Potential Role of Early-life Rhinovirus Infection in the Development of Asthma

by

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Dedication

To my wife, Jihye. For your patience and selfless commitment for me and family.

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List of Abbreviations

ANOVA, analysis of variance; **BAL**, bronchoalveolar lavage; **CD**, cluster of differentiation; **COPD**, chronic obstructive pulmonary disease; **DC**, dendritic cell; **ELISA**, enzyme-linked immunosorbent assay; **GAPDH**, Glyseraldehyde-3-phosphate dehydrogenase; **ICAM**, intercellular adhesion molecule**;** **IFN-γ**, interferon gamma, **IL,** interleukin**;** **ILC2**, type 2 innate lymphoid cells; **OVA**, ovalbumin; **PBS**, phosphate-buffered saline; **PVM**, pneumonia virus of mouse; **RSV**, respiratory syncytial virus, **RT-PCR**, reverse transcriptase polymerase chain reaction; **RV**, Rhinovirus; **TSLP**, thymic stromal lymphopoietin; **TSLPR KO**, thymic stromal lymphopoietin receptor knock-out; **UV**, ultraviolet.

Chapter 1

Introduction

Rhinovirus

Rhinovirus (RV) is small, non-enveloped, positive-stranded RNA virus belonging to the *Picornaviridae* family. To date, more than 100 serotypes were identified, and the major group serotypes, such as RV14, 16 and 39, bind to intercellular adhesion molecule (ICAM)-1 (1), while minor group viruses, for example RV1A, 1B and 2, bind to low density lipoprotein family receptors (LDLR) (2). Based on their sequence, RV has been further classified into two main phylogenetic species, RV-A and RV-B (3, 4). A novel RV-C species has recently been identified (5, 6). After binding, endocytosis, and endosomal acidification, RV releases their viral RNA into cytosol. RV rapidly replicates, requiring approximately 8 hours for the full cycle of replication. High mutation rate during replication results in distinct genetic diversity.

RV has been indicated as the most frequent cause of the common cold. The symptoms of RV infection are usually mild in normal healthy persons compared to the infection with other respiratory viruses like influenza and respiratory syncytial virus (RSV). However, RV infection in patients with chronic respiratory diseases such as asthma or chronic obstructive pulmonary disease (COPD) causes severe exacerbations of the disease state. Asthma exacerbations are characterized by increased airway

inflammation, excessive mucus production, and bronchoconstriction which together result in airflow obstruction. Viral infections are responsible for 80% of asthma exacerbations in children and roughly 50% of exacerbations in adults (7, 8). RV accounts for the most virus-induced asthma exacerbations (9) although the specific mechanisms of RV triggered asthma exacerbations are not completely known.

Potential role of early-life rhinovirus infection in asthma initiation

Asthma is a chronic inflammatory disease characterized by airway inflammation, mucus hyperplasia, and airway hyperresponsinvess which together result in airflow obstruction (10). Asthma is one of the most common chronic diseases worldwide, affecting about 3 million people, with the highest prevalence in westernized countries (11). Results from studies of young children with wheezing have uncovered strong associations between both lung function and immune responses in early life and the subsequent development of persistent asthma to adulthood (12). On this basis, attention has been focused on the origins of asthma in infancy and childhood.

Early-life wheezing-associated respiratory tract infections have long been considered risk factors for asthma. Initial attention focused on the potential role of respiratory syncytial virus (RSV). Data from the Tucson Children's Respiratory Study showed that RSV lower respiratory tract illness in early childhood was an independent risk factor for the subsequent development of wheezing up to age 11 years, but not at age 13 (13). In Sweden, children hospitalized with RSV bronchiolitis have a greater risk of asthma and allergy at ages 7 and 13 (14, 15). In a St. Louis cohort, about 50% of children who required emergency department treatment or hospitalization for RSV

bronchiolitis had a diagnosis of asthma at age 3-7 years (16).

Evidence also exists for an association between early-life rhinovirus (RV) infection and asthma. In Finnish infants hospitalized for respiratory infection-associated wheezing, RV was associated with asthma development in contrast to RSV, which was negatively associated (17). Data from a birth cohort of high-risk infants from Madison, Wisconsin (one parent with respiratory allergies or a history of physician-diagnosed asthma) showed that wheezing-associated illness with RV is the most important risk factor for asthma development, higher than that of infants with allergen sensitization or RSV infection (18, 19). A population-based retrospective analysis of a birth cohort of 90,000 Tennessee children showed an increased risk of early childhood asthma following bronchiolitis during RV-predominant non-winter months vs. RSV-predominant winter months (20). Together, these data are consistent with the notion that early-life viral infections, including those with RV, perhaps in combination with other factors such as genetic background, allergen exposure and microbiome, modulate the immune response, increasing the likelihood of childhood asthma development.

Alternatively, RV infections may simply reveal a pre-existing tendency for asthma. For example, further analysis of the Wisconsin birth cohort showed that allergic sensitization precedes RV-associated wheeze more often than the converse (21). Also, Danish children developing asthma by age seven had a lung function deficit and bronchial hyperresponsiveness as neonates (22) suggesting that asthma precedes RV infection. However, none of these studies, which correlate respiratory tract infection, airway function and allergen sensitization, provide causal information regarding the potential effects of RV infection on asthma development or the underlying mechanisms

involved. Such data can only be provided using an animal model (Figure 1-1).

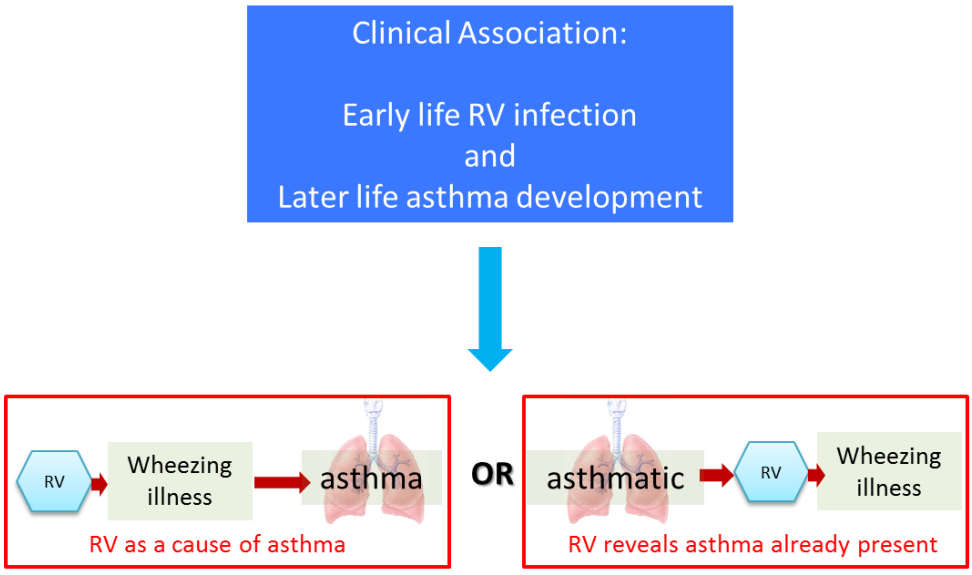

Figure 1-1. Possible implications of the clinical association between early-life RV infection and later-life asthma development. RV infection in early life can modulate the developing immune response, increasing the likelihood of asthma development. On the other hand, wheezing with RV infection may simply reveal a pre-existing tendency for asthma. An improved understanding of immune responses to RV in early life is needed to resolve this issue.

Neonatal immunity

Neonates are more vulnerable to infection than adults. Studies have found that both innate and adaptive immunity of neonates are qualitatively different from that of adults (23, 24). One of the key features of neonatal immunity is a diminished pro-inflammatory type 1 immune response (including cytokines such as IL-12 and interferons), which make them susceptible to viral and microbial infection (25-32). Moreover, type 2-polarized immune responses have been observed in neonates. For example, mice initially exposed or immunized as neonates showed type 2-biased memory responses when re-exposed to the same infection or antigens as adults (33-35). These suppressed type 1and enhanced type 2 immune responses in neonates might be attributed to several factors including age-related epigenetic changes (36), increased specific plasma factors such as adenosine (37), heightened immune tolerance via regulatory T cells (38) or CD71+ erythroid cells (39). After birth, infants undergo a significant transition from the sterile *in utero* environment to a world full of foreign antigens. It has been suggested that the decreased type 1 immune response helps neonates to avoid an unnecessary pro-inflammatory response when colonized by commensal microorganisms in the skin and intestinal tracts (40). However, prolonged maintenance of this type 2-biased immature immunity and the lack of strong type 1 immune stimuli could lead to the development of allergy and asthma, in accord with hygiene hypothesis (41).

Asthma and type 2 immune response

Asthma has been long considered as a cluster of differentiation (CD)-4+ T-helper-2 (Th2)-driven allergic airways disease. In the presence of allergen, antigen

presenting cells (APC) such as dendritic cells and macrophages residing in the peripheral tissues process the antigen and present the antigenic peptide to naïve CD4 T cells in the lymph nodes. In the presence of pro-allergic cytokines IL-4 and IL-13, or with the expression of cell surface molecules such as OX-40L on APC, naïve CD4 T cells differentiate into the Th2 cell lineage. Then, CD4+ Th2 cells secrete more type 2 cytokines, including IL-4, IL-5, and IL-13, as well as granulocyte macrophage colony stimulating factor (GM-CSF) (42). Among those cytokines, the role of IL-13 in asthma pathogenesis has been highlighted. Transgenic mice overexpressing IL-13 show enhanced airway hyperresponsiveness and type 2 cytokine production, suggesting that IL-13 plays a critical role in exacerbating allergic airway inflammation (43). In humans, IL-4, IL-5, and IL-13, have been found in bronchial biopsy specimens from asthmatics (44, 45). These type 2 cytokines promotes eosinophil infiltration, mucus secretion (46, 47) and contribute to overall airway inflammation and airflow obstruction.

TSLP and IL-25

TSLP, IL-25 and IL-33 are epithelium-derived cytokines which play a role in the maturation of Th2 cells via DC activation (48-53). These cytokines also induce the activation and IL-13 production from innate immune cells, namely type 2 innate lymphoid cells (ILC2s) (54-60), iNKT cells (61-63) and type 2 myeloid cells (64). TSLP is an IL-7-like cytokine which exerts its biological activity via a high-affinity TSLPR complex that is a heterodimer of the TSLPR chain and IL-7 receptor-α. Transgenic mice that overexpress TSLP in the lungs show an augmented Th2 inflammatory response and airway remodeling (goblet cell hyperplasia and subepithelial fibrosis) when challenged

with ovalbumin, whereas TSLPR-deficient mice show an attenuated Th2 inflammatory response and remodeling (65). TSLP and IL-25 are secreted from airway epithelial cells upon house dust mite challenge, leading to dendritic cell (DC) activation and adaptive Th2 response (66). IL-25/IL-17E is a member of the structurally related IL-17 cytokine family which binds to the IL-17B receptor (IL-17BR). Overabundance of IL-25 leads to an allergic phenotype characterized by high levels of IL-4, IL-5, and IL-13, airways hyperresponsiveness and enhanced IgE (67, 68), whereas IL-25 deficiency leads to reduced Th2 cytokine production (69).

Because the airway epithelium is the primary target of respiratory viral infection, TSLP, IL-25, IL-33 and their downstream cellular targets are uniquely positioned to play a role in viral-induced chronic airways disease. RV16 induces TSLP expression in primary human bronchial epithelial cells (53, 70), and RSV and influenza have similar effects (71, 72). RV infection in young children is associated with elevated airway TSLP levels (73). Immature mice infected with pneumonia virus of mice (PVM), like RSV a member of the of the *Paramyxoviridae* family, show increased lung IL-25 mRNA expression (74), and administration of anti-IL-25 decreased PVM-induced lymphocytic and eosinophilic airway inflammation (75). IL-17RB^{+} iNKT cells are required for the development of RSV-induced airways hyperresponsiveness (76) and IL-25-dependent ILC2s have been shown to be required for PVM-induced airways responses in immature TLR7 knockout mice (77).

Little is known about changes in viral-induced TSLP and IL-25 expression with development. However, there is reason to believe that expression of these cytokines might be accentuated in newborns and infants. As noted above, immature animals are

more susceptible to viral infection, in part due to impaired IL-12 and IFN production (49-56). As shown previously with neonatal adenovirus infection (78), baby mice show impaired IFN-γ responses to human rhinovirus 1B (RV1B) infection compared to adults, as well as reduced levels of IL-12 and TNF-α. We found that RV-infected immature 6 day-old mice maintained high levels of viral RNA ≥ 72 h after infection, representing at least three rounds of viral replication, in contrast to 8-week-old mature mice in which vRNA and titer peak within 24 h after infection (79). IFN-γ has been shown to inhibit double-stranded RNA-induced TSLP production in fibroblasts (80) and keratinocytes (81). Thus, immature animals with relatively impaired IFN responses would be expected to express higher levels of TSLP in response to viral infection.

Type 2 innate lymhphoid cells

Innate lymphoid cells (ILCs) have morphological characteristics similar to those of lymphocytes, but they do not express T cell or B cell antigen receptors and lack cell surface markers associated with other cell lineages of the immune system. ILCs express CD90 (Thy1 antigen), CD25 (IL-2Rα) and CD127 (IL-7Rα), and their development is partially or wholly dependent on the common γ-chain (γc or CD132), IL-7, Notch and the transcription factor Id2. Lineage (Lin)-negative, c-Kit$^+$, Sca-1$^+$ "natural helper cells" are required for IL-13 production and goblet cell hyperplasia during intestinal infection with *N. brasiliensis* (54) and IL-13 production and airways hyperresponsiveness following influenza A infection (56). In addition, non-T, non-B, c-kit$^+$, IL-17RB$^+$, ST2L$^+$ "nuocytes" are responsible for IL-13 production in *N. brasiliensis*-infected (55) and ovalbumin-sensitized and -challenged mice (59). Lin-, c;Kit$^+$, Sca-1$^+$, IL-2Rα/CD25$^+$,

IL-7Rα/CD127^{+} "lung natural helper" cells are required and sufficient for IL-13 production, airways hyperresponsiveness and mucus secretion in papain-treated mice (60). Although there are some differences among natural helper cells, nuocytes and lung natural helper cells in terms of their phenotypes and tissue distribution, it has been agreed that these cells should all be referred to as type 2 innate lymphoid cells (ILC2 cells). ILC2s produce IL-5, IL-9, IL-13, and/or amphiregulin and are dependent on RORα, GATA3 and TCF-1 for their development. (82).

Invariant natural killer T cells (iNKTs)

iNKT cells, an unusual group of T lymphocytes that recognize glycolipid antigens presented by the major histocompatibility complex (MHC) class I-related protein CD1d, are also an important source of type 2 cytokines. *Cd1d* -/- mice do not develop ovalbumin-induced airways hyperresponsiveness (83) and α-GalCer, a marine sponge glycolipid antigen that specifically activates CD1d-restricted iNKT cells, is sufficient to induce IL-4/13-dependent airways hyperresponsiveness (84). iNKT cells express TSLPR and IL-7R α-chain, preferentially increase IL-13 production upon TSLP stimulation, and are required for development of ovalbumin-induced airway hyperreactivity in transgenic mice expressing TSLP under the control of the surfactant protein C promoter (61). Other iNKT cells express IL-17RB, produce large amounts of type 2 cytokines upon IL-25 stimulation, and are required for IL-25-mediated airways hyperresponsiveness (62).

Animal models of RV infection

Species differences in ICAM-1 represent the main challenge in developing an

animal model of a human major group RV infection. Recently, we (85) and others (86) showed that minor group serotype RV1B, which binds proteins of the low-density lipoprotein receptor family, infects C57BL/6 and Balb/c mice, thereby providing an animal model to study RV-induced airway inflammation. We reported evidence of human RV1B replication in mouse lungs as follows: 1) the presence of negative-strand viral RNA in the lungs of inoculated mice, 2) the transmissibility of RV infection from the lung homogenates of inoculated mice to cultured HeLa cells, and 3) the induction of a robust lung IFN response (85). Replication-deficient UV-irradiated virus elicited none of these effects. RV infection also caused a moderate increase in airway resistance to methacholine, suggesting a role for RV-induced airway inflammation in airway hyper-reactivity.

The airway response to the major group virus RV16 was recently studied using a transgenic mouse expressing humanized Intercellular Adhesion Molecule 1 (ICAM-1) (86). The effects induced by RV16 were indistinguishable from those induced by RV1B. Studies indicate that major and minor group viruses induce nearly identical patterns of gene expression in cultured airway epithelial cells (87). Furthermore, recent analysis of all known HRV genomes has revealed that HRV1 and HRV16 are highly homologous and respond similarly to small-molecule antiviral compounds (88). Thus, the distinction between at least some major and minor group strains may not be clinically relevant. Therefore, we believe that mouse models of human RV1B infection hold promise for the study of RV-induced exacerbations of chronic airway diseases, such as asthma.

Significance

The onset of asthma is highly influenced by gene-environment interactions during the neonatal period, but the factors involved are not fully identified (41, 89). Recent studies suggest a possible role for the common cold virus, rhinovirus (RV), in the pathogenesis of asthma. Epidemiologic studies in children with a family history of asthma now show a strong association between the development of childhood asthma and wheezing-associated infections with rhinovirus (18, 19). This association with asthma was stronger than those with RSV infection or allergic sensitization (19).

Nevertheless, the question of whether rhinoviral infections can cause asthma is still controversial. While it is possible that early-life RV infection promotes asthma by modulating immune system that is under development, wheezing-associated with RV may simply be a marker of pre-existing airways disease. An improved understanding of immune responses to RV in early-life is needed to resolve this issue.

Recent studies have found that the immunity of newborns is qualitatively different from that of adults. When faced with an infection, neonatal animals are refractory to so-called type 1 pro-inflammatory responses and permissive to type 2 allergic responses (33, 90). It is therefore possible that early-life RV infection promotes allergic asthma by augmenting or maintaining this immature response.

Thus, we attempted to generate a neonatal RV infection model to test the role of early-life RV infection in the development of asthma. We sought to examine this issue with the following specific aims (Figure 1-2):

1. Determine the role of IL-25 and ILC2s in RV-induced asthma phenotype development in neonatal mice (Chapter 2);
2. Determine the contribution of TSLP and ILC2s in RV-induced asthma phenotype

development in neonatal mice (Chapter 3);

3. Determine the role of IL-4R signaling in RV-induced asthma exacerbation (Chapter 4).

This research is significant because it shows a direct casual role of RV infection in the inception of an asthma-like phenotype. Completion of subsequent dissertation work may facilitate the development of effective clinical methods for prevention and treatment of asthma.

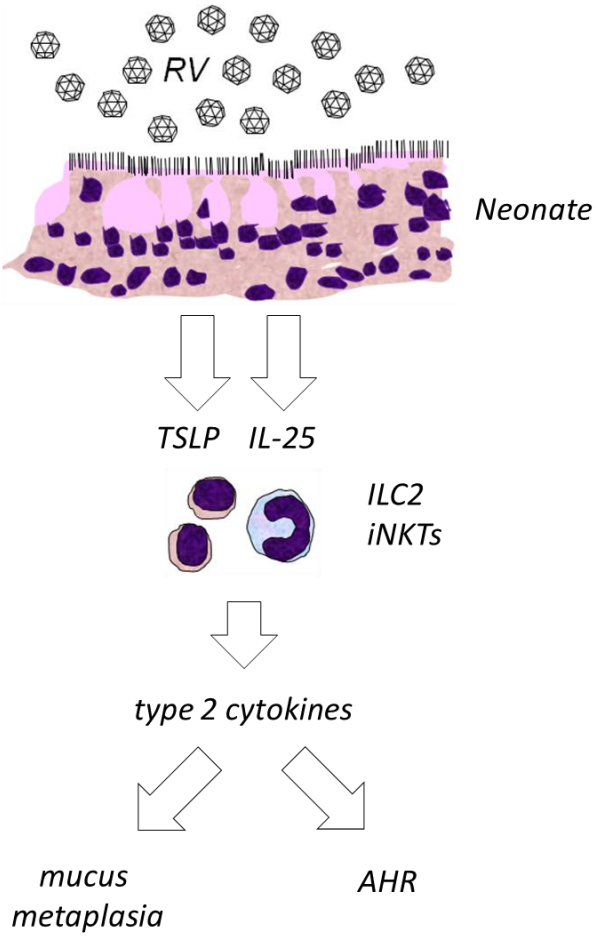

Figure 1-2. Model of neonatal RV-induced mucous metaplasia and airways hyperresponsiveness. RV infection of the airway epithelium induces expression of TSLP (Chapter 1) and IL-25 (Chapter 2), stimulating expansion and/or activation of type 2 cytokine-producing innate immune cells (Chapter 1-3).

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Chapter 2

Neonatal rhinovirus induces mucous metaplasia and airways hyperresponsiveness via IL-25 and ILC2s

Summary

Early-life rhinovirus (RV) infection has been linked to asthma development in high risk infants and children. Nevertheless, the role of RV infection in the initiation of asthma is still controversial. We hypothesized that, in contrast to infection of mature BALB/c mice, early-life infection with RV promotes an IL-25-driven type 2 response which causes persistent mucous metaplasia and airway hyperresponsiveness (AHR). Compared to mature mice, RV infection in neonatal mice increased lung IL-13 and IL-25 production whereas IFN-γ, IL-12p40 and TNF-α expression were suppressed. We also found that the population of IL-13-secreting type 2 innate lymphoid cells (ILC2s) was expanded with RV infection in neonatal but not in mature mice. ILC2 cells were the major cell type secreting IL-13 in neonatal mice. Finally, administration of an anti-IL-25 neutralizing antibody attenuated ILC2 expansion, mucous hypersecretion and airways responsiveness. These findings suggest that early-life viral infection could contribute to asthma development by provoking age-dependent, IL-25-driven type 2 immune responses.

Introduction

The precise mechanisms of asthma development are not fully understood. Initiation of asthma is highly associated with enhanced type 2 and reduced type 1 immunological responses which are in turn influenced by allergen exposure, respiratory infection and genetic susceptibility. Early-life exposures to immune-modulating factors in infancy may be particularly important in determining the vulnerability to lifelong asthma development.

Studies have found that the immature immune system is qualitatively different from that of adult, refractory to type 1 and permissive to type 2 responses. In contrast to mature T cells, human cord blood T cells demonstrate a permissive chromatin architecture at the IL-13 proximal promoter, favoring transcription (1). Murine neonatal CD4+ T cells harbor IL-4/IL-13 regulatory elements which are epigenetically modified to favor type 2 responses (2). With antigen challenge, secondary exposure to antigen causes IL-4-dependent depletion of T helper type 1 (Th1) cells in neonatal but not adult mice (3) Secretion of the type 1 cytokine IL-12 is suppressed in neonatal dendritic cells, thereby inhibiting Th1 cell differentiation (4-6). Finally, the innate type 1 response to TLR stimulation is significantly diminished in neonatal monocytes (7-9). Thus, in early life, type 2-biased neonatal adaptive and innate immune responses could provide a favorable environment for asthma development, particularly when maintained by appropriate stimuli.

Wheezing-associated acute respiratory viral infections in infancy, particularly those caused by respiratory syncytial virus (RSV), have long been considered risk factors for asthma (10, 11). However, recent studies suggest a possible role for the common cold

virus, rhinovirus (RV). Epidemiologic studies now show a strong association between early-life RV infection and the development of asthma in infants and children with a family history of asthma (12). In Finnish infants hospitalized for respiratory infection-associated wheezing, RV was associated with asthma development in contrast to RSV, which was negatively associated (13). Retrospective analysis of a birth cohort of 90,000 children showed an increased risk of early childhood asthma following bronchiolitis during RV-predominant non-winter months vs. RSV-predominant winter months (14). However, while it is possible that early-life RV infection promotes asthma by maintaining an immature type 2 immune response, wheezing associated with RV may simply be a marker of pre-existing airways disease. To examine this question, we tested the effects of RV infection in neonatal and mature BALB/c mice and found that, in contrast to adults, neonatal infection induced type 2 cytokine expression, airway hyperresponsiveness and mucous metaplasia (15). Nevertheless, the mechanisms by which RV infection may lead to chronic airways disease, including the roles of development, remain unclear.

Interleukin 25 (IL-25/IL-17E), a cytokine belonging to IL-17 family, is a potent inducer of type 2 immunity (16). Systemic injection of IL-25 induces eosinophilia, mucus hyperplasia and type 2 cytokines such as IL-4, IL-5, and IL-13 (16). Overexpression of IL-25 in epithelial cells induces goblet cell hyperplasia and a type 2 immune response (17), and intranasal IL-25 administration provokes airways hyperresponsiveness (18). Moreover, blocking of IL-25 reduces airways responsiveness in a mouse model of allergic asthma (19). Recent studies have found that, upon IL-25 stimulation, lineage-negative lymphoid cells expressing the IL-25 receptor IL-17RB

secrete the type 2 cytokines IL-5 and IL-13, promoting a type 2 immune response (20-24). These cells, originally termed natural helper cells, nuocytes, innate helper cells or type 2 multipotent progenitor cells, are now referred to as type 2 innate lymphoid cells (ILC2s) (25). On this basis, we hypothesized that early-life infection with RV promotes an IL-25-driven ILC2-mediated type 2 response, leading to persistent mucous metaplasia and airway hyperresponsiveness.

