13 showed increased expression of IL-13, IL-5, Gob5,

Abbreviations: ILC2s: group 2 innate lymphoid cells
RV: human rhinovirus

Received for publication September 3, 2019; revised March 11, 2020; accepted for publication March 26, 2020.

Available online April 25, 2020.
Muc5b, and Muc5ac mRNA; increased numbers of eosinophils and IL-13–producing ILC2s; and exaggerated mucus metaplasia and airway hyperresponsiveness. Compared with Rora<sup>fl/fl</sup> mice, Rora<sup>fl/fl</sup>Il7r<sup>cre</sup> mice showed complete suppression of bronchoalveolar lavage eosinophils and mucous metaplasia.

Conclusion: Early-life RV infection alters the response to subsequent heterologous infection, inducing an intensified asthma-like phenotype that is dependent on ILC2s. (J Allergy Clin Immunol 2020;146:571-82.)

Key words: Asthma, childhood, early-life, IL-13, rhinovirus, RV-A1B, RV-A2, trained immunity, type 2 innate lymphoid cell, ILC2

Early-life wheezing-associated respiratory tract infections by human rhinovirus (RV) and respiratory syncytial virus (RSV) are considered risk factors for asthma development. Children are infected with many different RV strains, with infants having 6 to 10 distinct RV infections per year. RV infections do not induce specific immunity to reinfection by heterologous serotypes, even if viruses are from the same species (eg, RV-A1A and RV-A2). RVs are divided into 3 species (A, B, and C), which comprise more than 160 antigenically distinct strains. Recurrent RV infections could result in greater degrees of airway inflammation and the potential for airway remodeling and loss of lung function over time.

In the Tucson Children’s Respiratory Study, which is a prospective birth cohort study, lower respiratory tract illness with RSV before 3 years of age was an independent risk factor for the development of wheezing up to age 11 years but not at 13 years. Infants with severe RSV<sup>1</sup> and RV<sup>1</sup> infections requiring hospitalization are more likely to have asthma at ages 13 and 7 years, respectively. In the University of Wisconsin Childhood Origins of Asthma birth cohort of infants at increased risk of asthma, wheezing with RV in the first 3 years of life was strongly associated with asthma at 6 and 13 years of age.<sup>2,4,11,12</sup> In contrast to RV, RSV had a lessened impact with time. RV and allergic sensitization had additive effects on asthma risk. In the Western Australian Pregnancy Cohort Study, wheezing lower respiratory illness in the first year of life increased the risk for asthma at the age of 6 years in both children without atopic disease and children with atopic disease.<sup>14</sup> In the Perth Childhood Asthma Study of children at high atopic risk, asthma at 5 years of age was associated with early-life wheezy and/or febrile lower respiratory tract infection.<sup>15</sup> However, the associations were restricted to children who displayed allergen sensitization. In The Netherlands Generation R study, those children with bronchitis, bronchiolitis, and pneumonia before 3 years of age were more likely to have asthma and lower lung function at 10 years of age.<sup>12</sup>

Allergic sensitization did not factor into the associations seen. Together, these data suggest that early-life respiratory viral infection, in particular with RV, may contribute to the development of asthma, in some cases in the absence of allergen sensitization.

Infection of immature mice with RV predisposes to the development of IL-13–dependent airway eosinophilia and airways hyperresponsiveness after homologous reinfection 5 weeks later, whereas infection at weaning protects against reinfection.<sup>16,17</sup> These data are consistent with the notion that early-life RSV infection polarizes the adaptive immune response in such a way that homologous reinfection stimulates type 2 immune responses. However, the effect of early-life heterologous RV infections, which are much more common, has not been studied. We have previously shown that RV infection of 6-day-old BALB/c mice, but not mature mice, induces an asthma-like phenotype that is associated with type 2 innate lymphoid cell (ILC2) expansion and dependent on IL-13, IL-25, and IL-33.<sup>18,19</sup> We found that ILC2s persisted longer than 3 weeks after infection.<sup>18</sup> These data are consistent with the notion that following early-life RV-induced expansion, ILC2s could form a stable population of innate immune cells capable of responding to subsequent heterologous infections, a form of trained immunity.<sup>20</sup>

To examine the effects of heterologous RV infections on airway responses, we infected immature mice with different RV strains (RV-A1B and RV-A2) on days 6 and 13 of life, measuring airway responses 1 week after the second infection. We hypothesized that RV-A1B infection on day 6 would alter the immune response to heterologous infection with RV-A2 on day 13 of life, inducing an exaggerated asthma-like phenotype. We found that early-life heterologous infection with RV-A1B and RV-A2 induced intensified type 2 cosinophilic inflammation and mucous metaplasia that was dependent, at least in part, on ILC2s.

