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The Innate Cytokines IL-25, IL-33, and TSLP Cooperate in the Induction of Type 2 Innate Lymphoid Cell Expansion and Mucous Metaplasia in Rhinovirus-Infected Immature Mice

Mingyuan Han,* Charu Rajput,* Jun Y. Hong,† Jing Lei,* Joanna L. Hinde,* Qian Wu,* J. Kelley Bentley,* and Marc B. Hershenson*,†

Early-life respiratory viral infection is a risk factor for asthma development. Rhinovirus (RV) infection of 6-d-old mice, but not mature mice, causes mucous metaplasia and airway hyperresponsiveness that are associated with the expansion of lung type 2 innate lymphoid cells (ILC2s) and are dependent on IL-13 and the innate cytokine IL-25. However, contributions of the other innate cytokines, IL-33 and thymic stromal lymphopoietin (TSLP), to the observed asthma-like phenotype have not been examined. We reasoned that IL-33 and TSLP expression are also induced by RV infection in immature mice and are required for maximum ILC2 expansion and mucous metaplasia. We inoculated 6-d-old BALB/c (wild-type) and TSLP receptor–knockout mice with sham HeLa cell lysate or RV. Selected mice were treated with neutralizing Abs to IL-33 or recombinant IL-33, IL-25, or TSLP. ILC2s were isolated from RV-infected immature mice and treated with innate cytokines ex vivo. RV infection of 6-d-old mice increased IL-33 and TSLP protein abundance. TSLP expression was localized to the airway epithelium, whereas IL-33 was expressed in epithelial and subepithelial cells. RV-induced mucous metaplasia, ILC2 expansion, airway hyperresponsiveness, and epithelial cell IL-25 expression were attenuated by anti–IL-33 treatment and in TSLP receptor–knockout mice. Administration of intranasal IL-33 and TSLP was sufficient for mucous metaplasia. Finally, TSLP was required for maximal ILC2 gene expression in response to IL-25 and IL-33. The generation of mucous metaplasia in immature RV-infected mice involves a complex interplay among the innate cytokines IL-25, IL-33, and TSLP. *The Journal of Immunology*, 2017, 199: 1308–1318.

The epithelial-derived innate cytokines IL-25 (or IL-17E) (1, 2), IL-33 (3), and thymic stromal lymphopoietin (TSLP) (4–7) play a role in the maturation of Th2 cells via dendritic cell activation. IL-25 (8–12), IL-33 (8–10, 13–16), and TSLP (16) also induce activation and IL-13 production from innate immune cells, including type 2 innate lymphoid cells (ILC2s).

The development of asthma is likely to be a result of genetic predisposition, immune dysfunction, and environmental exposures in early infancy. Epidemiologic studies in high-risk infants indicate that early-life respiratory viral infection, particularly with rhinovirus (RV), is a major predisposing factor for asthma development (17–20). 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. Previously, we have found that IL-25 plays a pivotal role in asthma phenotype development in immature mice following RV infection (21). RV infection of 6-d-old, but not mature, mice induced mucous metaplasia and airway hyperresponsiveness, which were accompanied by airway epithelial cell IL-25 production and expansion of IL-13–producing ILC2s. Neutralizing Ab against IL-25 abolished ILC2 expansion, mucous metaplasia, and airway hyperresponsiveness (21). However, we did not examine the roles of IL-33 and TSLP in this model.

The roles of IL-33 and TSLP in the pathogenesis of asthma have recently been highlighted. IL-33 is member of the IL-1 family of cytokines that signals through the IL-1R homolog ST2L, is constitutively expressed as a nuclear precursor in alveolar epithelial cells (22), and is released from producing cells upon cellular damage (23). In mice, IL-33 is expressed in airway epithelial and inflammatory cells after allergen challenge (24) and is required for OVA-, papain- and *Alternaria*-induced airway inflammation (16, 22, 25, 26). Allergic asthma, in particular childhood asthma and wheezing syndromes, has been associated with genetic variation in the genes encoding IL-33 and its receptor, IL-1 receptor-like 1/ST2L (27–30). Clinically, expression of IL-33 is increased in the airway epithelium, endothelium, and smooth muscle of adult patients with severe asthma (31), as well as the submucosal inflammatory cells of children with steroid-resistant asthma (32). In patients with asthma, experimental RV infection induces airway IL-33 production that is proportional to exacerbation severity and viral load (33).

TSLP is an IL-7–like cytokine that exerts its biological activity via a high-affinity TSLP receptor (TSLPR) complex that is a heterodimer of the TSLPR chain and IL-7R-α. Transgenic mice that overexpress TSLP in the lungs show an augmented type 2

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Abbreviations used in this article: HDM, house dust mite; ILC2, type 2 innate lymphoid cell; KO, knockout; PAS, periodic acid–Schiff; RSV, respiratory syncytial virus; rTSLP, recombinant TSLP; RV, rhinovirus; SPDEF, SAM-pointed domain–containing ETS-like factor; TSLP, thymic stromal lymphopoietin; TSLPR, TSLP receptor.

immune response, goblet cell hyperplasia, and subepithelial fibrosis when challenged with OVA, whereas TSLPR-deficient mice show attenuated responses (6). IL-25 and TSLP are secreted from airway epithelial cells upon house dust mite (HDM) challenge, leading to dendritic cell activation and an adaptive Th2 response (34). Genome-wide association studies have found that the TSLP gene locus is associated with asthma risk (35, 36). Several single-nucleotide polymorphisms of the TSLP gene locus are associated with asthma development (37–40). TSLP levels are increased in the airways of asthmatic patients and correlate with asthma severity (41, 42). Treatment of human asthmatic patients with anti-TSLP Ab blunts airway responses triggered by allergen inhalation (43). However, less is known about the role of TSLP in the development of asthma in childhood. RV infection increases nasal aspirate TSLP expression in young children (44), consistent with the notion that RV-induced TSLP production could play a role in the initiation of asthma. Therefore, we examined the effect of RV infection on IL-33 and TSLP expression in immature mice and the requirement for IL-33 and TSLP in RV-induced mucous metaplasia and ILC2 expansion.

Materials and Methods

Generation of RV and infection of immature mice

RV1B (American Type Culture Collection, Manassas, VA) was partially purified from infected HeLa cell lysates by ultrafiltration using a 100 kDa cut-off filter and titered by plaque assay (45, 46). Similarly concentrated and purified HeLa cell lysates were used for sham infection. BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) or TSLPR-knockout (KO) mice backcrossed to BALB/c mice for seven generations (6) (graciously supplied by Dr. S. Ziegler, Benaroya Research Institute, Seattle, WA) were inoculated via the intranasal route under isoflurane anesthesia with 20 μl RV1B (1×10^8 PFU/ml) or sham HeLa cell lysates. Selected mice were treated with 0.5 μg of recombinant murine IL-33, IL-25, or TSLP (PeproTech, Rocky Hill, NJ) intranasally on day 0. Experiments were approved by the University of Michigan Institutional Animal Care and Use Committee.