Methods

Generation of RV.

RV1B (ATCC, Manassas, VA) were grown in HeLa cells, concentrated and partially purified (26). Similarly concentrated and purified HeLa cell lysates were used for sham infection. Viral titer was measured by fifty percent tissue culture infectivity doses ($TCID_{50}$) using the Spearman-Karber method (27) or by plaque assay (28).

RV infection.

Experiments were approved by the University of Michigan Institutional Animal Care and Use Committee. BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were inoculated through the intranasal route under Forane anesthesia with RV1B ($1x10^8$ PFU/ml) or sham HeLa cell lysates. To 5-24 day-old mice, 20 μl of RV1B ($2x10^6$ PFU) or an equal volume of sham was given. To 21 day-old mice, 30 μl of RV1B ($3x10^6$ PFU) or sham was given. To eight week-old mice, 50 μl of RV1B ($5x10^6$ PFU) or sham was administered.

Anti-IL-25 neutralizing antibody treatment.

Six-day old neonatal mice were treated with either 100 μg of neutralizing antibody to IL-25 (clone 35B, Biolegend, San Diego, CA) or isotype control (rat, IgG1κ) intraperitoneally at days 0, 7 and 14 of infection. Mice were sacrificed 3-4 weeks after infection for analysis.

Flow cytometric analysis.

Lungs were perfused with PBS containing EDTA, minced and digested in collagenase IV. Cells were filtered and washed with RBC lysis buffer, and dead cells were stained with Pac-Orange Live/Dead fixable dead staining dye (Invitrogen, Carlsbad, CA). Cells were then stained with FITC-conjugated antibodies for lineage markers (CD3ε, TCRβ, B220/CD45R, Ter-119, Gr-1/Ly-6G/Ly-6C, CD11b, CD11c, F4/80 and FcεRIα, from Biolegend), anti-CD25-PerCP-Cy5.5 (Biolegend), anti-CD127-PE-Cy5 (eBioscience, San Diego, CA), anti-c-kit/CD117-APC (eBioscience), anti-sca-1-PE-Cy7 (eBioscience), anti-T1/ST2-PE (R&D Systems, Minneapolis, MN) and anti-IL-17RB (R&D Systems) conjugated with AF750. Cells were fixed, subjected to flow cytometry and analyzed on a FACSAria II (BD Biosciences, San Jose, CA). Data were collected using FACSDiva software (BD Biosciences) and analyzed using FlowJo software.

Assessment of airway responsiveness.

Airway cholinergic responsiveness was assessed by measuring changes in total respiratory system resistance in response to increasing doses of nebulized methacholine (15). Mice were anesthetized with sodium pentobarbital (50 mg/kg mouse,

intraperitoneal injection) and a tracheostomy performed. Mechanical ventilation was conducted and total respiratory system measured using a Buxco FinePointe operating system (Buxco, Wilmington, NC). Airway responsiveness was assessed by measuring changes in resistance in response to increasing doses of nebulized methacholine. Statistical analysis was performed using two-way ANOVA with repeated measures, employing Graph Pad Prism 6.0 software program.

Histology and immunohistochemistry.

Lungs were collected and fixed with 10% formaldehyde and paraffin embedded. Blocks were sectioned at 500-μm intervals at a thickness of 5 μm, and each section was deparaffinized and hydrated. After antigen demasking and permeabilization, sections were incubated with Alexa Fluor (AF)-488-conjugated rabbit anti-mouse IL-25/IL-17E (Millipore, Billerica, MA), guinea pig antiserum against HRV1B (ATCC), or AF-conjugated isotype control IgGs. Antiserum was partially purified by incubation with nitrocellulose-bound HeLa cell proteins and passing through an affinity resin containing nondenatured mouse lung protein, as described previously (29). Repurified antibody was directly conjugated to AF594. The control used was AF594-conjugated guinea pig antiserum. Nuclei were stained with 4′,6-diamidino-2-phenylindole. Images were visualized using a Zeiss Axioplan microscope equipped with an ApoTome and digital AxioCamMR charge-coupled device camera. To visualize mucus, deparaffinized sections were stained with periodic acid-Schiff (Sigma-Aldrich, St. Louis, MO).

Intracellular cytokine analysis with flow cytometry.

For analysis of intracellular IL-13 production, freshly isolated aliquots of lung mince were stimulated for 5 h with cell stimulation cocktail (PMA (40.5 uM), Ionomycin (670 uM), Brefeldin A (5.3 mM), Monensin (1 mM), eBioscience, San Diego, CA). Cells were then stained for live/dead and surface markers, fixed, permeabilized and incubated with anti-mouse IL-13 (eBioscience, San Diego, CA). Cells were fixed and analyzed utilizing the FACSAria II (BD Biosciences, San Diego, CA).

Fluorescence-activated cell sorting of ILC2s and treatment.

After staining for lineage markers, lineage-negative CD25 and CD127 double-positive ILC2s or lineage-negative CD25 and CD127 double-negative cells were sorted at 9000 cells/200 μl concentration in each well of 96 well plates and stimulated with different factors. To visualize ILC2s, cells were stained with Diff-Quick (Dade Behring, Newark, DE). Cells were stimulated with media, IL-25 (20 ng/ml), IL-2 (50 ng/ml) + IL-25 (20 ng/ml) or PMA + Ionomycin for 3 days. IL-13 protein concentration was measured by ELISA.

Measurement of IL-13 and IL-25.

IL-13 and IL-25 concentrations were measured with ELISA (eBioscience, San Diego, CA). The amount of IL-25 per lung weight was calculated by multiplying the concentration by the volume of lung homogenate divided by the weight of lungs.

Real-time quantitative PCR.

Lung RNA was extracted with Trizol method (Invitrogen, Carlsbad, CA) with the combination of on-column digestion of genomic DNA (Qiagen, Valencia, CA). cDNA was synthesized from 1 μg of RNA and subjected to quantitative real-time PCR using specific mRNA primers for IL-4, IL-5, IL-13, IFN-γ, IL-12p40, TNF-α, Muc5ac, Muc5b, Gob5 and IL-17RB. The sequences of specific primers are provided (Table 2-1).The level of gene expression was normalized to mRNA of GAPDH.

TABLE 2-1. Sequence of primers for quantitative PCR.

Gene name	*Forward primer (5'->3')*	*Reverse primer (3'->5')*
GAPDH	GTC GGT GTG AAC GGA TTT G	GTC GTT GAT GGC AAC AAT CTC
Gob5	CTG TCT TCC TCT TGA TCC TCC A	CGT GGT CTA TGG CGA TGA CG
IFN-g	TGG CTG TTT CTG GCT GTT AC	TCC ACA TCT ATG CCA CTT GAG TT
IL-12p40	CTC CTG GTT TGC CAT CGT TT	GGG AGT CCA GTC CAC CTC TA
IL-13	CCT GGC TCT TGC TTG CGT	GGT CTT GTG TGA TGT TGC TCA
IL-17RB	ACC TTC CGG CGG CAA ATG GAC	GCA TTG GGG ATG TTA TGG GCG CT
IL-25	ACA GGG ACT TGA ATC GGG TC	TGG TAA AGT GGG ACG GAG TTG
IL-33	GGC TGC ATG CCA AGG ACA AGG	AAG GCC TGT TCC GGA GGC GA
IL-4	GGT CTC AAC CCC CAG CTA GT	GCC GAT GAT CTC TCT CAA GTG AT
IL-5	CTC TGT TGA CAA GCA ATG AGA CG	TCT TCA GTA TGT CTA GCC CCT G
Mub5b	GAG CAG TGG CTA TGT GAA AAT CAG	CAG GGC GCT GTC TTC TTC AT
Muc5ac	AAA GAC ACC AGT AGT CAC TCA GCA A	CTG GGA AGT CAG TGT CAA ACC
TNF-a	ATG CAC CAC CAT CAA GGA CTC AA	ACC ACT CTC CCT TTG CAG AAC TC

Results

Compared to adult mice, RV infection of neonatal mice provokes an enhanced type 2 immune response and attenuated type 1 response.

We infected 6 day-old and 8 week-old mice with RV1B and analyzed cytokine gene expression. Unlike mature mice, RV infection of neonatal mice increased expression of the type 2 cytokines IL-13, IL-4 and IL-5 immediately post-infection, with slightly different kinetics (Figure 2-1A). In contrast, induction of type 1 cytokines IFN-γ and IL-12p40 gene was blunted in neonatal mice, whereas expression was increased in mature mice (Figure 2-1B). Compared to sham infection, TNF-α gene expression was significantly increased in both neonates and adults after RV infection, but induction was significantly less in neonatal mice. Consistent with the attenuated type 1 response, viral replication and load tended to be greater 3-7 days after inoculation in neonatal mice compared to adults (Figure 2-2). We performed additional studies examining the age-dependency of RV-induced cytokine responses. RV-induced IL-13 expression was increased, and IFN-γ expression decreased up to 8 days of age (Figure 2-1*C*). These results show that early-life RV infection elicits exaggerated type 2 responses and mitigated type 1 responses.

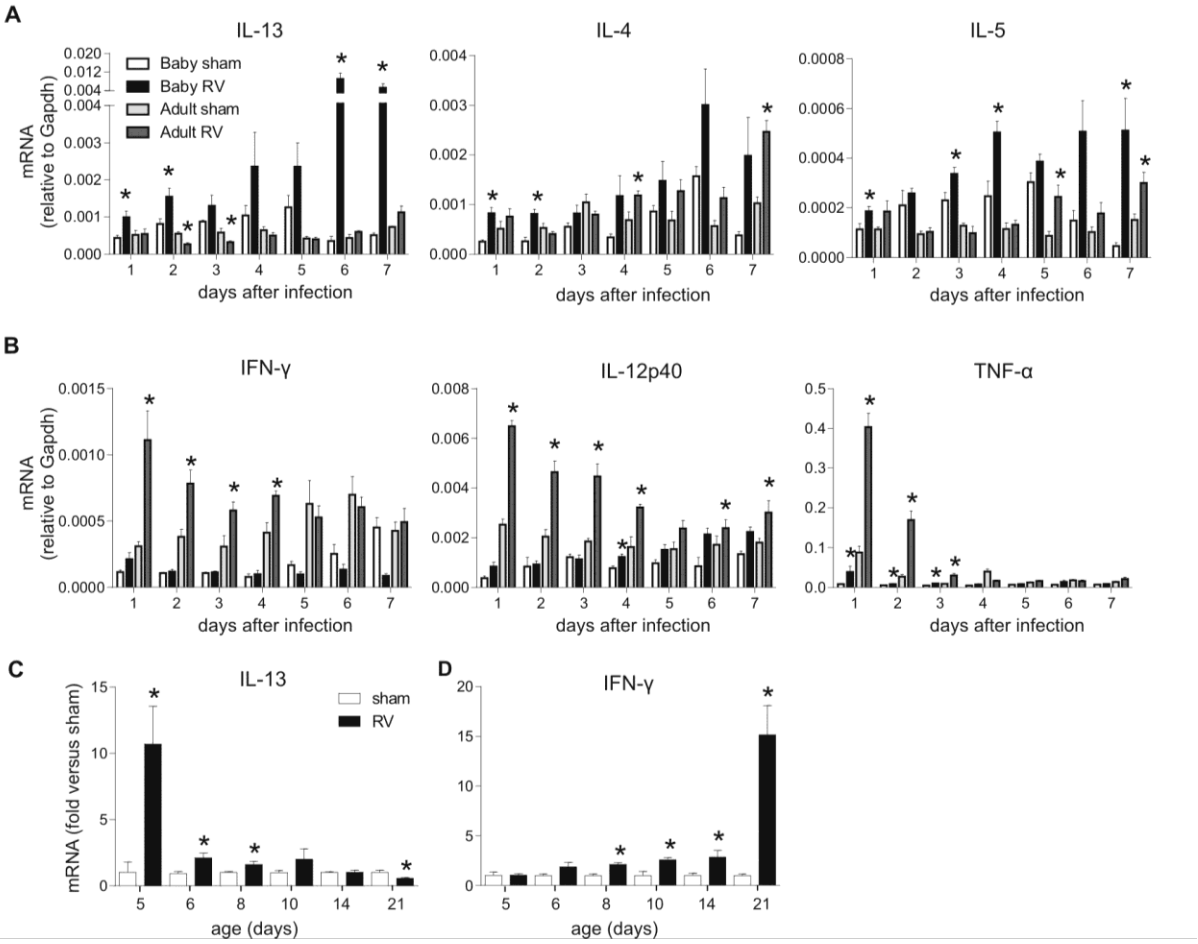

Figure 2-1. Cytokine expression after RV infection. A and B, Six-day-old and eight-week-old mice were inoculated with sham or RV (n=4-8 sham, n=5-14 RV) and lung mRNA measured 1-7 days later. *P<0.05 compared to sham (unpaired t-test). C and D, Mice of different ages (n=3-10/group) were inoculated with sham or RV and mRNA expression measured one day later. *P<0.05 versus sham (unpaired t-test).

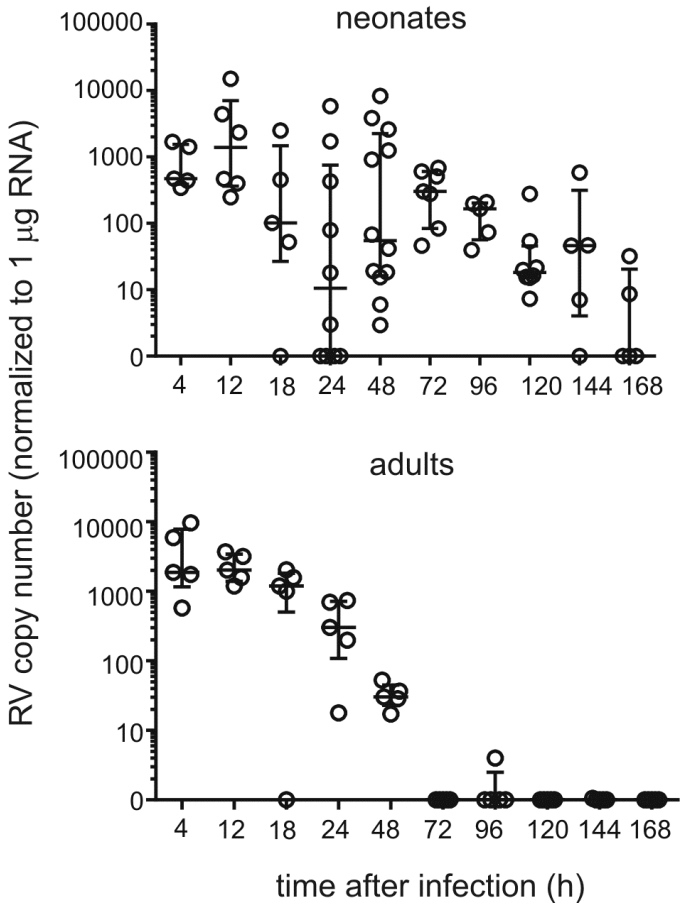

Figure 2-2. Viral copy number in RV-infected neonatal and adult mice. Six-day-old and eight week-old mice were inoculated with sham or RV intranasally. At specified times, lungs were harvested for analysis. Viral copy number was analyzed by qPCR. Shown are individual data, medians and interquartile range for each time point.

Infection of neonatal mice with RV provokes long-term mucus metaplasia and airways hyperresponsiveness.

Neonatal RV infection led to persistent asthma-like pathophysiological changes including IL-13 expression, mucous metaplasia and airways hyperresponsiveness (Figure 2-3*A*-*C*). The mucus-related genes *Muc5ac*, *Muc5b* and *Gob5* were increased with neonatal but not adult RV infection (Figure 2-3*D*). Induction of *Muc5ac* and *Gob5* was maintained after 8 weeks of infection (Figure 2-4). We also found that IL-17RB gene expression was increased (Figure 2-3*E*), suggesting a possible role for IL-25 in promoting the type 2 immune response.

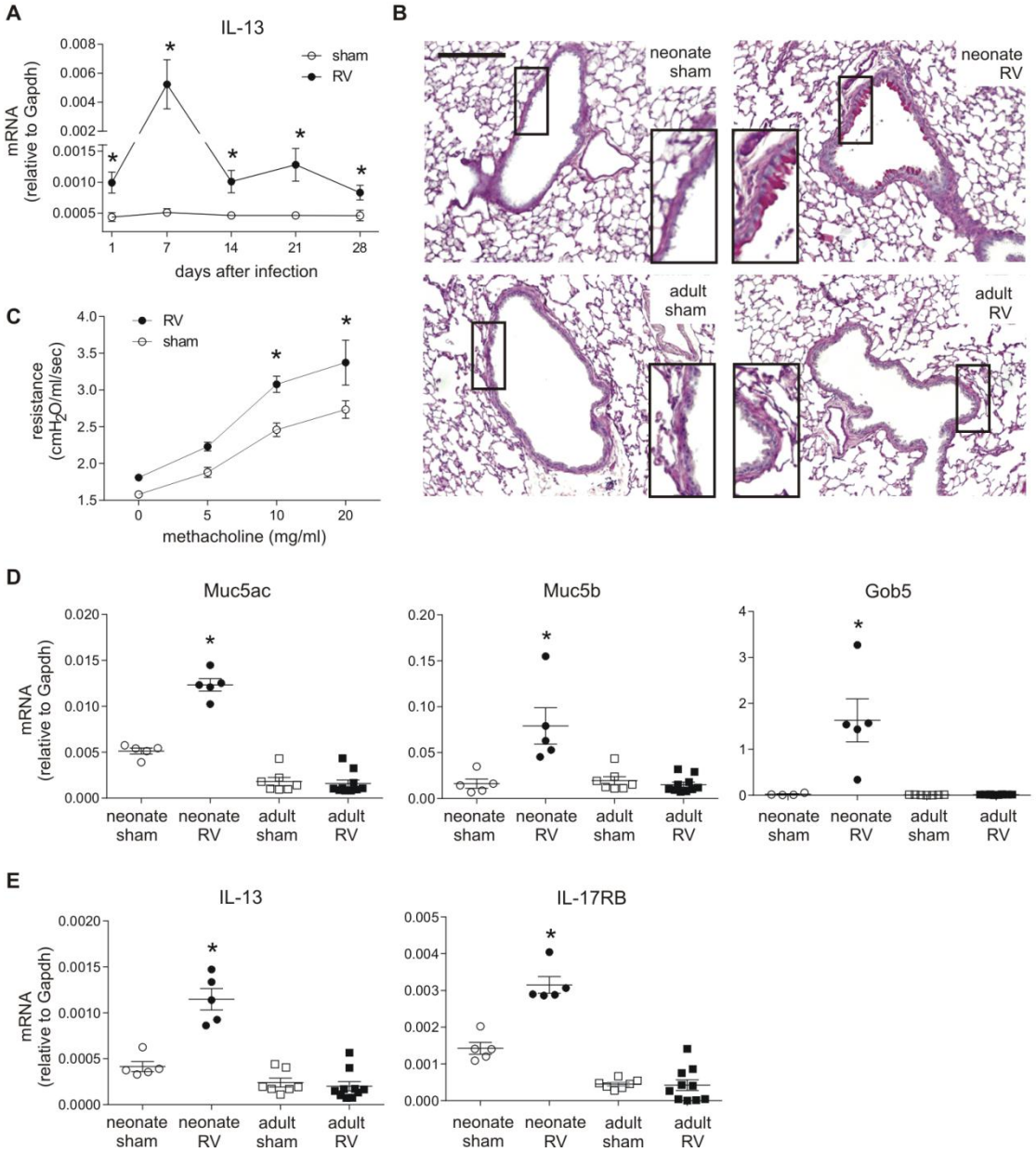

Figure 2-3. Mucous metaplasia and airway hyperresponsiveness after neonatal RV infection. A, Lung IL-13 from six day-old mice. *P<0.05 versus sham (unpaired t-test). B, PAS-stained lung sections prepared 3 weeks after inoculation of six day-old and eight week-old mice (magnification, 100X; bar. 200 μm). C, Airway responsiveness four weeks after inoculation of neonatal mice (n=4/group). * P<0.05 versus sham (two-way ANOVA). D and E, Lung mRNA expression three weeks after inoculation. *P<0.05 versus sham (unpaired t-test).

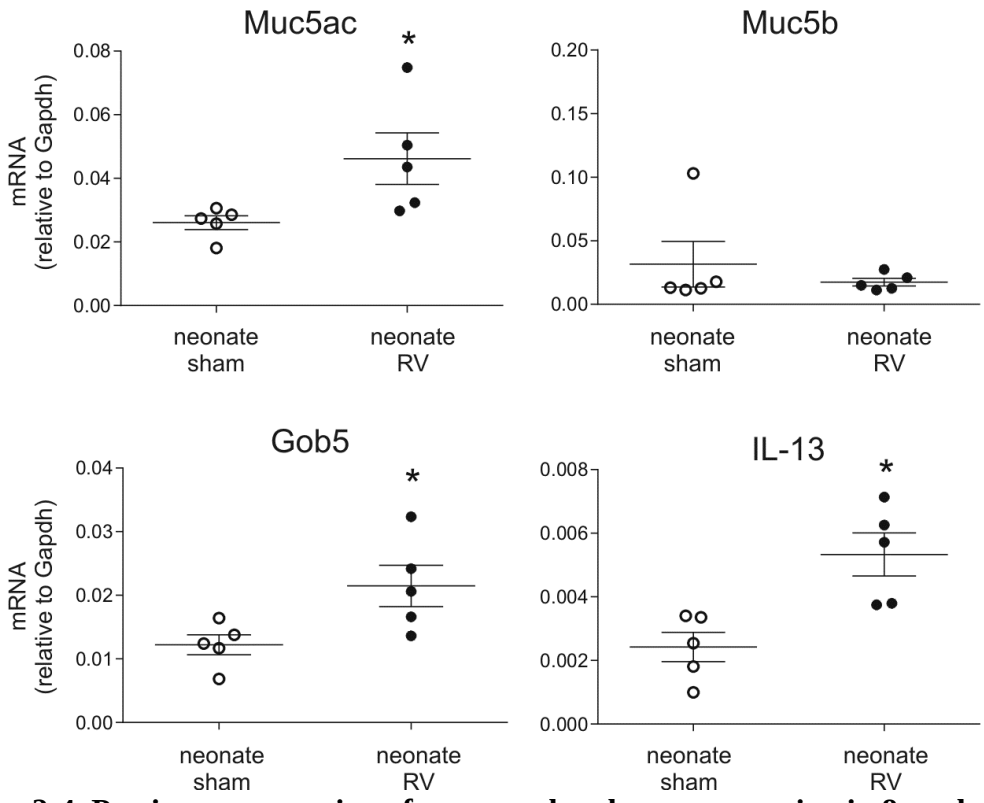

Figure 2-4. Persistent expression of mucus-related gene expression in 8 week-old mice infected with RV. Gene expression of Muc5ac, Muc5b, Gob5, and IL-13 was analyzed with quantitative PCR. * $P < 0.05$ versus sham (unpaired t-test).

Epithelial IL-25 is increased with neonatal RV infection.

We next asked whether IL-25 expression is increased following neonatal RV infection and differentially regulated with age. Similar to the induction of type 2 cytokines, IL-25 mRNA expression was increased in RV-infected neonatal mice (6-day-old) but not mature mice (8-10 weeks old) (Figure 2-5A). IL-25 protein production was induced with RV infection in neonates but not adults (Figure 2-5B). Expression of IL-33 did not change with infection (Figure 2-6). Additional studies examining the age-dependency of RV-induced IL-25 expression showed significant induction only in mice younger than 6 days-old (Figure 2-5C). UV-irradiated, replication-deficient virus did not generate a response (data not shown). We also asked whether viral dosage affects the neonatal type 2 cytokine response. Low-dose RV significantly increased IL-25 but not IFN-γ mRNA 24 h after infection (Figure 2-7). These results show that RV-induced type 2 cytokine production in neonates is not dose-dependent. Lung immunofluorescent staining showed that RV-infected epithelial cells were the major source of IL-25 (Figure 2-5D). Lungs of RV-infected mature mice showed minimal IL-25.

