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TSLP and IL-33 reciprocally promote each other's lung protein expression and ILC2 receptor expression to enhance innate type-2 airway inflammation

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Abstract

Background: The epithelial cell-derived danger signal mediators thymic stromal lymphopoietin (TSLP) and IL-33 are consistently associated with adaptive Th2 immune responses in asthma. In addition, TSLP and IL-33 synergistically promoted group 2 innate lymphoid cell (ILC2) activation to induce innate allergic inflammation. However, the mechanism of this synergistic ILC2 activation is unknown.

Methods: BALB/c WT and TSLP receptor-deficient (TSLPR^{-/-}) mice were challenged intranasally with *Alternaria* extract (Alt-Ext) or PBS for 4 consecutive days to evaluate innate airway allergic inflammation. WT mice pre-administered with rTSLP or vehicle, TSLPR^{-/-} mice, and IL-33 receptor-deficient (ST2^{-/-}) mice were challenged intranasally with Alt-Ext or vehicle once or twice to evaluate IL-33 release and TSLP expression in the lung. TSLPR and ST2 expression on lung ILC2 were measured by flow cytometry after treatment of rTSLP, rIL-33, rTSLP + rIL-33, or vehicle.

Results: Thymic stromal lymphopoietin receptor deficient mice had significantly decreased the number of lung ILC2 expressing IL-5 and IL-13 following Alt-Ext-challenge compared to WT mice. Further, eosinophilia, protein level of lung IL-4, IL-5, and IL-13, and airway mucus score were also significantly decreased in TSLPR^{-/-} mice compared to WT mice. Endogenous and exogenous TSLP increased Alt-Ext-induced IL-33 release into BALF, and ST2 deficiency decreased Alt-Ext-induced TSLP expression in the lung. Further, rTSLP and rIL-33 treatment reciprocally increased each other's receptor expression on lung ILC2 in vivo and in vitro.

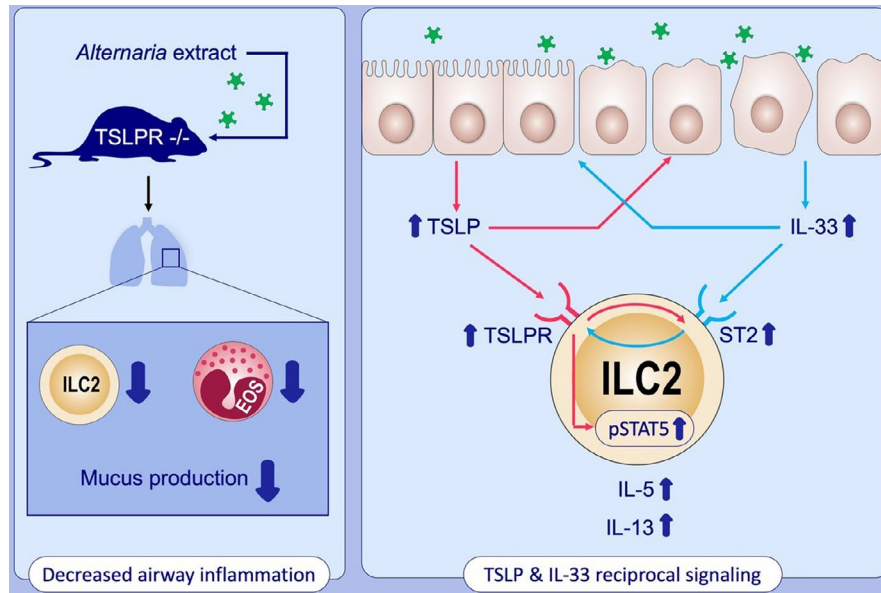
Conclusion: Thymic stromal lymphopoietin and IL-33 signaling reciprocally enhanced each other's protein release and expression in the lung following Alt-Ext-challenge and each other's receptor expression on lung ILC2 to enhance ILC2 activation.

