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Rhinovirus Infection Interferes with the Induction of Tolerance to Aeroantigens through OX40L, TSLP and IL-33

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Abstract

Background—Rhinovirus (RV) infection during an early age has been associated with development of asthma, but how RV influences the immune response is not clear.

Objective—Tolerance to inhaled antigen is mediated via the induction of regulatory T cells (Treg), and we asked whether RV infection of the respiratory tract might block airway tolerance by modulating Treg cells.

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

Results—RV1B infection of mice abrogated tolerance induced by inhalation of soluble OVA, suppressing the normal generation of $Foxp3^+$ Treg cells while promoting Th2 cells. Furthermore, RV1B infection led to susceptibility to develop asthmatic lung disease when mice subsequently reencountered aeroantigen. RV1B promoted early *in vivo* expression of the TNF family protein, OX40L, 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 Th2 differentiation, and

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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 RV can antagonize tolerance to inhaled antigen through a combined induction of TSLP, IL-33 and OX40L, and this may lead to susceptibility to developing asthmatic lung inflammation.

Keywords

Asthma; epithelial cell; OX40L; RV1B; RV16; TSLP; pTreg

INTRODUCTION

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 (RV) 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(s) by which RV promotes or enhances allergic or asthmatic reactions is not clear and is the subject of great interest.

RV 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 ICAM-1, whereas the minor group viruses, such as RV1B and RV2, bind to low-density lipoprotein receptors (LDLR) [9]. Rhinovirus mainly infects and replicates inside airway epithelial cells [5, 10], suggesting factors produced by epithelial cells that influence the immune response may explain why RV 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, RV infection also has the potential to alter this natural balance.

Recently, we have made strong progress in understanding how tolerance and regulatory T cells (Treg) are promoted in the lung and how allergens or pathogen-derived molecules might antagonize tolerance and suppress Treg cells [11–14]. In particular, several pieces of evidence suggest that induction of OX40L (TNFSF4), a TNF family ligand expressed on antigen-presenting cells (APCs), may be critical for promoting the initial differentiation of Th2 cells, as well as expanding existing Th2 cells, through signals from its receptor OX40 (TNFRSF4/CD134) [15–18]. Concomitantly, OX40-OX40L activity can antagonize the generation of Treg cells that mediate airway tolerance [11, 12, 19]. As OX40L can be induced by the bronchial epithelial-derived cytokine TSLP [12, 20, 21], we hypothesized that RV infection might promote this pathway and this could be a mechanism by 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 via suppressing the generation of inducible Foxp3$^+$ peripheral regulatory CD4 T cells (pTreg). The combined activity of TSLP and OX40L did not however fully explain the effect of RV, and we also found another innate cytokine, IL-33, was involved. Neutralizing or deleting TSLPR, IL-33R, or OX40L prevented the block of tolerance induced by RV and led to reduced susceptibility to develop allergic airway disease. In line with this being a likely mechanism applicable to human RV-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 (PBEC). These results suggest that respiratory tract exposure to RV can promote a program of inflammatory molecules that includes OX40L, and neutralizing OX40-OX40L interactions together with TSLP and/or IL-33 might be applicable to controlling asthma exacerbations that are driven by RV or other similar viruses.

METHODS

See Methods section in the 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 TCR transgenic and TSLPR knockout mice bred in-house as described in online repository.

RV Generation and Use

RV1B was generated as described[22] . Mice were infected i.n. with 5×10^7 plaque forming units (PFU) per ml of RV1B in 50 μl with or without OVA. Human RV-16 was propagated as described[23] . Bronchial epithelial cells were exposed to HRV-16 at $10^{5.5}$ 50% tissue culture-infective dose ($TCID_{50}$) U/ml (MOI of ~1) [24].

Airway tolerance and allergic airway inflammation

Airway tolerance was induced with i.n. OVA as before [11,12] and described in the online section. Briefly, mice were exposed to 100 μg soluble OVA (Low Endo™, Purified; ≤ 1 EU/mg, Worthington Biochemical Corp, Lakewood, NJ) for 3 consecutive days (day −10 to −8). Rhinovirus was given i.n. at day −10 with antigen. Mice were subsequently sensitized with OVA/alum i.p, and challenged later with 20 μg 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. BAL fluid (BALF) was examined for cytokine content by ELISA. For lung histology, 5–6 μm sections were stained with hematoxylin and eosin (H & E). Magnification ×200 was used for histologic scoring, and at least 6–8 fields were scored to obtain the average for each mouse.

Adoptive transfer to track Treg 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, CA). 5×10^6

OT-II cells were injected i.v. into B6.PL Thy1.1 congenic mice and then 1 day later mice were exposed to soluble OVA, or OVA with RV1B, given i.n. After 5 days, Foxp3 expression was assessed in gated OT-II cells by flow cytometry as described in online repository.

RT-PCR

Total RNA was isolated from lung tissue or PBEC using TRIzol reagent (Life Technologies, NY) and from NHBE using RNeasy Kit from Qiagen (Valencia, CA). RNA was reverse transcribed to cDNA by using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Corporation, Indianapolis, IN). Data are presented as normalized to ribosomal protein housekeeping gene L32 or GAPDH.

ELISA and ELISPOT

Murine cytokines in BALF were assayed by ELISA by using paired antibodies according to the manufacturers' recommendations. ELISPOT assay for IL-4 and IL-13-secreting cells in lymph nodes (LN) was performed at the time of analysis of Foxp3 expression, as described before [11, 12].

Statistical analyses

Statistical analyses were performed with GraphPad Prism software (La Jolla, CA). Data are expressed as means ± SEMs and were analyzed by using student t test and one-way ANOVA with bonferroni *post-hoc* tests where applicable. $*P < 0.05$.