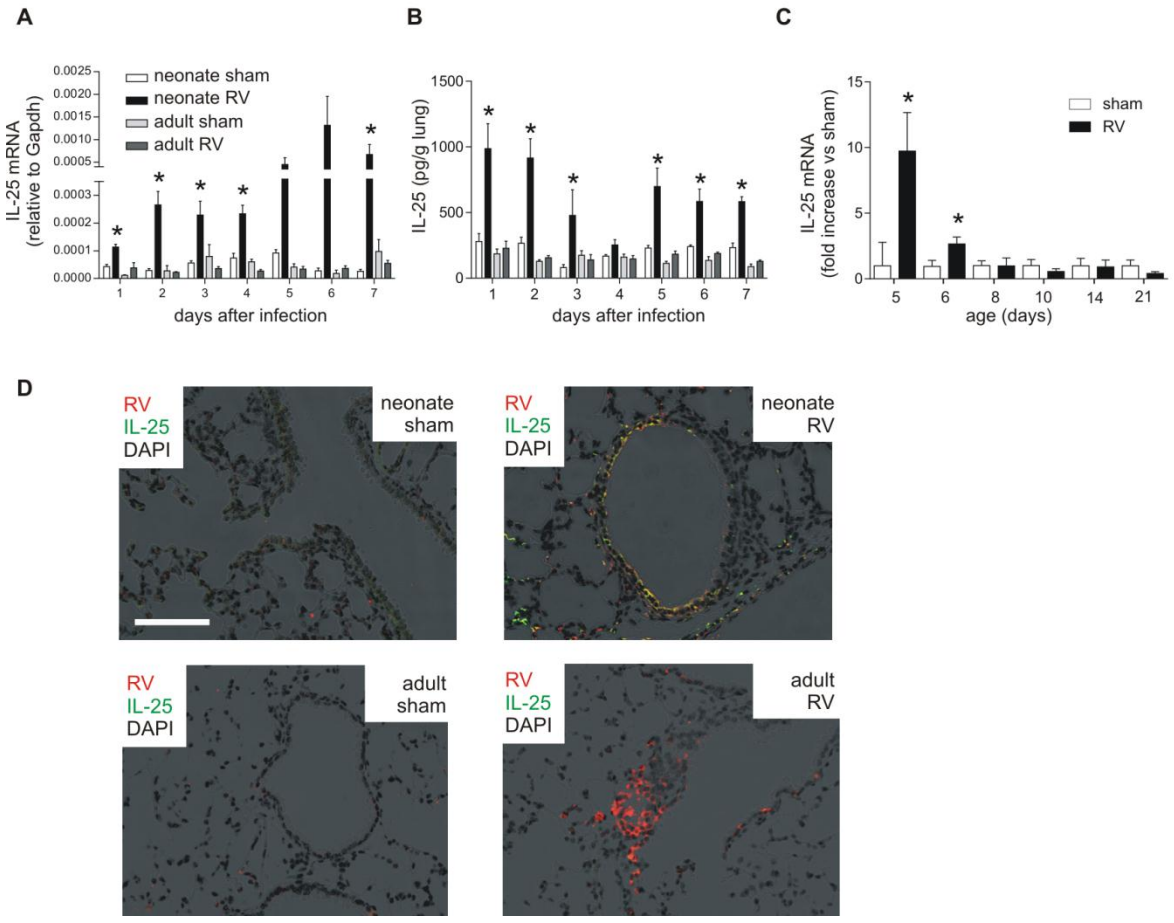

Figure 2-5. Lung IL-25 after RV infection. A, Six-day-old and eight-week-old mice were inoculated with sham or RV (n=4-7/group) and mRNA measured 1-7 days after infection. *P<0.05 versus sham (unpaired t-test). B, IL-25 protein. *P≤0.05 versus sham (one-way ANOVA). C, Mice were inoculated at different ages (n = 3-10/group) and mRNA measured one day after treatment. *P<0.05 versus sham (unpaired t-test). D, Two days after infection, lungs were stained for IL-25 (green), RV (red) and nuclei (DAPI, black). (Bar, 200 μm; magnification, 200X).

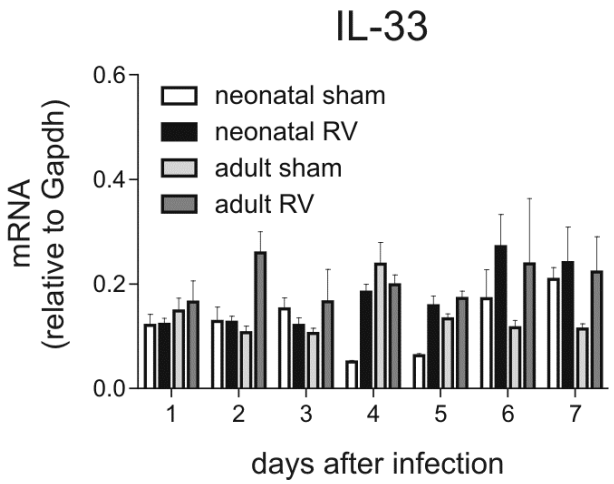

Figure 2-6. Effect of RV infection on the expression of IL-33. Six-day-old neonatal BALB/c mice and eight-week-old mature mice were inoculated with sham or RV. Whole lung gene expression of IL-33 was measured 1-7 days after infection with quantitative PCR. *P < 0.01 versus sham (unpaired t-test).

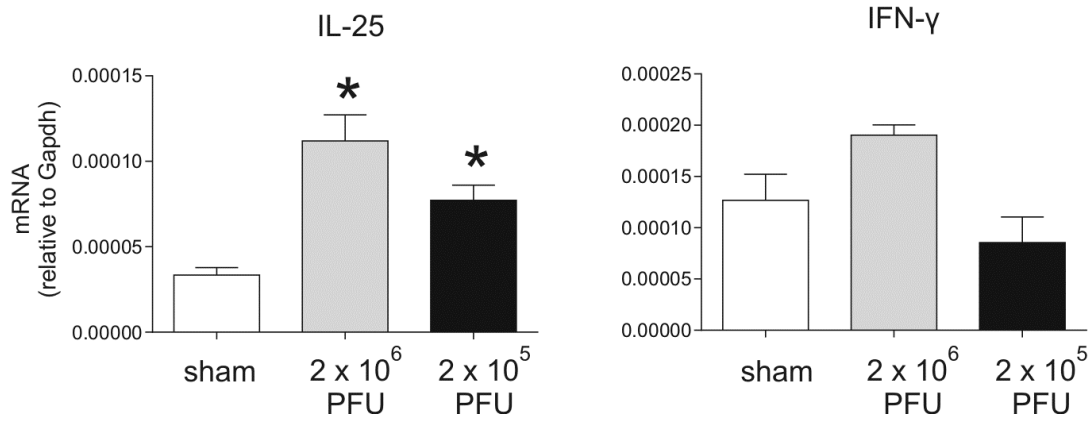

Figure 2-7. Effect of low-dosage RV infection in the induction of IL-25 and IFN-γ. Six-day-old neonatal BALB/c mice were inoculated with sham, or RV (normal dosage), or RV (10-fold lower dosage). Whole lung gene expression of IL-25 and IFN-γ mRNA was measured one day after infection with quantitative PCR. *$P < 0.05$ versus sham (unpaired t-test).

RV infection of neonatal but not mature mice expands the population of IL-17RB-expressing ILC2s.

We collected lungs of neonates and adults two weeks after infection and analyzed ILC2s using flow cytometry. We gated on small cells, live cells and lineage-negative cells using mixture of hematopoietic lineage markers (CD3ε, TCRβ, B220, Ter-119, Gr-1, CD11b, CD11c, F4/80, FcεRIα) (Figure 2-8A). Compared to adults, neonatal mice had nearly 2-fold more lineage-negative cells (Figure 2-9). After gating on the lineage-negative population, a discrete population of CD25 and CD127 double-positive ILC2s was found (Figure 2-8A-B). The basal level of Lin-negative, CD25, CD127 double-positive cells in adult mice was comparable to the previous findings in C57BL/6 mice (30). Following RV infection, ILC2s were increased in neonates but not in adults, both in percentage and number. Further analysis showed that Lin-negative, CD25, CD127 double-positive cells express c-kit (CD117), sca-1, IL-17RB, and ST2L (Figure 2-8*C*), suggesting that they are more closely related to lung natural helper cells than nuocytes, innate helper cells or type 2 multipotent progenitor cells, which are heterogeneous in c-kit or CD127 expression (22, 24, 31). ILC2s were increased as early as one day after infection and maintained three weeks after infection (Figure 2-8D). Taken together, these results suggest that, following neonatal RV infection, the population of lung ILC2s is expanded in neonates, possibly through the induction of IL-25.

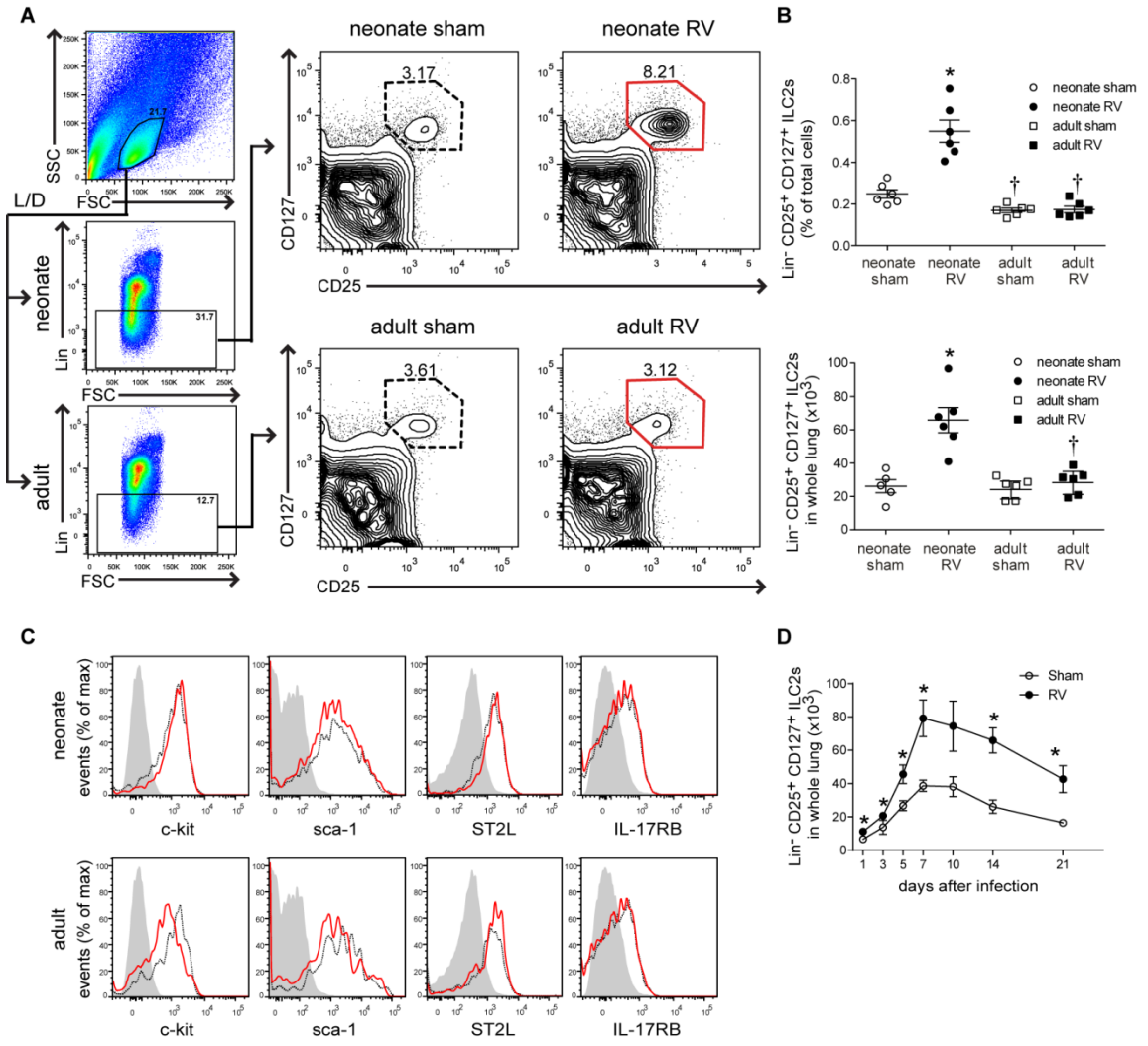

Figure 2-8. Lung lineage-, CD25+, CD127+ ILC2s. A, Six day-old and eight week-old mice were inoculated with sham or RV and live ILC2s identified fourteen days later. B, Percentage (upper panel) and total (lower panel) ILC2s for each group. *P<0.05 versus sham, †P<0.05 versus mature mice (unpaired t-test). C, C-kit/CD117, Sca-1, T1/ST2 and IL-17RB expression in ILC2s from sham- (black, dotted) and RV-treated mice (red, solid). (Isotype control is grey, filled). D, ILC2 time course after neonatal infection (n = 3-6/group). *P<0.05 versus sham (unpaired t-test).

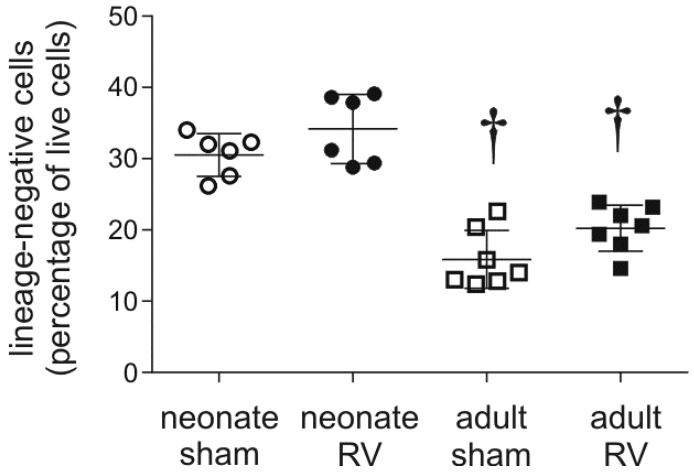

Figure 2-9. Lineage-negative cells in immature and mature mice. Six-day-old neonatal BALB/c mice and eight-week-old mature mice were inoculated with sham or RV. Lungs were collected 14 days after infection. Cell suspensions were stained with a cocktail of lineage antibodies (CD3ε, TCRβ, B220/CD45R, Ter-119, Gr-1/Ly-6G/Ly-6C, CD11b, CD11c, F4/80, and FcεRIα) and subjected to flow cytometry. The percentage of lineage-negative live cells was calculated. †$P < 0.05$ versus neonates (unpaired t-test).

ILC2s are a major IL-13-producing cell in RV-infected neonatal mice.

We asked whether Lin-negative CD25, CD127 double-positive ILC2 cells produce IL-13 after RV infection. First, we analyzed the role of T cells utilizing flow cytometric analysis. Lung samples were collected two weeks after infection and stimulated with phorbol 12-myristate 13-acetate, ionomycin, brefeldin A and monensin. The main population of IL-13-producing cells was TCRβ-negative (Figure 2-10A). After RV stimulation, the number of IL-13-secreting TCRβ-negative cells was approximately 18-fold higher than the number of IL-13-secreting TCRβ-positive cells. To determine if ILC2s produced IL-13, lineage-positive and -negative cell populations were stimulated as above. The major IL-13-secreting cells were lineage negative (Figure 2-10B). Among lineage-negative cells, CD25, CD127 double-positive cells contained a high IL-13-producing population (Figure 2-10C) suggesting that ILC2s are a major source of IL-13 in the lungs of RV-infected neonatal mice. To further examine the capacity of these cells to produce IL-13, we sorted Lin-negative CD25, CD127 double-positive cells and CD25, CD127 double-negative cells (Figure 2-11). Double positive cells expressed c-kit and sca-1, whereas double-negative cells were negative for both markers (Figure 2-10D). Similar to other ILC2s, double-positive cells were small cells with circular nuclei and scanty cytoplasm (Figure 2-10E) (20, 32). Stimulation with IL-25 and IL-2 or PMA and ionomycin induced large amounts of IL-13 (Figure 2-10F). These results show that an expanded population of ILC2s is the major source of IL-13 in RV-infected neonatal mice and likely contribute to the observed asthma-like phenotype.

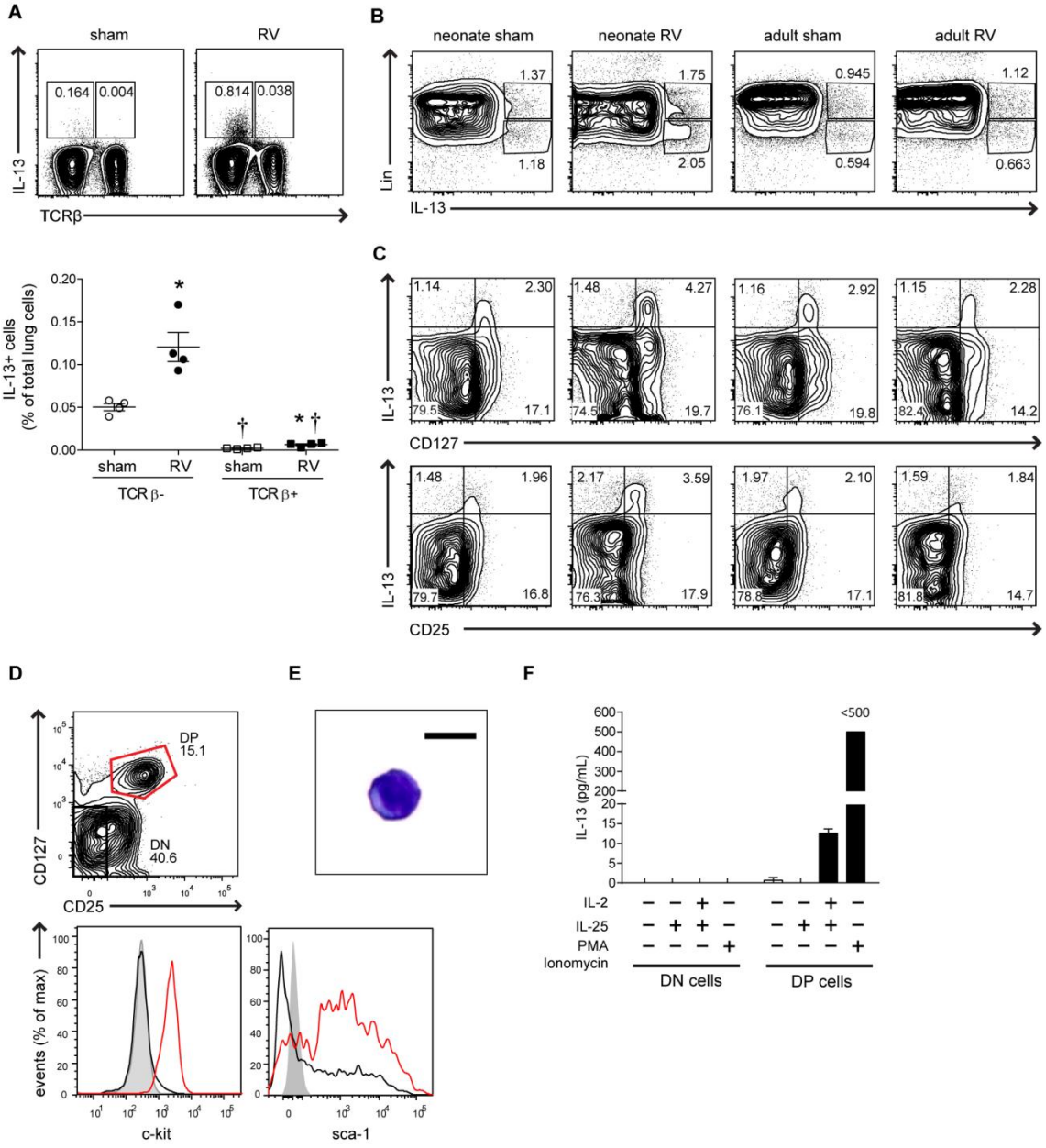

Figure 2-10. IL-13 producing cells. A, Percentages of IL-13+, TCRβ- and TCRβ+ cells two weeks after neonatal sham or RV. *P<0.05 versus sham, †P<0.05 versus TCRβ- cells (unpaired t-test). B and C, Percentages of lineage+ and lineage- IL-13+ cells (B). Percentages of lineage- IL-13+, CD127+ and CD25+ cells (C). D-F, Eight days after infection, Lin- CD25+ CD127+ double-positive (DP) and CD25- CD127- double-negative (DN) ILC2s were characterized for c-kit and Sca-1 (D). Image of ILC2 (E). IL-13 production by stimulated DP and DN cells (F).

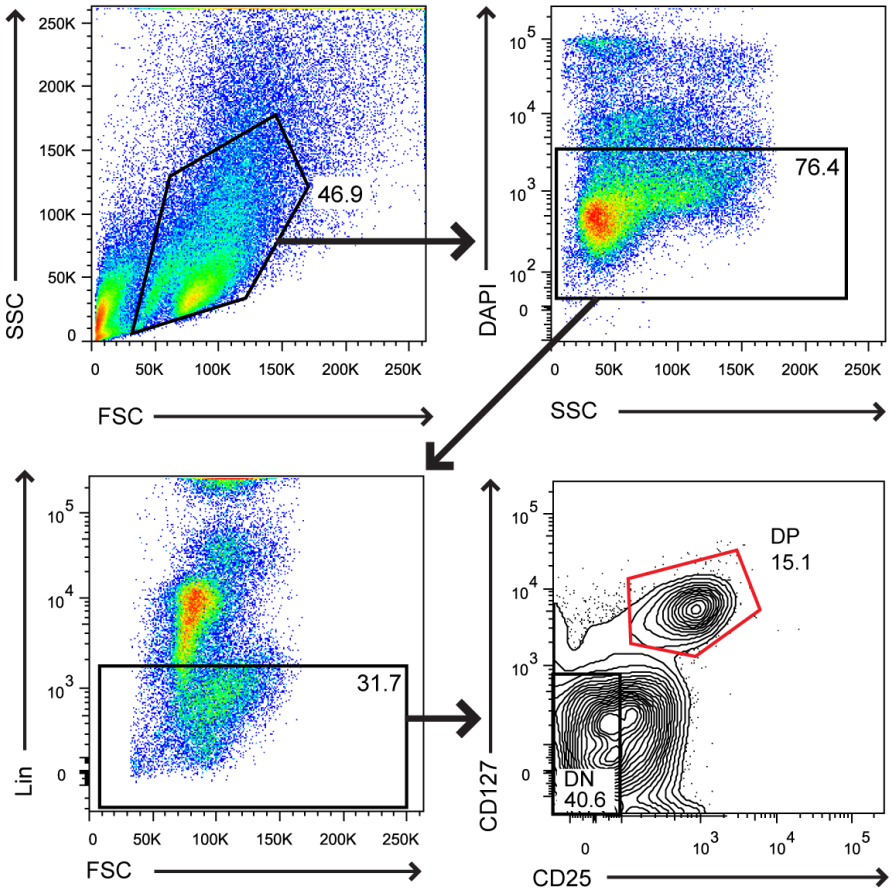

Figure 2-11. Gating strategy for sorting ILC2s. Six-day-old neonatal BALB/c mice were infected with RV. After 8 days, lungs were processed for cell sorting. To sort ILC2s, low FSC, low SSC, DAPI-negative live cells were gated and incubated with lineage cocktail antibodies (CD3ε, TCRβ, B220/CD45R, Ter-119, Gr-1/Ly-6G/Ly-6C, CD11b, CD11c, F4/80, and FcεRIα). Finally, CD25 and CD127 double positive cells were identified.

IL-25 mediates the development of mucus metaplasia and airways hyperresponsiveness in RV-infected neonatal mice.

To test whether IL-25 is required for the development of an asthma-like phenotype, we treated RV-infected neonatal mice with a neutralizing antibody against IL-25 (Figure 2-12A). Treatment with anti-IL-25 reduced mucus metaplasia (Figure 2-12B). The mucus-related genes Muc5ac, Muc5b, Gob5 decreased with anti-IL-25 treatment (Figure 2-12C). Anti-IL-25 blocked the development of airways hyperresponsiveness (Figure 2-12D). Finally, anti-IL-25 decreased the expansion of ILC2s in RV-infected neonates (Figure 2-12E), suggesting that ILC2 expansion is dependent on IL-25. These results show that IL-25 plays a key role in the development of mucous metaplasia and airways hyperresponsiveness in RV-infected neonatal mice, at least in part by increasing the number of ILC2s.