METHODS

Animals

All animal use was approved by the Institutional Animal Care and Use Committee and followed guidelines set forth in the Principles of Laboratory Animal Care from the National Society for Medical Research. BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and bred in-house in pathogen-free facility within the Unit for Laboratory Animal Medicine at the University of Michigan. The Rora<sup>fl/fl</sup>, Rora<sup>fl/fl</sup>Il7r<sup>cre</sup>, and IL7r<sup>cre</sup> mice were a gift from Dr Andrew Mckenzie (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom). Six day-old mice were used for the experiments.

Generation of RV-A1B and RV-A2

RV-A1B and RV-A2 (ATCC, Manassas, Va), which are minor group viruses that infect mouse cells,<sup>21</sup> were partially purified from infected HeLa cell lysates by means of ultracentrifugation with a 100-kDa cutoff filter and titered by measuring airway responses 1 week after the injection. They were partially purified. The Rora<sup>fl/fl</sup>, Rora<sup>fl/fl</sup>Il7r<sup>cre</sup>, and IL7r<sup>cre</sup> mice were a gift from Dr Andrew Mckenzie (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom). Six day-old mice were used for the experiments.

RV infections

Mice were inoculated with 20 μL of 1 × 10<sup>8</sup> plaque-forming units (pfu) or sham HeLa cell lysate through the intranasal route under isoflurane anesthesia. Mice were treated on day 6 of life with RV-A1B and day 13 of life with RV-A2.
Heterologous RV infection induces exaggerated airway responses. A, Baby mice were inoculated with sham or RV-A1B on day 6 of life and with sham or RV-A2 on day 13 of life. B, Lungs from sham plus sham–, sham plus RV-A1B–, sham plus RV-A2–, and RV-A1B plus RV-A2–infected mice were harvested on day 20 of life. Shown is lung expression of IL-5, IL-13, IL-5, IFN-γ, Gob5 (Clca1), Muc5b, Muc5ac, TNF-α, and IL-12b mRNA. C, BAL inflammatory cell counts for sham plus sham–, sham plus RV-A1B–, sham plus RV-A2–, and RV-A1B plus RV-A2–infected mice. Data shown are means ± SEMs; n = 3 to 9 per group from 2 different experiments; *indicates different from sham plus sham; †indicates different from RV-A1B plus sham; P < .05 by 1-way ANOVA and the Tukey multiple comparison test. D, RV positive-strand RNA was measured on days 14, 15, or 20 of age (1, 2, or 7 days after RV-A2 infection), and presented as viral copy number in total lung. Data shown are means ± SEMs; n = 4 to 9; *indicates different from day 6 sham plus day 13 RV-A2: P < .05, 1-way ANOVA and the Tukey multiple comparison test. E, mRNA expression in male and female mice.
as follows: (1) sham on day 6 plus sham on day 13, (2) RV-A1B on day 6 plus sham on day 13, (3) sham on day 6 plus RV-A2 on day 13, and (4) RV-A1B on day 6 plus RV-A2 on day 13 (Fig 1, A).

**Real-time quantitative PCR**

Lungs were harvested at day 20 of life (7 days after the last treatment), and RNA was extracted with Trizol (Invitrogen, Carlsbad, Calif). Lung RNA was isolated by using an RNAeasy kit (Qiagen Germantown, Md). cDNA was synthesized from 2 μg of RNA by using a high-capacity cDNA synthase kit (Applied Biosystems, Foster City, Calif) and subjected to quantitative real-time PCR using specific primers for mRNA (Table E1). The level of gene expression for each sample was normalized to glyceraldehyde-3-phosphate dehydrogenase. Sex was determined by sex-determining region Y (SRY) gene expression. To quantify virus particles, quantitative PCR for positive-strand viral RNA (vRNA) was conducted by using RV-specific primers and probes (forward primer, 5’-GTGAAGAGCCSCRGTGCT-3’; reverse primer, 5’-GCTSCAGGGTTAAGGTTAGCC-3’; and probe, 5’-FAM-TGAGTCCCTCCGCCCTGAAATG-TAMRA-3’).