RESULTS

Rhinovirus infection prevents airway tolerance and initiates Th2 lung inflammation

Recent studies have shown that the minor group RV serotype, RV1B, that infects both humans and mice by binding to LDLR, induces airway neutrophilic inflammation and airway hyper-responsiveness in mice reminiscent of the response in humans, thereby providing a suitable model to study how RV affects adaptive immunity to model allergens [25, 26]. Of particular interest is whether RV infection might antagonize the development of tolerance in the lungs to inhaled protein antigens. To address this, mice were exposed i.n. to pure soluble OVA protein as a nominal innocuous antigen for three successive days. These are the conditions that we have shown results in the conversion of antigen-reactive naïve T cells into peripheral iTreg 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 i.n. OVA [11, 12] (see protocol Fig. 1, *A*). 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 Th2 allergic airway disease upon 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 elevated levels of Th2 cytokines in BALF (Fig 1, *F* and *G*; upper panel) and a Th2 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 Th2 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 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 to mice that were challenged with OVA (Fig 1, *H*). Thus, RV infection abrogated the induction of airway tolerance to inhaled protein antigen, which resulted in susceptibility to mount a Th2-biased lung inflammatory response upon re-encounter with the inhaled antigen.

We furthermore observed that rhinovirus infection had a small effect when inhaled by mice that had already been tolerized, albeit 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 LN (see Fig E1 in this article's Online Repository at www.jacionline.org and data not shown). However, this was not statistically significant compared to controls or compared to the response in mice receiving RV1B at the time of tolerization. This suggests that RV likely exerts its major effect at the time of establishing tolerance. However, as some activity was evident, these data lead to the hypothesis that RV 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 TLR or protease activity.

Rhinovirus infection antagonizes airway tolerance through OX40L

We then pursued the critical molecules that RV infection can promote 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 Th2-driven airway inflammation [15, 16]. We also found that OX40L is induced by signaling through the pattern recognition receptors TLR4 and Nod2 in the lung resulting in a loss of tolerance [11, 12]. OX40L is not constitutively expressed, but suggesting RV 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*). To examine if OX40L was active, mice were treated with blocking antibody once at the time of i.n. exposure to antigen and RV1B. 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, albeit not completely, inhibited the OVA-induced airway eosinophilia and neutrophilia, bronchovascular inflammation, and Th2 cytokine response (Fig 2, *C-F*). The dose of the antibody was chosen to ensure that OX40L was only blocked within a 5–7 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, Th2 cytokines, and other

hallmarks of inflammation (see Fig E2 in this article's Online Repository at www.jacionline.org and data not shown). Thus, RV led to susceptibility to developing antigen-induced airway inflammation via the OX40-OX40L pathway.

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

We previously reported that Foxp3$^+$ pTreg cells are part of the tolerogenic mechanism that results upon inhalation of soluble antigen and that OX40 signaling can inhibit the generation of these pTreg cells [11, 12, 19]. We therefore tracked their generation by adoptive transfer of naive (CD25-Foxp3-) Thy1.2$^+$ OVA-specific OT-II TCR transgenic CD4 T cells into Thy1.1 recipients, assessing conversion by staining for Foxp3 expression [11, 12]. Flow analysis demonstrated that ~3–4% of OT-II CD4 cells converted into Foxp3$^+$ cells *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 following the initial exposure to soluble OVA, both in the lung draining LN (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 or IL-13-secreting cells was approximately 250:1 and 500:1, respectively, under tolerizing conditions. In contrast, upon 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 the generation of pTreg cells (Fig 3, *A*) and also reduced the development of IL-4 and IL-13-secreting T cells by ~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 DCs to induce Th2 differentiation [29, 30] through the induction of OX40L [20]. As RV 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 TSLP receptor-deficient mice were given soluble i.n. OVA and infected with RV1B, and then sensitized with OVA i.p. in alum 10 days later and subsequently challenged with OVA (protocol Fig. 1a), 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 Th2 inflammatory response to OVA when it is given in alum i.p. [12, 31]. We therefore assessed early parameters in TSLPR-deficient mice, before alum sensitization, to determine if TSLP was involved in the induction of OX40L by RV1B, and suppression of the development of pTreg cells. Notably, RV1B failed to promote OX40L expression in the lungs of TSLPR-deficient mice as

assessed at 5 hours post 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 i.n. OVA (Fig 4, *D* and *E*). This resulted in a less pronounced shift in the Treg/Th2 cell balance, in favor of the tolerogenic response (Fig 4, *F*). These data suggest that RV exposure induces TSLP that is active in regulating OX40L expression on APCs and this process then in part contributes to blocking the development of a suppressive environment and tolerance. In the pTreg cell analysis, the induction of Foxp3 in WT OT-II cells was assessed in TSLPR−/− mice, suggesting that TSLP activity was required on cells other than the responding T cells, 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 naïve T cells [43].

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

As blocking OX40L or TSLPR did not completely restore a state of tolerance in the context of RV exposure, 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 [32]. IL-33 might have several functions and targets, but interestingly was described to promote DC maturation that led to the differentiation of Th2 cells [33]. 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 RV as the antibody given to a naïve mouse 10 days before OVA sensitization and challenge have 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, RV 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 are upregulated in airway epithelial cells after rhinovirus infection

To provide clinical significance, we found that RV16, a major group RV, also induced TSLP mRNA *in vitro* in a time dependent manner in NHBE cells, although interestingly TSLP was not induced in PBEC obtained from non-transplanted normal human lungs (Fig 7, *A* and *B*). This correlated with prior data that also described TSLP expression in NHBE cells infected with RV16 [34]. Corresponding to the idea that IL-33 was also derived from bronchial epithelial cells, we found a time dependent increase of IL-33 mRNA in PBEC 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 PBEC (data not shown). Thus, rhinovirus infection of the lung epithelium can antagonize airway tolerance to inhaled antigen through the combined induction of TSLP and IL-33.