Anti–IL-25– and anti–IL-33–neutralizing Ab treatment

Six-day-old mice were treated i.p. with 100 μg of neutralizing Ab to IL-25 (clone 35B; BioLegend, San Diego, CA), neutralizing Ab to IL-33 (R&D Systems, Minneapolis, MN), or isotype control (rat, IgG1κ; BioLegend) 2 h prior to RV infection (day 0). Lungs were harvested 2–21 d postinfection for analysis.

Histology and immunofluorescence microscopy

Lungs were perfused through the pulmonary artery with PBS containing 5 mM EDTA. Next, lungs were fixed with 4% paraformaldehyde overnight. Five-micrometer-thick paraffin sections were processed for histology or fluorescence microscopy, as described (47). Lung sections were stained with periodic acid–Schiff (PAS) or Alexa Fluor 488–conjugated anti-Muc5ac (Thermo Fisher Scientific, Rockford, IL) to visualize mucus (Sigma-Aldrich, St Louis, MO). Levels of Muc5ac staining in the airway epithelium were quantified by NIH ImageJ software (Bethesda, MD), as described (48). Muc5ac expression was represented as the fraction of $Muc5ac^+$ epithelium compared with the total basement membrane length. Other lung sections were incubated with Alexa Fluor 555–conjugated rabbit anti-mouse TSLP (Thermo Fisher Scientific), Alexa Fluor 555–conjugated goat anti-mouse IL-33 (R&D Systems), Alexa Fluor 633–conjugated rabbit anti-mouse IL-25/IL-17E (Millipore, Billerica, MA), Alexa Fluor 488–conjugated mouse anti-RV16 (clone R16-7; QED Bioscience, San Diego, CA), Alexa Fluor 555–conjugated anti-eosinophil major basic protein (Mayo Clinic, Rochester, MN), Alexa Fluor 488–conjugated anti-Gr1 (R&D Systems), Alexa Fluor 647–conjugated anti-F4/80 (BioLegend), or Alexa Fluor 488–conjugated isotype control IgGs. The anti-RV16 Ab recognizes capsid protein VP2 of several RV serotypes, including RV1B, as well as the VP2 precursors VP0 and P1.

Quantitative real-time PCR

Lung or ILC2 RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA) combined with 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 encoding for IL-13, Muc5ac, Gob5, IL-25, IL-33, TSLP, IL-17RB, and ST2L (Supplemental Table I). For each sample, the level of gene expression was normalized to its own *GAPDH* mRNA.

Measurement of IL-13, IL-25, IL-33, and TSLP protein levels

Lung cytokine levels were measured by ELISA (eBioscience catalog numbers 88-7137-22, 88-7002-22, 88-7333-22, and 88-7490-22). ELISA data were analyzed using Gen5 software (BioTek, Winooski, VT).

Flow cytometric analysis

Lungs from sham- and RV-treated immature 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 Pacific Orange LIVE/DEAD Fixable Dead Cell Stain (Invitrogen). To identify ILC2s, cells were then stained with fluorescent-tagged Abs 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 (BioLegend), and anti-CD127 (eBioscience), as described (21). Cells were fixed, subjected to flow cytometry, and analyzed on an LSR Fortessa (BD Biosciences, San Jose, CA). Data were collected using FACSDiva software (BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR).

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 (49). Mechanical ventilation was conducted and the total respiratory system was 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.

ILC2 culture

To study lung ILC2s ex vivo, lung cells were stained with biotin-conjugated Abs for lineage markers (CD3ε, TCRβ, B220/CD45R, Ter-119, Gr-1/Ly-6G/Ly-6C, CD11b, CD11c, F4/80, and FcεRIα) and mixed with anti-biotin MicroBeads (Miltenyi Biotec, Auburn, CA). The cell mixture was processed using a MACS Separator (Miltenyi Biotec). The flow-through was collected for FACS. Lineage-negative CD25 and CD127 double-positive ILC2s were plated on round-bottom 96-well plates at 5000 cells per well and cultured in RPMI 1640 supplemented with 10% FBS, IL-2, and IL-7 (20 ng/ml each) (R&D Systems). Twenty-four hours later, the cells were stimulated with different combinations of IL-2, IL-7, IL-25, IL-33, and TSLP (20 ng/ml each, all from R&D Systems). After 48 h, the plates were centrifuged, and supernatants were tested for IL-13 using ELISA (eBioscience). Finally, cell pellet RNA was extracted for quantitative real-time PCR, as described above.

For measurement of BrdU uptake, cells were treated with BrdU (10 μg/ml; Sigma-Aldrich) for 24 h. Forty-eight hours later, cytospin preparations were fixed and stained with Alexa Fluor 555–conjugated anti-BrdU Ab (Thermo Fisher Scientific), and cells were visualized using confocal microscopy.

Data analysis

Data are represented as mean ± SE. Statistical significance was assessed using an unpaired *t* test or one-way ANOVA, as appropriate. Group differences were pinpointed by a Tukey multiple-comparison test.

Results

RV induces expression of epithelial-derived innate cytokines

In a previous study, we found that epithelial IL-25 is increased in RV-infected immature mice and is required for the development of mucous metaplasia and airway hyperresponsiveness (21). In the current study, we determined the effects of RV infection on IL-33 and TSLP mRNA expression and protein abundance. We collected lungs from RV-infected 6-d-old mice at day 2, 7, or 14 postinfection and measured IL-25, IL-33, and TSLP mRNA and protein. Consistent with our previous study, IL-25 mRNA and protein expression was increased in RV-infected 6-d-old mice (Fig. 1A, 1B). IL-33 mRNA and protein expression was increased at the early time points of infection. RV also increased the protein, but not the mRNA, level of TSLP.

