

Rhinovirus infection interferes with induction of tolerance to aeroantigens through OX40 ligand, thymic stromal lymphopoietin, and IL-33

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Background: Rhinovirus infection at an early age has been associated with development of asthma, but how rhinovirus influences the immune response is not clear.

Objective: Tolerance to inhaled antigen is mediated through induction of regulatory T (Treg) cells, and we examined whether rhinovirus infection of the respiratory tract can block airway tolerance by modulating Treg cells.

Methods: The immune response to intranasal ovalbumin in mice was assessed with concomitant infection with RV1B, and the factors induced *in vivo* were compared with those made by human lung epithelial cells infected *in vitro* with RV16.

Results: RV1B infection of mice abrogated tolerance induced by inhalation of soluble ovalbumin, suppressing the normal generation of forkhead box protein 3–positive Treg cells while promoting T_H2 cells. Furthermore, RV1B infection led to susceptibility to asthmatic lung disease when mice subsequently re-encountered aeroantigen. RV1B promoted early *in vivo* expression of the TNF family protein OX40 ligand on lung dendritic cells that was dependent on the innate cytokine thymic stromal lymphopoietin (TSLP) and also induced another innate cytokine, IL-33. Inhibiting each of these pathways allowed the natural development of Treg cells while minimizing T_H2 differentiation and restored tolerance in the face of RV1B infection. In accordance, RV16 infection of human lung epithelial cells upregulated TSLP and IL-33 expression.

Conclusions: These results suggest that infection of the respiratory epithelium with rhinovirus can antagonize tolerance to inhaled antigen through combined induction of TSLP, IL-33, and OX40 ligand and that this can lead to susceptibility to asthmatic lung inflammation. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Asthma, epithelial cell, OX40 ligand, RV1B, RV16, thymic stromal lymphopoietin, peripherally induced regulatory T cell

Abbreviations used

APC:	Antigen-presenting cell
BAL:	Bronchoalveolar lavage
BALF:	Bronchoalveolar lavage fluid
DC:	Dendritic cell
Foxp3:	Forkhead box protein 3
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
HDM:	House dust mite
IL-33R:	IL-33 receptor
LDLR:	Low-density lipoprotein receptor
LN:	Lymph node
NHBE:	Normal human bronchial epithelial
OVA:	Ovalbumin
OX40L:	OX40 ligand
PBEC:	Primary bronchial epithelial cell
PFU:	Plaque-forming unit
pTreg:	Peripherally induced regulatory T cell
TCR:	T-cell receptor
TLR:	Toll-like receptor
Treg:	Regulatory T
TSLP:	Thymic stromal lymphopoietin
TSLPR:	Thymic stromal lymphopoietin receptor

The development of asthma has been linked to respiratory tract infections in early childhood. Viral infections trigger 80% of asthma exacerbations in children and nearly 50% in adults, with rhinovirus being the most common virus identified.¹⁻³ Rhinoviruses not only infect the upper respiratory tract but also lead to lower respiratory tract disease in infants and young children,⁴⁻⁶ and there is a strong correlation with the development of respiratory allergies or asthma later in life.^{7,8} However, the mechanism or mechanisms through which rhinovirus promotes or enhances allergic or asthmatic reactions is not clear and is the subject of great interest.

Rhinovirus is a positive-stranded RNA virus of the Picornaviridae family. Ninety percent of more than 100 serotypes that comprise the genetic HRV-A and HRV-B clades, such as RV16 and RV39, comprise the major group and bind to intercellular adhesion molecule 1, whereas the minor group viruses, such as RV1B and RV2, bind to low-density lipoprotein receptors (LDLRs).⁹ Rhinovirus mainly infects and replicates inside airway epithelial cells,^{5,10} suggesting factors produced by epithelial cells that influence the immune response might explain why rhinovirus infection triggers asthmatic and allergic reactions. Immune response in the lung mucosal system is a balance between inflammatory activity against dangerous antigens and regulatory cell activity that maintains immune tolerance. Rhinovirus infection also has the potential to alter this natural balance.

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Recently, we have made strong progress in understanding how tolerance and regulatory T (Treg) cells are promoted in the lung and how allergens or pathogen-derived molecules might antagonize tolerance and suppress regulatory T (Treg) cells.^{11–14} In particular, several pieces of evidence suggest that induction of OX40 ligand (OX40L; TNFSF4), a TNF family ligand expressed on antigen-presenting cells (APCs), might be critical for promoting the initial differentiation of T_H2 cells, as well as expanding existing T_H2 cells, through signals from its receptor, OX40 (TNFRSF4/CD134).^{15–18} Concomitantly, OX40-OX40L activity can antagonize the generation of Treg cells, which mediate airway tolerance.^{11,12,19} Because OX40L can be induced by the bronchial epithelium-derived cytokine thymic stromal lymphopoietin (TSLP),^{12,20,21} we hypothesized that rhinovirus infection might promote this pathway and that this could be a mechanism through which this virus contributes to asthmatic responses.

Here we show in mouse models that infection with RV1B at the time of inhalation of soluble antigen resulted in lung expression of TSLP and OX40L-dependent antagonism of lung tolerance through suppressing the generation of inducible forkhead box protein 3 (Foxp3)⁺ peripherally induced regulatory T cells (pTregs). However, the combined activity of TSLP and OX40L did not fully explain the effect of rhinovirus, and we also found that another innate cytokine, IL-33, was involved. Neutralizing or deleting thymic stromal lymphopoietin receptor (TSLPR), IL-33 receptor (IL-33R), or OX40L prevented the block of tolerance induced by rhinovirus and led to reduced susceptibility to allergic airway disease. In line with this being a likely mechanism applicable to human rhinovirus-related lung inflammation, infection with the major serotype RV16 promoted TSLP and IL-33 production by either normal human bronchial epithelial (NHBE) cells or primary bronchial epithelial cells (PBECs). These results suggest that respiratory tract exposure to rhinovirus can promote a program of inflammatory molecules that includes OX40L, and neutralizing OX40-OX40L interactions together with TSLP, IL-33, or both might be applicable to controlling asthma exacerbations that are driven by rhinovirus or other similar viruses.

METHODS

See the [Methods](#) section in this article's Online Repository at www.jacionline.org for full details.

Mice

C57BL/6 and B6.PLThy1a (Thy1.1) mice were purchased (Jackson Laboratory, Bar Harbor, Me), and OT-II T-cell receptor (TCR)-transgenic and TSLPR knockout mice were bred in house, as described in the [Methods](#) section in this article's Online Repository.

Rhinovirus generation and use

RV1B was generated, as previously described.²² Mice were infected intranasally with 5×10^7 plaque-forming units (PFU) per milliliter of RV1B in 50 μ L with or without ovalbumin (OVA). Human RV-16 was propagated, as previously described.²³ Bronchial epithelial cells were exposed to HRV-16 at $10^{5.5}$ U/mL 50% tissue culture infective dose (multiplicity of infection of approximately 1).²⁴

Airway tolerance and allergic airway inflammation

Airway tolerance was induced with intranasal OVA, as previously described^{11,12} and described in the [Methods](#) section in this article's Online

Repository at www.jacionline.org. Briefly, mice were exposed to 100 μ g of soluble OVA (Low Endo, Purified; ≤ 1 EU/mg; Worthington Biochemical Corp, Lakewood, NJ) for 3 consecutive days (days -10 to -8). Rhinovirus was administered intranasally at day -10 with antigen. Mice were subsequently sensitized with OVA/alum intraperitoneally and challenged later with 20 μ g of OVA for 4 consecutive days, as depicted in [Fig 1](#), to test the ability to modulate airway tolerance.

Bronchoalveolar lavage and lung histology

Bronchoalveolar lavage (BAL) was performed 24 hours after the last OVA challenge. Bronchoalveolar lavage fluid (BALF) was examined for cytokine content by means of ELISA. For lung histology, 5- to 6- μ m sections were stained with hematoxylin and eosin. Magnification $\times 200$ was used for histologic scoring, and at least 6 to 8 fields were scored to obtain the average for each mouse.

Adoptive transfer to track Treg cell development

Naive OVA-specific CD25⁺CD4⁺ T cells were isolated from spleens of OT-II TCR-transgenic mice with CD4 T Cell Isolation Kits (Miltenyi Biotec, San Diego, Calif). OT-II cells (5×10^6) were injected intravenously into B6.PL Thy1.1 congenic mice, and then 1 day later, mice were exposed to soluble OVA alone or OVA with RV1B administered intranasally. After 5 days, Foxp3 expression was assessed in gated OT-II cells by means of flow cytometry, as described in the [Methods](#) section in this article's Online Repository.

RT-PCR

Total RNA was isolated from lung tissue or PBECs by using TRIzol reagent (Life Technologies, Grand Island, NY) and from NHBE cells by using the RNeasy Kit from Qiagen (Valencia, Calif). RNA was reverse transcribed to cDNA by using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, Ind). Data are presented as normalized to the ribosomal protein housekeeping genes L32 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

ELISA and ELISPOT

Murine cytokines in BALF were assayed by means of ELISA with paired antibodies, according to the manufacturers' recommendations. The ELISPOT assay for IL-4- and IL-13-secreting cells in lymph nodes (LNs) was performed at the time of analysis of Foxp3 expression, as described previously.^{11,12}

Statistical analyses

Statistical analyses were performed with GraphPad Prism software (GraphPad Software, La Jolla, Calif). Data are expressed as means $\pm$ SEMs and analyzed by using the Student *t* test and 1-way ANOVA with Bonferroni *post hoc* tests, where applicable.