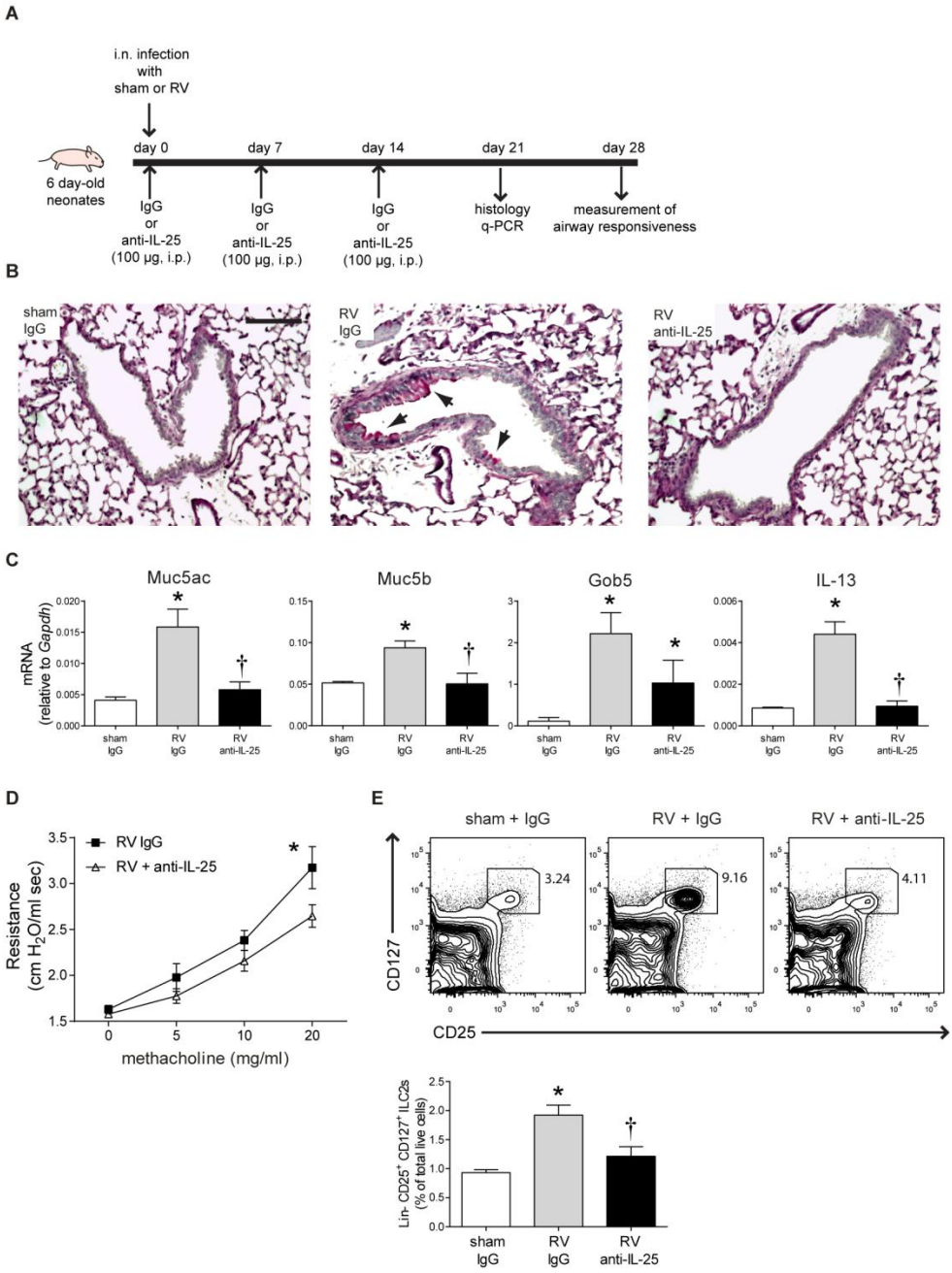

Figure 2-12. Effect of IL-25 neutralization on RV-infected neonatal mice. A, Protocol for anti-IL-25 treatment. B, Three weeks after inoculation, lungs were harvested and stained with PAS. Bar, 100 µm. C, Lung mRNA expression (n = 4-10/group). *P<0.05 versus sham, †P<0.05 versus RV+IgG (unpaired t-test). D, Airway resistance four weeks after RV infection and antibody treatment (n = 4-5 in each group). *P<0.05 versus RV+IgG (two-way ANOVA). E, Lineage-negative CD25+ CD127+ ILC2s four weeks after infection (top). Group percentages of live ILC2s (n = 3-8/group, bottom). *P<0.05 versus sham, †P<0.05 versus RV+IgG (unpaired t-test).

Discussion

In this study, we showed that infection of mice with rhinovirus induces an age-dependent type 2 immune response in the airways. Neonatal RV infection, but not adult infection, increased expression of IL-13 and IL-25. In contrast, induction of the type 1 cytokines IFN-γ, IL-12 p40 and TNF-α was diminished in neonates compared to adults. The increase in IL-25 production in neonatal mice was associated with long-term expansion of IL-25-responsive ILC2s in the lungs. Further, ILC2s were a significant source of IL-13 after RV infection. Finally, RV-induced mucous cell metaplasia and airways hyperresponsiveness were attenuated by anti-IL-25. Together, these studies indicate that RV induces an age-dependent asthma-like phenotype which is driven by IL-25 and ILC2s. These studies provide a mechanism by which viral infection in early-life could lead to persistent type 2 immune responses and asthma development.

The immature immune system is qualitatively different from that of adult, refractory to type 1 and permissive to type 2 responses (1-9). In our experiments, RV-induced IL-25 was regulated in an age-dependent manner and required for the development of mucous metaplasia and airways hyperresponsiveness. IL-25 appeared to be produced by RV-infected epithelial cells, though uninfected cells, including submucosal cells, may also have been involved. To our knowledge, this is the first report showing a developmental difference in the IL-25 response. Considering the epigenetic modification favoring type 2 cytokine induction in T cells (2), it is possible that the regulatory region of IL-25 is also epigenetically favored transcription in neonates compared to adults. Alternatively, blunted induction of type 1 cytokine IFN-γ in RV-infected neonates could be permissive for IL-25 induction. In NK cell-deficient mice,

RSV infection leads to an exaggerated IL-25 response which is blocked by recombinant IFN-γ treatment, consistent with the notion that IFN-γ blocks IL-25 expression (33). Finally, it is possible that neonates experienced a greater total IL-25 response based on a higher viral load 3-7 days after inoculation. However, treatment of neonatal mice with low-dose RV also induced lung IL-25 expression, and NK cell-deficient mice with exaggerated IL-25 production and attenuated IFN-γ responses have similar viral loads as wild-type mice (33), suggesting the primacy of IFN regulation.

The cytokine IL-33 has also been associated with development of lung ILC2s and type 2 cytokine responses in mice (30). However, IL-33 was not increased with RV infection. Thymic stromal lymphopoietin (TSLP) has also been shown to expand skin ILC2s in mice (34). RV16 infection increases TSLP expression in human airway epithelial cells (35). It is therefore conceivable that TSLP plays a role in RV-induced ILC2 expansion.

We have previously shown that this IL-13 induction is required for the development of RV-induced mucous metaplasia and airways hyperresponsiveness in neonatal mice (15). Persistent induction of IL-13-driven changes in airway inflammation and function following viral infection were first reported in Sendai-infected C57BL/6J mature mice (36). Subsequently, persistent IL-13 production has been noted following neonatal infection by RSV, pneumonia virus of mice (PVM) and influenza exposure (37-39). In the case of mature Sendai-infected mice, IL-13 was secreted by a combination of M2-polarized macrophages and invariant NKT cells (36). In the present study, the major cells persistently secreting IL-13 were lineage-negative, CD25, CD127 double-positive ILC2s. These cells expressed c-kit, Sca-1, ST2, and IL-17RB, closely resembling lung

natural helper cells and dissimilar from nuocytes, innate helper cells or type 2 multipotent progenitor cells (21-24).. Induction of ILC2s with viral infection has previously been shown following in mature mice with influenza virus (40). Following H1N1 infection, ILC2s restored gas exchange and helped tissue repair by secreting amphiregulin.' After H3N1 infection, ILC2s induced IL-13-mediated airway hyperreactivity (41). Finally, an acute increase in ILC2s was recently shown in neonatal TLR7 null mice with a severe PVM infection (42). However, IL-13 production by ILC2s was not assessed, and virus-induced airway inflammation and airway hyperreactivity was dependent on memory CD4+ T cells. Our experiments extend previous reports in the following respects. First, we found for the first time that ILC2s are expanded following infection with RV, perhaps the most common infection of humans. Second, we established that ILC2s make IL-13 in response to viral infection in neonatal animals. Third, we found a developmental difference in the ILC2 response between neonates and adults (see above). This difference was based on RV-induced production of ILC2-activating cytokines such as IL-25, rather than the infection itself. Fourth, following neonatal RV infection, IL-13 secretion and ILC2 expansion were long-lived, at least 21 days after infection. It is therefore conceivable that ILC2s could produce type 2 cytokines for extended periods, perhaps in response to subsequent infection or allergen exposure. Finally, we showed for the first time that, in addition to IL-33, IL-25 is required for ILC2 expansion following viral infection.

Our finding that RV infection elicits a significant ILC2 response in immature but not mature mice, one which is based on the age-dependent expression of IL-25 and other cytokines, suggests that it is the stage of development, rather than the specific virus, that

drives establishment of the asthma-like phenotype. Accordingly, it is possible that other early-life viral infections also induce mucous metaplasia and airways responsiveness through the early expansion of the ILC2 cells. As noted above, neonatal infection by RSV, PVM and influenza have been shown to induce a persistent asthma-like phenotype in mice (37-39). IL-25 production has been noted following neonatal infection with RSV (38). Recently, neonatal PVM infection of $TLR7^{-/-}$ mice showed an IFN-low, IL-25-high cytokine response similar to that we observed, which was associated with recruitment of ILC2 cells (42). In humans, early-life infection with RV (13, 14, 43) and RSV (10, 11) have each been associated with asthma development.

The association between respiratory viral infection and asthma is likely to be complex, with asthma development requiring the repeated infections, appropriate genetic background and allergen exposure (44-48). Indeed, studies have shown additive or synergistic effects of allergen exposure and neonatal RSV (37), PVM (38, 42), influenza (39) and RV infection (15). Further studies are needed to characterize the role of innate immune cells, including the ILC2, in combined responses.

We would like to add a few caveats about our mouse model of RV infection. We (49) and others (50) have found that a much higher viral titer is required to infect mice compared with humans. This finding is to be expected, because differences in the homology of viral receptors and intracellular signaling mechanisms are likely to restrict viral infection and replication in mice. Nevertheless, we have clearly shown that human RV1B replicates in mouse lungs, as evidenced by: 1) the presence of negative-strand viral RNA in the lungs of inoculated mice, 2) transmissibility of RV infection from the lung homogenates of inoculated mice to cultured HeLa cells; and 3) the induction of a robust

lung interferon response (49). RV replication appears to be augmented in neonatal mice. Nevertheless, as in humans, RV infection was not associated with neonatal mortality. In fact, viral titers were relatively low, suggesting that even mild respiratory viral infections may elicit an ILC2 response. These data are consistent with recent speculation by workers in the field that relatively innocuous RV infections in early childhood lead to sustained changes in the immune response which contribute to the development of asthma.

We conclude that early-life rhinoviral infection could contribute to asthma development by provoking age-dependent, IL-25-driven type 2 immune response. IL-25 induction was regulated in an age-dependent manner and required for ILC2 expansion and the development of asthma phenotype in neonates. Further characterization of this immune pathway may lead to new molecular and cellular targets for the prevention of asthma.

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Chapter 3

TSLP is required for mucous metaplasia, airways hyperresponsiveness and ILC2 expansion in RV-infected neonatal mice

Summary

Early-life respiratory viral infection, in particular with rhinovirus (RV), has been identified as a risk factor for asthma development in later life. We have shown that RV infection of six-day old but not mature mice causes mucous metaplasia and airway hyperresponsiveness which is dependent on IL-13 and IL-25. In this study, we examined the contribution of thymic stromal lymphopoietin (TSLP) to the observed asthma-like phenotype. We hypothesized that, like IL-25, TSLP expression is induced by RV infection in immature but not mature mice, and required for mucous metaplasia and airways hyperresponsiveness. We inoculated six day- and eight week-old BALB/c (wild-type) and TSLP receptor knockout (TSLPR KO) mice with sham HeLa cell lysate or RV. Airway responses were assessed by quantitative PCR, ELISA, histology, immunofluorescence microscopy and resistance changes to inhaled methacholine. RV infection increased TSLP protein abundance after early-life RV infection while reducing TSLP mRNA and protein expression in mature mice. RV-induced TSLP expression was localized to the airway epithelium. Neonatal RV infection induced persistent mucus hyperplasia and airway hyperresponsiveness in wild type mice whereas the phenotype

was blocked in TSLPR KO mice. Expansion of type 2 innate lymphoid cells (ILC2s) following neonatal RV infection was also significantly inhibited in TSLPR KO mice. Finally, when combined with IL-25 and IL-33, TSLP had a direct synergistic effect on ILC2 GATA3, IL-13, IL-5, ST2L and IL-17RB mRNA expression. These results show that TSLP plays a necessary role in the development of mucous metaplasia, airways hyperresponsiveness and ILC2 expansion in RV-infected immature mice.

Introduction

The onset of asthma is likely to be a result of genetic predisposition, immune dysfunction and environmental exposures in early infancy. One such environmental exposure may be viral infection; epidemiologic studies in high-risk infants indicate that early-life respiratory viral infection, particularly with rhinovirus (RV), is a major predisposing factor for subsequent asthma development (1-4).

Because the airway epithelium is a major target of respiratory viral infection, the epithelium-derived cytokines IL-25, IL-33 and TSLP and their downstream cellular targets are uniquely positioned to play a role in viral-induced chronic airways disease. IL-25 (5, 6), IL-33 (7) and TSLP (8-11), play a role in the maturation of Th2 cells via dendritic cell activation. IL-25 (12-15), IL-33 (12-14, 16-19) and TSLP (19) also induce activation and IL-13 production from innate immune cells including type 2 innate lymphoid cells (ILC2s). Previously, we have found that IL-25 plays pivotal role in asthma phenotype development in neonatal mice following RV infection (20). RV infection of six day-old but not mature mice induced mucous metaplasia and airways hyperresponsiveness which was accompanied by airway epithelial cell IL-25 production

and expansion of IL-13-producing type 2 innate lymphoid cells. Airway responses were blocked by neutralizing antibody against IL-25. However, we did not examine the role of TSLP in this model.

TSLP is an IL-7-like cytokine which exerts its biological activity via a high-affinity TSLPR complex that is a heterodimer of the TSLPR chain and IL-7 receptor-α. The role of TSLP in the pathogenesis of asthma has been recently highlighted. Several single-nucleotide polymorphisms of the TSLP gene locus are associated with asthma development (21-24). TSLP levels are increased in the airways of asthmatic patients, and this level correlates with asthma severity (25, 26). Genome-wide association studies have found that the TSLP gene locus is associated with asthma risk (27, 28). Treatment of human asthmatic patients with anti-TSLP antibody blunted airway responses triggered by allergen inhalation (29). In mice, transgenic mice that overexpress TSLP in the lungs show an augmented type 2 immune response, goblet cell hyperplasia and subepithelial fibrosis when challenged with ovalbumin, whereas TSLPR-deficient mice show an attenuated responses (30). However, less is known about the role of TSLP in the development of asthma in childhood. A recent study showed that RV infection increased nasal aspirate TSLP expression in young children (31), consistent with the notion that RV-induced TSLP production could play a role in the initiation of asthma.

We therefore examined the effect of RV infection on TSLP expression in immature and mature mice, and the requirement of TSLP for RV-induced mucus metaplasia, airway hyperresponsiveness and ILC2 expansion.

Methods

Generation of RV.

RV1B (ATCC, Manassas, VA) was grown in HeLa cells, concentrated and partially purified by ultrafiltration, as described (32). Similarly concentrated and purified HeLa cell lysates were used for sham infection. Viral titer was measured by fifty percent tissue culture infectivity doses ($TCID_{50}$) using the Spearman-Karber method (33) or plaque assay (34). For the plaque assay, HeLa cell monolayers were infected with serially-diluted RV and overlayed with a 0.6% agarose solution. Plaque growth was monitored by light microscopy and was confirmed by staining with crystal violet.

RV infection.

Experiments were approved by the Institutional Animal Care and Use Committee. BALB/c mice (Jackson Laboratories, Bar Harbor, ME), or TSLPR KO mice (graciously supplied by Dr. Steven Ziegler, Benaroya Research Institute, Seattle) were inoculated through intranasal route under Forane anesthesia with RV1B ($1x10^8$ PFU/ml) or sham HeLa cell lysates. To the mice aged from 5-14 days, 20 μl of RV1B or an equal volume of sham were given. To the mice aged 21 days, 30 μl of RV1B or sham were treated. To the mice aged 8 weeks, 50 μl of RV1B or sham were administered.

Assessment of airway responsiveness.

Airway cholinergic responsiveness was assessed by measuring changes in total respiratory system resistance in response to increasing doses of nebulized methacholine (35). Mice were anesthetized with sodium pentobarbital (50 mg/kg mouse, intraperitoneal injection) and a tracheostomy performed. Mechanical ventilation was

conducted and total respiratory system measured using a Buxco FinePointe operating system (Buxco, Wilmington, NC). Airway responsiveness was assessed by measuring changes in resistance in response to increasing doses of nebulized methacholine.

Histology and immunohistochemistry.

Lungs were collected and fixed with 10% formaldehyde and paraffin embedded. Blocks were sectioned at 500-μm intervals at a thickness of 5 μm, and each section was deparaffinized, hydrated, and stained. To visualize mucus, deparaffinized sections were stained with periodic acid-Schiff (Sigma-Aldrich, St. Louis, MO). After antigen demasking and permeabilization, sections were also incubated with Alexa Fluor (AF)-conjugated rabbit anti-mouse TSLP (Thermo Fisher Scientific, Rockford, IL), guinea pig antiserum against RV1B (ATCC) or AF-conjugated isotype control IgGs. Antiserum was partially purified by incubation with nitrocellulose-bound HeLa cell proteins and passing through an affinity resin containing nondenatured mouse lung protein (36). Repurified antibody was directly conjugated to AF488. The control used was AF488-conjugated guinea pig antiserum. Nuclei were stained with 4′,6-diamidino-2-phenylindole. Images were visualized using a Zeiss Axioplan microscope (Thornwood, NY) equipped with an ApoTome and digital AxioCamMR charge-coupled device camera.

Measurement of TSLP.

Whole lung homogenates were collected and centrifuged at full speed for supernatant preparation. The amount of total protein was measured by Pierce BCA protein assay kit (Thermo Fisher Scientific). TSLP concentrations were measured by

ELISA (eBioscience, San Diego, CA). The amount of TSLP in total lung was normalized to the total protein in each sample.

Real-time quantitative PCR.

Lung RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) and genomic DNA digested using with the combination of on-column digestion of genomic DNA (Qiagen, Gaithersburg, MD). cDNA was synthesized from 1 μg of RNA and subjected to quantitative real-time PCR using specific primers for mRNA as described previously (20). The level of gene expression was normalized to GAPDH.

Flow cytometric analysis.

Lungs from sham- and RV-treated neonatal wild-type BALB/c or TSLPR KO mice were perfused with PBS containing EDTA, minced and digested in collagenase IV. Cells were filtered and washed with RBC lysis buffer, and dead cells were stained with Pac-Orange Live/Dead fixable dead staining dye (Invitrogen). To identify ILC2s, cells were then stained with FITC-conjugated antibodies for lineage markers (CD3ε, TCRβ, B220/CD45R, Ter-119, Gr-1/Ly-6G/Ly-6C, CD11b, CD11c, F4/80 and FcεRIα, all from Biolegend), anti-CD25-PerCP-Cy5.5 (Biolegend), anti-CD127-PE-Cy5 (eBioscience), and anti-c-kit/CD117-APC (eBioscience), as described (20). Cells were fixed, subjected to flow cytometry and analyzed on a BD Biosciences FACSAria II (BD Biosciences, San Jose, CA). Data were collected using FACSDiva software (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Fluorescence-activated cell sorting of ILC2s.

After staining for lineage markers, lineage-negative CD25 and CD127 double-positive ILC2s were sorted at 6.5 X 10^3 cells/100 μl concentration in each well of 96 well plates and stimulated with different factors. Three days after stimulation, plates were centrifuged and supernatant were tested for IL-13 with ELISA (eBioscience). With cell pellet, RNA were extracted using RNeasy mini kit (Qiagen), and RNA were concentrated using RNA stable (Sigma-Aldrich). Then, cDNA was synthesized and subjected to quantitative real-time PCR using specific primers for mRNA. The level of gene expression was normalized to mRNA of GAPDH.

Data analysis.

All data were represented as mean ± standard error (SE). Statistical significance was assessed by unpaired t-test, one-way analysis of variance (ANOVA) or two-way ANOVA, as appropriate. Group differences were pinpointed by the Student-Newman-Keuls multiple comparison test.

Results

TSLP is increased with RV infection in neonatal mice but not in mature mice

In our previous report, we found that the immune response to RV in neonatal mice is qualitatively different from that of mature mice, showing exaggerated type 2 immune responses leading to mucous metaplasia and airways hyperresponsiveness (20). In this study, we hypothesized that TSLP contributes to the observed asthma phenotype. We first tested whether the TSLP expression pattern induced by RV is distinctive

between immature and mature mice. While TSLP mRNA expression was decreased with RV infection in mature mice, TSLP mRNA level was unchanged in neonatal mice (Figure 3-1A). Similar to mRNA, RV infection decreased TSLP protein level in mature mice (Figure 3-1B). In contrast, TSLP protein levels were enhanced in neonatal mice with RV infection. Together, these data provide evidence of both transcriptional and post-transcriptional regulation of TSLP expression by RV infection.

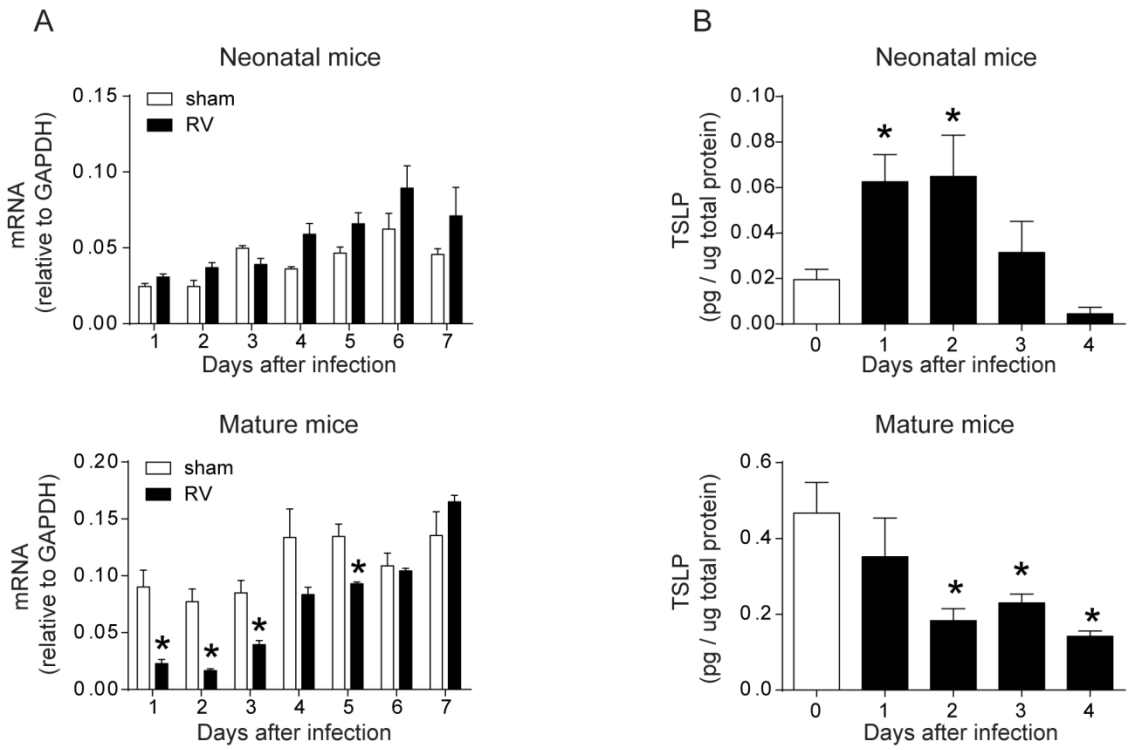

Figure 3-1. Differential TSLP regulation between immature and mature mice with RV infection. Six-day-old and 8-week-old BALB/c mice were inoculated with sham or RV (*n*= 3-8 /each group). A, Lung TSLP mRNA expression was measured 1 to 7 days after RV infection with quantitative PCR. B, Lung TSLP protein level was measured with ELISA and normalized to total protein. *P < 0.05 compared with sham (unpaired t test).

TSLP induction was regulated in an age-dependent manner with RV infection

To further examine developmental changes in the TSLP response to RV infection, we infected different age mice with RV and measured TSLP mRNA and protein. We found that TSLP mRNA was decreased with RV infection in the mice age 8 days or older (Figure 3-2A). Induction of TSLP protein expression was present only in mice ≤ 6 days of age (Figure 3-2B).