DISCUSSION

Rhinovirus infections can contribute to asthma and COPD exacerbations [9], but whether RVs can initiate Th2-driven inflammation has not been clear. RV infects epithelium in both the upper and lower respiratory tract [9, 35], and RV-induced exacerbations in asthmatics have been linked to defective expression of protective type I and III interferons in epithelial cells [36, 37]. However, epithelial cells are capable of producing many inflammatory mediators [35] after stimulation of pattern recognition receptors [38, 39], which may be critical for leading to Th2-biased disease. We now show in an experimental animal model that RV 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 developing 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, 40]. 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 Th2 cell development through signals transmitted from its receptor OX40 that is expressed on antigen-stimulated T cells [11, 12]. Our studies here show that RV can promote the same OX40L-dependent mechanism leading to susceptibility to developing allergic lung inflammation. RV may target TLR3 and/or RIG-I and MDA-5, suggesting a similar cascade of molecular events may be downstream of several pattern recognition receptors.

OX40L can be directly induced in APCs by TLR signals [18], however it was likely that RV induction of OX40L was indirect. Innate cytokines, particularly TSLP [12, 20], that are mostly the product of epithelial and fibroblast cells, have been reported to participate in promoting OX40L expression in DCs. Thus, cross-talk between RV-infected epithelial cells and DCs may be central to blocking airway tolerance and driving CD4 T cells into the Th2 pathway. In line with this, we found that RV1B infection induced lung expression of TSLP in mice, and also that RV16 induced TSLP 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 Th2 differentiation [30, 41, 42], but recently injection of TSLP *in vivo* was also shown to antagonize airway tolerance against OVA and inhibit the generation of pTreg cells [43] 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 RV. TSLP may have both direct and indirect activities on T cells [43, 44], however, with RV here, and in our previous study *in vivo* with pattern recognition receptor ligands, we found

OX40L expression in $CD11c^+$ DCs in the lung was dependent on TSLP. Moreover, much of the activity of RV in suppressing naïve T cell conversion into pTreg cells and promoting IL-4-secreting effector T cells was lost when wild-type (TSLPR+) transgenic T cells were responding in TSLPR-deficient hosts. This therefore supports the idea that the primary action of TSLP in these scenarios is mediated by cross talk with APCs, albeit 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, 42], and when house dust mite (HDM) extract was used as the allergen [45]. Additionally, a recent clinical study [46], supported these data and found that anti-TSLP reduced allergen-induced bronchoconstriction and eosinophilia in mild allergic asthmatics, including patients allergic to HDM, cat dander, grass pollen, ragweed, horse, and *alternaria*. Thus, TSLP may be central to many allergic/type 2 responses that develop in the lungs. Surprisingly, one report found that TSLP was not important for airway inflammation, also with HDM as the antigen [47], in this case using a protocol of administration that was markedly shorter (10 days versus 32 days) than in the aforementioned study where 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 Th2 immunity [47]. In our study here with RV infection, IL-33 was active, but it synergized with TSLP in controlling the Treg/Th2 cell balance. Epithelial cells in the lung are again the most likely candidates for producing IL-33 [48, 49] and we showed that RV infection could lead to production of IL-33 in primary epithelial cell cultures. Interestingly RV infection did not induce IL-33 from NHBE cells, contrasting with TSLP that 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 RV to induce OX40L expression in the lung, contrasting with the study described above where HDM-driven OX40L was IL-33 dependent in a system where 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. Likely, 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 the pTreg cell balance. IL-33 can stimulate mast cells, basophils, iNKT, ILC2, and NK cells [50–53] but has been suggested to contribute directly to differentiation of naive CD4 T cells into Th2 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 RV can alter the respiratory environment to disrupt the normal ability to induce T cell unresponsiveness. RV infection induces TSLP and IL-33 expression, and TSLP-dependent upregulation of OX40L, and all

three of these molecules contribute to the development of an adaptive immune response that is characterized by an altered balance between suppressive pTreg cells and Th2 effector cells. This may be a common response of viruses associated with allergic disease as 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, may represent a potential strategy for limiting the effects of these respiratory viruses in contributing to asthma and allergic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

APCs	Antigen presenting cells
BAL	Bronchoalveolar lavage
DC	Dendritic cell
Foxp3	Forkhead box protein 3
H & E	Hematoxylin and eosin
I.n.	intranasal
LN	Lymph node
NHBE	Normal human bronchial epithelial
OVA	Ovalbumin
OX40L	OX40 ligand
PBEC	Primary bronchial epithelial cell
RV	Rhinovirus
pTreg	peripherally-induced Regulatory T cell
TSLP	Thymic stromal lymphopoietin
WT	Wild-type

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Key Messages

1. RV1B infection initiates Th2 responses in the lung and antagonizes $Foxp3^+$ Treg cells and induction of tolerance.
2. RV1B induces IL-33 and TSLP-dependent OX40L in the lung.
3. RV16 upregulates TSLP and IL-33 in human lung epithelial cells.