RESULTS

Rhinovirus infection prevents airway tolerance and initiates T_H2 lung inflammation

Recent studies have shown that the minor group rhinovirus serotype RV1B, which infects both human beings and mice by binding to LDLRs, induces airway neutrophilic inflammation and airway hyperresponsiveness in mice reminiscent of the response in human beings, thereby providing a suitable model to study how rhinovirus affects adaptive immunity to model allergens.^{25,26} Of particular interest is whether rhinovirus infection can antagonize the development of tolerance in the lungs to inhaled

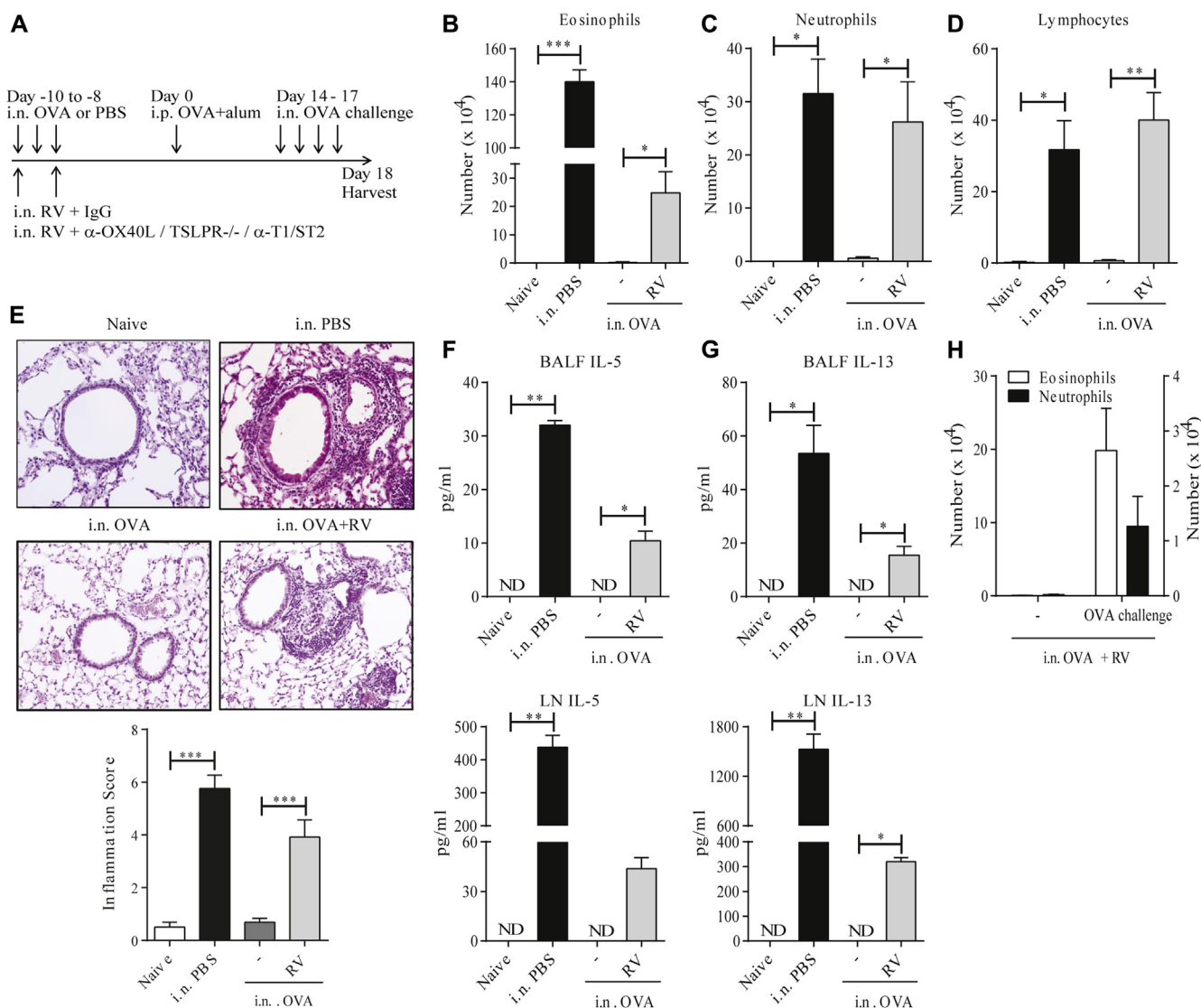


FIG 1. Rhinovirus blocks induction of tolerance to inhaled antigen. **A**, Schematic of experimental protocols. Mice were sensitized with intranasal OVA with or without RV1B. Nontolerized mice received PBS intranasally. All mice were sensitized with OVA/alum intraperitoneally on day 10 and challenged with intranasal OVA 2 weeks later to assess lung inflammation. **B-D**, BAL eosinophils, neutrophils, and lymphocytes. **E**, Hematoxylin and eosin-stained lung sections (upper) and inflammation scores (lower). **F** and **G**, IL-5 and IL-13 in BALF (upper) and LN culture supernatant (lower). **H**, BAL eosinophils and neutrophils in mice given intranasal OVA with rhinovirus that were not challenged or challenged with OVA. Results are means $\pm$ SEMs from 4 mice per group and representative of 3 experiments. *i.n.*, intranasal; *i.p.*, intraperitoneal; ND, not determined; RV, rhinovirus. * $P < .05$, ** $P < .01$, and *** $P < .001$.

protein antigens. To address this, mice were exposed intranasally to pure soluble OVA protein as a nominal innocuous antigen for 3 successive days. These are the conditions that have shown results in the conversion of antigen-reactive naive T cells into pTreg cells and the development of tolerance to the inhaled antigen, as assessed by the absence of response when mice are subsequently sensitized with OVA in alum and challenged with intranasal OVA (see protocol in Fig 1, A).^{11,12} Significantly, we observed that infection with RV1B at the time of inhalation of soluble OVA antagonized tolerance in that it led to the development of T_H2 allergic airway disease on subsequent exposure to OVA (Fig 1). Rhinovirus infection led to eosinophilia (Fig 1, B), neutrophilia (Fig 1, C), increased lymphocyte infiltration (Fig 1, D), and

peribronchial inflammation in the lungs (Fig 1, E), as well as increased T_H2 cytokine levels in BALF (Fig 1, F and G, upper panel) and a T_H2 response in LN T cells revealed after restimulation *in vitro* with OVA (Fig 1, F and G, lower panel). These phenotypes almost recapitulated those that developed in mice that were not tolerized with soluble OVA but were only exposed to PBS, although some evidence of a tolerogenic effect was still seen in eosinophilia and with T_H2 cytokines.

Intranasal RV1B infection in mice has previously been found to result in transient neutrophilia in the lungs along with influx of other inflammatory cells over the initial week of infection.²⁵ To show that our results represented a true block of tolerance to inhaled protein antigen and not simply a residual response to