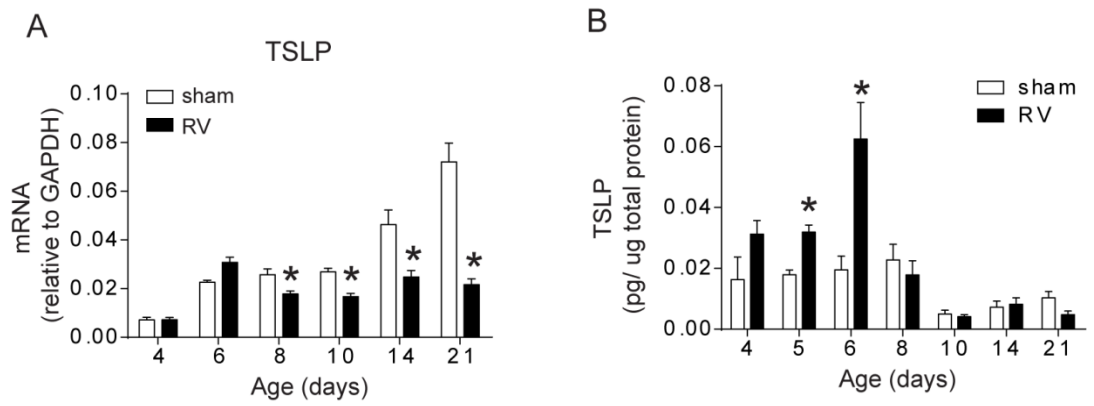

Figure 3-2. Age-dependent changes in TSLP expression after RV infection. BALB/c mice of different ages (*n*= 3-8/group) were inoculated with sham or RV. A, Lung TSLP mRNA expression was measured 1 day after infection with quantitative PCR. B, Lung TSLP protein level was measured with ELISA and normalized to total protein. **P*<0.05 versus sham (unpaired t-test)

Airway epithelium was the primary source of TSLP with RV infection neonatal mice

To determine the cellular source of TSLP, we stained the histological sections of lungs with anti-TSLP antibody. RV infection increased TSLP staining in the airways of neonatal mice (Figure 3-3). Both subepithelial and epithelial cells produced TSLP; the strongest signals were in the airway epithelial cells, infected with RV.

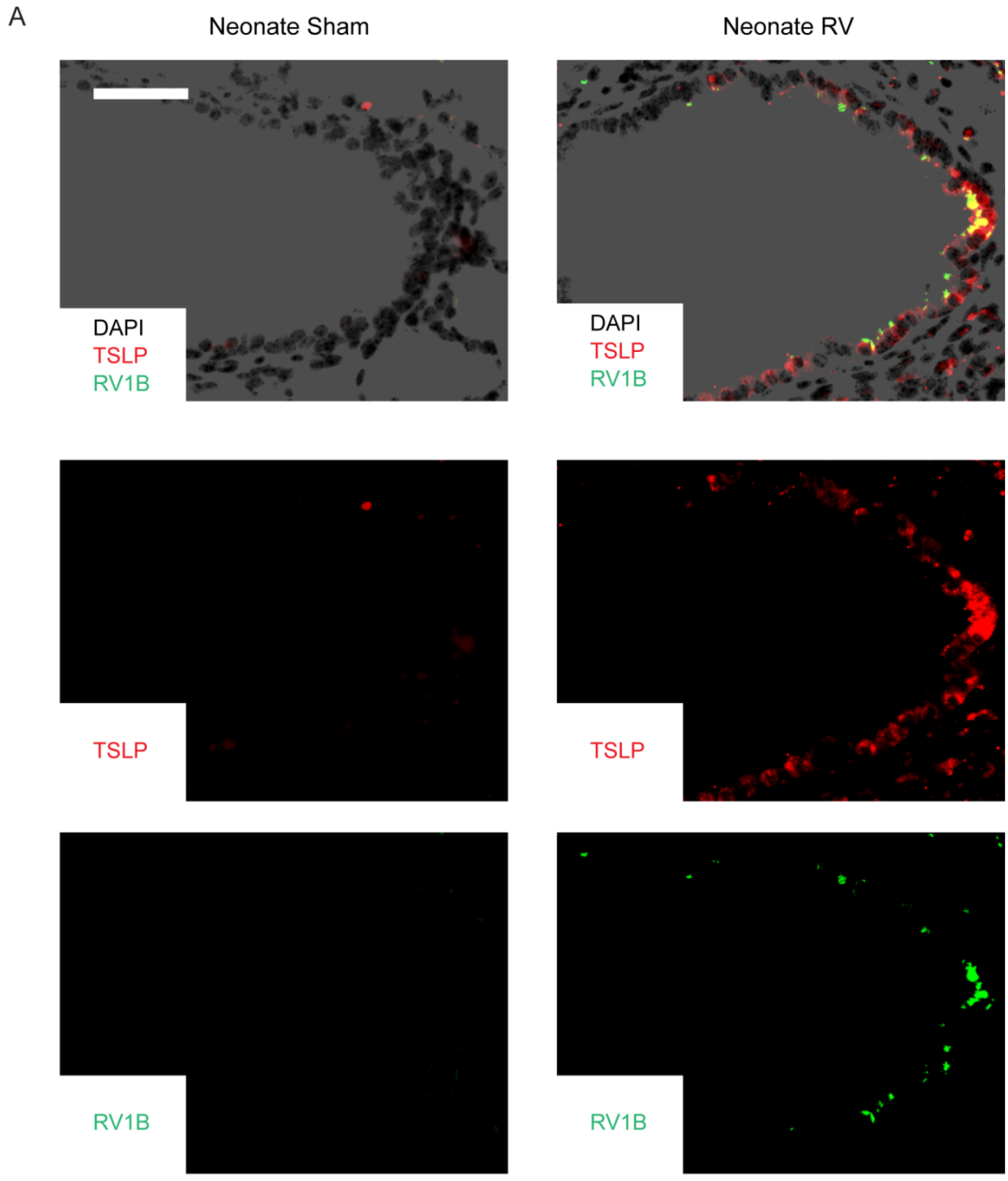

Figure 3-3. RV-induced TSLP expression in neonatal airway epithelium. A, BALB/c mice were inoculated with either sham or RV1B. Two days after infection, lungs were stained for TSLP (red), RV (green) and nuclei (DAPI, black). (Bar, 100 μm; magnification, 400X)

TSLPR KO mice showed attenuated mucous metaplasia and airway hyperresponsiveness with neonatal RV infection

Next, we tested the requirement of TSLP for RV-induced mucous metaplasia and airway hyperresponsiveness. RV infection increased PAS staining in wild-type mice, but no induction of mucus staining was found in TSLP receptor deficient (TSLPR KO) mice (Figure 3-4A). Consistent with the reduction in PAS staining, induction of IL-13 and the mucus-related genes muc5ac, muc5b and gob5 was significantly lower in TSLPR KO mice than in wild-type mice (Figure 3-4B). We next measured airway responsiveness. Consistent with our previous findings, wild-type mice showed airway hyperresponsiveness four weeks after infection. However, TSLPR KO mice were protected against the development of airway hyperresponsiveness (Figure 3-4C). These results demonstrate that TSLP is required for development of the RV-induced asthma phenotype.

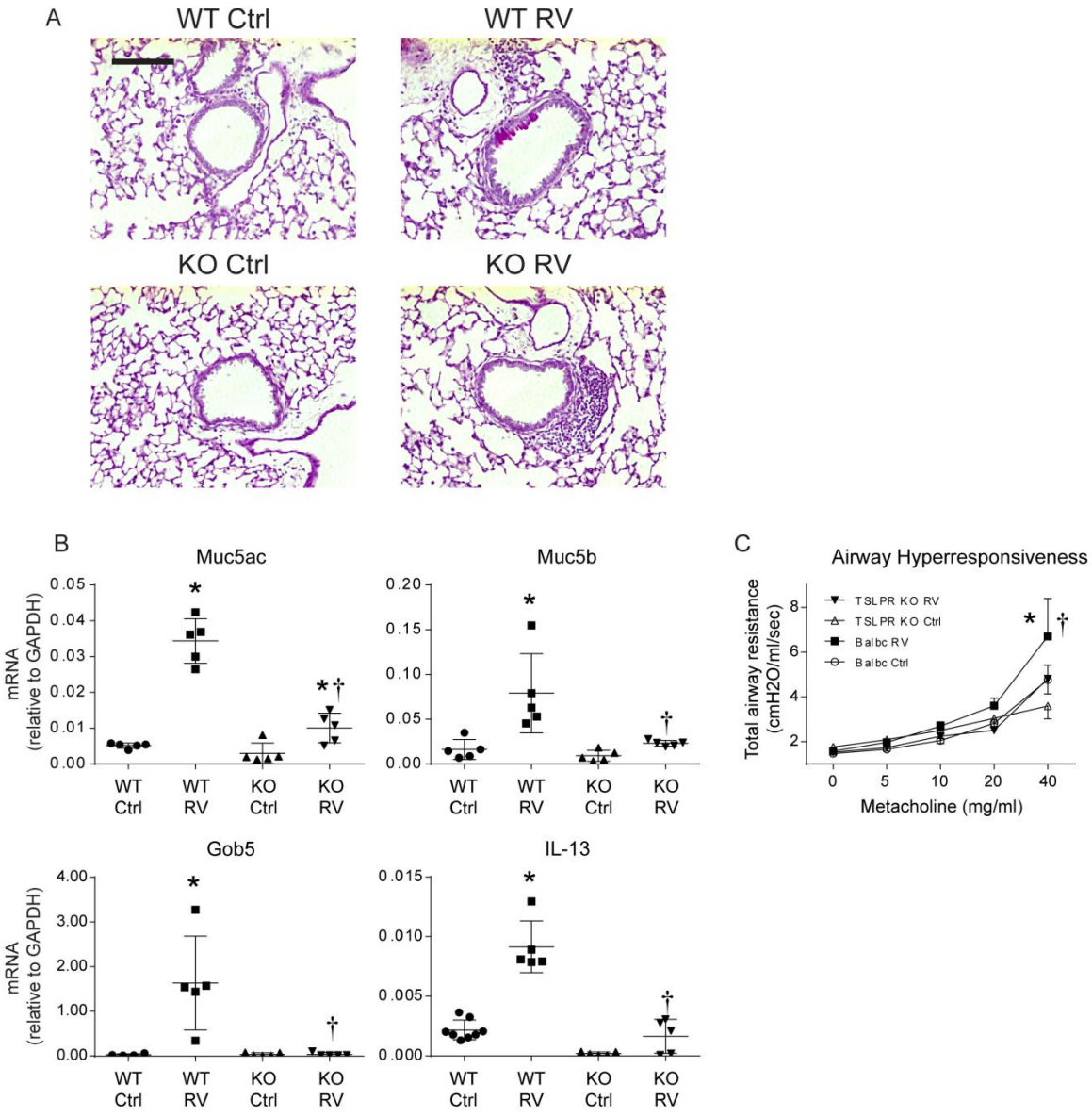

Figure 3-4. Inhibition of mucous metaplasia and airways hyperresponsiveness in TSLPR KO mice. Six-day-old BALB/c (WT) mice or TSLPR KO (KO) mice were inoculated with sham or RV. A, Lung sections were prepared 3 weeks after infection and stained with PAS solution. Representative lung sections of small airways are shown (original magnification, 160X). Scale bar: 100 μm. B, Whole lung gene expression of Muc5ac, Muc5b, Gob5 and IL-13 was measured with quantitative PCR. * $P < 0.05$ versus sham. C, Six-day-old neonatal BALB/c mice or TSLPR KO mice were inoculated with sham or RV and airway cholinergic responsiveness measured 4 weeks after initial inoculation. * $P < 0.05$ versus sham, † $P < 0.05$ versus TSLPR KO RV (two-way ANOVA).

Neonatal RV-induced ILC2 expansion was blocked TSLPR KO mice

In our previous report, we found that ILC2s are increased with neonatal RV infection and produce large amounts of IL-13. Neutralizing antibody against IL-25 decreased IL-13 production and ILC2 expansion while attenuating mucous metaplasia and airways hyperresponsiveness (20). Utilizing TSLPR KO mice, we tested the requirement of TSLP for ILC2 expansion associated with neonatal RV infection. Compared to wild-type mice, in which ILC2 was increased with neonatal RV infection, the ILC2 population was not expanded in TSLPR KO mice (Figure 3-5).

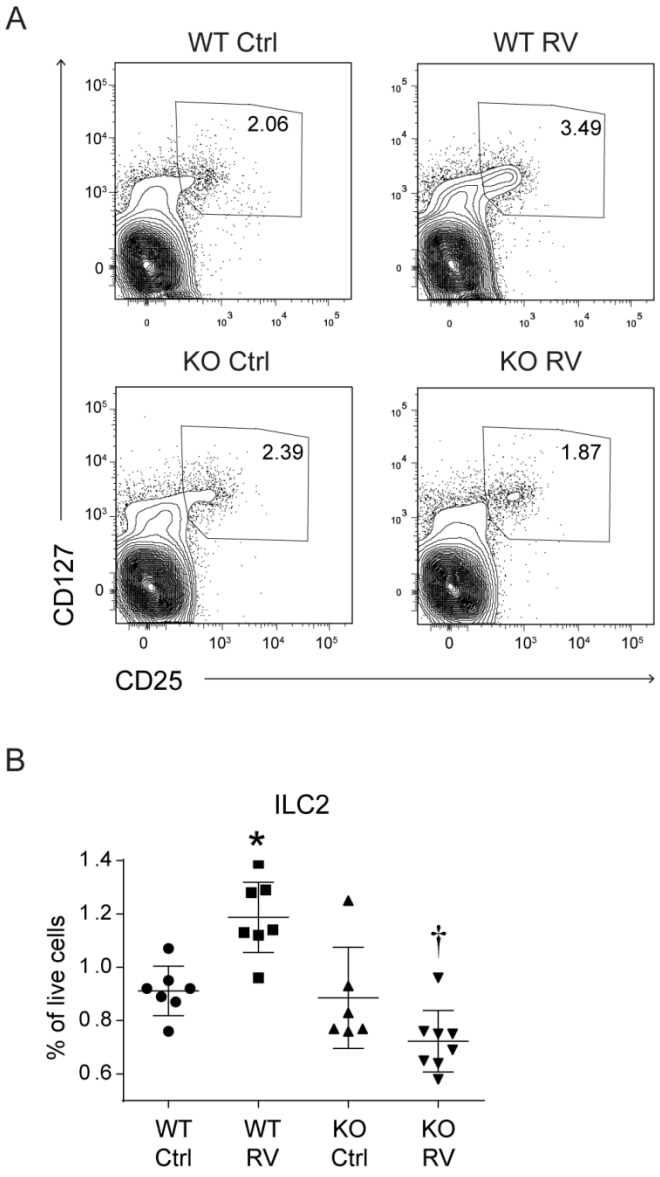

Figure 3-5. Reduction of RV-induced ILC2 expansion in neonatal TSLPR KO mice. Six-day-old neonatal BALB/c mice or TSLPR KO mice were inoculated with sham or RV. Lungs were collected 14 days after infection, minced and digested in collagenase IV. Filtered and washed cells were stained and subjected to flow cytometry. ILC2s were identified as lineage-negative, CD25+ CD127+ live cells with low FSC and SSC, as described previously (20). A, Representative contour plots and the percentage of lineage-negative, CD25- and CD127-double positive cells from each group are shown. B, The percentage of ILC2s for each group is shown. * $P < 0.05$ versus sham, † $P < 0.05$ versus mature mice.

TSLP synergistically increase ILC2 maturation and function with IL-25

To better assess the role of TSLP in ILC2 maturation and function, we sorted ILC2s and cultured them in the presence or absence of TSLP *ex vivo*. Lineage-negative, CD25 and CD127 double-positive ILC2s were sorted from the lungs of RV-infected baby mice. As reported previously (12, 37), IL-25 or IL-33, in combination with IL-2, increased ILC2 gene expression (Figure 3-6). Treatment of TSLP further enhanced IL-2- and IL-25-induced mRNA expression of IL-17RB, ST2L (the receptors for IL-25 and IL-33, respectively), GATA3, IL-5 and IL-13. TSLP also increased IL-2- and IL-33-induced IL-5, IL-13 and GATA3 expression, but had no effect on IL-17RB or ST2L. These results suggest that TSLP is not only involved in ILC2 expansion *in vivo* but also has an additive effect with IL-25 on ILC2 maturation and function.

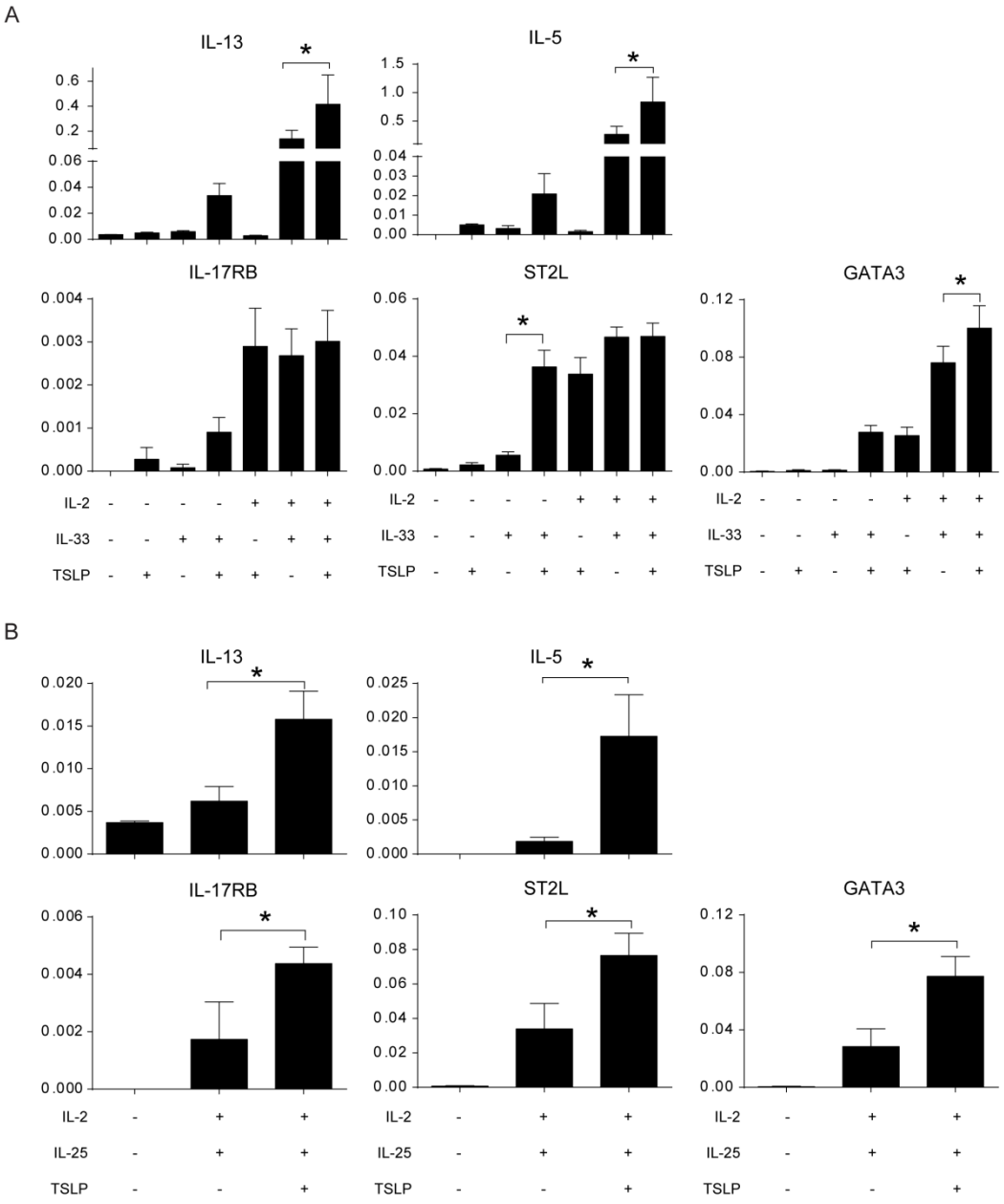

Figure 3-6. Effect of TSLP on the gene expression of ILC2s. Six-day-old neonatal BALB/c mice were infected with RV. Lungs were collected 7 to 10 days after treatment, and lung cell suspensions were sorted for Lin- CD25+ CD127+ ILC2s with FACS. Sorted ILC2s were seeded at 6.5 x 10^3 cells/100 µl in 96 well plates. Cells were stimulated with the indicated combination of cytokines using the following concentrations: IL-2 (50 ng/ml), IL-7 (20 ng/ml), IL-25 (20 ng/ml), IL-33 (20 ng/ml) and TSLP (20 ng/ml). After 3 days of stimulation, supernatants were tested for IL-13 protein and cell pellets were tested for mRNA expression with quantitative PCR. A, Combined effects of TSLP and IL-33 on ILC2 gene expression. B, Combined effects of TSLP and IL-25 on ILC2 gene expression. * $P < 0.05$ versus sham (one-way ANOVA)

Discussion

We have demonstrated previously that neonatal RV infection, but not adulthood infection, causes persistent mucus metaplasia and airway hyperresponsiveness (20, 35). In the present study, we showed that TSLP is required for full development of the observed asthma-like phenotype. RV infection increased lung TSLP levels in immature mice but decreased TSLP level in mature mice. Specific enhancement of TSLP expression was observed in the airway epithelium. Utilizing TSLPR KO mice, we showed that, compared to wild-type mice, RV-induced mucus metaplasia and airway hyperresponsiveness is significantly blocked in TSLPR KO neonatal mice. RV-induced ILC2 expansion was also significantly blocked in TSLPR KO neonatal mice. Finally, we found that, in the presence TSLP, IL-25-stimulated ILC2s express higher levels of GATA3, IL-17RB, ST2L, IL-5 and IL-13 *ex vivo*, evidence of more potent activation. These results suggest that TSLP plays an indispensible role in development of airway responses with neonatal RV infection.

Previous cell culture and animal studies have linked viral infection, TSLP response and the development of airways disease. RV16 induces TSLP expression in primary human bronchial epithelial cells (38, 39), and RSV and influenza have similar effects (40, 41). Airway epithelial cells from asthmatic children produce greater levels of TSLP after RSV infection than cells from healthy children (42). RSV infection of mature mice increases IL-13 levels, mucus production and airways hyperresponsiveness which was reduced in TSLPR KO mice (42).

In contrast to RSV, we have found that the airway response of mice to RV infection is dependent on the age of the infection. We previously found that the levels of

IL-13 and IL-25, which are required for the development of asthma phenotype, are only increased in immature mice after RV infection (20). IFN-γ levels were only increased after adult infection. Further, there was dramatic switch in the immune response to RV at approximately 6-8 days of life. In this report, we found similar changes in the RV-induced TSLP response, with increased TSLP protein levels in 4-6 day-old mice and decreased expression thereafter. To our knowledge, this is the first report showing developmental regulation of the TSLP response after respiratory viral infection. This age-dependent induction of TSLP is consistent with the association between nasal aspirate TSLP protein levels and RV in young children (31). We have not established the specific mechanism underlying the observed switch in immune phenotype, Previous studies have found that the immature immune system is qualitatively different from that of adult, refractory to type 1 and permissive to type 2 responses (43-51). Interestingly, we found that TSLP induction in immature animals was in part regulated by post-transcriptional mechanisms (translation or secretion), in contrast to mature animals in which TSLP mRNA expression was suppressed. IFN-γ has been shown to inhibit double-stranded RNA-induced TSLP production in fibroblasts (52) and keratinocytes (53). It is therefore conceivable that the strong IFN-γ response to RV infection in mature mice blocked the induction of TSLP.

TSLP expression following RV infection was primarily localized to the airway epithelium. However, subepithelial cells were also identified, similar to human asthmatic airways (25). Other potential cellular sources include neutrophils, mast cells, macrophages and eosinophils.

In our previous work, we found that ILC2s are increased in immature mice following RV infection, and that ILC2 produce ample amounts of IL-13 *ex vivo* (20). Further, administration of anti-IL-25 decreased ILC2 number and lung IL-13 while preventing RV-induced mucous metaplasia and airways hyperresponsiveness. These data are consistent with the notion that IL-25 is required for ILC2 expansion and function, as has been shown previously (14). In this report, we present evidence that TSLP is also required for ILC2 expansion and function. RV infection failed to increased lung ILC2 cells in TSLPR KO mice, and TSLP increased ILC2 gene expression *ex vivo*. Early studies identifying ILC2s as critical regulators of allergic responses highlighted the cooperative roles of IL-25 and IL-33 in the ILC2 response (12, 13, 16). In contrast, TSLP was reported to be a driver of Th2 cell (8, 10, 11) and basophil development (54, 55). However, it was later determined that ILC2 cells produce large amounts of IL-5 and IL-13 when stimulated by IL-33 plus TSLP (11, 19, 37), and also that ILC2s express high levels of TSLPR (37). Most recently, it was shown that TSLP is required for IL-33-induced ILC2 proliferation and steroid-resistant airways hyperresponsiveness (56). We now provide further evidence that TSLP promotes ILC2 expansion *in vivo*. Further, our data demonstrating that TSLP has additive effects on IL-25-induced expression of IL-5, IL-13 and IL-17RB, combined with our previous work (20), support a cooperative relationship between TSLP and IL-25 in the response to early-life viral infection.

Early-life wheezing-associated respiratory tract infections have long been considered risk factors for asthma. While initial attention focused on the potential role of respiratory syncytial virus, evidence also exists for an association between early-life RV infection and asthma. In Finnish infants hospitalized for respiratory infection-associated

wheezing, RV was associated with asthma development in contrast to RSV, which was negatively associated (1). Data from a birth cohort of high-risk infants from Madison, Wisconsin showed that wheezing-associated illness with RV is the most important risk factor for asthma development, higher than that of infants with allergen sensitization or RSV infection (2, 3). A population-based retrospective analysis of a birth cohort of 90,000 Tennessee children showed an increased risk of early childhood asthma following bronchiolitis during RV-predominant non-winter months vs. RSV-predominant winter months (4). Together, these data are consistent with the notion that early-life viral infections, including those with RV, perhaps in combination with other factors such as genetic background, allergen exposure and microbiome, modulate the immune response, increasing the likelihood of childhood asthma development. Our data are consistent with the notion that infants are susceptible to asthma development following RV infection,

We conclude that TSLP is required for persistent mucus metaplasia and airway hyperresponsiveness as well as ILC2 expansion with neonatal RV infection. Similar to IL-13 and IL-25, the TSLP response to viral infection was developmentally regulated. This research further supports the notion that TSLP may play a role in asthma development.