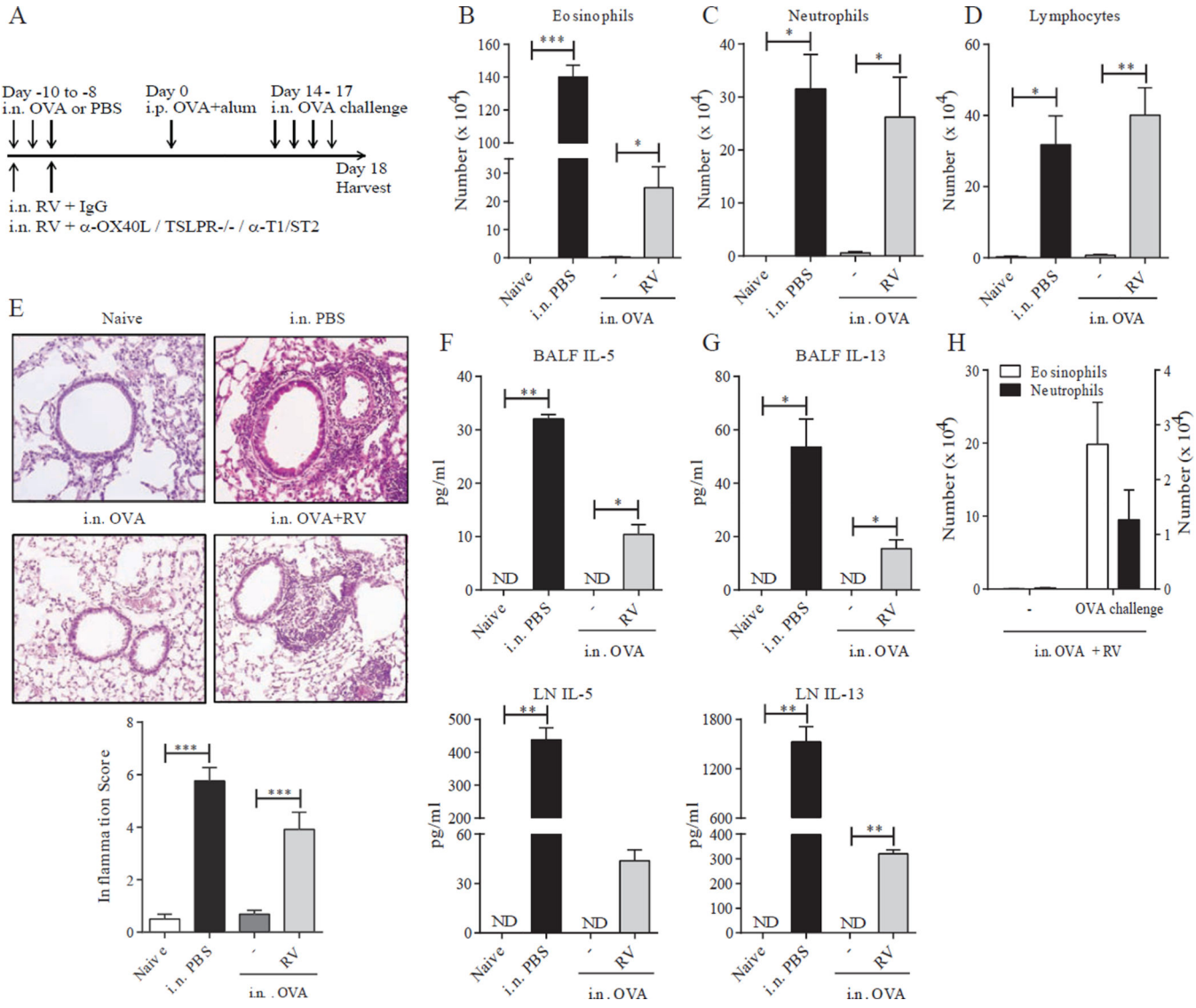

FIG 1.

Rhinovirus blocks induction of tolerance to inhaled antigen. **A**, Schematic of experimental protocols. Mice were tolerized with i.n. OVA, with or without RV1B. Non-tolerized mice received PBS i.n. All mice were sensitized with OVA/alum i.p. on day 10, and challenged with i.n. OVA 2 weeks later to assess lung inflammation. **B–D**, BAL eosinophils, neutrophils, and lymphocytes. **E**, H & E lung sections (upper) and inflammation score (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 i.n. OVA with RV that were not challenged or challenged with OVA. Results are means ± SEM from 4 mice/group, and representative of 3 experiments.