**Lung histology and immunofluorescence**

Lungs were harvested at day 20 of life, fixed with 10% formaldehyde overnight, and paraffin-embedded. Blocks were sectioned at 50-μm intervals at a thickness of 5 μm, and each section was deparaffinized, hydrated, and stained. To visualize mucus, sections were stained with periodic acid–Schiff (PAS) (Sigma-Aldrich, St Louis, Mo). Other lung sections were stained with 4’,6-diamidino-2-phenylindole (DAPI) and Alexa Fluor 488–conjugated mouse anti-Muc5ac (clone 45M1 [Thermo Fisher Scientific, Waltham, Mass]) or Alexa-Fluor 488–conjugated anti-CCL11 (Biolegend, San Diego, Calif). IL-13–producing ILC2s and T cells were identified in the airways by immunofluorescence. Lung sections were stained with DAPI, Alexa Fluor 488–conjugated mouse anti-GATA3 (Biolegend), Alexa Fluor 647–conjugated mouse anti-CD3 (Biolegend), and Alexa Fluor 750–conjugated mouse IL-13 (R&D Systems, Minneapolis, Minn). AlexaFluor N-hydroxy succinimidyl esters were purchased from Thermo Fisher. ILC2s were identified as IL-131GATA31, and T cells were identified as IL-131GATA31CD31.

Images were visualized by using an Axioplan ApoTome microscope with appropriate filters (Carl Zeiss, Thornwood, NY). Muc5ac staining in the
airway epithelium, ILC2s, and CD3+ T cells were quantified by National Institutes of Health ImageJ software (Bethesda, Md). Four separate mouse lungs from each of the 4 conditions were processed for sectioning. One section from the mid-left lung was analyzed from each mouse. Between 3 and 5 separate airways of similar size from each lung were chosen for analysis. Muc5ac expression was calculated as the fraction of Muc5ac+ epithelium compared with the total basement membrane length. For cell counts, the average number of cells per airway for each lung is shown.

FIG 3. RV infection induces ILC2 expansion. A, Figure showing flow cytometry analysis of live lineage-negative, CD25+ CD127+ ILC2s. B, Graph showing group mean data for ILC2s. Data shown are means ± SEMs; n = 7 per group from 3 different experiments; *indicates different from sham plus sham, †indicates different from RV-A1B plus sham; P < .05 by 1-way ANOVA and Tukey multiple comparison test. C, Flow cytometry analysis of lineage markers and IL-13 in sham, RV-A1B, RV-A2, and RV-A1B plus RV-A2 groups. D, Lineage-negative, IL-13+ cells (left panel) and lineage-positive, IL-13+ cells (right panel) for the 4 groups. Data are means ± SEMs; n = 3 per group from 1 experiment; *indicates different from sham plus sham; †indicates different from RV-A1B plus sham; P < .05 by 1-way ANOVA and the Tukey multiple comparison test. E, IL-13 production from sorted lineage-negative, CD25+ CD127+ ILC2s. ILC2s were stimulated with either phorbol myristate acetate and ionomycin (left panel) or IL-33 (right panel). Data shown are means ± SEMs; n = 9 or 10 per group from 3 experiments for phorbol myristate acetate and ionomycin stimulation; n = 3 per group from 1 experiment for IL-33 stimulation; *indicates different from sham plus sham; †indicates different from RV-A1B plus sham; P < .05 by 1-way ANOVA and the Tukey multiple comparison test.
**Flow cytometric analysis**