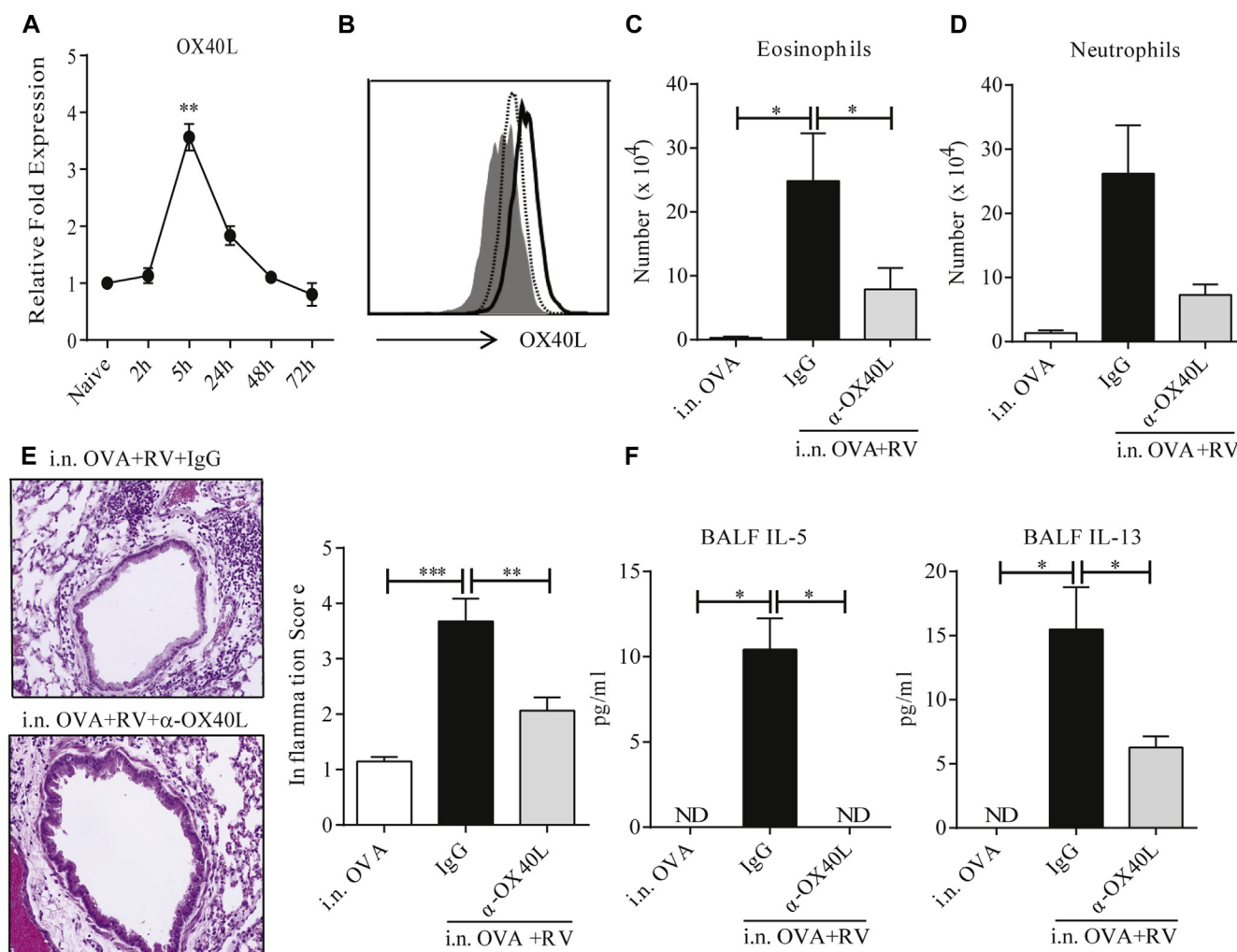


FIG 2. Rhinovirus infection inhibits airway tolerance through OX40L. **A**, OX40L mRNA in lung tissue after RV1B infection. **B**, OX40L on CD11c⁺ cells: isotype control (solid gray area), naive (dashed line), and RV1B (solid black line). **C–F**, Wild-type mice were tolerized, sensitized, and challenged as in Fig 1, **A**. Anti-OX40L or control antibody was administered intraperitoneally at the time of RV1B infection. Fig 2, **C** and **D**, BAL eosinophils and neutrophils. Fig 2, **E**, Hematoxylin and eosin-stained lung sections (left) and inflammation scores (right). Fig 2, **F**, BALF IL-5 and IL-13. Results are means $\pm$ SEMs from 3 to 4 mice per group and representative of 3 experiments. *i.n.*, Intranasal; *ND*, not determined; *RV*, rhinovirus. * $P < .05$, ** $P < .01$, and *** $P < .001$.

the virus, we monitored the extent of cellular infiltration in mice that were not subsequently challenged with OVA. These mice displayed no significant eosinophilia and neutrophilia compared with that seen in mice challenged with OVA (Fig 1, *H*). Thus rhinovirus infection abrogated induction of airway tolerance to inhaled protein antigen, which resulted in susceptibility to mount a T_H2-biased lung inflammatory response on re-encounter with the inhaled antigen.

Furthermore, we observed that rhinovirus infection had a small effect when inhaled by mice that had already been tolerized, although the action was not as striking. Rhinovirus exposure 10 days after inhalation of soluble OVA (day 0) led to a low level of peribronchial inflammation in the lungs (not shown), with some eosinophilia, neutrophilia, and IL-5 detectable in BALF and LNs (see Fig E1 in this article's Online Repository at www.jacionline.org and data not shown). However, this was not statistically significant compared with controls or compared with the response

in mice receiving RV1B at the time of tolerization. This suggests that rhinovirus likely exerts its major effect at the time of establishing tolerance. However, because some activity was evident, these data lead to the hypothesis that rhinovirus might lead to a break of established tolerance if infection was accompanied by another insult, such as from a second virus or an allergen with strong Toll-like receptor (TLR) or protease activity.

Rhinovirus infection antagonizes airway tolerance through OX40L

We then pursued the critical molecules promoted by rhinovirus infection that prevented tolerance induction. We previously reported that the interaction of the TNF family molecule OX40 on activated T cells with OX40L on APCs strongly promotes T-cell responses^{18,27,28} and is critical to the development of T_H2-driven airway inflammation.^{15,16} We also found that

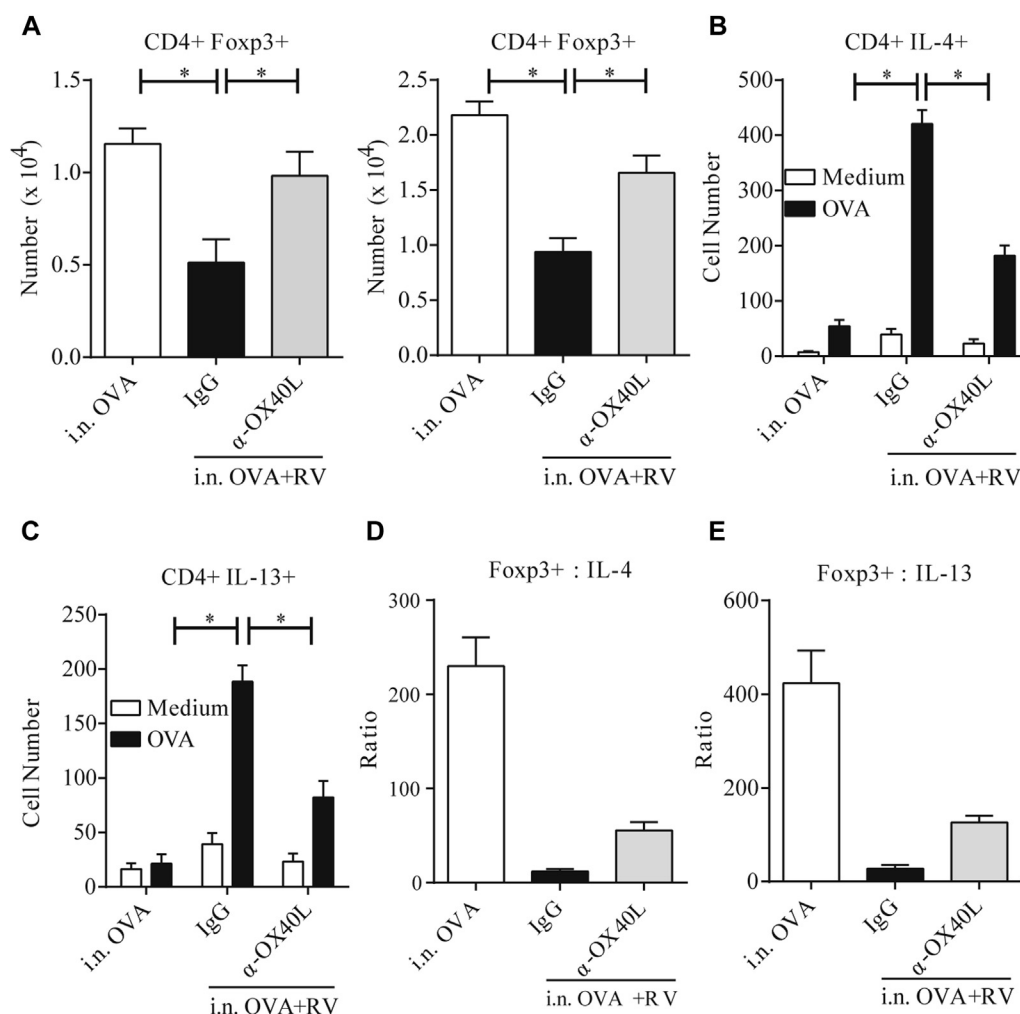


FIG 3. Rhinovirus suppresses generation of OVA-specific Foxp3⁺ T cells through OX40L. OVA-specific OT-II T cells were transferred into congenic mice that were then tolerized with OVA administered intranasally on 3 consecutive days with or without RV1B infection. Mice were treated with IgG or α -OX40L at the time of RV1B infection. **A**, Number of Foxp3⁺ OT-II T cells enumerated in LNs (left) and lung tissue (right) by means of flow cytometry 5 days after the first inhalation of soluble OVA. **B** and **C**, Number of IL-4- and IL-13-secreting CD4 T cells enumerated in LNs by using ELISPOT at the same time. **D** and **E**, Ratio of Foxp3⁺ and IL-4- and IL-13-secreting CD4 T cells. Results are means $\pm$ SEMs from 3 to 4 mice per group and representative of 2 experiments. i.n., Intranasal; RV, rhinovirus. * $P < .05$.

OX40L is induced by signaling through the pattern recognition receptors TLR4 and Nod2 in the lung, resulting in loss of tolerance.^{11,12} OX40L is not constitutively expressed, but suggesting that rhinovirus infection might promote the same pathway, we detected an increase in mRNA within 5 hours of RV1B infection and also visualized OX40L on CD11c⁺ within 24 hours (Fig 2, A and B). Mice were treated with blocking antibody at the time of intranasal exposure to antigen and RV1B to examine whether OX40L was active. Mice were then subsequently sensitized and challenged with OVA as before 10 days after the infection and treatment (see Fig 1, A). Anti-OX40L significantly, although not completely, inhibited OVA-induced airway eosinophilia and neutrophilia, bronchovascular inflammation, and T_H2 cytokine response (Fig 2, C-F). The antibody dose was chosen to ensure that OX40L was only blocked within a 5- to 7-day timeframe when the virus was active and that it was not affecting the response to OVA during the sensitization and challenge phases.¹¹ To confirm this, mice were given anti-OX40L without exposure to

soluble OVA or RV1B and then 10 days later sensitized and challenged with OVA. Importantly, there was no effect on airway eosinophilia, T_H2 cytokines, and other hallmarks of inflammation (see Fig E2 in this article's Online Repository at www.jacionline.org and data not shown). Thus rhinovirus led to susceptibility to antigen-induced airway inflammation through the OX40-OX40L pathway.