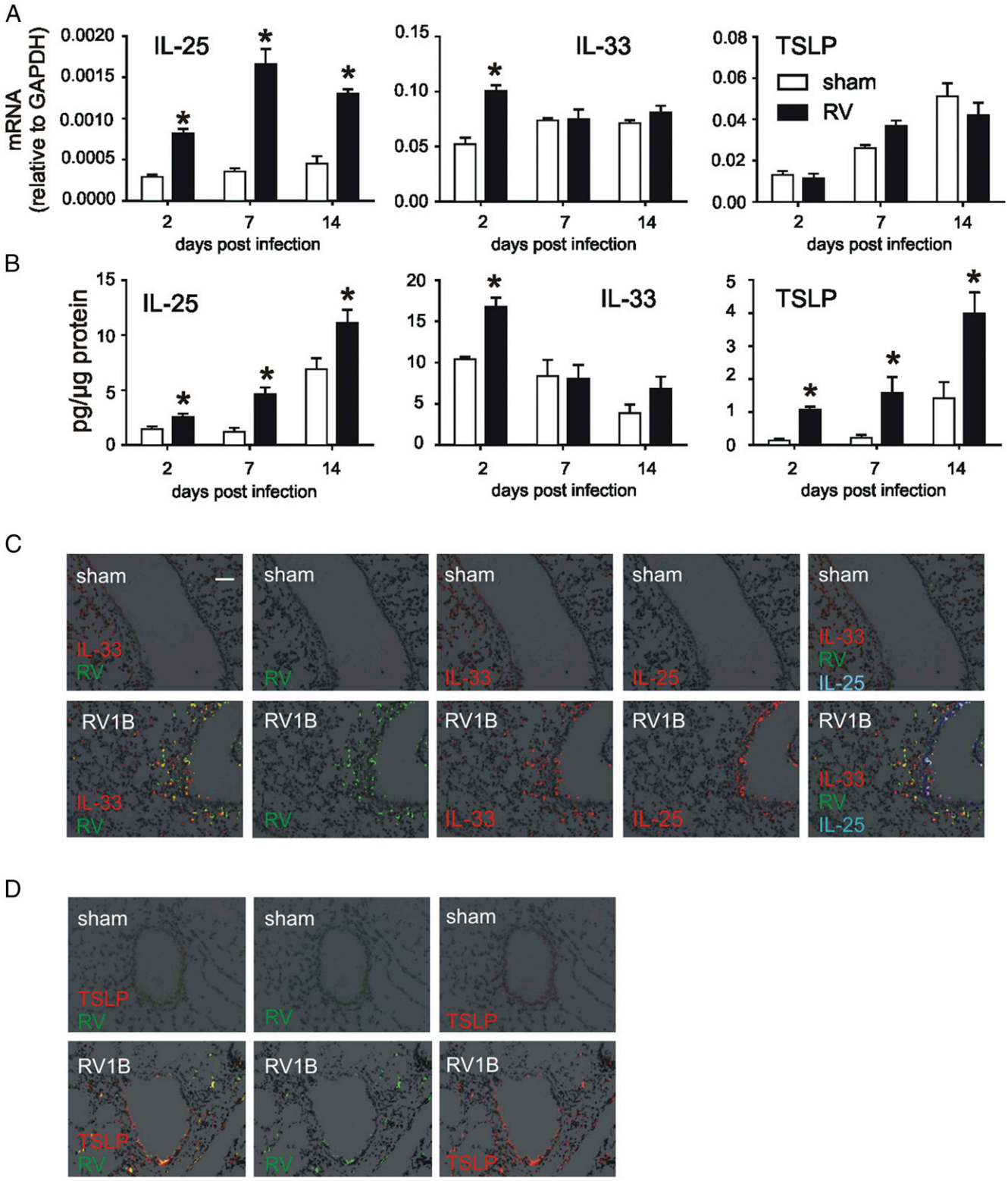

FIGURE 1. Innate cytokine expression after RV infection. Six-day-old BALB/c mice were inoculated with sham or RV. Lung mRNA (**A**) and protein (**B**) expression was measured 2, 7, or 14 d later (n = 3–5, mean ± SEM). *$p < 0.05$ versus sham, one-way ANOVA. Two days postinfection, lungs were stained for IL-33 (red), RV (green), IL-25 (shown separately in red, but shown in blue in the merged image [far right panel]), and nuclei (DAPI, black) (**C**) or were stained for TSLP (red), RV (green), and nuclei (DAPI, black) (**D**). (C) Scale bar, 50 µm. Original magnification ×200.

To better understand the expression pattern of epithelial-derived innate cytokines, we examined airway innate cytokine protein deposition by immunofluorescence. Infection with RV increased airway IL-33 expression, with the strongest signal found in the cytoplasm of RV-infected airway epithelial cells (Fig. 1C). IL-33 was also expressed in subepithelial cells. RV infection also increased airway epithelial cell expression of IL-25. Infection also increased TSLP staining (Fig. 1D). Both subepithelial and epi-

thelial cells produced TSLP; the strongest signals were found in airway epithelial cells infected with RV.

IL-33 is required for mucous metaplasia in RV-infected immature mice

To test whether IL-33 is required for the development of an asthma-like phenotype, we treated RV-infected 6-d-old mice with a neutralizing Ab against IL-33. Because previous studies showed development of mucous metaplasia 2–4 wk after RV infection (49), lung sections were analyzed 3 wk postinfection. Sham-infected animals showed no PAS staining in the noncartilaginous airways. RV infection induced PAS staining of the airway epithelium (Fig. 2A). Treatment with anti–IL-33 blocked PAS staining, whereas rIL-33 induced it. Like PAS staining, Muc5ac immunostaining in response to RV was reduced by anti–IL-33 and increased by rIL-33. Similarly, anti–IL-33 blocked RV-increased airway hyperresponsiveness, whereas rIL-33 induced increased airway responsiveness (Fig. 2B). Expression of