Lungs were perfused with PBS containing EDTA and minced and digested in collagenase IV. Cells were filtered and washed with RBC lysis buffer. For staining with anti–IL-13 antibody, the cells were incubated for 3 hours with cell stimulation cocktail and protein transport inhibitors. Nonspecific binding was blocked by 1% FBS with 1% LPS-free BSA in Dulbecco modified Eagle medium, and 5 μg of rat anti-mouse CD16/32 (Biolegend) was added. To identify ILC2s, cells were stained with FITC-conjugated antibodies for the lineage markers CD3ε, TCRβ, B220/CD45R, Ter-119, Gr-1/Ly-6G/Ly-6C, CD11b (Biolegend), CD11c (Biolegend), TCRβ (Biolegend), F4/80 (Biolegend), FcRγI (Biolegend), anti-CD25–peridinin-chlorophyll-protein complex (PerCP)-Cy5.5 (eBioscience), and anti-CD127–allophycocyanin (APC; eBioscience), as described. After the staining for cell surface antigens, dead cells were stained with DAPI for flow sorting with live cells or Pac-Blue Live/Dead fixable dead staining dye for further staining with anti–IL-13 antibody (eBioscience). For IL-13 staining, cells were fixed and permeabilized by using permeabilization buffer (eBioscience) and stained with phycocyanin (PE)-labeled anti-IL-13 antibody (eBioscience). Cells were subjected to flow cytometry on an LSR Fortessa flow cytometer (BD Biosciences, San Jose, Calif), or sorted on a FACS Aria II cell sorter (BD Biosciences). Sorted lung ILC2s were cultured in vitro and then stimulated with cell stimulation cocktail (phorbol myristate acetate and ionomycin from eBioscience) or recombinant mouse IL-33 (50 ng/mL, Biolegend). Data were collected by using FACSDiva software (BD Biosciences) and analyzed by using FlowJo software (Tree Star, Ashland, Ore).

**Data analysis**

All data were represented as means ± SEs. For studies of airway responsiveness, statistical significance was assessed by 2-way analysis of variance. For all other experiments, statistical significance was assessed by 1-way analysis of variance. Group differences were pinpointed by using the Tukey multiple comparison test.

**RESULTS**

Heterologous RV infection induces exaggerated airway responses

We found that RV infection of 6-day-old BALB/c mice, but not mature mice, induces an asthma-like phenotype that is associated with ILC2 expansion and dependent on IL-13, IL-25, and IL-33. However, the effects of early-life heterologous infection are unknown. Mice were treated on day 6 of life with RV-A1B and day 13 of life with RV-A2 as follows (Fig 1, A): (1) sham on day 6 plus sham on day 13, (2) RV-A1B on day 6 plus sham on day 13, (3) sham on day 6 plus RV-A2 on day 13, and (4) RV-A1B on day 6 plus RV-A2 on day 13. Mice were humanely killed and their lungs harvested at day 20. As seen previously,
early-life RV-A1B infection (at day 6 of age) increased total lung expression of the mRNA of the ILC2 products IL-5 and IL-13 and the mucus-related genes *Muc5ac*, *Muc5b*, and *Gob5* (Fig 1, B). In contrast, later infection with RV-A2 (on day 13 of life) failed to increase mRNA expression of these genes. IFN-γ mRNA was induced after late RV-A2 infection but not early RV-A1B infection, which is consistent with a mature antiviral response.18 When we did the reverse experiment and infected 6-day-old mice with RV-A2 and 13-day-old mice with RV-A1B, we got similar results (see Fig E1 in this article’s Online Repository at www.jacionline.org).

Next, we examined BAL inflammatory cell counts in RV-A1B– and RV-A2–treated mice. As shown previously, RV-A1B infection on day 6 of life significantly increased the number of eosinophils in the BAL fluid (Fig 1, C). Further, the numbers of eosinophils were significantly increased in mice undergoing heterologous infection compared to mice infected with RV-A1B on day 6 alone. RV-A2 infection on day 13 did not significantly increase eosinophils. There was an increase in lymphocytes in mice undergoing heterologous infection compared to the other groups (Fig 1, C).
To determine whether enhanced type 2 inflammatory responses in mice undergoing heterologous infection were due to an increased viral load, we measured lung vRNA levels. First, RV-A1B and RV-A2 infections showed a similar time course of vRNA (see Fig E2, A in this article’s Online Repository at www.jacionline.org) and infectious virions were detected from mouse lungs infected with either strain (see Fig E2, B). When we compared vRNA levels in mice infected with RV-A1B on day 6 and RV-A2 on day 13 with mice infected with RV-A2 on day 13 alone, mice undergoing heterologous infection unexpectedly showed a reduction in viral copies 1 day after RV-A2 infection (Fig 1, D), suggesting that early-life RV-A1B infection altered the immune response against subsequent RV-A2 infection. By days 2 and 7 after RV-A2 infection, there were no differences in vRNA level. Finally, there were no significant differences in Il5, Il13, Muc5ac, and Muc5b mRNA expression between male and female mice (Fig 1, E).