Rhinovirus suppresses generation of antigen-specific Foxp3⁺ T cells through OX40L

We previously reported that Foxp3⁺ pTreg cells are part of the tolerogenic mechanism that results on inhalation of soluble antigen and that OX40 signaling can inhibit the generation of these pTreg cells.^{11,12,19} Therefore, we tracked their generation by adoptively transferring the naive (CD25⁻Foxp3⁻) Thy1.2⁺ OVA-specific OT-II TCR-transgenic CD4 T cells into Thy1.1 recipients, assessing conversion by staining for Foxp3

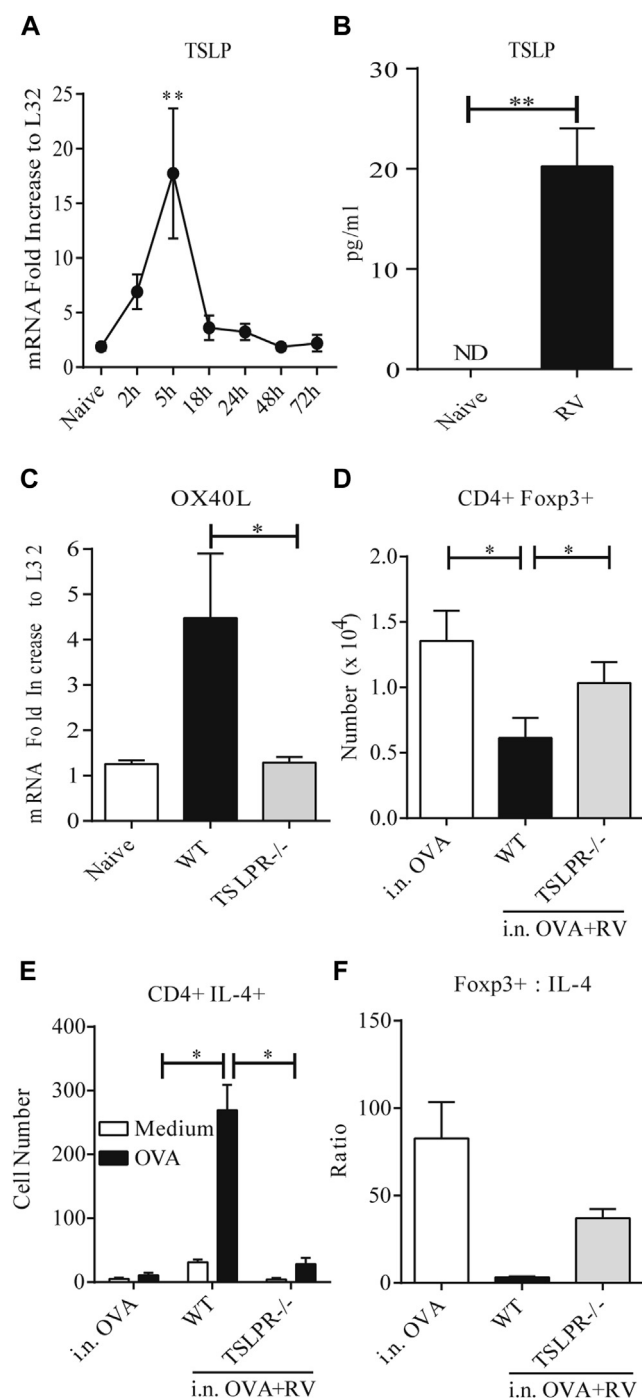


FIG 4. TSLP is required for rhinovirus to modulate the lung tolerogenic environment. **A**, TSLP mRNA in lung tissue after RV1B infection over 72 hours. **B**, TSLP protein in BALF after 24 hours. **C**, OX40L mRNA in lung tissue after RV1B infection of wild-type or TSLPR^{-/-} mice at 5 hours. **D-F**, OT-II T cells were transferred into wild-type or TSLPR^{-/-} mice, as in Fig 3. Recipients were tolerized with OVA and infected with RV1B as before. Fig 4, **D**, Number of Foxp3⁺ OT-II T cells in LNs 5 days after OVA inhalation. Fig 4, **E**, Number of IL-4-secreting CD4 T cells in LNs. Fig 4, **F**, Ratio of Foxp3⁺ and IL-4-secreting CD4 T cells in LNs. Results are means $\pm$ SEMs from 3 to 4 mice per group and representative of 2 experiments. *i.n.*, Intranasal; *ND*, not determined; *RV*, rhinovirus; *WT*, wild-type.

in vivo with soluble OVA inhalation, and this was reduced to less than 1% after RV1B infection. When converted to total numbers of cells, RV1B resulted in an approximate 50% decrease in OVA-specific Foxp3⁺ pTreg cells that developed over 5 days after the initial exposure to soluble OVA, both in the LNs (Fig 3, A, left panel) and lungs (Fig 3, A, right panel). Furthermore, RV1B promoted the appearance of OVA-specific IL-4- and IL-13-secreting CD4 T cells at the same time (Fig 3, B and C), whereas few were found under tolerizing conditions without RV1B infection. We estimated that the ratio of Treg cells to IL-4-secreting and IL-13-secreting cells was approximately 250:1 and 500:1, respectively, under tolerizing conditions. In contrast, on exposure to RV1B, the balance between these cells changed to approximately 10:1 and 30:1, respectively (Fig 3, D and E). Moreover, blocking OX40L partially prevented suppression of pTreg cell generation (Fig 3, A) and also reduced the development of IL-4- and IL-13-secreting T cells by approximately 50% (Fig 3, B and C). The approximate ratio of Foxp3⁺ to IL-4- or IL-13-secreting T cells was then skewed back in favor of the Treg cells, although the balance of these subsets was not restored to the level seen under conditions without RV1B infection (Fig 3, D and E).

TSLP participates in the rhinovirus response that blocks airway tolerance

Previous data suggested that TSLP, a product of epithelial cells, fibroblasts, and smooth muscle cells, stimulates dendritic cells (DCs) to induce T_H2 differentiation^{29,30} through induction of OX40L.²⁰ Because rhinovirus primarily infects and replicates inside airway epithelial cells,⁹ we tested whether RV1B induced TSLP in the lungs. Kinetic studies showed that RV1B induced TSLP mRNA within 2 hours, and this peaked after 5 hours, when OX40L mRNA was found. TSLP protein was also detected in BALF at 24 hours (Fig 4, A and B).

When TSLPR-deficient mice were given soluble intranasal OVA and infected with RV1B and then sensitized with OVA intraperitoneally in alum 10 days later and subsequently challenged with OVA (protocol in Fig 1, A), they failed to mount a strong lung inflammatory response (see Fig E3 in this article's Online Repository at www.jacionline.org). However, from this experiment, it was not possible to conclude that RV1B promoted TSLP activity because TSLPR-deficient mice are not able to mount a normal T_H2 inflammatory response to OVA when it is administered in alum intraperitoneally.^{12,31} Therefore we assessed early parameters in TSLPR-deficient mice before alum sensitization to determine whether TSLP was involved in induction of OX40L by RV1B and suppression of pTreg cell development. Notably, RV1B did not promote OX40L expression in the lungs of TSLPR-deficient mice, as assessed 5 hours after infection (Fig 4, C). Furthermore, the absence of TSLPR also resulted in RV1B being less active in suppressing the development of OVA-reactive Foxp3⁺ pTreg cells and in promoting the appearance of IL-4-secreting T cells when assessed 5 days after infection and exposure to intranasal OVA (Fig 4, D and E). This resulted in a less pronounced shift in the Treg/T_H2 cell balance in favor of the tolerogenic response (Fig 4, F). These data suggest that rhinovirus exposure induces TSLP, which is active in regulating OX40L expression on APCs, and therefore this process in part contributes to blocking the development of a suppressive environment and tolerance. In the pTreg cell analysis the induction of Foxp3 in wild-type

expression.^{11,12} Flow analysis demonstrated that approximately 3% to 4% of OT-II CD4 cells converted into Foxp3⁺ cells