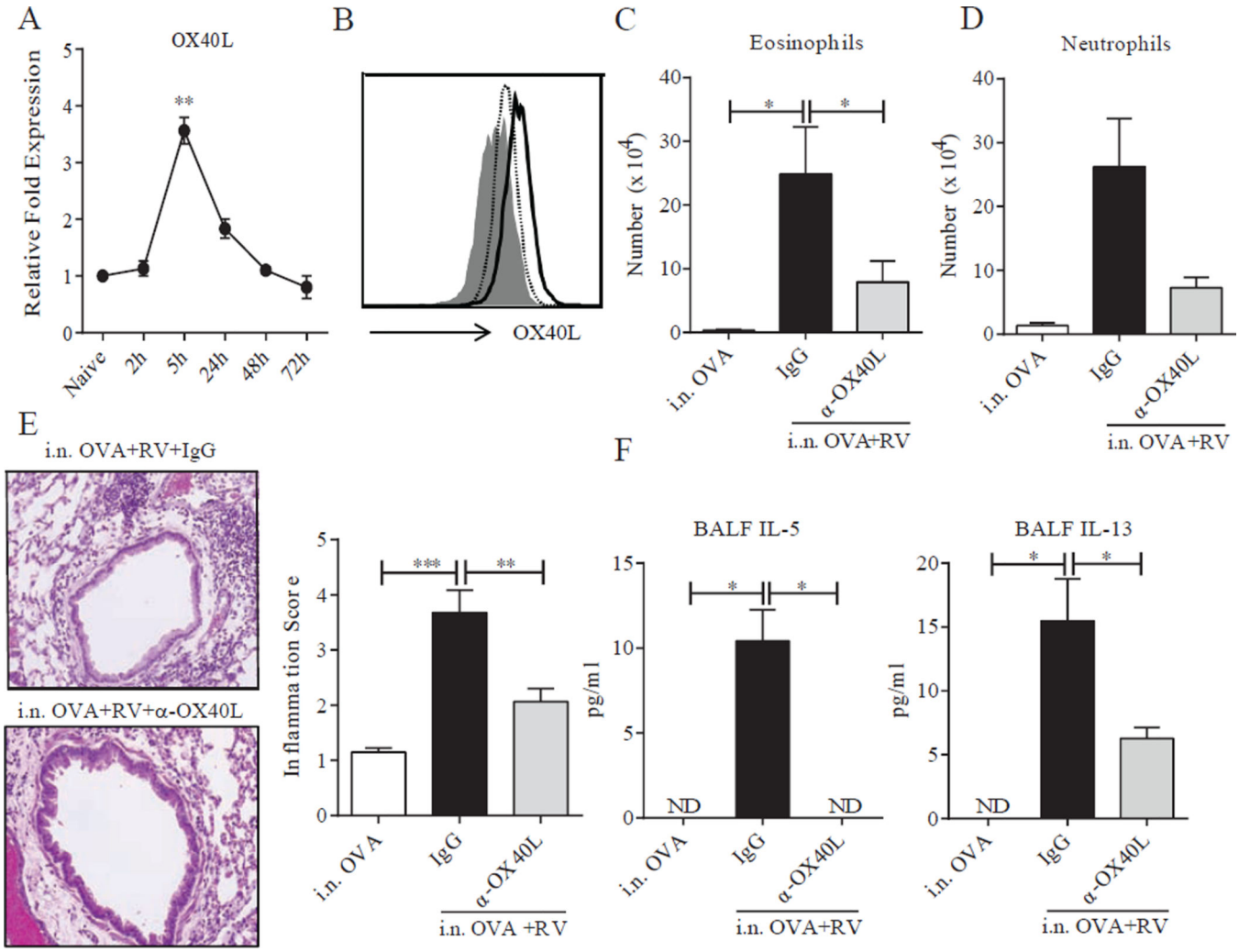

FIG 2.

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), naïve (dashed) and RV1B (solid black). **C–F**, WT mice were tolerized, sensitized and challenged as in Fig 1A. Anti-OX40L or control antibody were given i.p. at the time of RV1B infection. **CD**, BAL eosinophils and neutrophils. **E**, H & E lung sections (left) and inflammation score (right). **F**, BALF IL-5 and IL-13. Results are means ± SEM from 3–4 mice/group and representative of 3 experiments.

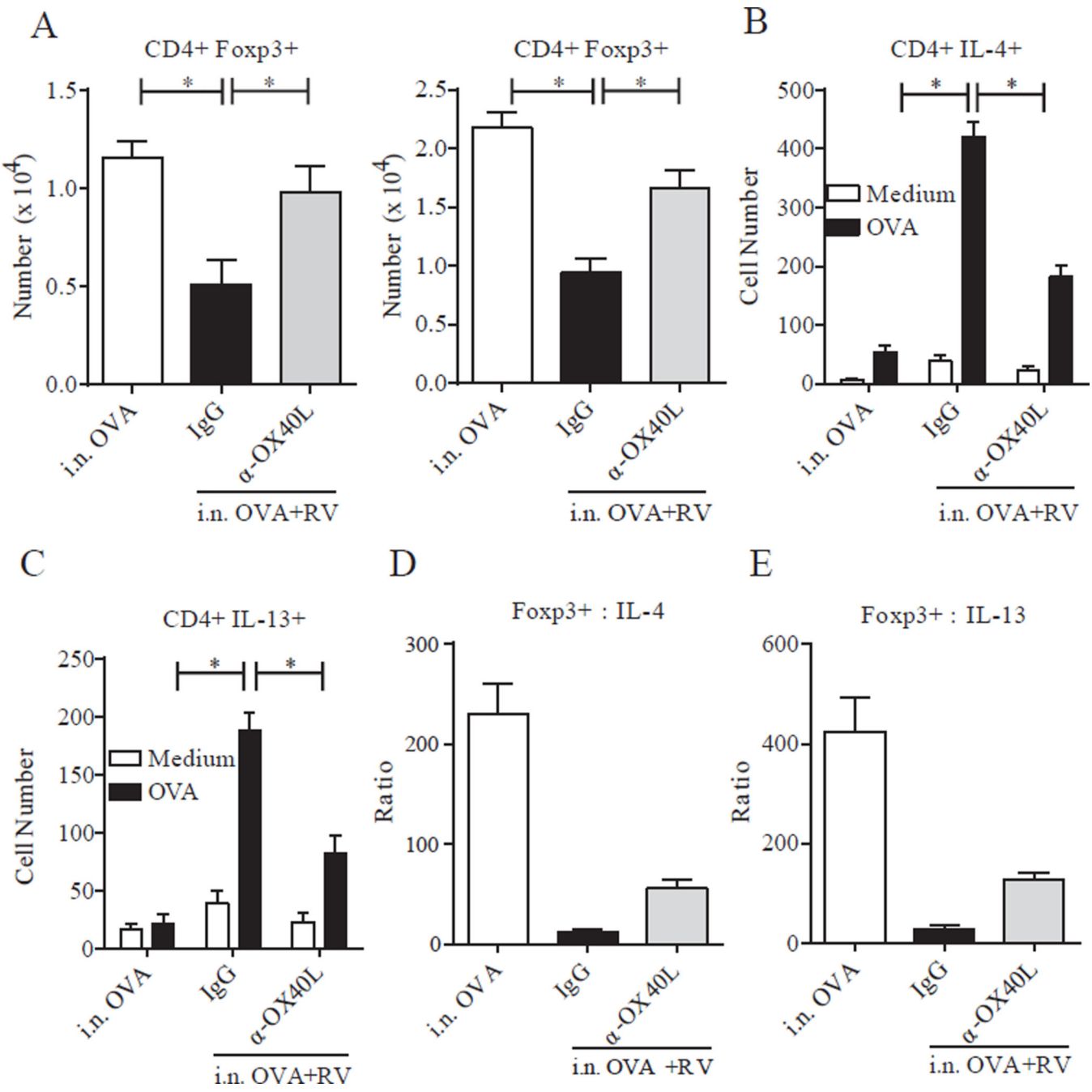

FIG 3.