We examined mice for PAS and Muc5ac staining, which is evidence of mucous metaplasia. As shown previously, compared with sham infection, RV-A1B infection on day 6 of life increased PAS staining (Fig 2, A). Because PAS detects glycoproteins, carbohydrates, and mucins, staining indicates mucous metaplasia. Infection with RV-A2 on day 13 had no effect on PAS staining. The mice treated with RV1B on day 6 plus RV-A2 on day 13 showed significantly greater PAS staining than did the mice treated with RV1B on day 6 (Fig 2, A). Similar results were obtained for deposition of the gel-forming mucin Muc5ac (Fig 2, B and C), which is further evidence of mucous hypersecretion.

**Heterologous infection with RV1B and RV-A2 induces ILC2 expansion and IL-13 production**

Next, we examined the effects of heterologous infection on lung ILC2 expansion.

As shown previously, compared with sham infection, RV-A1B infection on day 6 of life increased the number of lung lineage-negative CD25⁺ CD127⁺ ILC2s. Compared with infection with RV-A1B alone, heterologous infection with RV-A1B and RV-A2 increased lung ILC2 expansion further (Fig 3, A and B). Furthermore, when we stained cells for IL-13, most of the IL-13-expressing cells were lineage negative (Fig 3, C). Compared with infection with RV-A1B alone, heterologous infection with RV-A1B and RV-A2 increased the number of lung IL-13-expressing lineage-negative cells (Fig 3, D). However, heterologous infection also led to an increase in the number IL-13-expressing lineage-positive cells when compared with the number in other groups (Fig 3, D). When we measured IL-13 production from sorted lung ILC2s, phorbol myristate acetate– and ionomycin-stimulated ILC2s from lungs of mice treated with RV-A1B plus RV-A2 showed increased IL-13 production compared with that in cells from mice infected with RV-A1B alone (Fig 3, E). Similar results were obtained when cells were stimulated with IL-33, which is a physiologic agonist.

Finally, we assessed lung ILC2s by immunofluorescence microscopy. Mice infected with RV-A1B on day 6 of life demonstrated significantly increased lung IL-13⁺ GATA3⁺ CD3⁺ cells, which is consistent with lung ILC2 expansion (Fig 4, A-C). Heterologous infection with RV-A2 on day 13 of life further augmented lung ILC2s. Heterologous infection also increased the number of IL-13⁺, GATA3⁺, and CD3⁺ cells, suggesting IL-13 expression by T1h2 cells.

As already noted, compared with sham-treated mice, mice infected with RV-A1B 7 days before RV-A2 infection on day 13 of life failed to show a mature antiviral response, as was evidenced by the absence of IFN-γ mRNA expression. These data suggest that early-life RV-A1B infection shifted the response to subsequent RV-A2 infection from a type 1 to a type 2 response. To better characterize the effect of early-life RV-A1B infection on the immune response to subsequent heterologous RV infection, we compared airway responses of the sham plus RV-A2 group and the RV-A1B plus RV-A2 group 2 days after RV-A2 infection. Compared with sham infection, preceding RV-A1B infection significantly reduced RV-A2–induced lung expression of CXC1L1 and CXC1L2 mRNA (Fig 5, A). In contrast, prior RV-A1B infection increased mRNA expression of lung CCL11 and CCL24. Immunofluorescence imaging showed increased epithelial cell CCL11 protein expression (Fig 5, B).