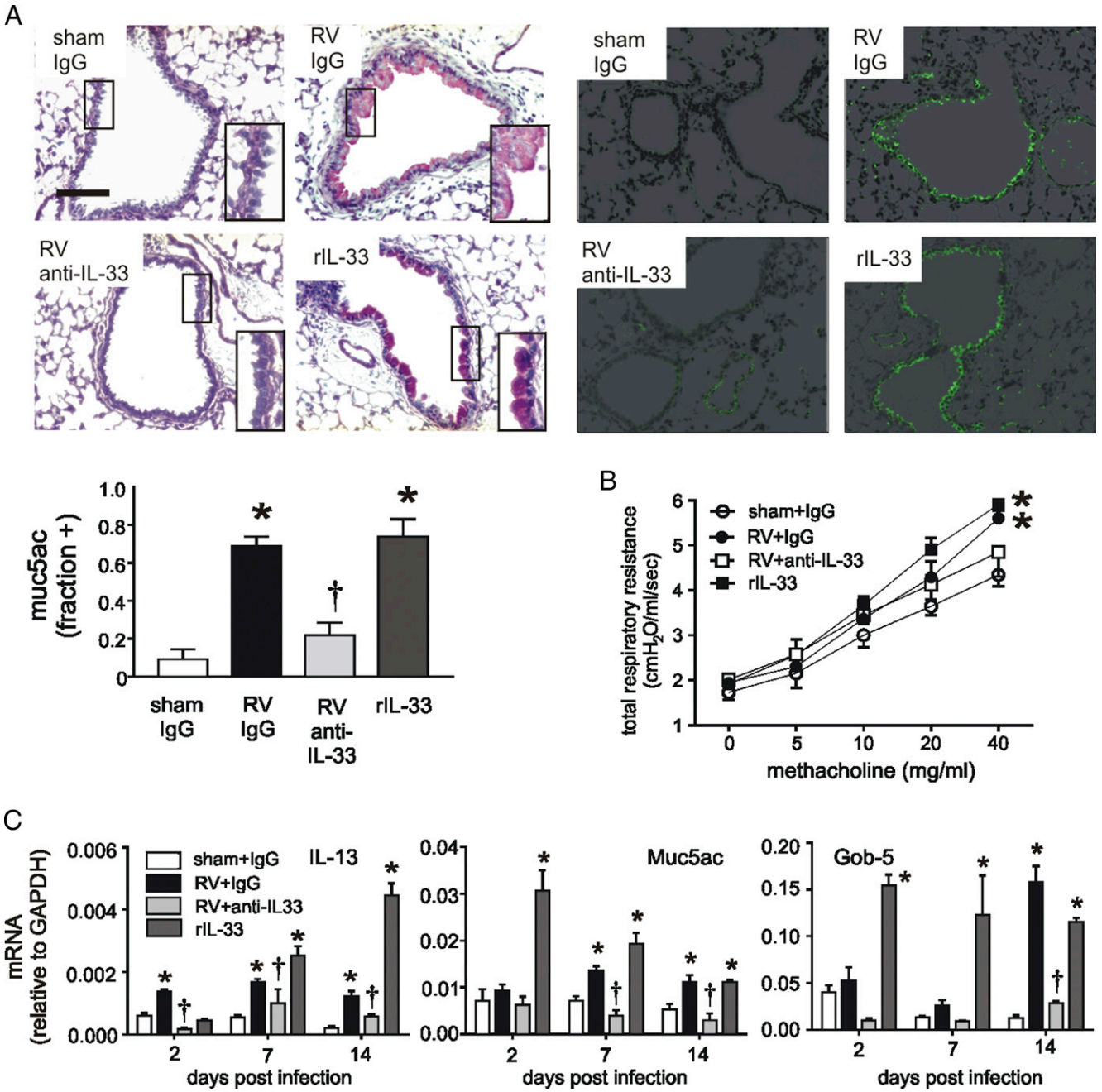

FIGURE 2. Mucous metaplasia and lung mRNA expression in anti–IL-33– and rIL-33–treated mice. Six-day-old BALB/c mice were inoculated with sham, RV, or rIL-33. Anti–IL-33 was given 1 h postinfection to selected RV-treated mice. (**A**) Mucous metaplasia was assessed by PAS staining and Muc5ac immunofluorescence. Lung sections prepared 3 wk after treatment of 6-d-old mice with sham + IgG, RV + IgG, RV + anti–IL-33, or rIL-33. The smaller box is magnified and shown as the inset in the lower right corner of each panel. Scale bar, 50 μm. Muc5ac was quantified as the fraction of epithelium that was positively stained, as measured by NIH ImageJ software ($n = 3$, mean ± SEM). *$p < 0.05$ versus sham + IgG, †$p < 0.05$ versus RV + IgG, one-way ANOVA. (**B**) Airway hyperresponsiveness of 4-wk-old baby mice, 21 d after sham infection, RV infection, RV + anti–IL-33, or rIL-33 ($n = 4$). *$p < 0.05$ versus sham, two-way ANOVA. (**C**) Whole-lung gene expression of the mucus-related genes *Muc5ac*, *Gob5*, and *IL-13* was measured using quantitative PCR ($n = 3$–5, mean ± SEM). *$p < 0.05$ versus sham, †$p < 0.05$ versus RV + IgG, one-way ANOVA.

mRNAs encoding IL-13 and the mucus-related genes Muc5ac and Gob5 also decreased with anti–IL-33 treatment (Fig. 2C). Taken together, these results show that IL-33 plays a key role in the development of mucous metaplasia in RV-infected immature mice.

IL-33 is required for expansion of ILC2s in RV-infected immature mice

RV infection of 6-d-old mice expands the population of lung ILC2s, a major source of IL-13 after RV infection (21). The level of ILC2s peaks at 7 d and plateaus at 7–21 d postinfection. We asked whether anti–IL-33 suppresses expansion of ILC2s using flow cytometry, collecting lungs 2 wk after sham or RV infection. Cells were incubated with a mixture of Abs against hematopoietic lineage markers (CD3ε, TCRβ, B220, Ter-119, Gr-1, CD11b, CD11c, F4/80, FcεRIα), anti-CD25, and anti-CD127. After gating on small lineage-negative cells, a discrete population of CD25 and CD127 double-positive ILC2s was found. RV infection and rIL-33 treatment significantly increased the