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Chapter 4

IL-4 receptor signaling determines macrophage activation state and response to rhinovirus infection in a mouse model of allergic asthma

Summary

In contrast to the potential role of early life viral infections in asthma pathogenesis, the contribution of viruses to asthma exacerbations is well-established. However, the mechanisms by which viruses cause asthma exacerbations are not precisely known. In this chapter, we examine the role of another innate immune cell, the macrophage, in asthma attacks. Previously, we showed that, in ovalbumin (OVA)-sensitized and -challenged mice with allergic airway inflammation, rhinovirus (RV) infection increases type 2 cytokine production from alternatively-activated (M2) airway macrophages, enhancing eosinophilic inflammation and airways hyperresponsiveness. In this paper, we tested the hypothesis that IL-4 signaling determines the state of macrophage activation and pattern of RV-induced exacerbation in mice with allergic airways disease. To resolve this issue, eight week-old wild type or IL-4 receptor knockout (IL-4R KO) mice were sensitized and challenged with OVA and inoculated with RV1B or sham HeLa cell lysate. In contrast to OVA-treated wild-type mice with both neutrophilic and eosinophilic airway inflammation, OVA-treated IL-4R KO mice showed increased neutrophilic inflammation with few eosinophils in the airways. Like

wild-type mice, IL-4R KO mice showed OVA-induced airway hyperreactivity which was further exacerbated by RV. There was a shift in lung cytokines from a type 2-predominant response to a type 1 response, including production of IL-12p40 and TNF-α. IL-17A was also increased. RV infection of OVA-treated IL-4R KO mice further increased neutrophilic inflammation. Bronchoalveolar macrophages showed an M1 polarization pattern and ex vivo RV infection increased macrophage production of TNF-α, IFN-γ and IL-12p40. Finally, lung cells from OVA-treated IL-4R KO mice showed reduced CD206+ CD301+ M2 macrophages, decreased IL-13 and increased TNF-α and IL-17A production by F4/80+, CD11b+ macrophages.

In conclusion, OVA-treated IL-4R KO mice show neutrophilic airway inflammation constituting a model of allergic, type 1 cytokine-driven neutrophilic asthma. In the absence of IL-4/IL-13 signaling, RV infection of OVA-treated mice increased type 1 cytokine and IL-17 production from conventionally-activated macrophages, augmenting neutrophilic rather than eosinophilic inflammation. In mice with allergic airways inflammation, IL-4R signaling determines macrophage activation state and the response to subsequent RV infection.

Introduction

Macrophages are innate immune cells that play a critical role in early phases of host defense against pathogens, coordination of the adaptive immune response, and the regulation of inflammation and tissue repair. Through activation signals by various ligands and environmental cues, macrophages may change their polarization state, leading to altered immune responses. In the presence of type 1 cytokines such as

interferon-γ and IL-12, macrophages are activated in a classical manner (M1-polarized) and produce pro-inflammatory cytokines and toxic mediators. In the presence of the type 2 cytokines IL-4 and IL-13, alternatively-activated (M2-polarized) macrophages express a distinct pattern of phagocytic receptors (1) and produce type 2 cytokines which play a role in anti-parasitic and allergic responses, including asthma. IL-13-overexpressing transgenic mice infected with *C. neoformans* demonstrate alternatively-activated macrophages expressing Arg-1, macrophage mannose receptor (CD206) and Ym-1, as well as lung eosinophilia, goblet cell metaplasia, elevated mucus production and airway hyperreactivity (2). Adoptive transfer of IL-4Rα^{+} alternatively-activated macrophages enhances eosinophilic inflammation in ovalbumin (OVA)-sensitized and -challenged mice (3). Moreover, CD206 is increased in the asthmatic patients and correlates with the severity of asthma (4).

Viral-induced exacerbations are a major cause of morbidity in asthma. Rhinovirus (RV), a single-stranded RNA virus belonging to *Picornavirus* family, is consistently the most frequent pathogen identified. However, the precise mechanisms underlying RV-induced asthma exacerbations are not known. Compared to normal subjects, asthmatic patients with RV infection show enhanced neutrophilic and eosinophilic inflammation in the lower airways (5), consistent with the notion that asthmatics have a qualitatively different immune response to RV infection than controls. To test this in an animal model, we exposed OVA-sensitized and -challenged mice with allergic airways disease to RV1B, a minor group virus which infects mouse epithelial cells (6). We found that RV increased airway hyperresponsiveness and eosinophilic inflammation, and that RV colocalized with eotaxin-producing, CD68-positive lung

macrophages *in vivo.* Macrophages from OVA-treated mice showed increased expression of arginase-1, Ym-1 and Mgl-2, indicating a shift in macrophage activation status, and RV inoculation of lung macrophages from OVA-treated mice induced expression of eotaxin-1, IL-4, and IL-13 *ex vivo*. Depletion of macrophages from OVA-sensitized and -challenged mice reduced eosinophilic inflammation following RV infection. Together, these results suggest that RV causes asthma exacerbations in part by infection of alternatively-activated macrophages. Finally, exposure of macrophages from naïve mice to IL-4 and IL-13 significantly increased RV-induced eotaxin mRNA expression, consistent with the notion that type 2 cytokines are sufficient to alter the response of macrophages to RV infection.

In the present study, we hypothesized that IL-4 signaling regulates the state of macrophage activation and the pattern of RV-induced exacerbation in mice with allergic airways disease. We anticipated that, in the absence of IL-4/IL-13 signaling, RV infection would preferentially increase type 1 cytokine production from conventionally-activated macrophages, augmenting neutrophilic rather than eosinophilic inflammation. To test this, we sensitized and challenged wild-type and IL-4 receptor knockout (IL-4R KO) mice with OVA and then infected these animals with RV. We found that OVA-treated IL-4R KO mice showed neutrophilic airway inflammation which was exacerbated by RV infection. Macrophages from OVA-treated mice showed an M1 polarization pattern and expressed type 1 cytokines and IL-17 in response to *ex vivo* RV infection. OVA-treated IL-4R KO mice showed decreased IL-13 and increased TNF-α and IL-17 production with RV infection by F4/80+, CD11b+ macrophages, demonstrating that IL-

4R signaling determines macrophage activation state and the response to subsequent RV infection.

Methods

Generation of HRV.

HRV1B (ATCC, Manassas, VA) were grown in cultured HeLa cells, concentrated, partially purified and titered as described previously (7). Similarly concentrated and purified HeLa cell lysates were used for sham infection.

OVA sensitization and challenge.

All animal experiments were approved by the University of Michigan Institutional Animal Care and Use Committee. Female 8 wk-old BALB/c mice (Jackson Laboratories, Bar Harbor, MA) or age-matched BALB/c-*Il4ra*^{tm1Sz}/J IL-4R KO mice (Jackson Laboratories) were injected intraperitoneally on days 0 and 7 with 0.2 ml PBS or a solution of alum and 100 µg endotoxin-free OVA (Sigma-Aldrich, St. Louis, MO). Next, mice were challenged intranasally with 50 µl of PBS or 100 µg OVA on days 12 and 13. Selected mice were inoculated with sham or RV1B on day 14, and mice were harvested for analysis on day 15.

RV exposure.

Selected mice were inoculated intranasally with 50 ul of $1x10^8$ $TCID_{50}$/ml RV1B, or an equal volume of sham control immediately following the last OVA treatment. Lungs were collected 24 hr post infection for further analysis.

Assessment of airway responsiveness.

Airway cholinergic responsiveness was assessed by measuring changes in total respiratory system resistance in response to increasing doses of nebulized methacholine, as described previously (8). Mice were anesthetized with sodium pentobarbital (50 mg/kg mouse, intraperitoneal injection) and a tracheostomy performed. Mechanical ventilation was conducted and total respiratory system measured using a Buxco FinePointe operating system (Buxco, Wilmington, NC). Airway responsiveness was assessed by measuring changes in resistance in response to increasing doses of nebulized methacholine.

Bone marrow-derived macrophage cultures.

Femurs were harvested from naïve wild-type BALB/c and IL-4R KO mice and the bone marrow was used to expand macrophages for *in vitro* analyses. Dissociated marrow cells were plated onto 12-well culture dishes at $1x10^6$ cells/ml and cultured for 6 days in RPMI supplemented with 30% L929-conditioned medium and 10% heat-inactivated fetal bovine serum (9). Cells were stimulated for 1.5 h with medium or RV1B (multiplicity of infection, 10) and RNA harvested 8 h after infection.

Mouse bronchoalveolar inflammatory cells and macrophage culture.

Bronchoalveolar lavage (BAL) was performed using 1 ml PBS aliquots, and differential cell counts were performed using the DiffQuick method. BAL fluid from PBS- and OVA-treated mice was seeded in 24 well plates. BAL macrophages were purified by plastic adherence, which yielded >90% purity. Cells were stimulated for 1.5

h in the presence or absence of HRV1B (multiplicity of infection, 10), and harvested 8 or 24 h after infection for RNA and protein analysis.

Cytokine/chemokine expression.

Lung RNA was extracted with Trizol (Sigma-Aldrich) and analyzed for cytokine and chemokine gene expression by quantitative real-time PCR using specific primers and probes. Signals were normalized to GAPDH. BAL fluid was spun for 15 min at 1500 rpm, and the supernatants were analyzed for cytokine protein by multiplex immune assay (Bio-Rad, Hercules, CA) or ELISA (R&D Systems, Minneapolis, MN).

Histology, immunohistochemistry and immunofluorescence microscopy.

Lungs were fixed with 10% formaldehyde overnight and paraffin embedded. Blocks were sectioned at 500 µm intervals at a thickness of 5 µm and each section was deparaffinized, hydrated and stained with hematoxylin and eosin. Other sections were stained with rabbit anti-mouse IL-17 (Abcam, Cambridge, MA). For immunohistochemistry, sections were incubated with biotinylated secondary goat-IgG, ABC reagent (Vector Laboratories, Burlingame, CA), diaminobenzidine (DAB, Sigma-Aldrich) and Gill's hematoxylin (Fisher Scientific, Kalamazoo, MI). For fluorescence microscopy, slides were incubated with Alexa Fluor (AF)-555-conjugated rat anti-mouse CD68 and AF488-conjugated rabbit anti-mouse IL-17 or AF-conjugated isotype control IgGs. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Images were visualized using a Olympus IX71inverted phase/epifluorescence microscope and digital CCD camera.

Flow cytometric analysis.

Lungs from mice were perfused with PBS containing EDTA, minced and digested in collagenase IV. Cells were filtered and washed with RBC lysis buffer, and stimulated with for 5 h with cell stimulation cocktail (eBioscience, San Diego, CA) containing PMA, ionomycin and protein export blockers. After stimulation, dead cells were stained with Pac-Orange Live/Dead fixable dead staining dye (Invitrogen, Carlsbad, CA). Cells were then stained with anti-CD45-PacBlue, anti-TCRβ-FITC, anti-F4/80-PE-Cy5, anti-CD11c-APC, anti-CD11b-APC-Cy7, anti-CD68-PerCP-Cy5.5, anti-CD301 conjugated with Alexa Fluor (AF)-633 and anti-CD206 conjugated with AF488 (all antibodies from Biolegend, San Diego, CA). Cells were fixed, permeabilized and incubated with anti-IL-13-PE (eBioscience), anti-IL-17A-PE-Cy7 antibody (Biolegend) or anti-TNF-α-PE-Cy7 antibody (Biolegend). Stained cells were subjected to flow cytometry and analyzed on a BD Biosciences FACSAria II (BD Biosciences, San Jose, CA). Data were collected using FACSDiva software and analyzed using FlowJo software (Tree Star, Ashland, OR).

Data analysis.

Data are represented as mean±SEM. Statistical significance was assessed by student's t test, unpaired t test, one-way analysis of variance (ANOVA), two-way ANOVA, ANOVA based on ranks, as appropriate. Differences were pinpointed by the Bonferroni or Newman-Keuls multiple comparisons test.

Results

RV infection elicit differential cytokine response from wild-type and IL-4R KO macrophages.

We previously found that, in OVA-sensitized and -challenged mice with allergic airway inflammation, RV infection increases eotaxin-1, IL-4 and IL-13 production from alternatively-activated (M2 polarized) airway macrophages, further enhancing eosinophilic inflammation and airways hyperresponsiveness (6). We also found that *in vitro* exposure of macrophages from naïve mice to IL-4 and IL-13 significantly increased RV-induced eotaxin mRNA expression, consistent with the notion that type 2 cytokines are sufficient to alter the response of macrophages to RV infection. In the present study, we hypothesized that IL-4 signaling determines the state of macrophage activation and pattern of RV-induced exacerbation in mice with allergic airways disease. First, we tested the responses of bone marrow-derived macrophages from naïve wild-type and IL-4R KO mice. Dissociated marrow cells were differentiated to macrophages in L929-conditioned medium (9). Macrophages from naïve wild-type mice showed significant increases in IL-1β, TNF-α, CXCL1 and CXCL2 mRNA expression in response to RV infection *ex vivo* (Figure 4-1). Bone marrow-derived macrophages from naïve IL-4R KO cells also showed increases in IL-1β, TNF-α, CXCL1 and CXCL2 mRNA expression. However, compared to control cells, IL-4R KO cells demonstrated significantly increased IL-1β and CXCL2 in response to RV infection. Further, in contrast to cells from wild-type mice, RV infection of macrophages from naïve IL-4R KO mice significantly increased mRNA expression of IL-17A and IL-6. These results suggest that, in the

absence of IL-4R signaling, macrophages show an exaggerated type 1 phenotype and increased IL-17 production in response to RV infection.

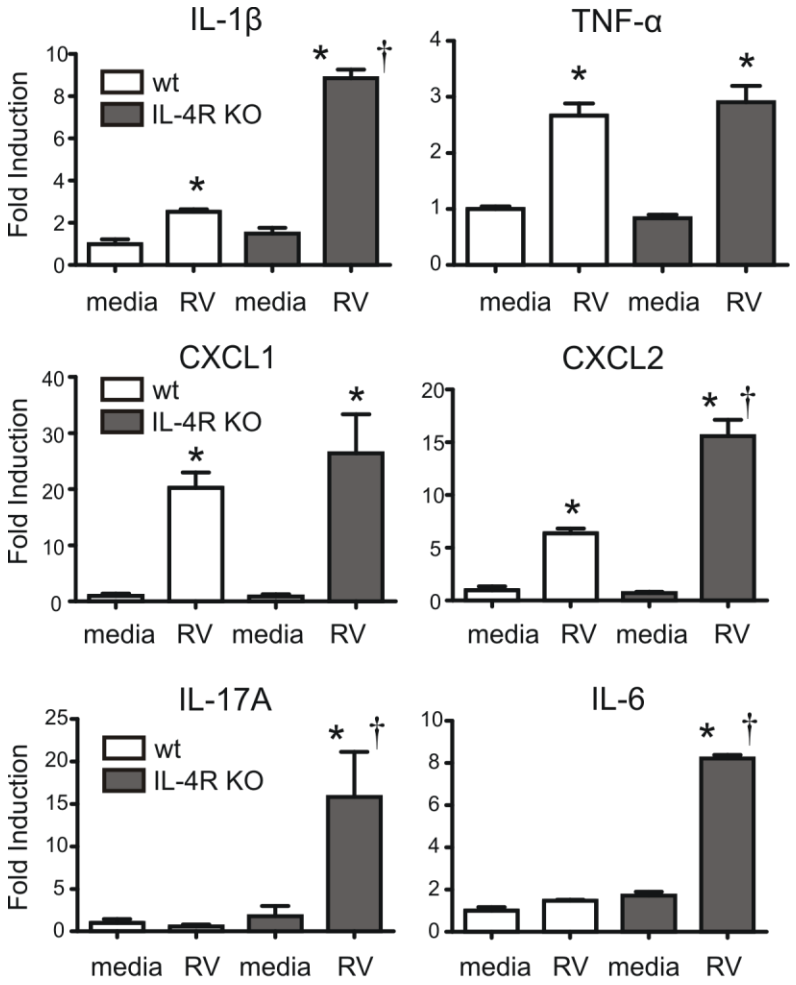

Figure 4-1. Responses of bone marrow-derived macrophages to RV infection. Dissociated marrow cells from wild-type and IL-4R KO mice were differentiated to macrophages in L929-conditioned medium. Compared to control cells, IL-4R KO cells demonstrated increased RV-induced cytokine responses. (Mean±SEM, n=3, *different from medium, $P < 0.05$, one-way ANOVA; †different from wild-type, $P < 0.05$, one-way ANOVA)

Immune responses to OVA challenge and RV infection were differentially regulated in the absence of IL-4R signaling

Next, we examined the effect of IL-4R KO in mice sensitized and challenged with OVA. As expected, OVA treatment of wild-type mice significantly increased the total number of BAL cells (Figure 4-2A), augmenting the number of neutrophils and eosinophils (Figure 4-2B and C). RV infection of wild-type mice further increased lung inflammation, resulting in an additional 2-fold increase in BAL cells. Both neutrophils and eosinophils were increased in the BAL following RV infection. In IL-4R KO mice, OVA sensitization and challenge was also accompanied by a significant increase in BAL inflammatory cells (Figure 4-2A). The inflammatory cells consisted nearly exclusively of neutrophils, and the number of eosinophils in the airways was significantly reduced compared to wild-type mice (Figure 4-2B and C). When OVA-treated IL-4R KO mice were infected with RV, neutrophil infiltration further increased (Figure 4-2D). In contrast to wild-type mice, RV did not induce eosinophilic inflammation. Finally, we found that, similar to wild type mice, IL-4R KO mice showed OVA-induced airway hyperreactivity which was further exacerbated by RV (Figure 4-2E and F). Together, these results show that IL-4 receptor signaling is not required for allergen-induced airway inflammation or hyperresponsiveness. Instead, the immune responses to OVA challenge and RV infection were differentially regulated in the absence of IL-4R signaling, accentuating neutrophilic rather than eosinophilic inflammation.

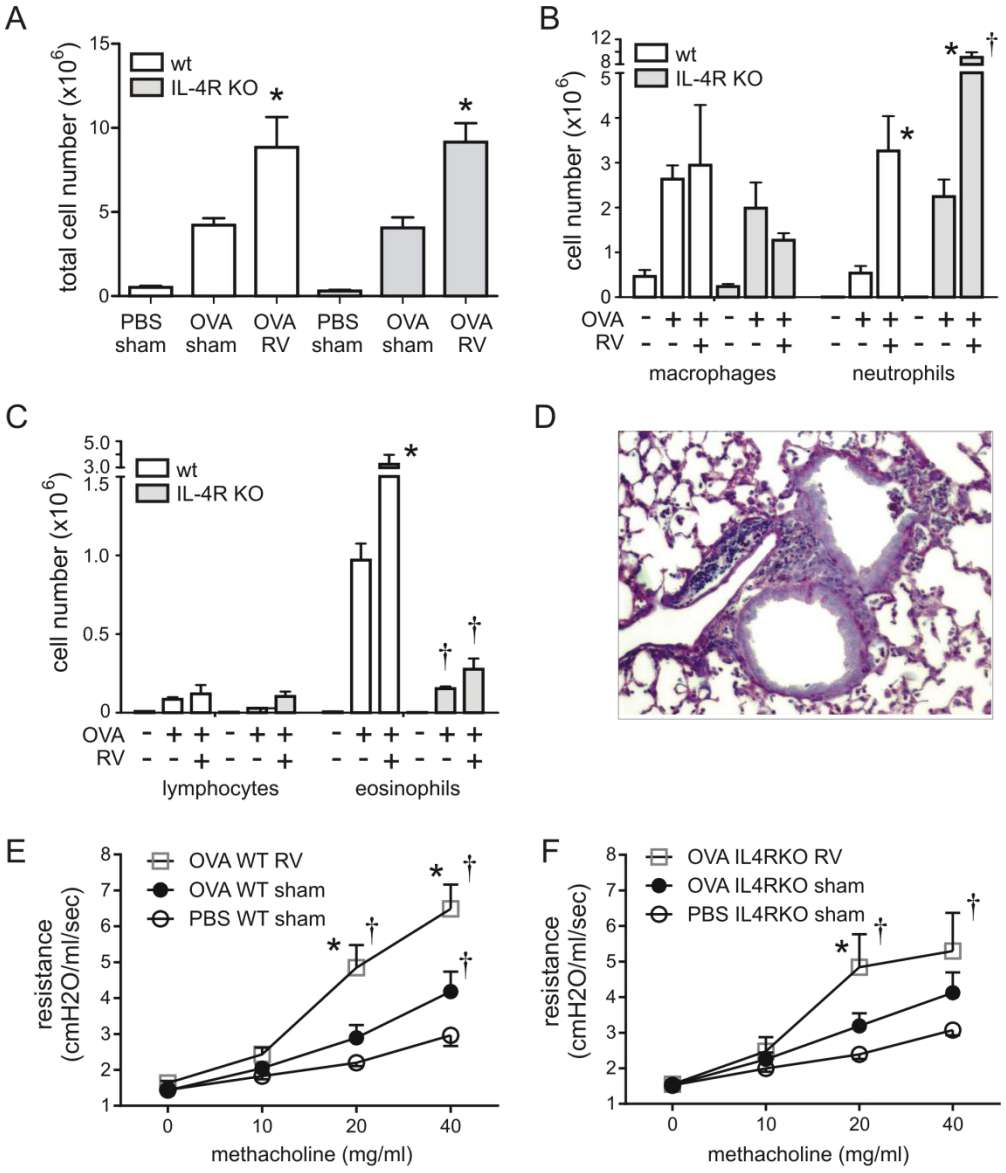

Figure 4-2. Airway inflammation and airway hyperresponsiveness in OVA-treated wild-type and IL-4R KO mice. Eight-week old wild-type or IL-4R KO mice were treated with PBS or OVA and inoculated with sham or RV. Bronchoalveolar lavage was performed 24 hour post-inoculation. After counting the total number of cells, cytospins were performed and stained with Diff-Quick, and differential counts determined from 200 cells. **(A)** RV infection increases the total number of BAL cells in OVA-sensitized and -challenged wild-type and IL-4R KO mice. **(B)** RV infection increases the number of airway neutrophils in OVA-treated wild-type and IL-4R KO mice. The neutrophil response was significantly higher in IL-4R KO mice. **(C)** RV infection increases the number of airway eosinophils in OVA-treated wild-type mice. The eosinophil response was significantly attenuated in IL-4R KO mice. (Mean±SEM, n=3, *different from medium, $p<0.05$, one-way ANOVA; †different from wild-type, $p<0.05$, one-way ANOVA.) **(D)** Hematoxlin and eosin staining of airway from OVA- and RV-treated IL-

4R KO mouse. Neutrophils are present in the airway lumen, bronchovascular bundle and some alveolar spaces. **(E & F)** Airway cholinergic responsiveness was assessed by measuring changes in total respiratory system resistance in response to increasing doses of nebulized methacholine. Data from wild type (**E**) and IL-4R KO mice (**F**) are shown. (Mean±SEM, n=4-6 in each group, *different from sham, $P < 0.05$, two-way ANOVA; †different from PBS, $P < 0.05$, two-way ANOVA).

Exaggerated type 1 immune response to RV in OVA-treated IL-4R KO mice.

To determine the factors driving neutrophilic inflammation in IL-4R KO mice, we analyzed lung mRNA expression by qPCR (Figure 4-3A). In OVA-treated wild-type mice, RV treatment increased TNF-α, IFN-γ, CCL2, CCL3 and IL-4 mRNA expression. RV infection of OVA-treated IL-4R KO mice significantly increased TNF-α, IFN-γ, CXCL1, IL-12p40, CCL2, CCL3 and IL-17 mRNA levels. Finally, compared to OVA-treated, RV-infected wild-type mice, OVA-treated, RV-infected IL-4R KO mice showed increased IL-12p40 and IL-17A mRNA expression. Considering the neutrophil-recruiting ability of TNF-α and CXCL1 (10, 11), and the capacity of IL-17A to indirectly stimulate recruitment of neutrophils into the airways via the induction of C-X-C chemokines (12-17), these results suggest that neutrophilic inflammation in the airways of OVA-treated IL-4R KO mice was mediated, at least in part, by the induction of TNF-α, CXCL1 and IL-17A. We also examined BAL fluid protein levels (Figure 4-3B). We found that, compared to similarly-treated wild type mice, TNF-α and IL-12p40 levels were significantly higher in the lungs of OVA-treated, RV-infected IL-4R KO mice, consistent with an enhanced type I immune response.