Abbreviations: ab, antibody; Alt-Ext, *Alternaria* extract; BALF, bronchoalveolar lavage fluid; ECs, epithelial cells; hBE33, human bronchial epithelial cells stably expressing IL-33; ILC2, group 2 innate lymphoid cells; lin, lineage; MFI, mean fluorescence intensity; PAR-2, protease-activated receptor 2; pSTAT5, phosphorylated signal transducer and activator of transcription 5; rIL-33, mouse recombinant IL-33; rTSLP, mouse recombinant TSLP; ST2, IL-33 receptor; ST2^{-/-}, ST2 deficient; STAT5, signal transducer and activator of transcription 5; TSLP, thymic stromal lymphopoietin; TSLPR^{-/-}, TSLP receptor deficient; WT, wild type.

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KEYWORDS

Alternaria-extract (Alt-Ext), Group 2 innate lymphoid cells (ILC2), IL-33, TSLP

GRAPHICAL ABSTRACT

We found that TSLPR deficiency significantly decrease the number of lung ILC2, airway eosinophils, and mucus production in mouse challenged with *Alternaria* extract. TSLP-TSLPR and IL-33-ST2 signaling augments each other's protein release and expression in the lung epithelial cells after aeroallergen challenge. TSLP and IL-33 signaling increases each other's receptor expression on lung ILC2, and the IL-33-augmented TSLPR expression enhances pSTAT5 in the ILC2.

1 | INTRODUCTION

The epithelial cell-derived danger signal mediators thymic stromal lymphopoietin (TSLP) and IL-33 are consistently associated with adaptive Th2 immune responses in allergic diseases.^{1,2}

Genome-wide association studies (GWAS) have shown the association of allergic diseases and genetic polymorphisms in the *TSLP*, *IL33*, and *IL1RL1* (receptor for IL-33).³

Thymic stromal lymphopoietin, an IL-7-like cytokine, is expressed mainly by epithelial cells (ECs) and epidermal keratinocytes (KCs). Previous animal studies reported that mice deficient in TSLPR (TSLPR^{-/-}) had significantly decreased airway inflammation, serum IgE, and airway hyperresponsiveness (AHR) in chronic models of adaptive immunity-mediated allergic airway inflammation with house dust mite antigen,⁴ OVA,^{5,6} or a mixture of allergen extracts.² In addition, subcutaneous TSLP or intranasal TSLP + OVA administration induced ear thickness or allergic airway inflammation with serum IgE induction.^{7,8} IL-33 normally resides in the nucleus of structural cells in the lung.⁹ Unlike the release of classic cytokines, IL-33 is rapidly released from epithelial or endothelial cells upon cellular damage or stress.¹⁰ Previous studies reported that IL-33 deficiency significantly decreased eosinophilia and AHR in the mouse model of OVA- or HDM-induced adaptive allergic inflammation.¹¹ However,

the interaction of TSLP and IL-33 in regulating the innate allergic responses is unknown.

Group 2 innate lymphoid cells (ILC2), as well as Th2 cells, are an important source of type-2 cytokines to initiate and propagate allergic immune responses.^{12,13} ILC2 are defined by the absence of lineage (lin) markers (for T cells, B cells, macrophages, dendritic cells, neutrophils, NK cells, or erythrocytes) and expression of CD45 (lymphocyte common antigen), ST2 (IL-33 receptor), CD25 (IL-2R α), CD127 (IL-7R α), and ICOS.^{14,15} ILC2 are resident in the lung, skin, gut, and the other organs¹⁶ and produce type-2 cytokines in response to IL-33, or a combination of IL-33 and the other epithelium-derived cytokines such as TSLP and IL-25 in the peripheral tissues.¹⁴ These danger signals initiate ILC2 activation prior to adaptive immune responses.^{16,17} While IL-33 is an essential cytokine in the activation of ILC2,¹⁵ TSLP synergistically promoted the proliferation and type-2 cytokine production by IL-33-stimulated ILC2 in vitro.¹⁴ Although TSLP itself did not induce IL-5 and IL-13 by ILC2, combined stimulation with TSLP and IL-33 elicited an approximate 10-fold increase in the cytokine production by ILC2 compared with stimulation by IL-33 alone.¹⁴ However, the mechanisms of this synergistic ILC2 activation in the setting of the innate immune responses to an inhaled aeroallergen are not fully elucidated.