**Rora<sup>fl/fl</sup>Il7rcre<sup>mice lacking functional ILC2s demonstrate suppressed type 2 inflammation, eosinophilic inflammation, mucous metaplasia, and airway responsiveness on heterologous infection**

The Rora<sup>fl/fl</sup>Il7rcre<sup>mice lack functional ILC2s.**<sup>25</sup> We infected the Rora<sup>fl/fl</sup>Il7rcre and Rora<sup>fl/fl</sup> mice with RV-A1B and determined the number of ILC2s by flow cytometry. Rora<sup>fl/fl</sup> mice showed expansion of the number of lung ILC2s in response to RV-A1B treatment. Rora<sup>fl/fl</sup>IL7rcre showed almost a complete lack of ILC2s (Fig 6, A). Next, we determined BAL inflammatory cell...
counts after RV-A1B and RV-A2 treatment. The numbers of eosinophils were significantly increased in RV-A1B–infected 
Rora \(^{fl/fl}\) mice and further increased in Rora \(^{fl/fl}\) mice infected 
with RV-A1B on day 6 and with RV-A2 on day 13 (Fig 6, B). In 
contrast, Rora \(^{fl/fl}\) Ilt7 \(^{cre}\) mice showed almost a complete absence 
of eosinophils and mucus gene expression. On heterologous 
infected, Rora \(^{fl/fl}\) Ilt7 \(^{cre}\) mice also showed significant but 
incomplete suppression of IL-5, IL-13, and IL-4 compared to 
Rora \(^{fl/fl}\) mice (Fig 6, C). Rora \(^{fl/fl}\) Ilt7 \(^{cre}\) mice showed no decrement 
in TNF-\(\alpha\) or IL-12 mRNA expression, demonstrating that ILC2s 
are not responsible for these cytokines. Rora \(^{fl/fl}\) Ilt7 \(^{cre}\) mice 
appeared to show a slight increase in viral copy number 7 days after 
infection (Fig 6, D). Finally, compared with similarly treated Rora 
\(^{fl/fl}\) mice, Rora \(^{fl/fl}\) Ilt7 \(^{cre}\) mice infected with RV1B on day 6 plus 
RV-A2 on day 13 showed an absence of PAS staining (Fig 6, E) 
or airway responsiveness (Fig 6, F).

**DISCUSSION**

Early-life wheezing-associated respiratory tract infection by RV 
is considered a risk factor for asthma development. \(^{1-3}\) Children are 
infected with many different RV strains, with infants having 6 to 10 
distinct RV infections per year. \(^{4}\) RV infections do not induce spe-
cific immunity to reinfection by heterologous serotypes, even if vi-
ruses are from the same species (eg, RV-A1A and RV-A2). \(^{5,6}\) 
Recurrent RV infections could result in greater degrees of airway 
inflammation and the potential for airway remodeling and loss of 
lung function over time. To test this, we infected wild-type 
BALB/c mice with RV-A1B on day 6 of life and RV-A2 on day 
13 of life. RV infection of 6-day-old mice, but not mature mice, 
induces an asthma-like phenotype that is associated with ILC2 
expression and dependent on IL-3, IL-25, and IL-33. \(^{18}\) We found 
that compared with mice undergoing RV-A1B infection alone, 
mice undergoing heterologous infection with RV-A1B and 
RV-A2 showed additive increases in IL-13, IL-5, IL-4, Gob5, 
Muc5b, and Muc5ac mRNA expression; additive expansion of eo-
sinophils; and exaggerated mucus metaplasia. These data demon-
strate that successive RV infections can result in greater degrees 
of inflammation and mucus production than a single infection does.

These results are more significant when examined in the 
context of development. By examining the age dependency of 
RV responses, we previously found that RV induced IL-13 and IL-
25 expression in 6-day-old mice, whereas mice aged 8 days and 
older showed significant IFN-\(\gamma\) expression. Thus, as expected, 
mice infected with sham on day 6 of life and RV-A2 at day 13 of 
life showed mature antiviral responses. However, infection with 
RV-A1B on day 6 life shifted the response to later RV-A2 
infected from a type 1 to a type 2 response, as evidenced by 
increased IL-5 and IL-13 mRNA expression and reduced IFN-\(\gamma\) 
expression. In addition, compared with sham-infected mice, mice 
injected with RV-A1B on day 6 and RV-A2 on day 13 of life 
showed increased lung CCL11 and CCL24 and reduced CXCL1 
and CXCL2 mRNA responses, which is consistent with the notion 
that early-life RV infection alters the host response to 
heterologous infection, causing a shift toward type 2 
inflammation. However, mRNA expression of TNF and IL-12b 
was also exaggerated in mice infected with both RV-A1B and 
RV-A2, suggesting that heterologous infection does not strictly fit 
a type 2 model.