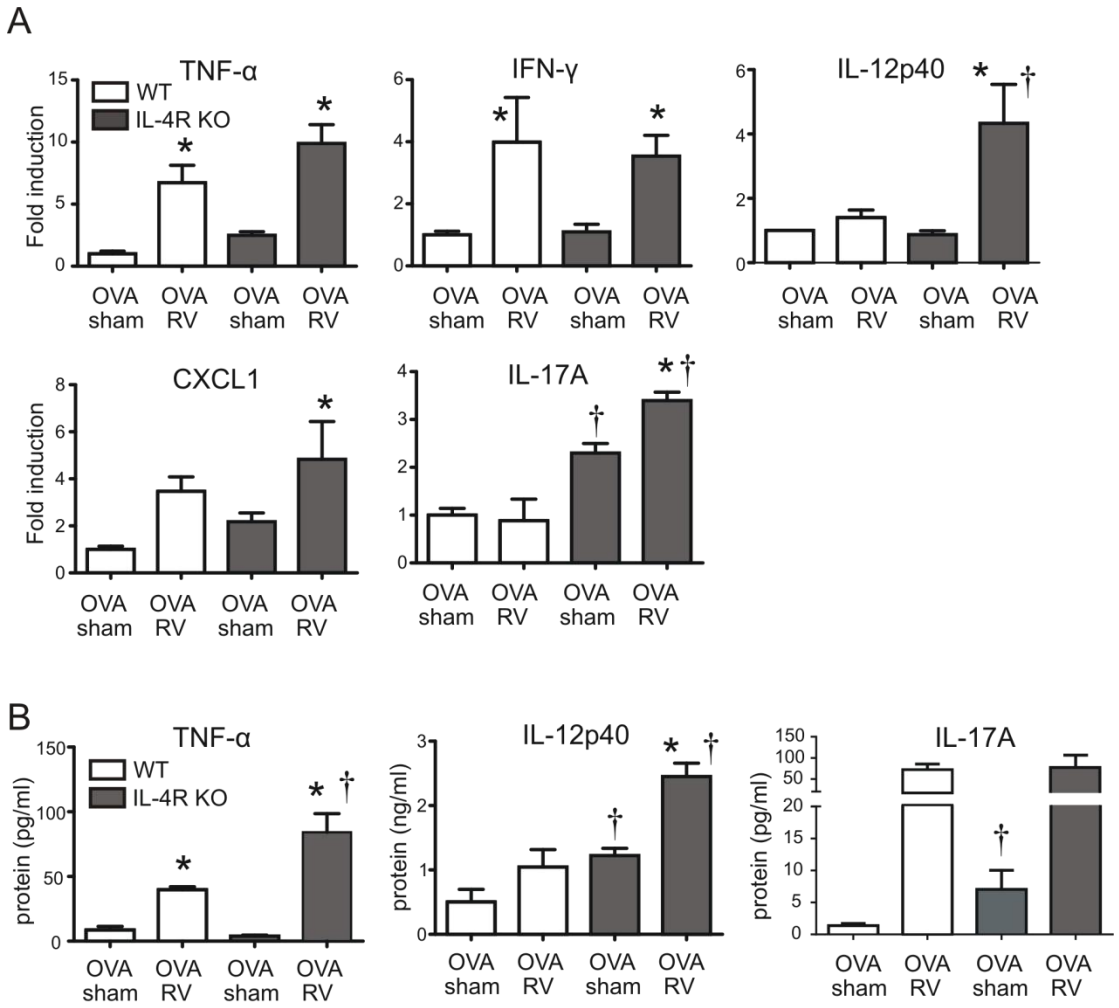

Figure 4-3. Cytokine expression in lungs of OVA-treated wild-type and IL-4R KO mice. Wild-type or IL-4R KO mice were sensitized and challenged with OVA and inoculated with sham or RV. **(A)** After 24 h, lungs were collected and gene expression measured by qPCR. **(B)** TNF-α and IL-12p40 protein in the BAL fluid was assessed with multiplex immune assay. (Mean±SEM, n=3-6 each group, *different from sham, $P < 0.05$, one-way ANOVA; †different from wild-type, $P < 0.05$, one-way ANOVA.)

IL-4R KO macrophages are polarized towards an M1 phenotype, leading to a differential response to RV infection compared to wild-type mice.

To examine the specific role of the macrophage in airway neutrophilic responses, we isolated adherent BAL cells (>90% macrophages) from OVA-sensitized and -challenged wild-type and IL-4R KO mice and infected them with RV *ex vivo*. As shown previously (6), macrophages from wild-type mice treated with OVA expressed high levels of the M2 marker Arg 1 and Ym-1, and expressed the type 2 cytokines IL-4, IL-13 and CCL-24 upon RV infection, typical of alternative activation (Figure 4-4A). RV infection also stimulated mRNA expression of CCL2 in macrophages from wild-type mice. In contrast, macrophages from OVA-treated IL-4R KO mice failed to express significant levels of Arg-1, Ym-1 or and CCL24, and did not express IL-4 or IL-13 mRNA with RV infection. Compared to cells from wild-type mice, CCL2 expression was decreased in cells from IL-4R KO mice. On the other hand, IL-4R KO macrophages showed more potent type 1 cytokine responses to RV infection, including TNF-α, IFN-γ and IL-12p40. Moreover, the basal level of IL-17A gene expression was increased in macrophages from OVA-treated IL-4R KO mice. Analysis of macrophage supernatants confirmed increased production of TNF-α and IL-17A protein in cells from IL-4R KO mice (Figure 4-4B). These results suggest that, upon OVA treatment, IL-4R KO macrophages are polarized towards an M1 phenotype, leading to a differential response to RV infection compared to wild-type mice.

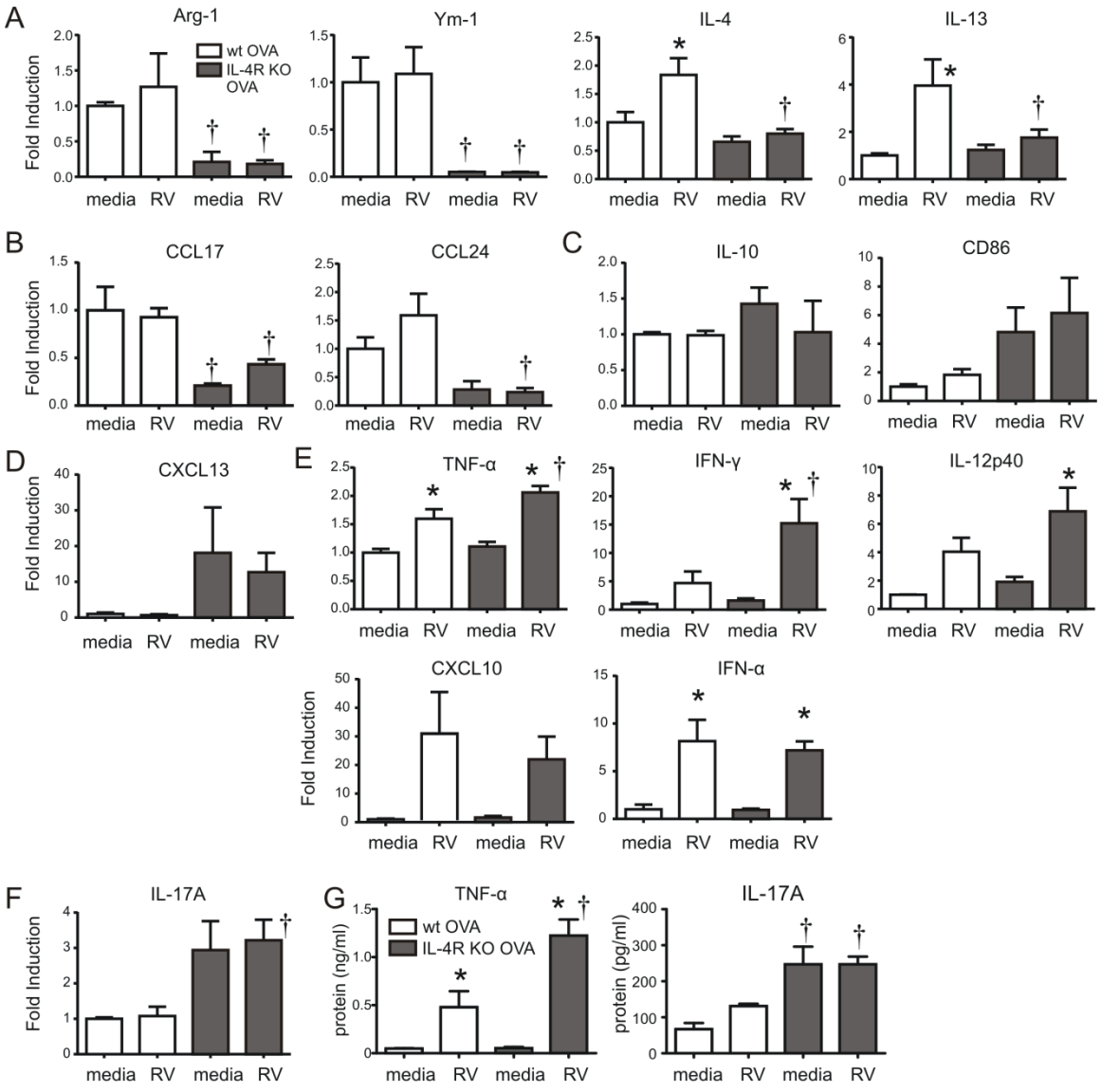

Figure 4-4. Differential cytokine expression in RV-stimulated macrophages from OVA-treated wild-type and IL-4R KO mice. Macrophages were collected from the BAL of OVA-treated wild-type or IL-4RKO mice. Macrophages were selected by allowing adherence to plastic for 2 h. Macrophages were treated with medium or RV (multiplicity of infection, 5) for 1.5 hours. Cells were collected 8 h or 24 h after infection for RNA and protein analysis. **(A)** Gene expression in macrophage was measured by qPCR. **(B)** TNF-α and IL-17A protein levels were assessed with ELISA. (Data represent three independent experiments, mean±SEM, n=3-8 each group, *different from sham, $P < 0.05$, one-way ANOVA; †different from wild type, $P < 0.05$, one-way ANOVA).

IL-4 signaling is required for alternative activation of lung macrophages and production of IL-13 with OVA treatment.

Next, we examined the effect of IL-4R KO on macrophage activation *in vivo*. We reasoned that, in the absence of IL-4R signaling, RV infection of OVA-sensitized and -challenged mice would fail to induce type 2 cytokine production and, instead, increase type 1 and IL-17 cytokine production from conventionally-activated macrophages, augmenting neutrophilic rather than eosinophilic inflammation. Lungs of wild type and IL-4R KO mice were examined by flow cytometric analysis. Lung cell suspensions were stained with antibodies against macrophage surface markers. As we found previously (6), in wild-type mice, expression of the macrophage alternative activation markers CD206 and CD301 was enhanced after OVA challenge (Figure 4-5A). Further, IL-13 production by CD11b+ macrophages was increased with OVA treatment and further increased with RV infection (Figure 4-5B and C). In contrast, in IL-4R KO mice, neither CD206 and CD301 expression (Figure 4-5A) nor IL-13 (Figure 4-5B and C) increased after OVA treatment or RV infection. These data suggest that IL-4 signaling is required for alternative activation of lung macrophages and production of effector cytokine IL-13 in this experimental system.

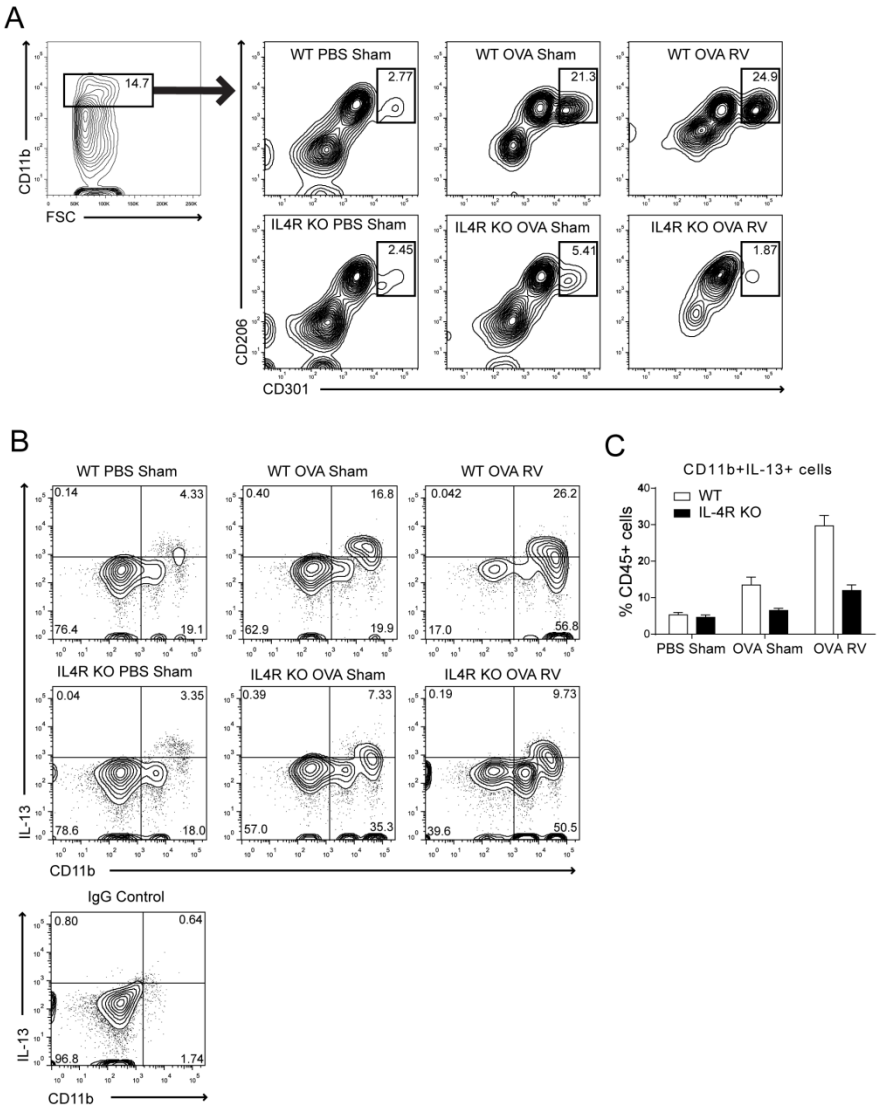

Figure 4-5. Differential expansion of CD206+ CD301+ M2-polarized macrophages and IL-13 production in wild-type and IL-4R KO mice. Eight-week old wild-type or IL-4R KO mice were treated with PBS or OVA by intraperitoneal injection (days 0, 7) and intranasal installation (days 12, 13). Mice were intranasally inoculated with sham or RV on day 14. Lungs were harvested and minced in collagenase IV solution. **(A)** Cells were stained with antibodies against macrophage surface markers and assessed with flow cytometric analysis. CD206- and CD301-double positive cells in the CD11b+ cell fraction are shown. **(B)** Cells were incubated with cell stimulation cocktail for 5 h, stained, and analyzed with flow cytometric method. Expression of CD11b and IL-13 was analyzed among CD45+ cells. The numbers represent the percentage of cells within each quadrant. **(C)** The percentage of CD11b+ IL-13+ cells were shown among CD45+ fraction.

RV infection increased TNF and IL-17A expression in macrophages of OVA-treated IL-4R KO mice.

Next, we asked whether type 1 cytokine production is increased in macrophages from OVA-treated IL-4R KO mice *in vivo*. We collected the lungs of wild type and IL-4R KO mice, stained for TNF-α and analyzed with flow cytometry. We found that, compared to wild-type mice, TNF-α producing CD45+, F4/80+, CD11c-, CD11b+ macrophages were increased in OVA-treated IL-4R KO mice, and these cells were further increased with RV infection (Figure 4-6A and B).

To test whether macrophages secrete IL-17A *in vivo*, we examined the lungs of wild type and IL-4R KO mice by flow cytometric analysis. We found that CD45+, TCRβ-, CD68+, F4/80+, CD11c-, CD11b+, IL-17A+ macrophages were increased in naïve IL-4R KO mice compared to wild-type mice, and further enhanced with RV infection (Figure 4-6C and D). We also employed immunohistochemistry and immunofluorescence microscopy to confirm IL-17 localization in lungs from OVA-treated IL-4R KO mice. IL-17-positive monocytic cells were evident in the subepithelium (Figure 4-6E). IL-17 co-localized with CD68, a macrophage marker (Figure 4-6F).

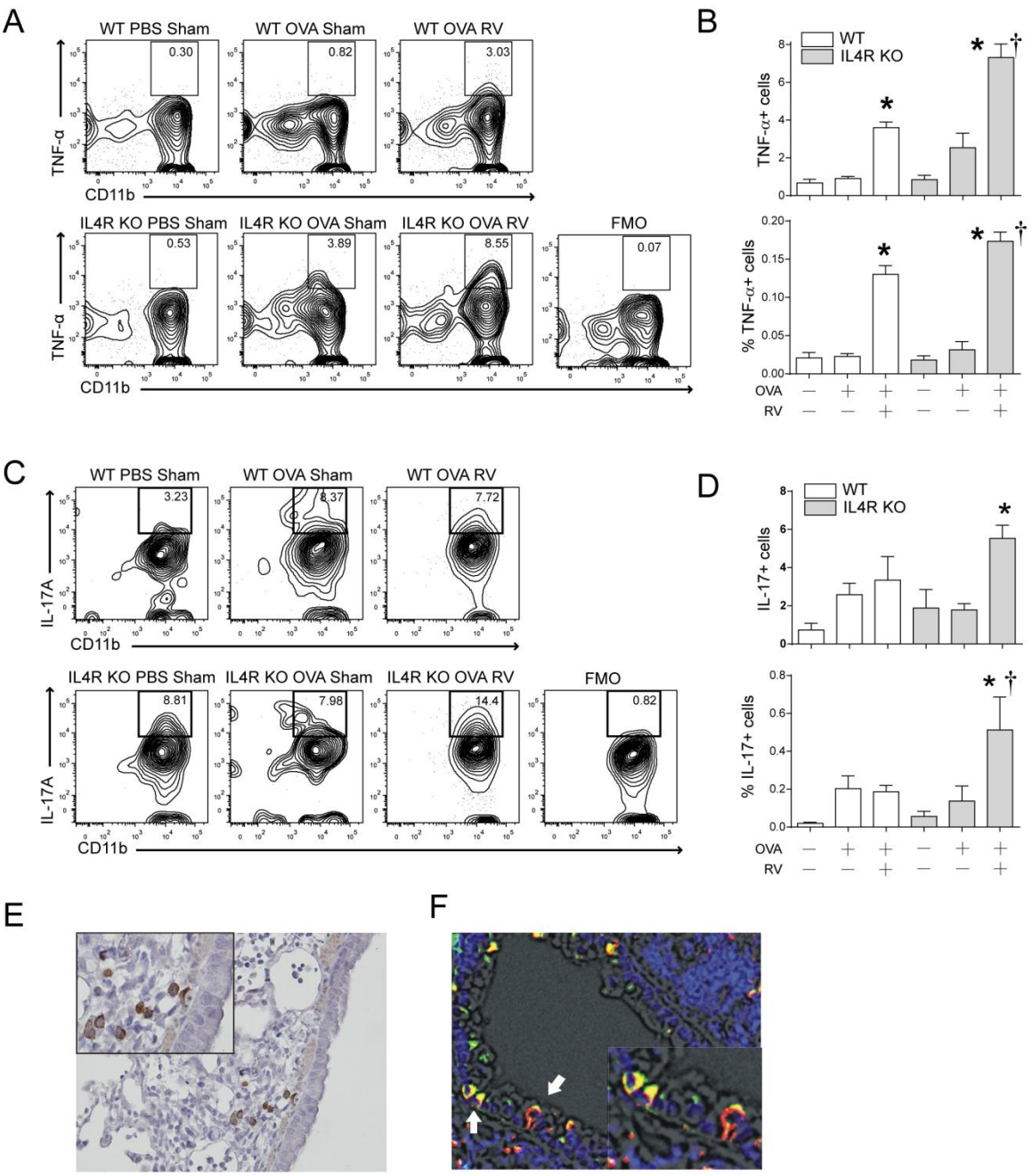

Figure 4-6. TNF-α and IL-17A expression in macrophages of wild-type and IL-4R KO mice. Wild-type or IL-4R KO mice were sensitized and challenged with OVA and inoculated with sham or RV. **(A)** Lungs were harvested and digested with collagenase IV. Cells were stimulated with cell stimulation cocktail for 5h and stained with antibodies against macrophage surface markers, fixed, permeabilized and incubated with anti-TNF-α. CD11b+ TNF-α+ cells were analyzed in the CD45+ F4/80+ CD11c- fraction. A fluorescent minus one (FMO) control was utilized to confirm TNF-α signals. **(B)** The percentage of CD45+, F4/80+, CD11c-, CD11b+, TNF-α cells in the CD45+ F4/80+ CD11c- fraction (upper panel) and total CD45+ TNF-α+ cells (lower panel) were

calculated. **(C)** IL-17A producing macrophages were assessed by flow cytometry. Lung cells were stained with anti-IL-17A. CD45+, CD68+, F4/80+, CD11c- cells were analyzed for CD11b and IL-17A. An FMO control was utilized to confirm IL-17A signals. **(D)** The percentage of CD45+, CD68+, F4/80+, CD11c-, CD11b+, IL-17A+ cells in the CD45+ CD68+ fraction (upper panel) and total CD45+ IL-17A+ cells (lower panel) were calculated. **(E)** Lung sections were stained with anti-IL-17A antibody. Immunohistochemistry shows DAB staining of round cells in the airway subepithelium. **(F)** Lungs were stained with AF555-conjugated anti-CD68 (red) and AF488-conjugated anti-IL-17A (green). Nuclei were stained with DAPI (blue). Immunofluorescence shows colocalization (yellow), indicating IL-17A production by CD68-positive macrophages. (Mean±SEM, n=3-5 each group, *different from sham, $P < 0.05$, one-way ANOVA; †different from wild-type, $P < 0.05$, one-way ANOVA).

Discussion

Viral-induced exacerbations are a major cause of morbidity in asthma. RVs comprise approximately 50% of the viruses isolated. However, the precise mechanisms underlying RV-induced asthma exacerbations are not known. RV, unlike influenza and other viruses, causes minimal if any cytotoxicity (18, 19). The current explanation is that RV infection induces the release of chemokines from airway epithelial cells, thereby attracting inflammatory cells to the airways. However, it is also conceivable that RV directly infects airway inflammatory cells. Several studies have examined the infection of monocytic cells by RV *in vitro* (20-25). Recently, we found that, in both mice and humans, RV colocalizes with monocytes *in vivo* (6, 26). Following infection of OVA-sensitized and -challenged mice, we found that RV colocalized with eotaxin-producing, CD68-positive lung macrophages. Compared to cells from untreated mice, BAL macrophages from allergen-treated mice showed increased expression of type 2 and decreased expression of type I cytokines in response to ex vivo RV infection, indicating a shift from M1 to M2 activation status. Finally, depletion of macrophages using clodronate liposomes reduced RV-induced eosinophilic inflammation and airway hyperreactivity, suggesting that RV causes asthma exacerbations in part by infection of alternatively-activated macrophages.

We hypothesized that IL-4 signaling drives the state of macrophage activation and determines the pattern of RV-induced exacerbation in mice with allergic airways disease. To test this in an animal model, we examined the effect of RV infection on OVA-sensitized and -challenged wild-type and IL-4R KO mice. We anticipated that, in the absence of IL-4/IL-13 signaling, RV infection would increase type 1 cytokine production

from conventionally-activated macrophages, augmenting neutrophilic rather than eosinophilic inflammation. We found that OVA increased the number of CD206+, CD301+ M2-polarized lung macrophages and IL-13+ CD11b+ cells in wild type but not IL-4R KO mice. In addition, unlike OVA-exposed wild-type mice with mixed neutrophilic and eosinophilic inflammation, OVA treatment of IL-4R KO mice induced airway inflammation which was almost exclusively neutrophilic in character. When OVA-treated IL-4R KO mice were infected with RV, neutrophil infiltration further increased. Neutrophilic inflammation was associated with increased lung type 1 cytokine expression, and BAL macrophages expressed type 1 cytokines in response to RV infection *ex vivo.* Together, these results show that IL-4 signaling is required for lung macrophage M2 polarization, and that macrophage polarization state determines the response to RV infection (Figure 4-7).