In this study, we hypothesized that TSLPR signaling increased T cell-independent innate allergic inflammation mediated by lung ILC2 activation. To test the hypothesis, we used a mouse model of *Alternaria* extract (Alt-Ext)-challenge for 4 consecutive days. Further, to determine the mechanisms of synergistic lung ILC2 activation by TSLP and IL-33 during aeroallergen exposure, we evaluated the effects of TSLP and IL-33 signaling on each other's protein release or expression in the lung after Alt-Ext-challenge. In addition, we evaluated the effects of TSLP and IL-33 signaling to each other's receptor expression on lung ILC2.

2 | METHODS

2.1 | Mice

Nine- to 12-week old female wild-type (WT) BALB/c mice were obtained from Charles River laboratories. TSLPR^{-/-} mice and ST2-deficient (ST2^{-/-}) mice were generated on BALB/c background as previously described.^{5,18} IL-33^{Citrine/+} reporter mice were generated by crossbreeding WT BALB/c mice and IL-33^{Citrine/Citrine} mice which were the kind gift of Dr Andrew N. J. McKenzie (Cambridge, UK).¹⁹ Animal experiments were approved by the Institutional Animal Care and Use Committee at Vanderbilt University and were conducted according to the guidelines for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council.

2.2 | Alt-Ext-challenge in a mouse model of innate allergic airway inflammation

WT BALB/c and TSLPR^{-/-} mice were challenged intranasally with 5 µg (protein amount) of Alt-Ext (Stallergenes Greer) in 100 µL PBS or 100 µL PBS as the vehicle for 4 consecutive days. LPS contamination of the Alt-Ext was measured by PierceTM Chromogenic Endotoxin Quant kit (Thermo fisher scientific). LPS was detected as 0.03 endotoxin unit (EU), approximately 3 pg in 5 µg (protein amount) of Alt-Ext. The mice were killed, and bronchoalveolar lavage fluid (BALF) and whole lungs were harvested 24 hour after the last challenge of Alt-Ext or PBS for cell differentials, cytokine measurement, and flow cytometry. In the other protocol, whole lungs were harvested 48 hour after the last Alt-Ext-challenge or PBS challenge to evaluate mucus production in histological section.

2.3 | Endogenous TSLP neutralization

The 28F12 hybridoma was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA), and anti-TSLP antibodies (ab) from the hybridoma were purified by the Vanderbilt Antibody and Protein Resource.²⁰ WT BALB/c mice were injected intraperitoneally with

the anti-TSLP ab (200 µg) or isotype ab (200 µg) 1 hour prior to first Alt-Ext-challenge.

2.4 | Statistical analysis

All data were analyzed with GraphPad Prism 8 (GraphPad Software). The *P* values were calculated by t test or one-way analysis of variance (ANOVA) with Bonferroni-multiple pair's comparisons test. Values of *P* < .05 were considered significant between two groups.

Further experimental details are provided in online Appendix S1. All antibodies used in this study are listed in Table S1.

3 | RESULTS

3.1 | TSLPR deficiency suppresses Alt-Ext-induced lung ILC2 activation and innate allergic airway inflammation