Successive infections with RV-A1B and RV-A2 increased lung 
expression of IL-5, IL-13, and IL-4 mRNA. ILC2s produce IL-4, 
IL-5, and IL-13 and are dependent on ROR\(\gamma\) and GATA3 for their 
development. \(^{26-30}\) Accordingly, mice undergoing heterologous 
infected also showed significant expansion of CD25\(^{+}\) CD127\(^{+}\) 
ILC2s. Immunofluorescence imaging also showed expansion of 
IL-13\(^{+}\) GATA3\(^{+}\) CD3\(^{+}\) cells around the airways. To test the 
requirement of ILC2s for eosinophilic inflammation and mucus 
metaplasia, we infected Rora \(^{fl/fl}\) Ilt7 \(^{cre}\) mice lacking 
functional ILC2s \(^{25}\) as well as their Rora \(^{fl/fl}\) littermates. Compared with 
Rora \(^{fl/fl}\) mice, Rora \(^{fl/fl}\) Ilt7 \(^{cre}\) mice showed complete suppression of BAL eosinophils, mucus metaplasia, 
and airway hyperresponsiveness. We conclude that early-life 
heterologous infection with RV-A1B and RV-A2 induces 
an exaggerated asthma-like phenotype that is dependent on 
ILC2s.

ILC2s establish their presence in tissues primarily during early 
postnatal development, \(^{18,31}\) and increases in tissue ILC2s 
following infection are mediated through local expansion. \(^{31}\) 
Further, pulse-chase experiments in naïve adult mice show 
persistence of IL-5–producing ILC2s for at least 4 weeks, with 
a substantially lower rate of decay than in labeled CD4\(^{+}\) T cells. \(^{32}\) 
We found that ILC2s from lungs of mice treated with RV-A1B 
plus RV-A2 showed increased IL-13 production compared with 
that in cells from mice infected on with RV-A1B alone. It is 
therefore conceivable that lung ILC2s arising after the initial 
RV-A1B infection form a stable population of innate immune 
cells capable of responding to subsequent infections, albeit in a 
nonspecific manner. This concept of “trained immunity” (ie, 
heterologous immunity attributable to innate immune memory) 
has been reviewed elsewhere. \(^{20}\) Future studies, using a longer 
gap between infections and fate mapping approaches, \(^{31}\) could 
answer this question.

Other cells besides ILC2s may be involved in the altered 
response to heterologous RV infection. Successive infection 
with RV-A1B and RV-A2 increased the number of lineage-
positive IL-13–secreting GATA3\(^{+}\) CD3\(^{+}\) cells. In ILC2-
deficient mice, the adaptive T\(_{H2}\) cell response to protease 
agon was impaired because of the loss of ILC2-derived IL-13, 
which promoted dendritic cell migration to the draining lymph 
node \(^{33}\) and production of the \(\text{T}_{H2}\) cell–attracting chemokine 
CCL17. \(^{34}\) ILC2s also express the costimulator molecule 
OX40L, which is needed for tissue-restricted T-cell costimula-
tion. \(^{35}\) It is therefore possible that ILC2s cooperate with 
adaptive T\(_{H2}\) cells to drive pathologic airway inflammation in 
response to heterologous viral infection. M2-polarized macro-
phages could also produce IL-5 and IL-13 in response to 
heterologous viral infection. In ovalbumin-treated mice, 
macrophages may produce IL-17 or IL-13 in response to 
RV-A1B infection, depending on their activation state. \(^{36}\) 
Macrophages may also produce IL-5 and IL-13 in response to 
IL-25 and IL-33. \(^{37}\) IL-4 and/or IL-13 production by CD4\(^{+}\) T cells 
and ILC2s activate M2 macrophages required for lung immunity 
against hookworms. \(^{38}\) In sputum from patients with asthma, 
there is a positive correlation between numbers of ILC2s and 
numbers of M2-polarized macrophages, and coculture of 
ILC2s with alveolar macrophages induced expression of M2 
macrophage-related genes. \(^{39}\) Finally, we have also demon-
strated that compared with sham infection, prior RV-A1B infec-
tion increases RV-A2–induced epithelial cell expression of 
CCL11, which is consistent with the notion that early-life RV 
infection directly or indirectly alters the response of epithelial 
cells to subsequent infection.
We would like to mention 2 limitations to our study. First, replication of human RV is minimal in mice. Species differences restrict replication, requiring a high inoculum. However, infection with RV-A1B increases lung type 1 IFN production and negative-strand vRNA expression, which are markers of viral replication. Although replication is limited, the resulting host-induced innate immune response and immunopathology can still be studied. Indeed, replication-deficient viral vectors are a useful tool for studying the innate immune response to acute viral infection without ongoing cytopathic effects. Second, it is difficult to compare the maturity of immature mice and human infants. Although it may be surprising that 6- and 13-day-old-mice, which are still nursing, demonstrate qualitatively different immune responses to viral infection, we have noted previously that the response to respiratory viral infection shifts from an immature type 2 response (characterized by IL-13 and IL-25) to a mature type 1 response (IFN-γ) around 8 days of age. In a subsequent study, we showed that IFN-γ treatment inhibits RV-induced ILC2 function and expansion, suggesting deficient IFN-γ production in 6-day-old mice permits RV-induced type 2 immune responses. Thus, breast-feeding is just 1 indicator of maturity, and it may not reflect structural or functional maturity of the lungs or the development of pulmonary immune function.