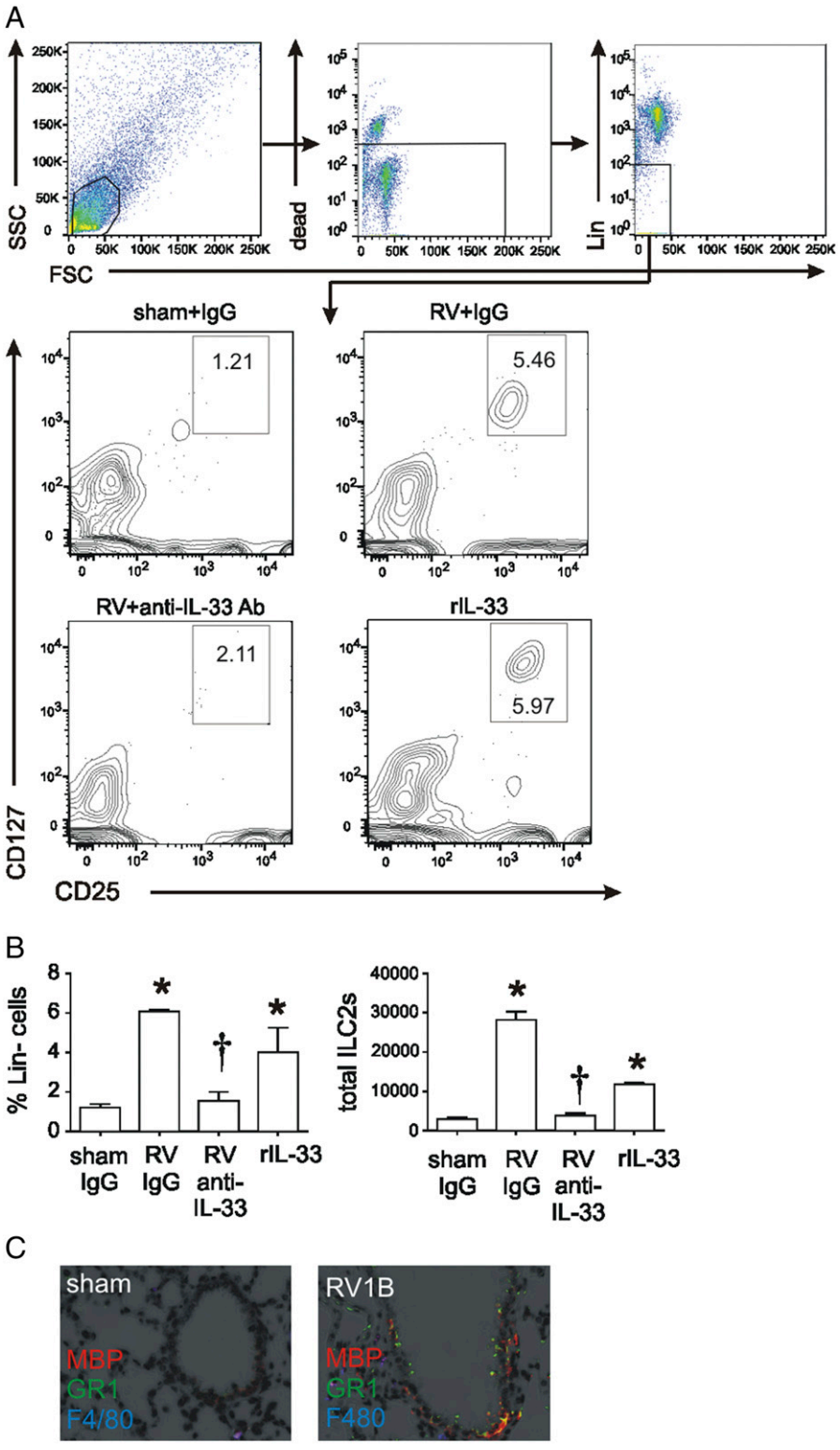

FIGURE 3. (**A** and **B**) Lung lineage-negative CD25$^+$ CD127$^+$ ILC2s in RV-infected BALB/c immature mice. Six-day-old mice were treated with sham + IgG, RV + IgG, RV + anti–IL-33, or rIL-33. Live ILC2s were identified 14 d later and analyzed as a percentage of lineage-negative and total cells (n = 3–5, mean ± SEM). (**C**) Eosinophil major basic protein (red), Gr-1/Ly6G (green), and F4/80 (blue) were examined 14 d post-RV infection. Original magnification ×200. *$p < 0.05$ versus sham, †$p < 0.05$ versus RV + IgG, one-way ANOVA.

number and percentage of lineage-negative $CD25^+$ $CD127^+$ cells (Fig. 3A, 3B). Anti–IL-33 treatment decreased the number and percentage of lineage-negative $CD25^+$ $CD127^+$ cells per lung. Finally, as shown previously (49), RV infection also increased airway granulocytes, including $Gr1^+$ MBP^+ eosinophils (Fig. 3C).

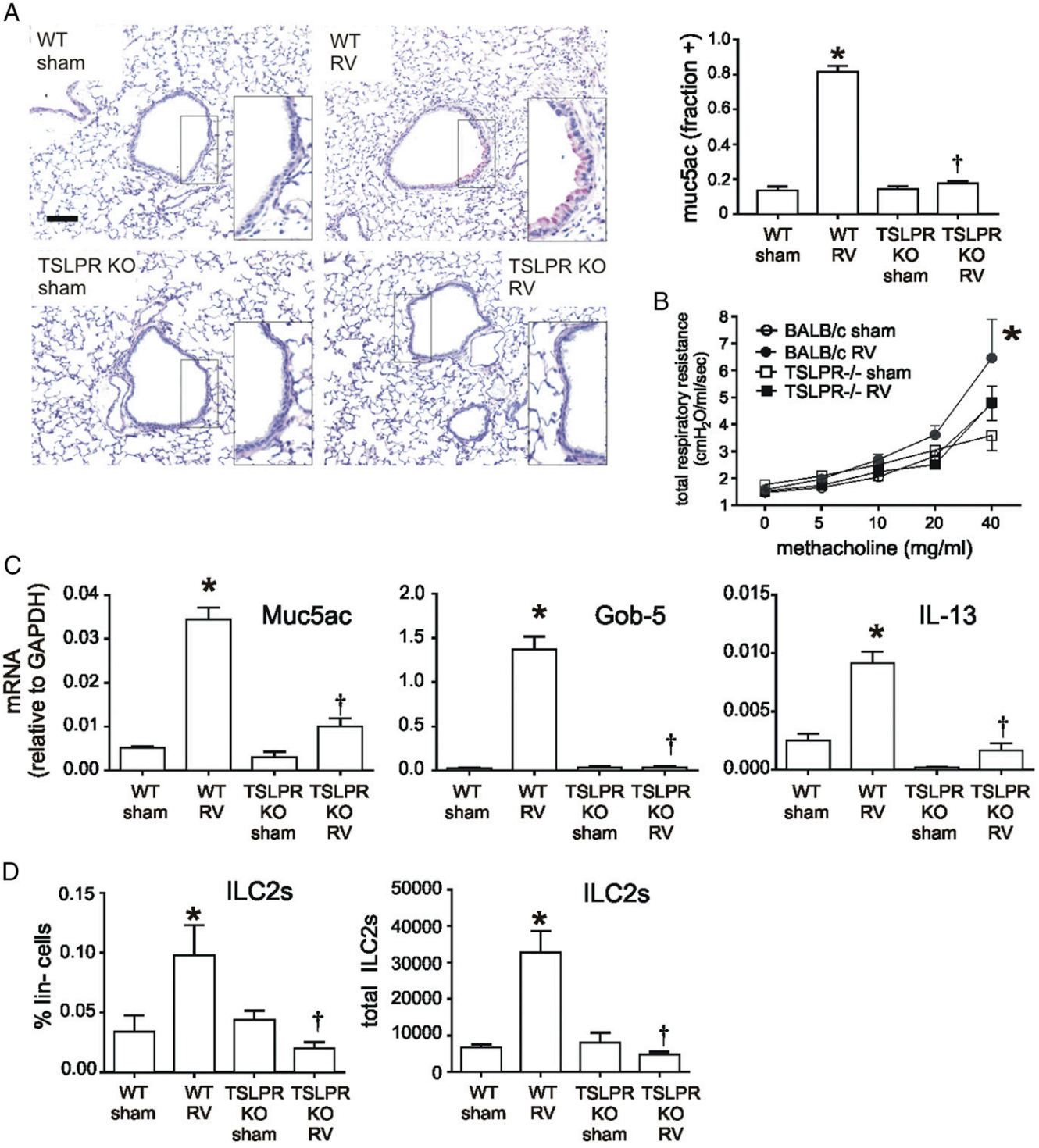

FIGURE 4. Inhibition of RV-induced mucous metaplasia and ILC2 expansion in TSLPR-KO mice. (**A**) Six-day-old BALB/c (wild-type [WT]) mice or TSLPR-KO mice were inoculated with sham or RV. Lung sections were prepared 3 wk postinfection and stained with PAS solution or anti-Muc5ac. Representative lung sections of PAS-stained small airways are shown (left panels). The smaller box is magnified and shown as the inset in the lower right corner of each panel. Scale bar, 50 μm. Muc5ac was quantified as the fraction of epithelium positively stained (right panel), as measured by NIH ImageJ software ($n = 3$, mean ± SEM). *$p < 0.05$ versus sham + IgG, †$p < 0.05$ versus RV + IgG, one-way ANOVA. (**B**) Airway hyperresponsiveness was measured in sham- or RV-treated wild-type BALB/c or TSLPR-KO mice 21 d after treatment ($n = 4$, mean ± SEM). *$p < 0.05$ versus sham, two-way ANOVA. (**C**) Whole-lung gene expression of the mucus-related genes *Muc5ac*, *Gob5*, and *IL-13* was measured using quantitative PCR ($n = 3$–5, mean ± SEM). *$p < 0.05$ versus sham, †$p < 0.05$ versus WT RV, one-way ANOVA. (**D**) Reduction in RV-induced ILC2 expansion in immature TSLPR-KO mice. Six-day-old BALB/c mice and TSLPR-KO mice were inoculated with sham or RV. Lung cells were collected 14 d postinfection, stained, and subjected to flow cytometry. Percentage of lineage-negative ILC2s (left panel) and total ILC2s (right panel) per lung for each group of mice ($n = 4$–5, mean ± SEM). *$p < 0.05$ versus sham, †$p < 0.05$ versus WT RV, one-way ANOVA.