Previous studies reported that deficiency of TSLPR signaling decreased adaptive allergic immune responses,^{5,7} but the effect of TSLPR deficiency on allergen-induced T cell-independent innate immune responses has not been reported. To evaluate the effects of TSLPR signaling during aeroallergen-induced innate type-2 airway inflammation, we performed Alt-Ext-challenge for 4 consecutive days in WT and TSLPR^{-/-} mice and killed them 24 or 48 hour after the last Alt-Ext-challenge (Figure 1A). Four consecutive days of Alt-Ext-challenge significantly increased the number of total BAL cells, macrophages, eosinophils, lymphocytes, and neutrophils compared with PBS-challenged groups. TSLPR^{-/-} mice challenged with Alt-Ext had a significant decrease in the number of total BAL cells, eosinophils, and lymphocytes compared with WT mice (Figure 1B). In contrast, there was no change in the number of macrophages and neutrophils between WT and TSLPR^{-/-} mice challenged with Alt-Ext (Figure 1B). Next, we evaluated the lung ILC2 expressing IL-5 and IL-13. Figure S1 showed the gating strategy of identifying lung ILC2 after intracellular staining on flow cytometry. The lung ILC2 were identified as lin⁻ CD3⁻ CD4⁻ CD45⁺ CD25⁺ ICOS⁺ cells to determine the IL-5 and IL-13 expression (Figure S1A,B). Alt-Ext-challenge significantly increased the number of lung ILC2 expressing IL-5 and IL-13 compared with PBS-challenged groups (Figure 1C). There was a significant decrease in the number of lung ILC2 expressing IL-5 and IL-13 in TSLPR^{-/-} mice compared with WT mice after the Alt-Ext-challenge (Figure 1C). Further, TSLPR^{-/-} mice had a lower percentage of IL-13⁺ cells in total ILC2 compared with WT mice after the Alt-Ext-challenge (Figure 1C). TSLPR^{-/-} mice had a significant decrease in Alt-Ext-challenge-induced protein expression of IL-4, IL-5, and IL-13 in the lung homogenates to almost the same level as the PBS-challenged mice (Figure 1D). TSLPR^{-/-} mice had a significant decrease in the mucus score compared with WT mice after the Alt-Ext-challenge (Figure 1E). These findings revealed that TSLPR deficiency significantly