We conclude that early-life heterologous infection with RV-A1B and RV-A2 induces an intensified asthma-like phenotype consisting of airway eosinophilic inflammation and mucous metaplasia. Furthermore, early RV infection shifted the immune response to subsequent infection with a heterologous RV strain toward a type 2 response. This model, which is the first preclinical model to combine 2 early-life respiratory viral infections, may provide insight into the development of childhood asthma.

Key messages
- Early-life (on day 6), RV infection of immature mice induces eosinophilic inflammation and mucous metaplasia, whereas later infection (on day 13) induces a mature type 1 antiviral response.
- Early-life infection with RV-A1B skews the immune response to subsequent heterologous infection with RV-A2, causing an exaggerated asthma phenotype unrelated to increased viral load.
- ILC2s are required for exaggerated eosinophilic inflammation and mucous metaplasia due to heterologous RV infections.

REFERENCES
5. Moraes TJ, Sears MR. Lower respiratory infections in early life are linked to later asthma. Thorax 2018;73:105-6.


FIG E1. Reversal of RV strain order yields similar airway responses. We infected 6-day-old mice with sham or RV-A2 and 13-day-old mice with sham or RV-A1B. Lungs were harvested on day 20 and processed for RNA analysis. Lung expression of IL-13, IL-5, Muc5ac, Muc5b, Gob5 (Clca1), IFN-γ, and TNF-α mRNA was quantified by quantitative PCR. Gene expression values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data shown are means ± SEMs; n = 3 to 5 per group from 2 different experiments; *indicates different from sham + sham; P < .05 by 1-way ANOVA; †indicates different from RV-A1B + sham; P < .05 by 1-way ANOVA and the Tukey multiple comparison test.
FIG E2. Detection of vRNA and infectious virions from RV-A1B– and RV-A2–infected mice lungs. Six-day-old mice were infected with RV-A1B and RV-2 and harvested at the indicated time points for vRNA analysis (A) and plaque assay (B). For vRNA, data represent means ± SDs for a sample size of 3 per group from 1 experiment.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>Gapdh</td>
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<td>5'-TTCCTTCACCTGGATTTGGG-3'</td>
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<td>5'-CGTGGTCTATGGGCGATGACG-3'</td>
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<tr>
<td>Ifng</td>
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<td>5'-GGAGTCCAGTCACCTCTCA-3'</td>
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<td>5'-CTCCTGTGGTTGCCCATTGTT-3'</td>
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<tr>
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<td>5'-AAAGACACCAGTAGACTAGCA-3'</td>
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<td>5'-CTGGGAAGTGGGCTATGTGTTCTC-3'</td>
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<td>SRY</td>
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<td>5'-CTTACACCTTCACGAGGAGG-3'</td>
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GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.