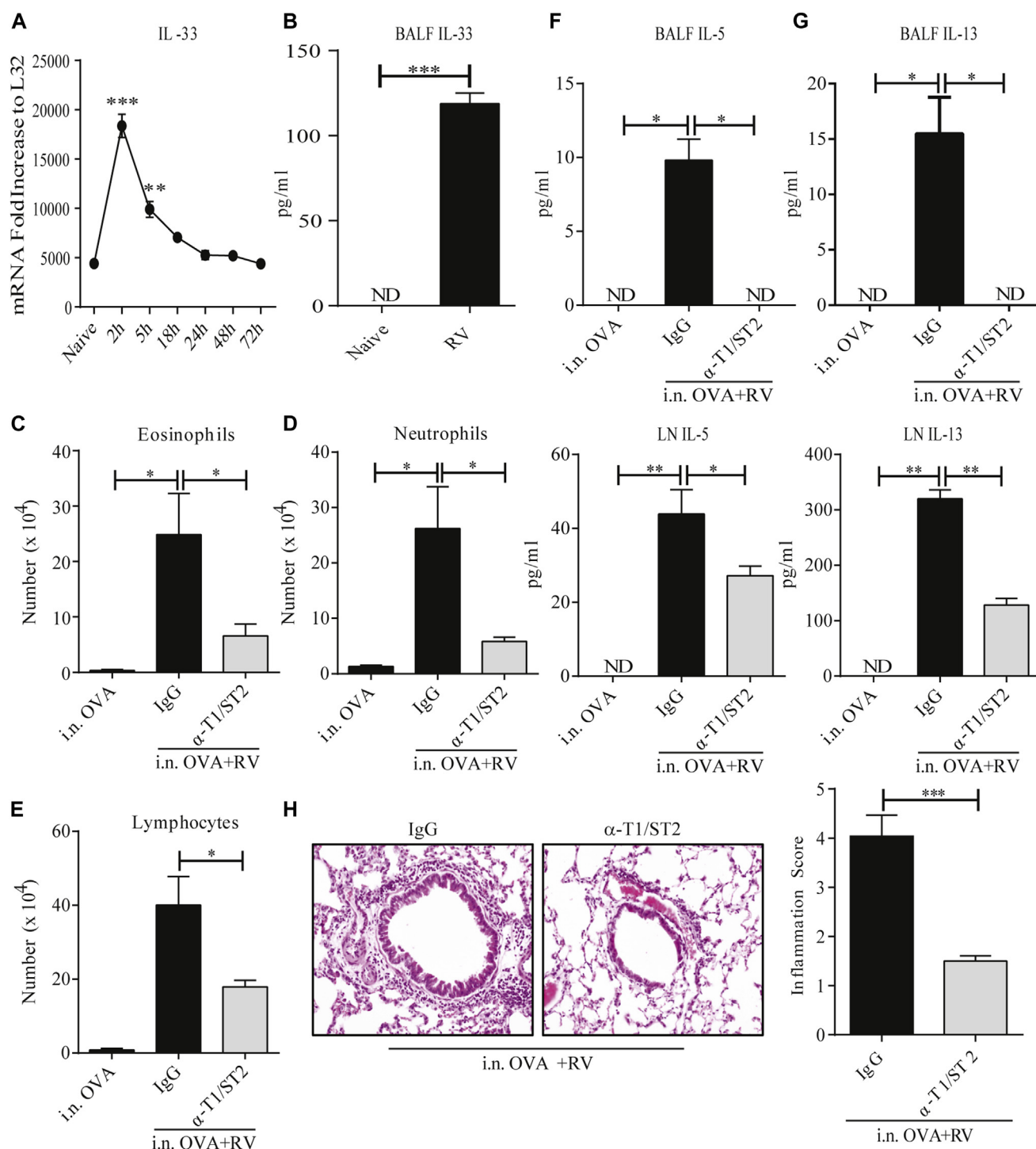


FIG 5. IL-33 is necessary for rhinovirus to block airway tolerance. **A**, IL-33 mRNA in lung tissue after RV1B infection over 72 hours. **B**, IL-33 protein in BALF after 24 hours. **C-H**, Wild-type mice were tolerized, sensitized, and challenged as in Fig 1, A, to assess lung inflammation. Anti-T1/ST2 or control antibody was administered intraperitoneally at the time of RV1B infection. Fig 5, C-E, BAL eosinophils, neutrophils, and lymphocytes. Fig 5, F and G, IL-5 and IL-13 in BALF (upper) and LN culture supernatant (lower). Fig 5, H, Hematoxylin and eosin-stained lung sections (left) and inflammation score (right). Results are means $\pm$ SEMs from 4 mice per group and representative of 2 to 3 independent experiments. *i.n.*, Intranasal; *ND*, not determined; *RV*, rhinovirus. * P < .05, ** P < .01, and *** P < .001.

OT-II cells was assessed in TSLPR^{-/-} mice, suggesting that TSLP activity was required on cells other than the responding T cells, which was in line with the reduced OX40L expression.

However, it was still possible that TSLP also directly acted on the T cells, as suggested by one study that found TSLP could suppress the induction of Foxp3 in isolated naive T cells.³²

IL-33 is essential to block airway tolerance after rhinovirus exposure

Blocking OX40L or TSLPR did not completely restore a state of tolerance in the context of rhinovirus exposure, therefore we sought another molecule that might synergize with these factors. IL-33 has been suggested to function as an alarmin that is expressed mostly by epithelial cells, fibroblasts, and endothelial cells.³³ IL-33 might have several functions and targets but, interestingly, was described to promote DC maturation that led to the differentiation of T_H2 cells.³⁴ Kinetic studies showed that IL-33 mRNA levels peaked in the lungs within 2 hours of RV1B infection, and the production of IL-33 protein was also detected in BALF at 24 hours (Fig 5, A and B).

Mimicking the phenotype observed when OX40L was blocked and in TSLPR-deficient mice, we found that inhibiting IL-33R with an antibody (α -T1/ST2) limited the activity of RV1B in enabling an OVA-specific lung inflammatory response to develop (Fig 5, C-H). Significantly and in contrast to the absence of TSLPR signaling, blocking IL-33R had no effect on OX40L expression induced by RV1B in the lung (Fig 6, A). However, IL-33 still participated in the suppression of pTreg cell development and promotion of early IL-4-secreting T cells that were generated over the initial 5 days after inhalation of RV1B (Fig 6, B-D). α -T1/ST2 treatment only affected the response generated to OVA during exposure to rhinovirus because the antibody given to a naive mouse 10 days before OVA sensitization and challenge has no effect on airway eosinophilia, neutrophilia, and other inflammatory parameters (see Fig E4 in this article's Online Repository at www.jacionline.org and data not shown). Thus rhinovirus infection in the lung also elicits IL-33 that synergizes with TSLP and OX40L to antagonize the development of pTreg cells and airway tolerance.

TSLP and IL-33 levels are upregulated in airway epithelial cells after rhinovirus infection

To provide clinical significance, we found that RV16, a major group rhinovirus, also induced TSLP mRNA *in vitro* in a time-dependent manner in NHBE cells, although interestingly, TSLP was not induced in PBECs obtained from nontransplanted normal human lungs (Fig 7, A and B). This correlated with prior data that also described TSLP expression in NHBE cells infected with RV16.³⁵ Corresponding to the idea that IL-33 was also derived from bronchial epithelial cells, we found a time-dependent increase of IL-33 mRNA expression in PBECs from normal human lungs (Fig 7, C and D), although in this case not from NHBE cells, contrasting with TSLP (Fig 7, A). However, in line with OX40L being derived from APCs, we did not detect OX40L mRNA induced by RV16 in NHBE cells or PBECs (data not shown). Thus rhinovirus infection of the lung epithelium can antagonize airway tolerance to inhaled antigen through combined induction of TSLP and IL-33.

DISCUSSION

Rhinovirus infections can contribute to asthma and COPD exacerbations,⁹ but whether rhinoviruses can initiate T_H2-driven inflammation has not been clear. Rhinovirus infects epithelium in both the upper and lower respiratory tract,^{9,36} and rhinovirus-induced exacerbations in asthmatic patients have been linked to defective expression of protective type I and III interferons in epithelial cells.^{37,38} However, epithelial cells are capable of

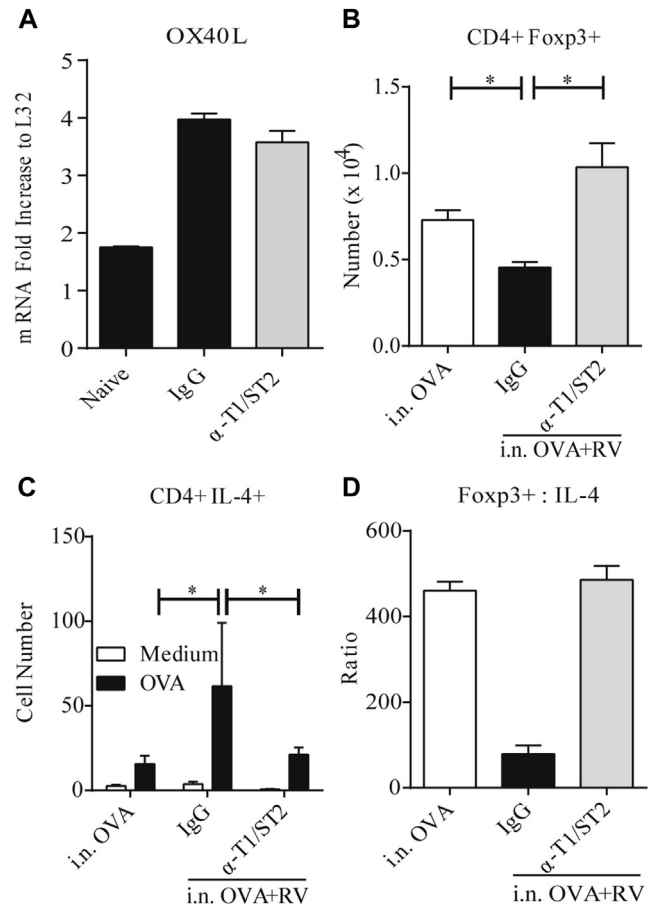


FIG 6. IL-33 is required for rhinovirus to modulate the Treg/T_H2 balance. **A**, OX40L mRNA at 5 hours in lung tissue of control IgG- and α -T1/ST2-treated mice after RV1B infection. **B-D**, OT-II T cells were transferred into wild-type congenic mice, as in Fig 3. Recipients were tolerized and infected with RV1B as before. Fig 6, B, Number of Foxp3⁺ OT-II T cells enumerated in LNs by using flow cytometry 5 days after OVA inhalation. Fig 6, C, Number of IL-4-secreting CD4 T cells in LNs by using ELISPOT. Fig 6, D, Ratio of Foxp3⁺ to IL-4-secreting CD4 T cells. Results are means $\pm$ SEMs from 3 to 4 mice per group and representative of 2 independent experiments. i.n., Intranasal; RV, rhinovirus. **P* < .05.

producing many inflammatory mediators³⁶ after stimulation of pattern recognition receptors,^{39,40} which might be critical for leading to T_H2-biased disease. We now show in an experimental animal model that rhinovirus infection of the respiratory tract can prevent the induction of tolerance to inhaled protein antigen. Significantly, this is mediated through several molecules, likely derived from airway epithelium, that link innate and adaptive immunity, and this results in susceptibility to acute allergic airway inflammation.