TSLP is required for RV-induced mucous metaplasia and ILC2 expansion

To examine the requirement of TSLP for RV-induced mucous metaplasia, we used TSLPR-KO mice. RV infection increased PAS staining and Muc5ac protein accumulation in wild-type mice, but no induction of mucus staining was found in TSLPR-deficient mice (Fig. 4A). Similarly, airway hyperresponsiveness to RV was blocked in TSLPR-KO mice (Fig. 4B). Consistent with the reduction in PAS staining, induction of IL-13 and the mucus-related genes Muc5ac and Gob5 was significantly lower in TSLPR-KO mice than in wild-type mice (Fig. 4C). Using TSLPR-KO mice, we tested the requirement of TSLP for ILC2 expansion associated with early-life RV infection. In contrast to wild-type mice, in which ILC2s were increased, the ILC2 population was not expanded in TSLPR-KO mice (Fig. 4D). These results demonstrate that, like IL-33, TSLP is required for development of the RV-induced asthma phenotypes via regulation of ILC2 expansion.

Requirement and sufficiency of IL-25, IL-33, and TSLP for innate cytokine production

To better assess the overlapping contributions of IL-33, TSLP (above), and IL-25 (21) to RV-induced mucous metaplasia, we examined the effects of anti–IL-33 and TSLPR KO on RV-induced innate cytokine expression. As shown previously (21), immunofluorescence staining 2 d after early-life RV infection showed increased epithelial cell expression of IL-25 (Fig. 5A). Administration of rIL-33 was sufficient for IL-25 and TSLP staining. Anti–IL-33 blocked RV-induced IL-25, but not TSLP, expression (Fig. 5B). (TSLP levels, which peaked 14 d postinfection, showed a trend toward inhibition at this time point.) Similarly, TSLPR-KO mice showed no increase in RV-induced IL-25 protein expression (Fig. 6A, 6B). BALB/c mice exhibited IL-33 mRNA and protein induction following RV infection, whereas TSLPR-KO mice showed constitutive IL-33 in airway epithelial and subepithelial cells, with epithelial cells showing perinuclear and cytoplasmic staining. Together, these results demonstrate that IL-33 and TSLP are required for RV-induced airway epithelial cell IL-25 expression. Finally, administration of recombinant TSLP (rTSLP) was sufficient to induce mucous metaplasia, as evidenced by PAS staining and Muc5ac expression (Fig. 6C).

Airway mucous cell differentiation is regulated by the transcription factor SAM-pointed domain–containing ETS-like factor (SPDEF). SPDEF, in turn, regulates the transcription of several mRNAs, including *IL13*, *Muc5ac*, *CCL20*, *CCL24*, *IL25*, *IL33*, and *TSLP* (50). We examined the effects of RV infection on *SPDEF* mRNA expression in wild-type and TSLPR-KO mice. In wild-type mice, *SPDEF* mRNA expression was significantly increased 2 d after RV infection (Fig. 6D). Induction of SPDEF was absent in TSLPR-KO mice, suggesting a mechanism by which TSLP may regulate airway epithelial cell IL-25 expression.

Requirement of innate cytokines for ILC2 proliferation, IL-13 production, and innate cytokine receptor expression

Interrelated requirements of IL-33, TSLP, and IL-25 for RV-induced mucous metaplasia may also relate to effects on ILC2 expansion and function. To test this, lineage-negative CD25 and CD127 double-positive ILC2s were sorted from the lungs of RV-infected baby mice and cultured ex vivo in the presence or absence of these cytokines, as well as IL-2 and IL-7. DNA synthesis was measured by BrdU

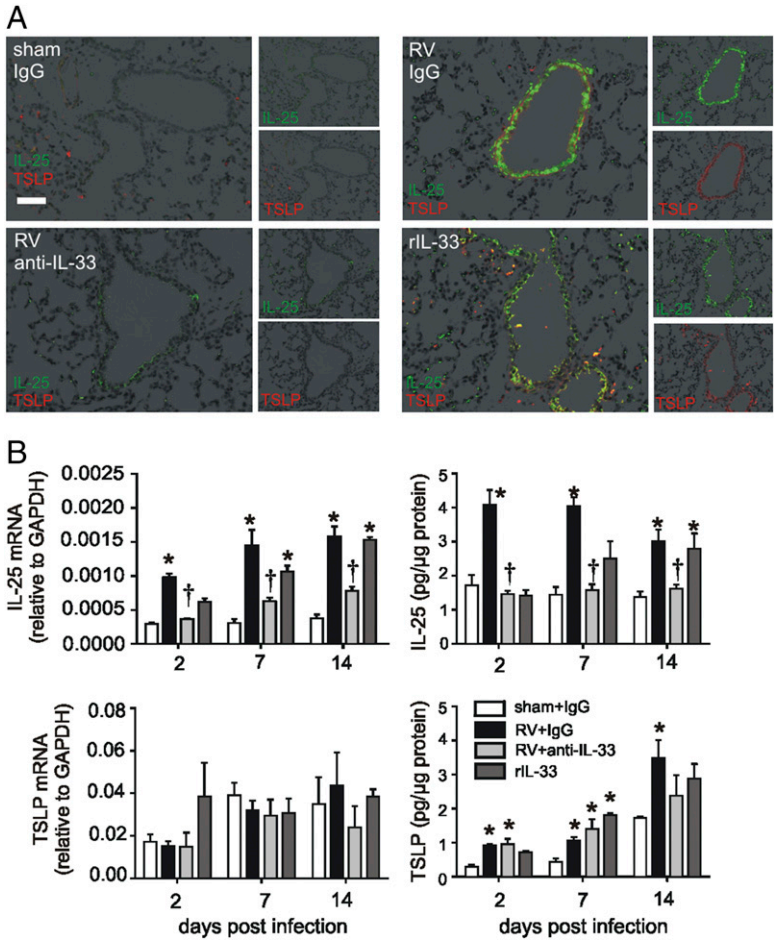

FIGURE 5. Innate cytokine expression in anti–IL-33– and rIL-33–treated immature mice after RV infection. Six-day-old BALB/c mice were inoculated with sham + IgG, RV + IgG, or rIL-33. Anti–IL-33 was given 1 h postinfection to selected RV-treated mice. (**A**) Two days postinfection, lungs were stained for TSLP (red) and IL-25 (green). Scale bar, 50 μm. (**B**) Lung mRNA and protein expression were measured 2, 7, or 14 d later (n = 3–5, mean ± SEM). *$p < 0.05$ versus sham, †$p < 0.05$ versus RV, one-way ANOVA.