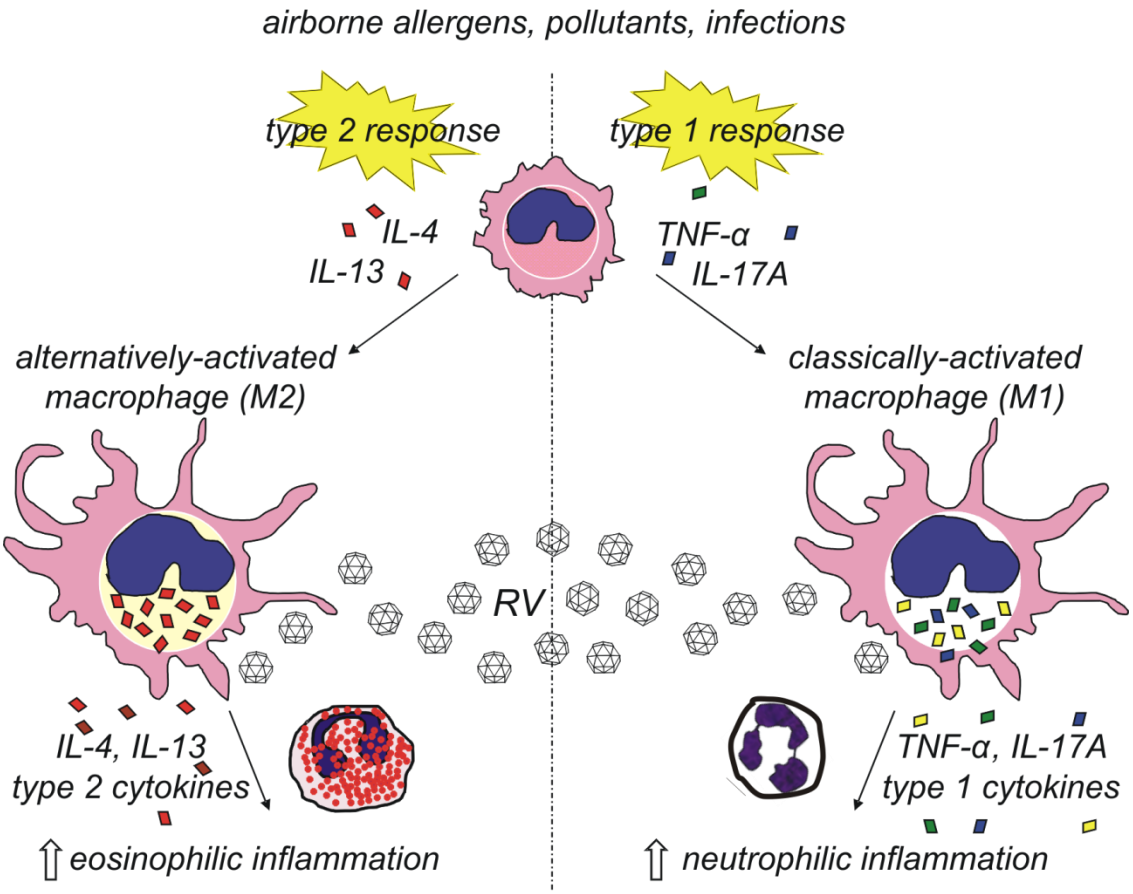

Figure 4-7. Macrophage activation state determines the response to subsequent rhinovirus infection. Upon airway injury, individuals with a type 2-predominant immune response experience eosinophilic airway inflammation which is heightened by RV infection. Alternatively. individuals with a type 1/IL-17-predominant response (analogous to IL-4R KO mice) demonstrate neutrophilic inflammation which is exacerbated by RV infection.

In this study, we found that IL-17A was increased in the lungs of OVA-treated IL-4R KO mice and expressed by BAL and lung macrophages infected with RV. IL-17A plays an important role in the recruitment and activation of neutrophils following bacterial infection (13). IL-17A indirectly stimulates recruitment of neutrophils into the airways via the induction of C-X-C chemokines (12-17). Production of IL-17A was first reported in $CD4^+$ cells (27). IL-17A-producing Th17 cells are regarded as a distinct subset of T cells, divergent from Th1 and Th2 cells. It is now established that neutrophils, eosinophils and macrophages also produce IL-17A (15, 28, 29). It was recently shown that IL-13 signaling inhibits IL-17A production from $CD4^+$ Th17 cells (30). Thus, it is likely that, in our study, IL-17A production was derepressed in IL-4R KO mice deficient in IL-13 signaling. Further, we found that, in the absence of IL-4R signaling, BAL macrophages from OVA-treated mice were polarized to produce IL-17A as well as type 1 cytokines. Macrophage IL-17A production likely contributed to the observed neutrophilic inflammation.

We found that OVA-treated IL-4R KO mice showed neutrophilic airway inflammation, essentially constituting a model of allergic, type 1 cytokine-driven neutrophilic asthma. Although the allergic type 2 immune response has been considered as a hallmark of asthma, only 50% of asthma cases are due to eosinophilic inflammation, the rest showing airway neutrophils (31). This type of asthma has been associated with specific asthma phenotypes including severe asthma, corticosteroid-resistant asthma, nocturnal asthma and occupational asthma (32-40). Although non-allergic stimuli, for example, lipopolysaccharide and ozone (41-43), have been associated with neutrophilic airway inflammation, our data are consistent with the notion that a subset of allergic

patients may experience neutrophilic rather than eosinophilic airway inflammation due to the influence of type 1 cytokines and IL-17A.

In conclusion, we showed that IL-4R KO mice sensitized and challenged with OVA have significant neutrophilic inflammation which is further enhanced by RV infection. Macrophages from OVA-treated IL-4R KO mice showed an M1 polarization pattern and expressed type 1 cytokines and IL-17 in response to RV infection. We conclude that, in mice with allergic airways disease, the macrophage activation state determine the response to RV infection.

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Chapter 5

Summary, Limitations, and Future Directions

Summary

Asthma, the most common chronic illness in Westernized countries, is characterized by recurrent attacks of airflow obstruction, airway inflammation and airways hyperresponsiveness (AHR) (1, 2). Airway changes in asthma have been linked to allergy and type 2 immune responses. However, the mechanisms of asthma development are not fully understood. Recent studies indicate that early-life respiratory viral infection is a major predisposing factor for subsequent asthma development (3). In high risk infants, wheezing-associated illness with rhinovirus (RV) is the most significant risk factor for asthma development, more so than allergen sensitization or infection with respiratory syncytial virus (RSV) (4, 5). In hospitalized infants, RV infection was also associated with asthma development, in contrast to RSV which was negatively associated (6). Thus, with the appropriate genetic background and allergen exposure, RV infection in early life may modulate the immune response, increasing the likelihood of asthma development. Alternatively, RV may simply reveal a pre-existing tendency for asthma (7, 8). An improved understanding of immune responses to RV in early life is needed to resolve this issue.

Recent studies have found that neonatal immunity is qualitatively different from that of adult. Neonatal animals are refractory to Th-1 responses and permissive to allergic Th2 responses (9, 10). It is therefore possible that early life RV infection promotes allergic asthma by augmenting or maintaining this response. Previously, to test this, we infected 7 day-old BALB/c mice with RV. In contrast to adults, neonatal infection induced airway hyperresponsiveness and mucous metaplasia in lungs. These physiological changes were dependent on IL-13, a canonical type 2 cytokine (11). Neonatal mice developed sustained airways hyperresponsiveness and mucous cell metaplasia. Neonatal RV infection also induced IL-13 production by invariant natural killer T cells (iNKT) cells, as well as lung infiltration by CD11b+ monocytes.

In this research, we hypothesized that neonatal RV infection induces type 2 cytokine production by innate immune cells, leading to mucous metaplasia and airways hyperresponsiveness. To study the developmental and immunologic mechanisms of RV-induced chronic airways disease, I intended to assess the role of TSLP and and IL-25, epithelial cytokines which play a role in the maturation and activation of Th2 (12-17) and iNKT cells (18, 19). TSLP and IL-25 also induce type 2 (Th2-like) cytokine production by type 2 innate lymphoid cells (ILC2s) (20-26).

Six day-old BALB/c mice and TSLPR KO mice as well as eight week-old BALB/c mice were inoculated with sham HeLa cell lysate or RV. Airway responses from 1 to 28 days after infection were assessed by qPCR, ELISA, histology, immunofluorescence microscopy, flow cytometry and methacholine responsiveness. Selected mice were treated with a neutralizing antibody to IL-25.

Compared to mature mice, RV infection of neonatal mice increased expression of the type 2 cytokines IL-13, IL-4 and IL-5. In contrast, the induction of type 1 cytokines IFN-γ, IL-12p40 and TNF-α gene was blunted in neonatal mice, whereas expression was increased in mature mice. Consistent with the attenuated type 1 response, viral replication and load tended to be greater 3-7 days after inoculation in neonatal mice compared to adults. Neonatal RV infection led to persistent asthma-like pathophysiological changes including IL-13 expression, mucous metaplasia and airways hyperresponsiveness. RV infection leads to the induction of IL-25 mRNA and protein in neonatal mice but not in mature mice. Additional studies examining the age-dependency of RV-induced IL-25 expression showed significant induction only in mice younger than 6 days-old. Lung immunofluorescent staining showed that RV-infected epithelial cells were the major source of IL-25. Flow cytometric analysis found that RV infection of neonatal but not mature mice expands the population of IL-17RB-expressing ILC2s. These cells were the major population secreting IL-13 in RV-infected neonatal mice. Neutralization antibody of IL-25 treatment in RV-infected neonates strongly inhibited ILC2 expansion, mucous hypersecretion and airways responsiveness. These findings suggest that early-life viral infection could contribute to asthma development by provoking age-dependent, IL-25-driven type 2 immune responses.

To test the role of TSLP, we infected neonatal BALB/c mice and TSLPR KO mice. RV infection increased TSLP protein abundance after early-life RV infection while reducing TSLP mRNA and protein expression in mature mice. We also found that TSLP mRNA was decreased with RV infection in the mice age 8 days or older. Induction of TSLP protein expression was present only in mice $\leq$ 6 days of age. RV infection

increased TSLP staining in the airways epithelium of neonatal mice, which may have infected with RV as well. Neonatal RV infection induced persistent mucus hyperplasia and airway hyperresponsiveness in wild type mice whereas the phenotype was blocked in TSLPR KO mice. Expansion of type 2 innate lymphoid cells (ILC2s) following neonatal RV infection was also significantly inhibited in TSLPR KO mice. Finally, when combined with IL-25 and IL-33, TSLP had a direct synergistic effect on ILC2 GATA3, IL-13, IL-5, ST2L and IL-17RB mRNA expression. These results show that TSLP plays a necessary role in the development of mucous metaplasia, airways hyperresponsiveness and ILC2 expansion in RV-infected immature mice.

We conclude that TSLP and IL-25 are required for persistent mucus metaplasia and airway hyperresponsiveness as well as ILC2 expansion with neonatal RV infection. Induction of type 2 cytokines, including IL-13, IL-25, and TSLP, in response to viral infection was developmentally regulated. These findings may suggest, with an appropriate genetic background, early-life RV infection may modulate and induce type 2 immune response, thereby leading to the development of persistent mucus metaplasia and airway hyperresponsiveness. Further characterization of this immune pathway may lead to new molecular and cellular targets for the prevention of asthma.

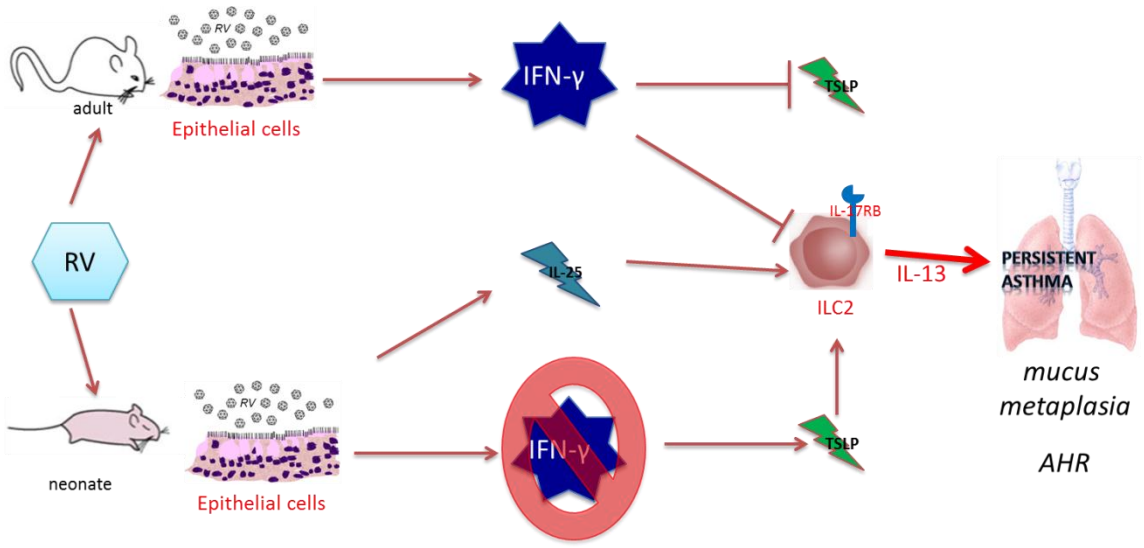

Figure 5-1. Proposed mechanism for neonatal RV-induced mucus metaplasia and airway hyperresponsiveness.

Limitations and Future directions

Mouse model of RV infection

We have successfully established a mouse model of RV infection and utilized the model to test the effect of RV in the context of respiratory disease and inflammation. We infected C57BL/6 mice with minor groups serotype RV1B and shown increased CXC chemokines, elevated neutrophils, and moderate changes in airway hyperreactivity (27). We understand the possibility of slightly different lung pathology between minor group RV and major group RV, for minor group RV binds to LDL-R compared to major group viruses including RV16 and RV39 which bind to the receptor to ICAM-1 (28). However, sequence analysis of RV serotypes (29) and studies in RV-infected cultured cells (30) and mice (31) suggest that , as well as sequence analysis suggest that the distinction between at least some major and minor group strains may not be clinically relevant.

We would like to add a few additional caveats about our mouse model of RV infection. We (27) and others (31) have found that a much higher viral inoculum is required to infect mice compared with humans. This finding is to be expected, because differences in the homology of viral receptors and intracellular signaling mechanisms are likely to restrict viral infection and replication in mice. Nevertheless, we have clearly shown that human RV1B replicates in mouse lungs, as evidenced by: 1) the presence of negative-strand viral RNA in the lungs of inoculated mice, 2) transmissibility of RV infection from the lung homogenates of inoculated mice to cultured HeLa cells; and 3) the induction of a robust lung interferon response (27). Also, despite a low viral titer, RV was associated with a significant ILC2 response. These data are consistent with recent speculation by workers in the field that relatively innocuous RV infections in early

childhood lead to sustained changes in the immune response which contribute to the development of asthma.

Interaction with other risk factors for asthma development

We recognize that asthma is a complex disease, and the effects of RV infection in early infancy are likely to be determined by interplay with genetic and environmental factors (allergen exposure, microbiome, viral re-infection). For example, a positive family history of asthma is a known risk factor for asthma development, and it has recently been found that infants of mothers with asthma are more likely to have severe respiratory tract infections with RV (8). Variants at the 17q21 locus were recently associated with asthma in children who had had RV-induced wheezing illnesses (32). In preliminary studies, we have found that C57BL/6 mice are less sensitive to RV infection than BALB/c mice. In addition, we have shown that early-life RV infection augments the effect to subsequent allergen exposure (33), and it has been speculated that viral infection may lead to atopic disease and asthma exacerbations via the production of virus-specific IgE (34). With regard to microbiome, detection of pathogenic bacteria during RV infections is associated with exacerbations (35), suggesting the importance of airway bacteria in the response to RV. In addition, early-life antibiotic treatment increases the response to ovalbumin in mature mice (36), and lung microbiota promote tolerance in immature mice exposed to house dust mite allergen (37). Finally, the timing of viral infection may play a role, with earlier infections increasing the risk of persistent airway responses.

Unfortunately, it is impossible to explore all these factors in a single thesis research. Therefore, we have chosen to focus the roles of innate cytokines and ILC2s in the response to early-life viral infection. Nevertheless, for future direction, it is plausible to test the effects of genetic background and microbiome with the following simple experiments. First, experiments examining the differential response to RV1B infection in immature BALB/c and C57BL/6 mice can be performed. If clear differences are found between the two strains, F2 progeny can be produced with cross breeding, infect the progeny with RV, phenotype the mice, and identify candidate genes regulating the RV response using quantitative trait locus (QTL) mapping. High-resolution SNP panels are widely available for this purpose (38). Second, germ-free and antibiotic-treated BALB/c and C57BL/6 immature mice can be infected with RV1B, anticipating that these mice will have exaggerated type 2 responses to RV infection compared to their SPF-pathogen free counterparts.

Recurrent heterologous infection and ILC2s

In our original report, we found that immature mice infected with RV on day 7 of life showed mucous metaplasia at 35 days of life (33). However, by 60 and 100 d after early-life RV infection, mucous metaplasia had mostly resolved. These data are consistent with studies showing that RSV was an independent risk factor for the subsequent development of wheezing up to age 11 years, but not at age 13 (39). Nevertheless, under certain circumstances, early life infection might induce long-term effects. It is therefore conceivable that early life RV infection alters the immune response to future viral infections, leading to type 2 rather than type 1 responses, and that these

responses are driven in part by ILC2s.

RV is the most common respiratory tract infection in infants, comprising up to 80% of all infections (40). Virtually all children are infected with RV during infancy. Longitudinal surveillance of RV infections by PCR has shown that pre-school age children have about six distinct RV infections per year (41). Given the large number of known RV serotypes (which do not include the new HRV-C lineage), it is almost certain that most children experience infections with many different RV strains. Naturally-acquired RV infections do not produce non-specific immunity to reinfection of the respiratory tract by heterologous serotypes, even if the viruses are from the same group, for example RV1A and RV2 (42, 43). Perhaps the second most common respiratory infection in infants is RSV. RSV isolates can be divided into 2 groups, A and B, which are distinct at the antigenic level. In addition, RSV groups can be subdivided into several strains or genotypes that co-circulate during epidemics. Immunity following primary exposure does not prevent subsequent infections (44).

While human studies have demonstrated the strong association between viral infections and asthma development (4, 5), few animal studies have examined the effects of reinfection with RV or RSV in an animal model. A recent study (45) using an RV1B mouse model similar to our own showed that multiple *homologous* infections were required to generate neutralizing antibodies. No inflammatory responses were assessed. With RSV, infection of immature mice predisposes to the development of IL-13-dependent airway eosinophilia and hyperresponsiveness after homologous reinfection, whereas infection at a later age protects against reinfection (46, 47): Early-life RSV infection combined with homologous reinfection five weeks later resulted in enhanced

airways responsiveness, airway eosinophilia and increased IL-13 levels after reinfection. Both CD4- and CD8-positive T cells were a source of IL-13 in the lung. In contrast, infection of mice at weaning elicited a protective airway response upon reinfection which was characterized by increased airway inflammation but without the development of airway hyperresponsiveness or eosinophilia, and decreased IL-13 levels. 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.

The situation with heterologous infections is likely to be different from that described above. Infection with distinct antigenic strains is less likely to promote a T cell-mediated memory response. An alternative mechanism by which organisms lacking adaptive immune responses – for example, plants, invertebrates and newborn animals -- are protected against reinfection with pathogens has been referred to as "trained immunity." In trained immunity, secondary infections induce a non-specific heightened activation state without the involvement of a T and B cell adaptive immune response (48). Due to limited antigen exposure and distinct adaptive immune function compared to the adult, the newborn animal has a significant dependence on innate immune function for host defense to microbial challenge (49). The mechanisms that mediate the heightened activation state of trained immunity may involve innate immune cells such as ILCs, macrophages and NK cells, as well as improved pathogen recognition by pattern recognition receptors.

Considering ILCs, there are a number of mechanisms by which ILC2s may drive the response to secondary infection. First, there could be a second expansion of ILC2s. Although developing mice "switch off" IL-25 production by 8 days after birth (50), ILC2

expansion could be stimulated by epithelial-derived TSLP or IL-33. Cysteinyl leukotrienes have recently been shown to promote *Alternaria* species-induced ILC2 accumulation and cytokine expression *in vivo* and *ex vivo* (51), and experimental RV infections double BAL cysteinyl leukotriene levels in adult volunteers (52). Second, heterotypic infection could elicit cytokine production from ILC2s which expanded after the first infection. We have shown that these cells persist in immature mouse lungs at least three weeks after initial infection (50), and other studies suggest even longer persistence (53). Thus it is even possible that secondary RV infection could directly elicit IL-13 production from ILC2s which persist in the lung after the original infection.

Potential role of IL-33

IL-33 is a pro-allergic cytokine that belongs to the IL-1 superfamily. It is predominantly released at the epithelial barrier when it is exposed to allergens, pathogens or injury-inducing stimuli. After secretion, IL-33 binds to a ST2L, which activate signaling pathway for type 2 cytokines and chemokines via MyD88, TRAF6, IRAK-1/4 and MAP kinases (54). Similar to IL-1β and IL-18, other cytokines in IL-1 superfamily, IL-33 lacks an apparent signal peptide for secretion. Therefore, IL-33 was first thought to be synthesized as an inactive precursor and get liberated as an active cytokine by caspase-1 (54). However, recent studies found that full-length IL-33 is biologically active, and IL-33 cleavage by caspases dramatically inactivates IL-33 function (55, 56). Thus, it has been suggested that full-length IL-33 is released from necrotic cells as danger-associated molecular pattern (DAMP) or an "alarmin" to induce type 2 immunity, whereas IL-33 is inactivated during apoptosis by caspase activity (57).

In chapter 2, we showed that IL-33 mRNA expression is not increased with RV infection neither in neonates nor in adults. However, considering the fact that full-length IL-33 can released during necrosis, it is conceivable that RV infection may increase lung IL-33 secretion. In our pilot study, we found that RV infection increases IL-33 protein level in neonates but not in adults, while there was no obvious difference in mRNA expression of IL-33 (data not shown). The role of IL-33 will be tested by using neutralizing antibody of IL-33 in BALB/c neonates in future.

Potential role of IFN-γ in regulation of IL-25 and TSLP

In chapter 2 and chapter 3, we have found the age-dependency of IL-25 and TSLP increase with RV infection. Induction of IL-25 and TSLP was only observed in the mice younger than day 6 of age with RV infection. Considering the epigenetic modification favoring type 2 cytokine induction in T cells (58), it is possible that the regulatory regions of IL-25 and TSLP are also epigenetically favored transcription in neonates compared to adults.

Alternatively, blunted induction of type 1 cytokine IFN-γ in RV-infected neonates could be permissive for IL-25 and TSLP induction. We and others showed that immature animals are more susceptible to viral infection, in part due to impaired IL-12 and IFN production (59-62). As shown previously with neonatal adenovirus infection (63), baby mice showed impaired IFN-γ responses to RV1B infection compared to adults, as well as reduced levels of IL-12 and TNF-α. We found that RV-infected immature 6 day-old mice maintained high levels of viral RNA ≥ 72 h after infection, representing at least three rounds of viral replication, in contrast to mature mice in which vRNA and titer peak

within 24 h after infection (64). IFN-γ has been shown to inhibit double-stranded RNA-induced TSLP production in fibroblasts (65) and keratinocytes (66). Thus, immature animals with relatively impaired IFN responses would be expected to express higher levels of TSLP in response to viral infection. Moreover, in NK cell-deficient mice, RSV infection leads to an exaggerated IL-25 response which is blocked by recombinant IFN-γ treatment. This result suggests the potential role of IFN-γ in blocking IL-25 expression (12). In a pilot study, we found that recombinant IFN-γ treatment reduces the RV-stimulated TSLP level *in vitro* and *in vivo* (data not shown). We also found that recombinant IFN-γ inhibited the development of mucus hyperplasia and airway hyperresponsiveness in neonatal mice (data not shown). Moreover, recombinant IFN-γ strongly blocked the activation of ILC2s (data not shown). In future, the role of IFN-γ in regulation of IL-25 and TSLP could be tested.

Physiological Relevance

In high risk infants, wheezing-associated illness with rhinovirus (RV) is the most significant risk factor for asthma development, more so than allergen sensitization or infection with respiratory syncytial virus (RSV)(4, 5). In hospitalized infants, RV infection was also associated with asthma development, in contrast to RSV which was negatively associated (6), Thus, with the appropriate genetic background and allergen exposure, RV infection in early life may modulate the immune response, increasing the likelihood of asthma development.

In this current study, we found that infection of neonatal but not adult mice with human rhinovirus (RV) induces mucous metaplasia and airways hyperresponsiveness

which is associated with enhanced expression of IL-13, IL-25 and TSLP, reduced expression of IFN-γ, IL-12p40 and TNF-α, and expansion of ILC2s. These findings suggest that early-life viral infection could contribute to asthma development by provoking age-dependent, IL-25- and TSLP-driven type 2 immune responses.

In spite of our findings in mice, situation in human may not be simple because of the complex etiology of asthma. In human, the effects of RV infection in early infancy are likely to be determined by interaction with genetic factors (family history of atopic diseases), and environmental factors (allergen exposure, microbiome, viral re-infection). Also, the timing of viral infection may play a role, with earlier infections increasing the risk of persistent airway responses. More clinical research studying the interplay between neonatal RV infection and other etiological factors of asthma, in the context of IL-25 and TSLP response, may be required to determine precise role of early-life RV infection in the development of asthma in human. Completion of this future work, which would include a new model of neonatal infection and human studies, will address the novel hypothesis that, in susceptible individuals, early viral infection contributes to asthma development.

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