In healthy lungs tissue macrophages and DCs promote immune tolerance to innocuous antigens, at least in part by promoting suppressive pTreg cells.^{14,41} We previously showed that stimulation of the TLR4 and Nod2 pattern recognition receptors in the lungs, both of which are expressed by airway epithelial cells, resulted in OX40L expression in lung and lung-draining LN DCs. Antigen presentation in the context of this pattern recognition receptor-induced OX40L then blocked pTreg cell formation and concomitantly directed T_H2 cell development through signals transmitted from its receptor, OX40, which is

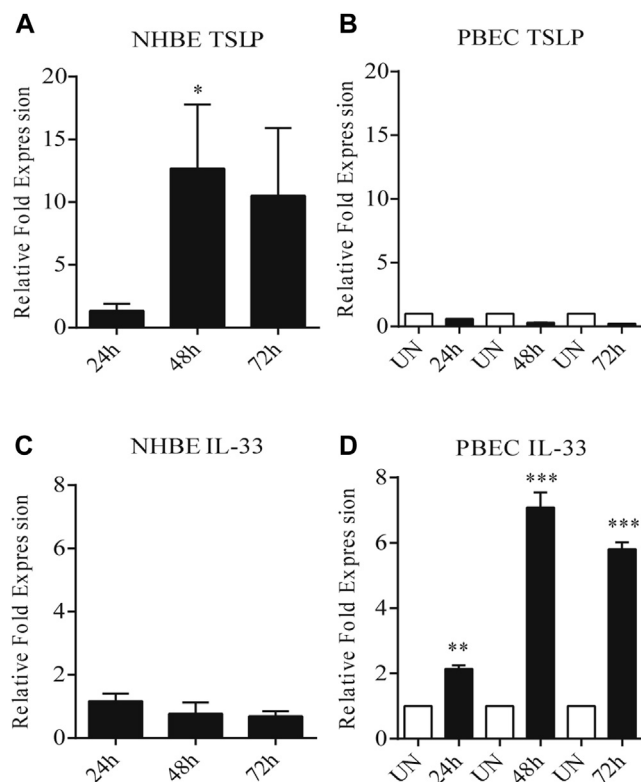


FIG 7. TSLP and IL-33 are upregulated in human airway epithelial cells by rhinovirus. TSLP mRNA (**A** and **B**) and IL-33 mRNA (**C** and **D**) from 24 to 72 hours in NHBE cells and PBECs after RV16 infection *in vitro* compared with unstimulated controls (UN). Results are means $\pm$ SEMs from 3 independent experiments with cells from a single donor for NHBE cells and means $\pm$ SEM from cells derived from 3 different donors for PBECs. PBEC results are representative of 2 experiments. * $P < .05$, ** $P < .01$, and *** $P < .001$.

expressed on antigen-stimulated T cells.^{11,12} Our studies here show that rhinovirus can promote the same OX40L-dependent mechanism, leading to susceptibility to allergic lung inflammation. Rhinovirus can target TLR3 and/or retinoic acid-inducible gene 1 and melanoma differentiation-associated protein-5, suggesting a similar cascade of molecular events might be downstream of several pattern recognition receptors.

OX40L can be directly induced in APCs by TLR signals¹⁸; however, it is likely that rhinovirus induction of OX40L was indirect. Innate cytokines, particularly TSLP,^{12,20} which are mostly the product of epithelial and fibroblast cells, have been reported to participate in promoting OX40L expression in DCs. Thus cross-talk between rhinovirus-infected epithelial cells and DCs might be central to blocking airway tolerance and driving CD4 T cells into the T_H2 pathway. In line with this, we found that RV1B infection induced lung expression of TSLP in mice and also that RV16 induced TSLP expression in NHBE cells. Furthermore, we also found that TSLP expression was promoted by RV1B infection of an alveolar epithelial cell line (A549; data not shown). TSLP has been well documented as a factor that promotes T_H2 differentiation,^{30,42,43} but recently, injection of TSLP *in vivo* was also shown to antagonize airway tolerance against OVA and inhibit the generation of pTreg cells,³² confirming our earlier results in TSLPR^{-/-} mice.¹² The data in the current study with TSLPR^{-/-} mice further support this

contention and suggest TSLP is central to the inflammatory activity of rhinovirus. TSLP can have both direct and indirect activities on T cells^{32,44}; however, with rhinovirus, both here and in our previous study *in vivo* with pattern recognition receptor ligands, we found that OX40L expression in CD11c⁺ DCs in the lung was dependent on TSLP. Moreover, much of the activity of rhinovirus in suppressing naive T-cell conversion into pTreg cells and promoting IL-4-secreting effector T cells was lost when wild-type (TSLPR⁺) transgenic T cells responded in TSLPR-deficient hosts. Therefore this supports the idea that the primary action of TSLP in these scenarios is mediated by cross-talk with APCs, although this does not rule out an additional direct action on the responding T cells.

TSLP has been shown to control the induction of lung inflammation in responses involving OVA when the adjuvant is alum or a Nod ligand^{12,31,43} and when house dust mite (HDM) extract was used as the allergen.⁴⁵ Additionally, a recent clinical study⁴⁶ supported these data and found that anti-TSLP reduced allergen-induced bronchoconstriction and eosinophilia in patients with mild allergic asthma, including patients allergic to HDM, cat dander, grass pollen, ragweed, horse, and *Alternaria* species. Thus TSLP might be central to many allergic/type 2 responses that develop in the lungs. Surprisingly, one report, also with HDM as the antigen,⁴⁷ found that TSLP was not important for airway inflammation, in this case using an administration protocol that was markedly shorter (10 vs 32 days) than in the aforementioned study in which TSLP was active with HDM.⁴⁵ However, interestingly, IL-33 was required for this HDM-driven response, and also IL-33 partially contributed to OX40L expression that additionally participated in driving T_H2 immunity.⁴⁷ In our study with rhinovirus infection, IL-33 was active, but it synergized with TSLP in controlling the Treg/ T_H2 cell balance. Epithelial cells in the lung are again the most likely candidates for producing IL-33,^{48,49} and we showed that rhinovirus infection could lead to production of IL-33 in primary epithelial cell cultures. Interestingly, rhinovirus infection did not induce IL-33 from NHBE cells, contrasting with TSLP, which was oppositely regulated. NHBE cells are usually isolated from normal donor airway tissue positioned above the branching of the lungs and often passaged before use, and the primary epithelial cells are established from mainstem to third-generation bronchi and used immediately. Thus the variability in producing IL-33 versus TSLP might then be due to alternate subsets of epithelial cells or simply phenotypic drift.

In contrast to blocking TSLP, we found no effect of blocking IL-33 on the ability of rhinovirus to induce OX40L expression in the lung, contrasting with the study described above in which HDM-driven OX40L was IL-33 dependent in a system in which TSLP was not active.⁴⁷ It is possible that both TSLP and IL-33 may have overlapping activities, but their relative concentrations *in vivo* could dictate which is dominant for promoting OX40L. It is likely that the variability in the model systems used to analyze lung inflammation contribute to which cytokine is active in this regard. However, in our study, even though OX40L was not controlled by IL-33, this cytokine still was critical in determining pTreg cell balance. IL-33 can stimulate mast cells, basophils, invariant natural killer T cells, type 2 innate lymphoid cells, and natural killer cells⁵⁰⁻⁵³ but has been suggested to contribute directly to differentiation of naive CD4 T cells into T_H2 cells.⁵⁴ Therefore it is possible that IL-33 acted in our model system at least in part directly on the antigen-responding T cells, providing

synergistic signals that integrated with those from OX40-OX40L interactions.

To summarize, the results of the present study demonstrate that rhinovirus can alter the respiratory environment to disrupt the normal ability to induce T-cell unresponsiveness. Rhinovirus infection induces TSLP and IL-33 expression and TSLP-dependent upregulation of OX40L, and all 3 of these molecules contribute to the development of an adaptive immune response that is characterized by an altered balance between suppressive pTreg cells and T_H2 effector cells. This might be a common response of viruses associated with allergic disease because recent data with respiratory syncytial virus also found that it could promote TSLP expression in the lung, with concomitant OX40L expression in DCs.⁵⁵ Neutralizing OX40L, TSLPR, or IL-33R alone or in combination might represent a potential strategy for limiting the effects of these respiratory viruses in contributing to asthma and allergic disease.

We thank Steve Ziegler for originally making available mice deficient in TSLPR.

Key messages

- RV1B infection initiates T_H2 responses in the lung and antagonizes Foxp3⁺ Treg cells and induction of tolerance.
- RV1B induces IL-33- and TSLP-dependent OX40L in the lung.
- RV16 upregulates TSLP and IL-33 in human lung epithelial cells.

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METHODS

Mice

C57BL/6 and B6.PLThy1a (Thy1.1) mice were from the Jackson Laboratory. OT-II TCR-transgenic mice and TSLPR knockout mice on the BL/6 background were bred in house.^{E1,E2} All experiments were conducted according to the guidelines of the La Jolla Institute for Allergy and Immunology's Institutional Animal Care and Use Committee.