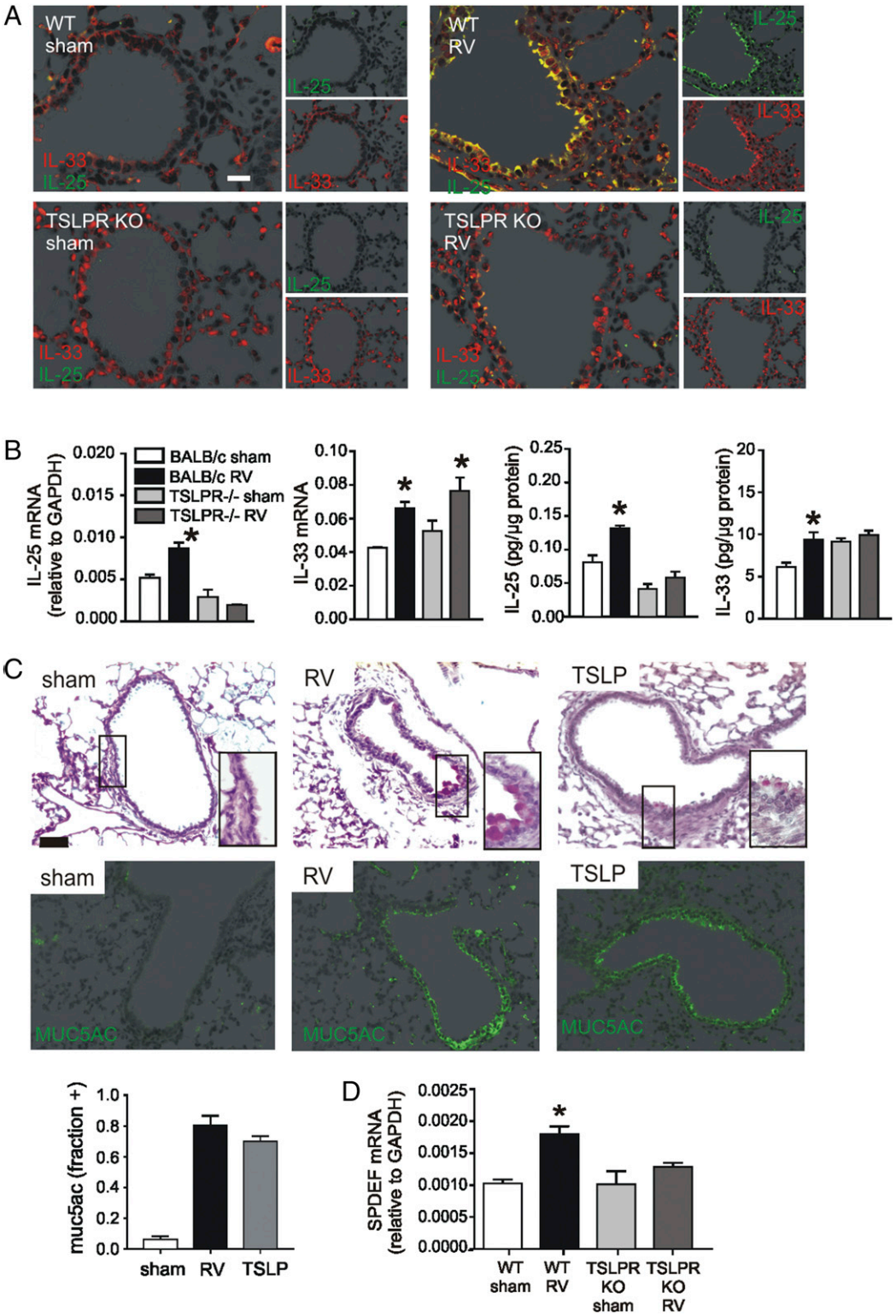

FIGURE 6. Innate cytokine expression in immature TSLPR-KO mice after RV infection. Six-day-old BALB/c or TSLPR-KO mice were inoculated with sham or RV. (**A**) Two days postinfection, lungs were stained for IL-33 (red) and IL-25 (green). Scale bar, 50 μm. (**B**) Lung mRNA and protein expression was measured 2 d postinfection (n = 3–5, mean ± SEM). (**C**) Six-day-old BALB/c mice were inoculated with sham, RV, or rTSLP. Lung sections were prepared 3 wk postinfection and stained with PAS solution or anti-Muc5ac. Fractional airway epithelial staining for Muc5ac was measured (n = 3, mean ± SEM). (**D**) Lung *SPDEF* mRNA was measured 2 d after RV infection (n = 4, mean ± SEM). *$p < 0.05$ versus sham, one-way ANOVA.

uptake, and IL-13 production was measured by ELISA. IL-33, but not TSLP or IL-25, significantly increased DNA synthesis and IL-13 production over that induced by IL-2 and IL-7 alone (Fig. 7A, 7B). However, addition of TSLP significantly increased IL-25– and IL-33–induced DNA synthesis and IL-13 production, with both combinations achieving a maximal response. IL-25 and IL-33, in combination with IL-2 and IL-7, also induced maximal ILC2 DNA synthesis and IL-13 production. Finally, we examined the effects of TSLP on ILC2 expression of *IL17RB* and *IL1RL1*, which encode the innate cytokine receptors IL-17RB and

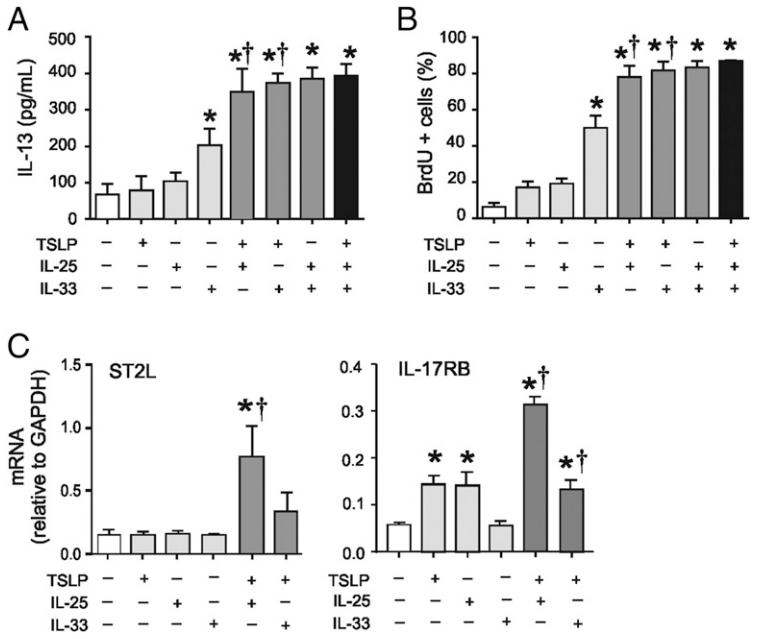

FIGURE 7. Cooperative effects of innate cytokines on ILC2 function. (**A**–**C**) Lungs were collected from RV-infected immature mice, and cell suspensions were sorted for lineage-negative $CD25^+$ $CD127^+$ ILC2s by FACS. Sorted ILC2s were stimulated with a combination of IL-2, IL-7, IL-25, IL-33, and TSLP. DNA synthesis (A) and IL-13 protein production (B) of ILC2s were tested after 3 d of stimulation. The cell pellet was collected for mRNA expression by quantitative PCR (n = 3–6 per group). Effect of innate cytokines on ILC2 mRNA expression of *IL17RB* and *IL1RL1*. *$p < 0.05$ versus IL-2 + IL-7, $^{\dagger}p < 0.05$ versus without rTSLP, one-way ANOVA.