Rhinovirus suppresses the generation of OVA-specific Foxp3$^+$ T cells through OX40L. **A–E**, OVA-specific OT-II T cells were transferred into congenic mice, that were then tolerized with OVA given i.n. 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 LN (left) and lung (right) by 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 LN by ELISPOT at the same time. **D and E**, Ratio of Foxp3$^+$ and IL-4 and

IL-13-secreting CD4 T cells. Results are means ± SEM from 3–4 mice/group and representative of 2 experiments.

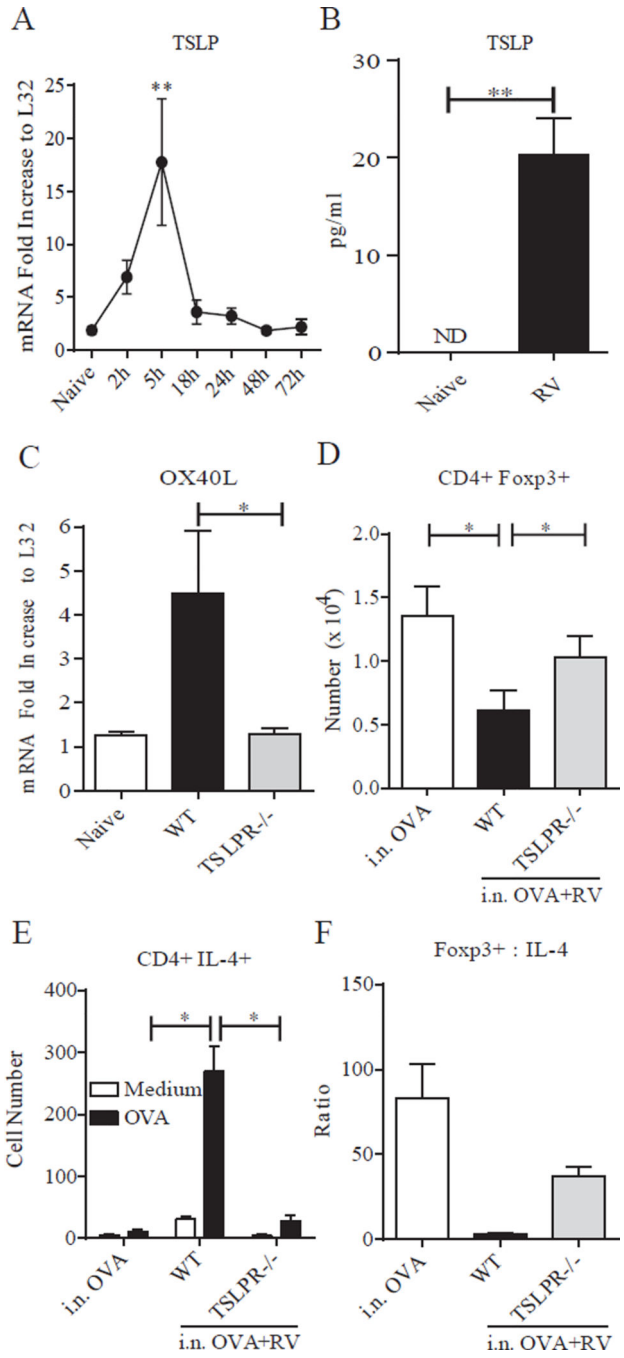

FIG 4.

TSLP is required for rhinovirus to modulate the lung tolerogenic environment. **A**, TSLP mRNA in lung tissue after RV1B infection over 72h. **B**, TSLP protein in BALF after 24 hours. **C**, OX40L mRNA in lung tissue after RV1B infection of WT or TSLPR−/− mice at 5h. **D–F**, OT-II T cells were transferred into WT or TSLPR−/− mice as in Fig. 3. Recipients were tolerized with OVA and infected with RV1B as before. **D**, Number of Foxp3^{+} OT-II T cells in LN, 5 days after OVA inhalation. **E**, Number of IL-4-secreting CD4 T cells in LN.

F, Ratio of Foxp3$^+$ and IL-4-secreting CD4 T cells in LN. Results are means ± SEM from 3–4 mice/group and representative of 2 experiments.