Rhinovirus generation and use

RV1B was generated, as previously described.^{E3} Ohio Hela cells (ECACC, UK) were infected until approximately 70% to 80% of cells were cytopathic. RV1B from cell lysates was concentrated and centrifuged with an M_r 100,000 cutoff centrifuge filter (Millipore, Billerica, Mass). Virus was titered on Ohio Hela monolayers. Cells were fixed with formalin, and plaques were visualized with 0.1% crystal violet and quantified. Mice were infected with 5×10^7 PFU/mL RV1B in 50 μ L intranasally with or without OVA.

Human RV-16 was propagated in WI-38 cells and purified by means of centrifugation over sucrose and then titered, as previously described.^{E4} NHBE cells were purchased (Lonza, Walkersville, Md), and PBECs were derived from normal human lungs that were not used for transplantation, as previously described.^{E5} Cells were from nonsmoking subjects. Cells were propagated in bronchial epithelial cell growth medium (Lonza, Walkersville, Md) and then cultured in bronchial epithelial cell growth medium without hydrocortisone, starting 24 hours before infection. Cells were exposed to purified HRV-16 at $10^{5.5}$ U/mL 50% tissue culture-infective dose (multiplicity of infection of approximately 1) and incubated at 34°C in 5% CO₂.

Airway tolerance and allergic airway inflammation

Airway tolerance was induced, as previously described.^{E6,E7} Briefly, on days -10, -9, and -8, mice were exposed to 100 μ g of soluble OVA (Low Endo, Purified, ≤ 1 EU/mg endotoxin, Worthington Biochemical) in 20 μ L of PBS (also low endotoxin) or to PBS alone administered intranasally. On day 0, mice were sensitized by means of intraperitoneal injection of 20 μ g of OVA protein (chicken egg albumin; Sigma-Aldrich, St Louis, Mo) adsorbed to 4 mg alum (Pierce Chemical, Rockford, Ill) to test whether tolerance was induced. On days 14 to 17, mice were challenged each day intranasally with 20 μ g of OVA (Sigma) in 20 μ L of PBS and killed at day 18 for further analyses. Mice were infected once intranasally with 5×10^7 PFU/mL RV1B in 50 μ L at the time of initial OVA inhalation on day -10 to assess block of tolerance. In some experiments RV1B infection was performed on day 0. Two doses of anti-OX40L (250 μ g), anti-T1/ST2 (0.1 mg/mL; clone DJ8, MD Biosciences, San Jose, Calif),^{E8} or control rat immunoglobulin (Millipore, Billerica, Mass) were injected intraperitoneally on days -10 and -8, and this occurred 30 minutes before RV1B and/or OVA administration.

Bronchoalveolar lavage and lung histology

BAL was performed 24 hours after the last OVA challenge. BALF was examined for cytokine content by using ELISA. For cytologic examination, BALF cells were spun on a slide (Cytospin; Thermo Shandon, Pittsburgh, Pa), fixed, and stained with Protocol HEMA3 (Fisher Scientific Company, Pittsburgh, Pa). A differential cell count was performed on at least 250 cells in each cytospin slide. For lung histology examination, 5- to 6- μ m paraformaldehyde-fixed lung sections were stained with hematoxylin and eosin. Magnification $\times 200$ was used for histologic scoring, and at least 6 to 8 fields were scored to obtain the average for each mouse. The degree of bronchovascular inflammation was evaluated on a subjective scale of 0 to 9, as described previously.^{E9}

Adoptive transfer to track Treg cell development

Naive OVA-specific CD25⁻CD4⁺ cells were isolated from spleens and LNs of OT-II transgenic mice with CD4 T Cell Isolation Kits (Miltenyi Biotec, San Diego, Calif). Cell suspensions were incubated with the biotinylated

antibody cocktail and supplemented with biotinylated anti-mouse CD25, followed by magnetic anti-biotin microbeads and negative selection on LS MACS columns, according to the manufacturer's instructions. The CD4 purity was greater than 90%, with more than 95% of resulting cells expressing V α 2 and V β 5 transgenes and less than 0.1% Foxp3⁺ cells. A total of 5×10^6 OT-II CD4 cells were injected intravenously into B6.PL Thy1.1 congenic mice. The next day, mice were intranasally exposed to soluble OVA (Low Endo, Purified; ≤ 1 EU/mg) for 3 consecutive days in the presence or absence of RV1B (given at day 0 only). Five days after the initial exposure to OVA or rhinovirus, induction of Foxp3 expression was assessed in gated OT-II (Thy1.2) cells by means of intracellular flow cytometry, as described previously.^{E6,E7} At the same time, LNs or lung cells were assayed for OVA-specific production of IL-4 and IL-13 by using ELISPOT, as described below.

Flow cytometry

LNs or lungs from individual mice were homogenized and treated with red blood cell lysing buffer (Sigma) to track inducible Treg cells expressing Foxp3⁺ after adoptive transfer. After Fc block with the 2.4G2 mAb, cells were stained with anti-Thy1.2 (53-2.1-fluorescein isothiocyanate), anti-CD4 (RM4-5-peridinin chlorophyll protein complex), and anti-CD25 (7D4-allophycocyanin, BD Biosciences) and then intracellularly for Foxp3 (FJK-16s-phycoerythrin) with a kit from eBioscience (San Diego, Calif). Appropriate rat IgG (BD Biosciences, San Diego, Calif) was used as an isotype control. For OX40L expression, BAL cells were collected 24 hours after exposure to RV1B, blocked with the 2.4G2, and stained with biotinylated OX40L (RM134L), phycoerythrin-conjugated Siglec-F (E5-2440) from BD Biosciences, and CD11c-eFluor 450 (N418), Gr-1-APC (RB6-8C5), and CD11b-phycoerythrin cyanine7 (M1/70) from eBioscience. Appropriate rat IgG was used as isotype control. All samples were analyzed with FACS LSRII (BD Biosciences) and FlowJo software (Tree Star, Ashland, Ore).

ELISA and ELISPOT

Murine cytokines in BALF were assayed by means of ELISA with paired antibodies, according to the manufacturers' recommendations. IL-5 (TRFK4) and IL-13 (eBio13A and eBio1316H) ELISAs were obtained from eBioscience. The TSLP and IL-33 Duo ELISA set was purchased from R&D Systems (Minneapolis, Minn) and performed as per instructions. The detection limit for IL-5 and IL-13 was 3.9 pg/mL.

For ELISPOT, flat-bottomed 96-well nitrocellulose plates (Immobilon-P membrane, Millipore) were prewetted with 35% ethanol and rinsed with PBS. Then plates were coated with anti-mouse IL-4 (BVD4-1D11 and BVD6-24G2; BD Biosciences) or IL-13 (eBio13A and eBio1316H) mAb overnight at 4°C. The next day, plates were washed and blocked, and 1×10^6 LN or lung cells were cultured with medium or 10 μ g/mL class II-restricted OVA peptide, as described previously.^{E6,E7} After 48 hours, plates were washed with PBS plus 0.05% Tween-20 and then incubated with biotinylated anti-mouse IL-4 and IL-13 mAb for 2 hours at 37°C. After additional washes, spots were developed by means of incubation with 3-amino-9-ethylcarbazole solution (Sigma-Aldrich) and counted by using computer-assisted image analysis (Zeiss KS ELISPOT Reader, Jena, Germany).

RT-PCR

Lung tissue was homogenized with TISSUE MASTER (OMNI International). Total RNA was isolated with the TRIzol reagent (Life Technologies). An aliquot of total RNA (1 μ g) was reverse transcribed to cDNA by using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). Real-time PCR assay was carried out with LightCycler (Roche Diagnostics, Basel, Switzerland) by using LightCycler 480 SYBR Green I master (Roche Diagnostics) and RT-PCR-specific primers. Data are presented as normalized to ribosomal protein housekeeping gene L32. For NHBE cells, RNA was isolated by using the RNeasy Kit from Qiagen. Stimulated NHBE cells and PBECs were compared with unstimulated controls, and data were normalized to the housekeeping gene GAPDH. The mouse oligonucleotide primer sequences are as follows: OX40L forward,

5'-GGGATGCTTCTGTGCTTCATCT- 3'; OX40L reverse, 5'-TTTGGA TTGGAGGGTCCTTTG- 3'; IL-33 forward, 5'-TGCCATGTCTACTGCAT GAGA- 3'; IL-33 reverse, 5'-CCTAGAATCCCGTGGATAGGC- 3'; TSLP forward, 5'-CGACAGCATGGTCTTCTCA- 3'; TSLP reverse, 5'-CGATT TGCTCGAACTTAGCC- 3'; L32 forward, 5'-GAAACTGGCGGAAAC CCA- 3'; and L32 reverse, 5'-GGATCTGGCCCTTGAACCTT- 3'. The human oligonucleotide primer sequences are as follows: IL-33 forward, 5'-GGAGTGCTTTGCCTTTGGTA- 3'; IL-33 reverse, 5'-TCATTTGAGGG GTGTTGAGA- 3'; TSLP forward, 5'-CCCAGGCTATTCGGAAGTCAAG- 3'; TSLP reverse, 5'-CGCCACAATCCTTGTAATTGTG- 3'; GAPDH forward, 5'-TGATGACATCAAGAAGGTGGTGAAG- 3'; and GAPDH reverse, 5'-TCCTTGGAGGCCATGTAGGCCAT- 3'.