ST2L. TSLP significantly increased IL-25– and IL-33–induced *IL17RB* and *IL1RL1* mRNA expression (Fig. 7C).

Discussion

We demonstrated previously that early-life RV infection causes persistent mucous metaplasia and airway hyperresponsiveness that is dependent on IL-13 and IL-25 and associated with the expansion of IL-13–producing lung ILC2s (21). In the current study, we examined the contributions of two alternative innate cytokines, IL-33 and TSLP, to the observed asthma-like phenotype. RV infection increased lung IL-33 and TSLP levels. Specific enhancement of IL-33 expression was observed in the airway epithelium and peribronchial cells, whereas TSLP expression was observed in the airway epithelium only. Using a neutralizing Ab to IL-33 and TSLPR-KO mice, we showed that, compared with wild-type mice, RV-induced mucous metaplasia and airway hyperresponsiveness are significantly attenuated in anti–IL-33–treated wild-type and TSLPR-KO mice. RV-induced ILC2 expansion was also significantly reduced. To understand the overlapping requirements of IL-25, IL-33, and TSLP for RV-induced mucous metaplasia, we examined the effects of anti–IL-33 and TSLPR KO on IL-25 expression, as well as the requirements of these innate cytokines for ILC2 expansion and IL-13 production ex vivo. IL-33 and TSLP were required for airway epithelial cell IL-25 expression. IL-33 was sufficient for IL-25 and TSLP expression, mucous metaplasia, and airway responsiveness. However, no single cytokine was sufficient for maximal ILC2 DNA synthesis or IL-13 expression. Together, these results demonstrate that IL-25, IL-33, and TSLP play cooperative roles in the development of airway responses in mice with early-life RV infection.

Previous studies in mice have linked viral infection, IL-33, TSLP, ILC2s, and the development of airway disease. In mice, IL-33–activated ILC2s mediate influenza-induced airway hyperresponsiveness (14). IL-33 is also required for ILC2-dependent restoration of airway epithelial integrity after influenza infection (51). Early-life pneumovirus infection induces an asthma-like phenotype in TLR7-KO mice that is accompanied by increased expression of IL-33 expression and expansion of lung ILC2s (52). Parainfluenza virus infection is followed by long-term induction of IL-33 expression and release from a subset of airway serous cells and alveolar type 2 cells linked to progenitor/stem cell function (53). IL-33 is necessary for respiratory syncytial virus (RSV)-induced bronchiolitis and ILC2s in neonatal mice (54). RSV infection activates IL-13–producing ILC2s through TSLP (55), and TSLP expression is required for IL-13 production, mucus production, and airway hyperresponsiveness (56). TSLP and IL-33 expression in the mouse lung is induced by human metapneumovirus infection, and TSLP is required for lung inflammation (57). We now report that IL-33 and TSLP are required for RV-induced ILC2 expansion in a mouse model of developing asthma.

Few studies have addressed the overlapping functions of IL-33, TSLP, and another epithelial-derived innate cytokine, IL-25, in models of airways disease. It has been suggested that the respective roles of IL-25, IL-33, and TSLP in allergic airway disease may vary depending on the type of Ag and route of Ag sensitization (i.e., i.p., intranasal, or epicutaneous). However, more recent studies suggest this may not be the case (58). Instead, IL-25, IL-33, and TSLP appear to regulate expression of each other. In an HDM model of asthma, neutralization of IL-25 moderately reduces pulmonary eosinophilia and levels of type 2 cytokines while blocking IL-33 and TSLP expression (59). Vaccination against IL-33 also inhibits HDM-induced airway inflammation, including lung expression of IL-25 and TSLP expression (60). In the current study, we found that IL-33 and TSLP are required for RV-induced IL-25 expression and deposition of IL-25 in the airway epithelium, localizing these cells as a site of cross-regulation. Anti–IL-33 had no effect on RV-induced lung TSLP levels, and TSLPR KO had no effect on IL-33, suggesting that IL-33 and TSLP sit atop the epithelial cell–derived innate cytokine hierarchy that promotes Th2 cytokine responses. Consistent with this notion, administration of rIL-33 and rTSLP each induced mucous metaplasia. TSLP signaling was also required for RV-induced *SPDEF* mRNA expression, suggesting that epithelial-derived TSLP targets the epithelium in an autocrine manner, thereby regulating the induction of other innate cytokines via SPDEF. IL-25 has been shown to increase TSLP expression in MLE12 epithelial cells (61), additional evidence of cross-regulation in the epithelium. Although RV has been noted to induce SPDEF expression in cultured airway epithelial cells (62), this has not been shown previously in vivo.

IL-25, IL-33, and TSLP may also have overlapping effects on immune target cells, such as ILC2s. ILC2 cells produce large amounts of IL-5 and IL-13 when stimulated by IL-33 plus TSLP (16, 29, 7). To test this in the context of RV infection, we isolated ILC2s from the lungs of immature mice infected with RV. We found that addition of TSLP significantly increased IL-25– and IL-33–induced DNA synthesis and IL-13 production, with both combinations achieving a maximal response. IL-25 and IL-33, in combination with IL-2 and IL-7, also induced maximal ILC2 DNA synthesis and IL-13 production. Such additive effects on IL-13 production may be based on the activation of common signaling pathways regulating ILC2 cytokine expression. For example, IL-33 and TSLP synergistically induce an IFN regulatory factor 4–IL-9 program in ILC2s (63). In addition, we found that TSLP had additive effects on IL-25– and IL-33–induced mRNA expression of *IL17RB* and *IL1RL1*, which encode the IL-25 and IL-33 receptors IL-17RB and ST2L. Taken together, these data suggest that TSLP, IL-25, and IL-33 have additive effects on immature ILC2 function and augment cellular responsiveness to each other via upregulation of their cellular receptors.

Early-life wheezing-associated respiratory tract infections have long been considered risk factors for asthma. Although initial attention focused on the potential role of RSV, 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, RSV was negatively associated with it (17). Data from a birth cohort of high-risk infants from Wisconsin showed that wheezing-associated illness with RV is the most important risk factor for asthma development and is higher than that of infants with allergen sensitization or RSV infection (18, 19). A population-based retrospective analysis of a Tennessee birth cohort showed an increased risk for early childhood asthma following bronchiolitis during RV-predominant nonwinter months versus RSV-predominant winter months (20). Finally, oral prednisolone treatment of infants hospitalized for their first episode of RV-induced wheezing decreased the risk for recurrent wheezing 7 y later (64), 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.

We conclude that IL-33 and TSLP are required for epithelial cell IL-25 expression, mucous metaplasia, and ILC2 expansion following early-life RV infection. Administration of intranasal IL-33 and TSLP, was sufficient for mucous metaplasia. Finally, TSLP was required for maximal ILC2 gene expression in response to IL-25 and IL-33. The generation of mucous metaplasia in immature mice involves a complex interplay among the innate cytokines IL-25, IL-33, and TSLP.

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Disclosures

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