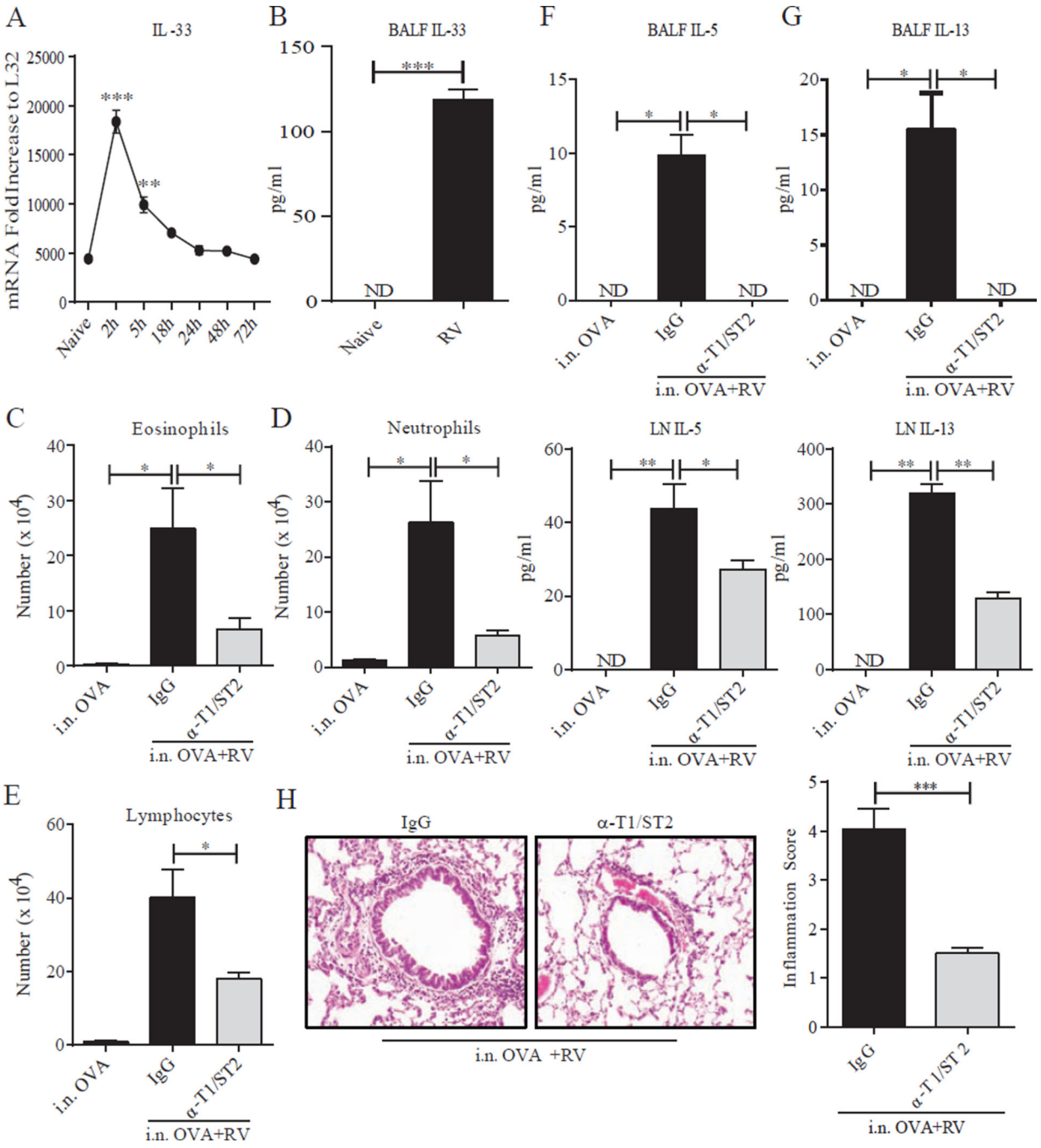

FIG 5.

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–G**, WT mice were tolerized, sensitized and challenged as in Fig. 1A, to assess lung inflammation. Anti-T1/ST2, or control antibody, were given i.p. at the time of RV1B infection. **C–E**, BAL eosinophils, neutrophils, and lymphocytes. **F and G**, IL-5 and IL-13 in BALF (upper) and LN culture supernatant (lower). **H**, H & E lung sections (left) and inflammation score

(right). Results are means ± SEM from 4 mice/group and representative of 2–3 independent experiments.