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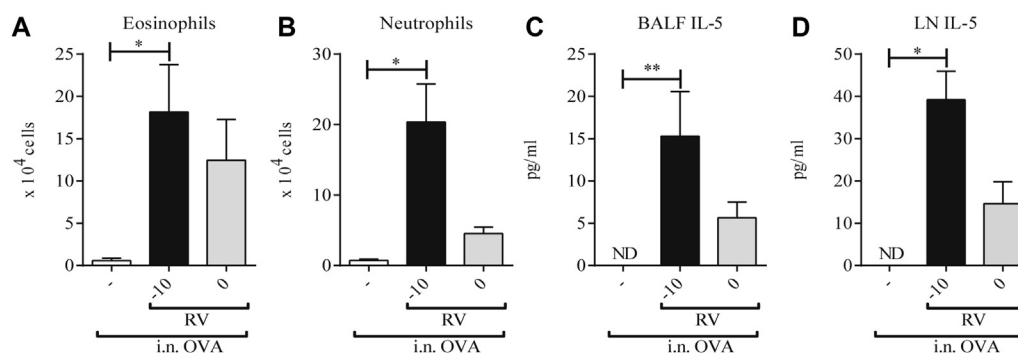


FIG E1. Effect of rhinovirus infection after tolerization. Wild-type mice were administered soluble OVA intranasally on days -10 to -8 and infected with RV1B either at day -10 or day 0 and sensitized and challenged as described in Fig 1, A. **A** and **B**, BAL eosinophils and neutrophils. **C** and **D**, BALF and LN IL-5 levels. Results are means $\pm$ SEMs from 5 to 8 mice per group, and data were pooled from 2 independent experiments. *i.n.*, Intranasal; *ND*, not determined; *RV*, rhinovirus. * $P < .05$ and ** $P < .01$.

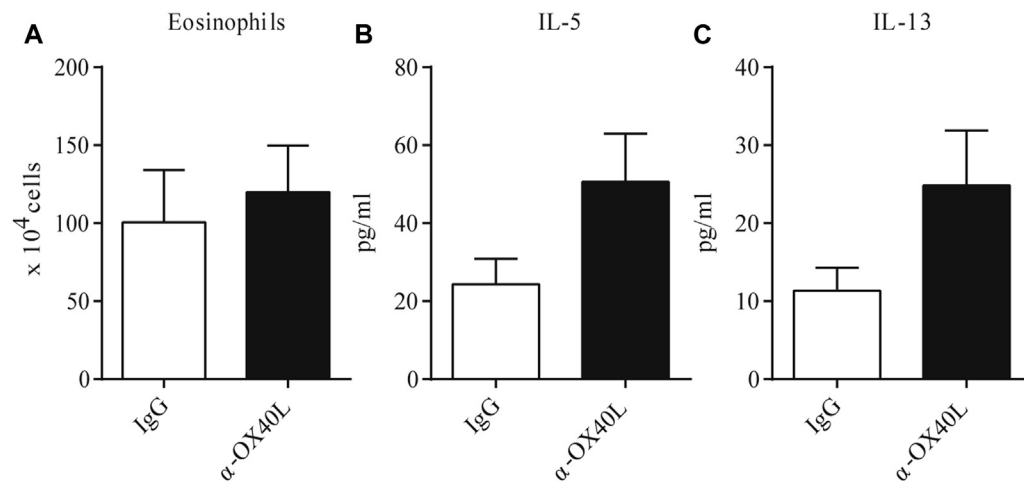


FIG E2. Effect of OX40L antibody without tolerance. Wild-type mice were administered α -OX40L or control antibody without RV1B infection or soluble intranasal OVA but sensitized 10 days later and challenged as described in Fig 1, A. **A**, BAL eosinophils. **B** and **C**, IL-5 and IL-13 in BALF. Results are means $\pm$ SEMs from 6 to 7 mice per group, and data were pooled from 2 independent experiments.

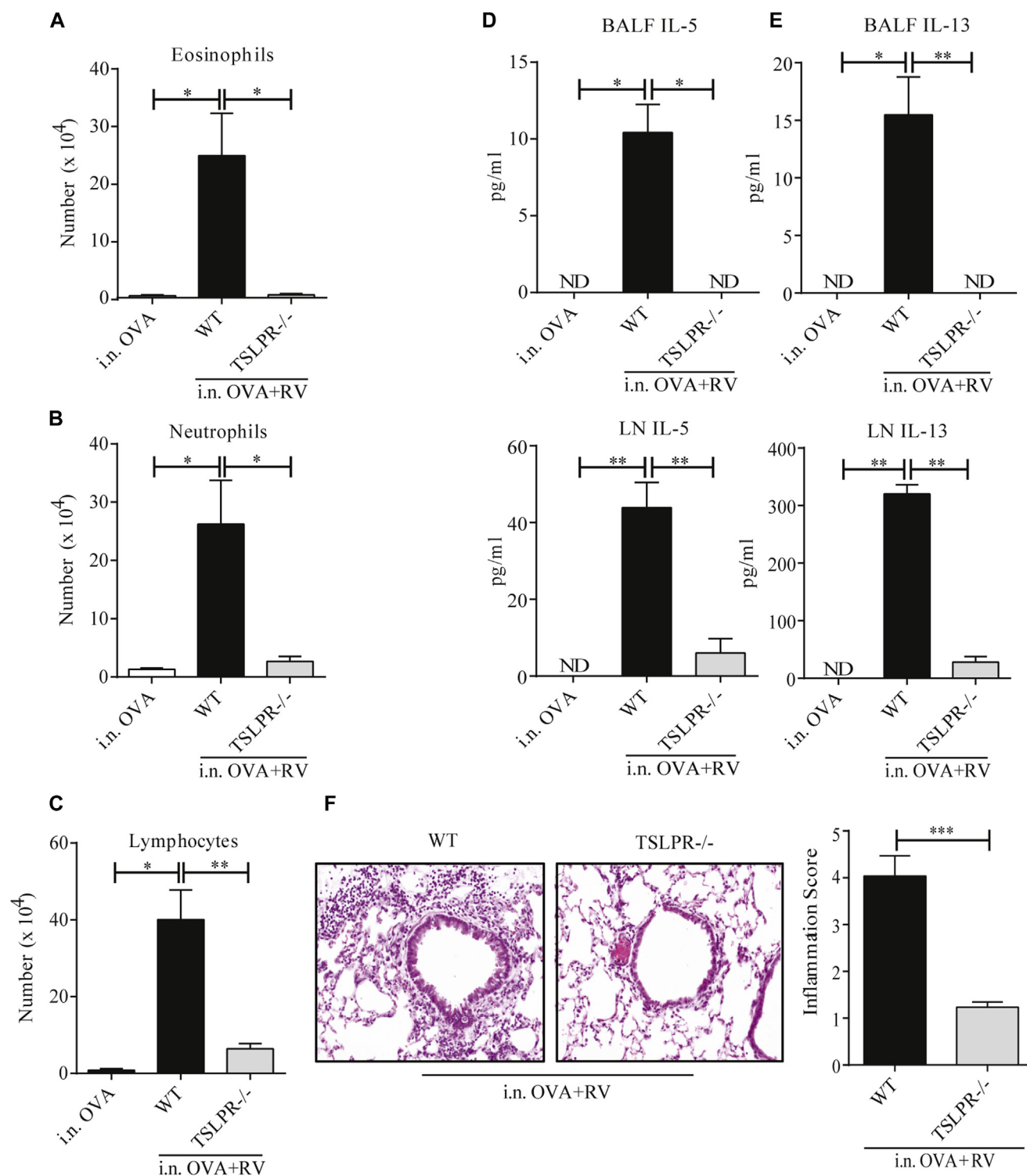


FIG E3. TSLP is required for rhinovirus to inhibit airway tolerance. Wild-type and TSLPR^{-/-} mice were tolerized, sensitized, and challenged as in Fig 1, A, to assess lung inflammation. **A-C**, BAL eosinophils, neutrophils, and lymphocytes. **D** and **E**, IL-5 and IL-13, respectively, in BALF (upper) and LN culture supernatant (lower). **F**, Hematoxylin and eosin-stained lung sections (left) and inflammation score (right). Results are means $\pm$ SEMs from 4 mice per group and representative of 2 to 3 independent experiments. i.n., Intranasal; ND, not determined; RV, rhinovirus. * $P < .05$, ** $P < .01$, and *** $P < .001$.

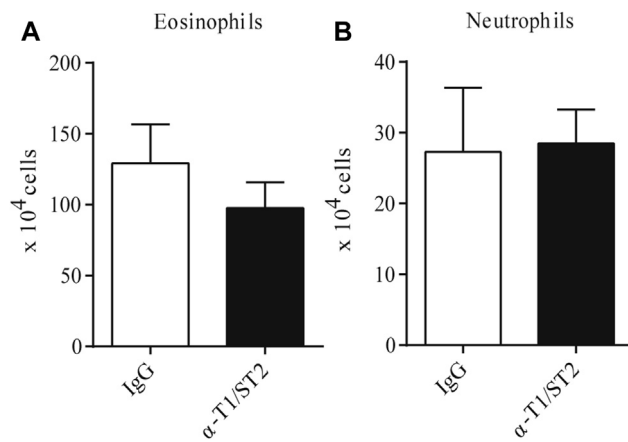


FIG E4. Effect of α -T1/ST2 antibody without tolerance. Wild-type mice were given α -T1/ST2 or control antibody without RV1B infection or soluble intranasal OVA but sensitized 10 days later and challenged as described in Fig 1, A. **A** and **B**, BAL eosinophils and neutrophils. Results are means $\pm$ SEMs from 6 to 7 mice per group, and data were pooled from 2 independent experiments.