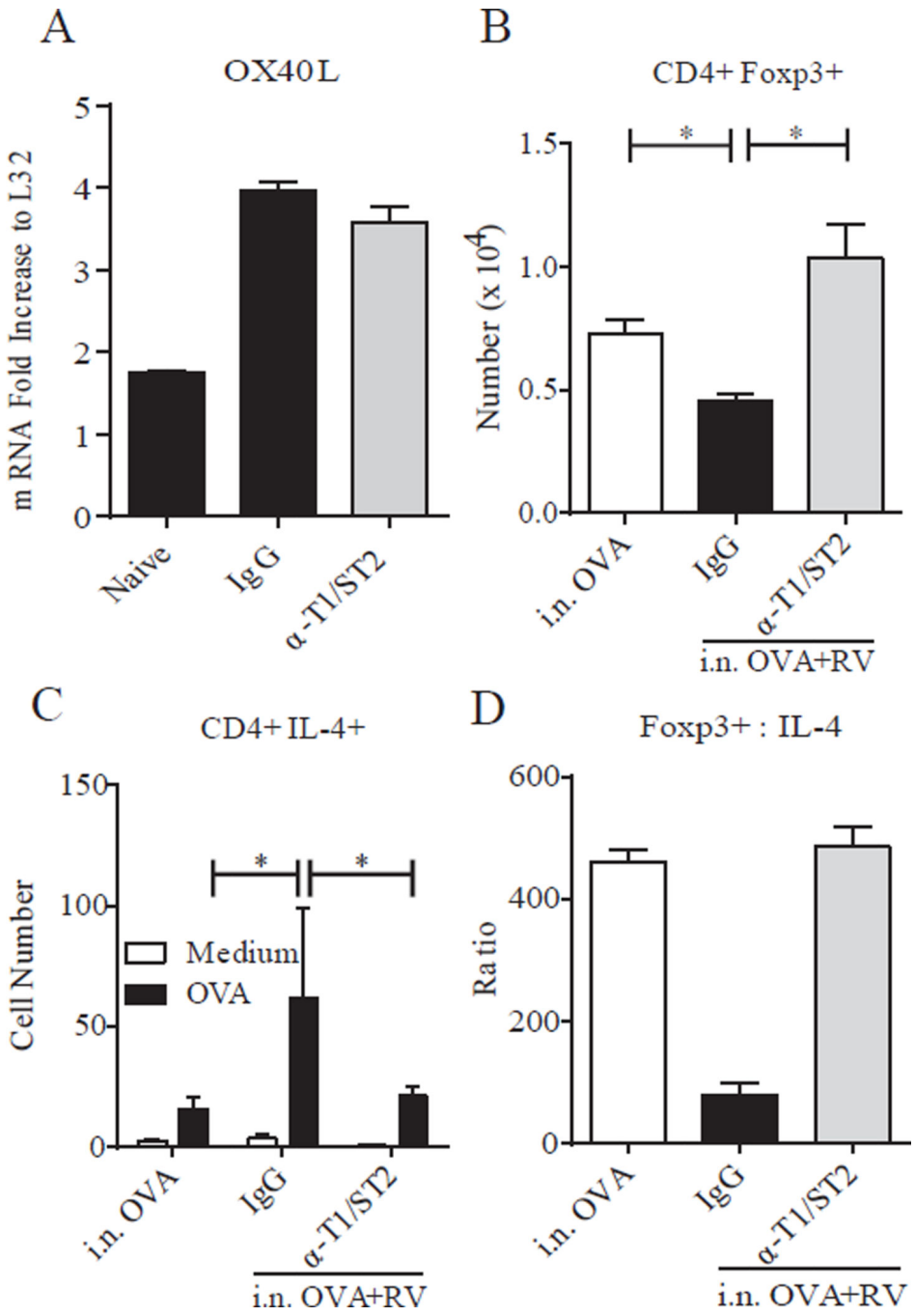

FIG 6.

IL-33 is required for rhinovirus to modulate the Treg/Th2 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. **B**, Number of $Foxp3^+$ OT-II T cells enumerated in LN by flow cytometry, 5 days after OVA inhalation. **C**, Number of IL-4-secreting CD4 T cells in LN by ELISPOT. **D**, Ratio of $Foxp3^+$ to IL-4-secreting CD4 T cells. Results are means ± SEM from 3–4 mice/group and representative of 2 independent experiments.

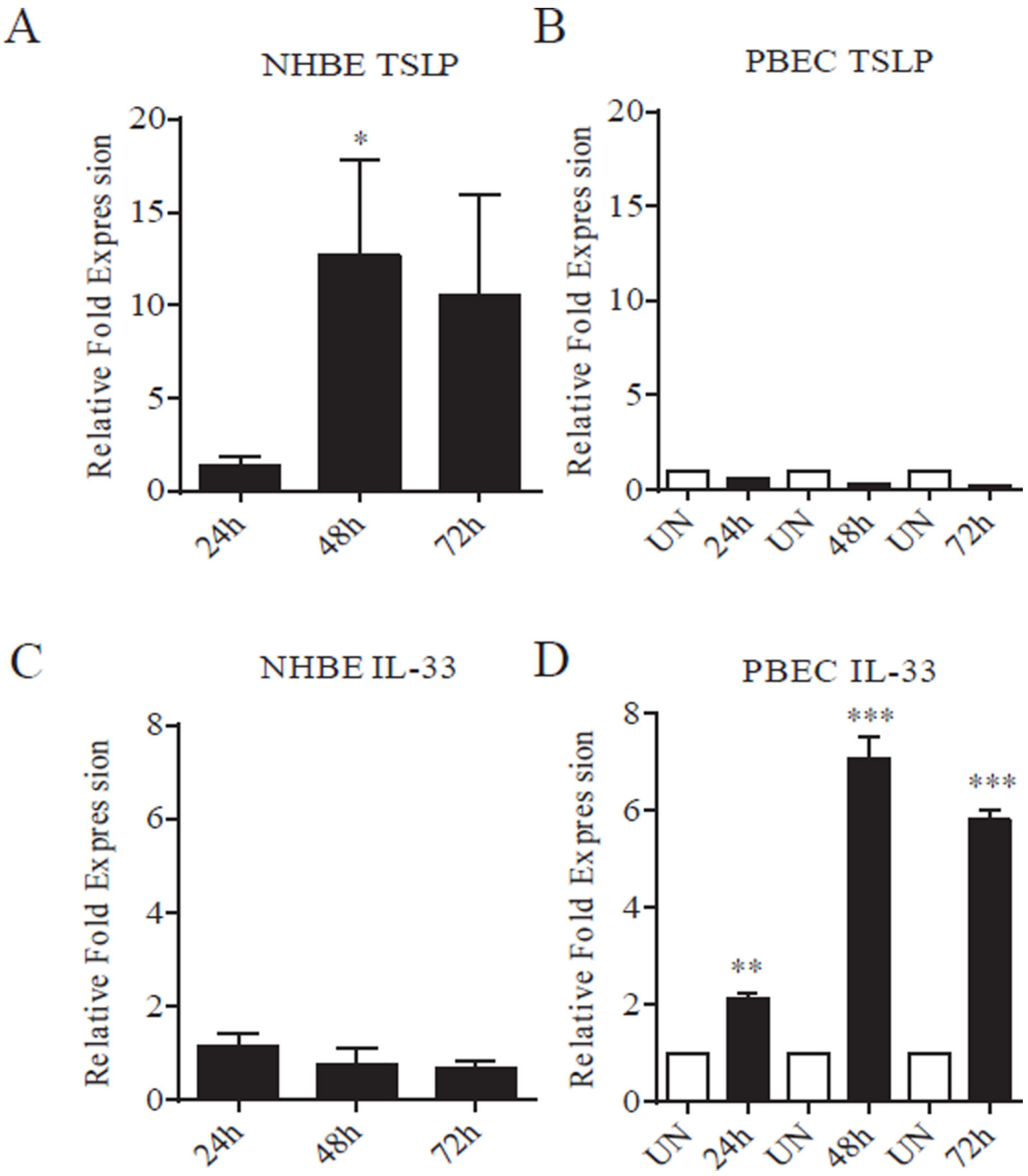

FIG 7.

TSLP and IL-33 are upregulated in human airway epithelial cells by rhinovirus. **A–B**, TSLP mRNA and **C–D**, IL-33 mRNA from 24–72 hours in NHBE and PBEC after RV16 infection *in vitro*, compared to unstimulated controls. Results are means ± SEM from 3 independent experiments with cells from a single donor for NHBE, and means ± SEM from cells derived from 3 different donors for PBEC. PBEC results are representative of 2 experiments.