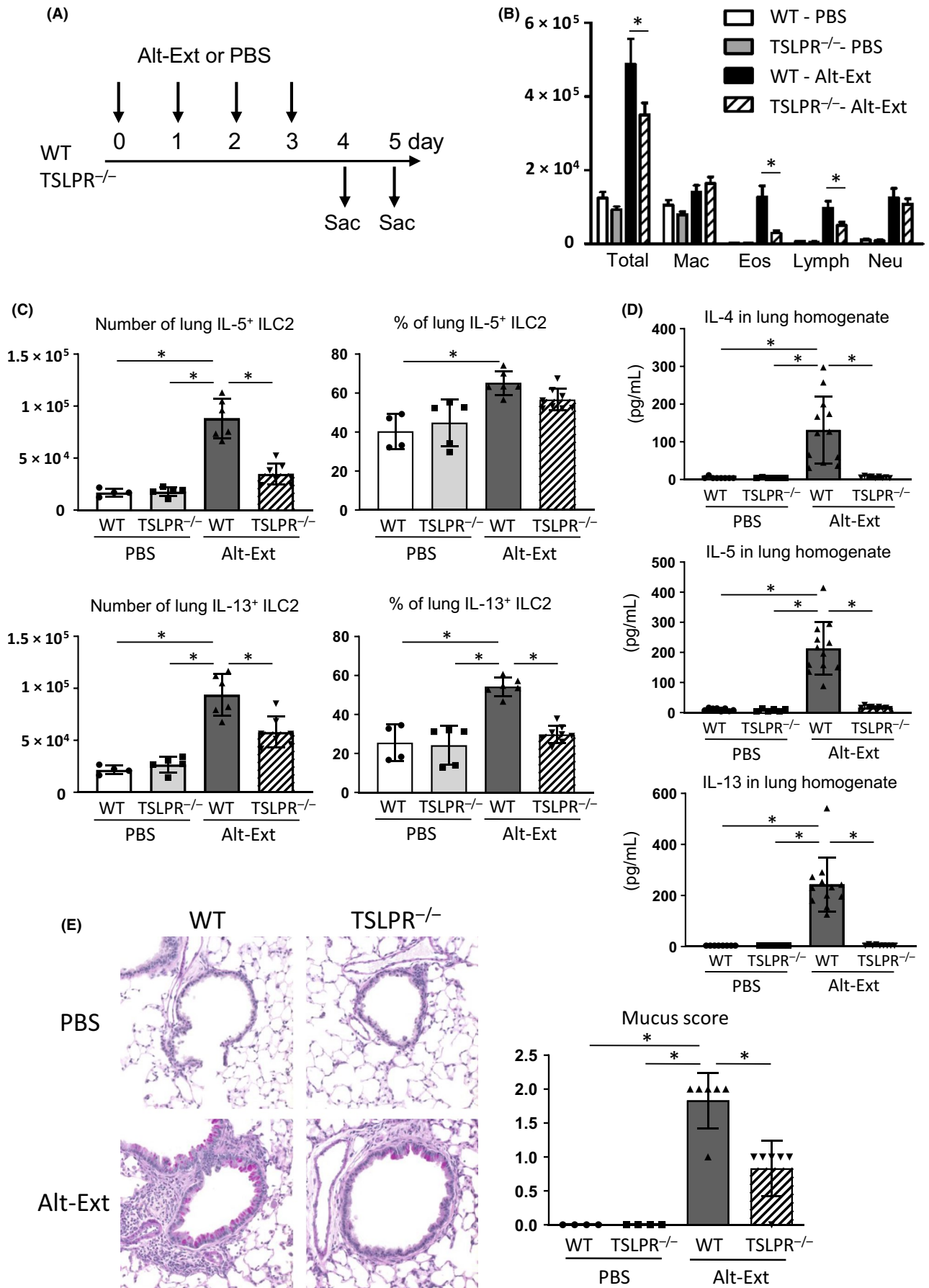


FIGURE 1 TSLPR deficiency decreased *Alternaria* extract (Alt-Ext)-induced type-2 airway inflammation. A, WT and TSLPR^{-/-} mice were challenged with Alt-Ext intranasally for 4 consecutive days. Bronchoalveolar lavage fluid (BALF) and lung were harvested 24 h after the last Alt-Ext-challenge. Whole lungs for histological mucus detection were harvested 48 h after the last Alt-Ext-challenge. B, Cell differentials in BALF. C, The number and percentage of lung group 2 innate lymphoid cells (ILC2) expressing IL-5 and IL-13. D, The protein level of IL-4, IL-5, and IL-13 in the lung homogenates. E, Representative sections and mucus score as determined by periodic acid-Schiff (PAS) staining. The data are a combination of 2 independent experiments and shown as mean $\pm$ SD (n = 4-9). *P < .05

decreased aeroallergen-induced lung ILC2 activation and innate allergic airway inflammation.

3.2 | Anti-TSLP ab treatment suppresses Alt-Ext-induced innate allergic airway inflammation

To confirm the effect of TSLPR signaling for Alt-Ext-induced T cell-independent innate allergic inflammation, we performed anti-TSLP ab treatment prior to Alt-Ext-challenge. WT mice were treated with anti-TSLP ab or isotype ab 1 hour prior to the first Alt-Ext-challenge to neutralize the Alt-Ext-induced endogenous TSLP. Twenty-four hours after the 4th daily Alt-Ext-challenge, BALF and lungs were harvested (Figure 2A) to evaluate innate allergic inflammation. Anti-TSLP ab treatment significantly decreased the number of total BAL cells, macrophages, eosinophils, and lymphocytes, but not neutrophils compared with isotype ab treatment in Alt-Ext-challenged groups (Figure 2B). Further, anti-TSLP ab treatment significantly decreased

Alt-Ext-induced IL-4, IL-5, and IL-13 in lung homogenates compared with isotype ab treatment (Figure 2C). While other studies have reported that TSLP antagonism reduced the adaptive allergic immune responses, to our knowledge, these results are the first to reveal that anti-TSLP ab treatment suppressed Alt-Ext-induced innate allergic airway inflammation.

3.3 | TSLPR signaling increases Alt-Ext-induced IL-33 release, and ST2 signaling increases Alt-Ext-induced TSLP expression in the lung

Previous studies reported that aeroallergen-induced IL-33 release from lung epithelial cell led to lung ILC2 activation.^{21,22} We, therefore, investigated the effects of TSLPR signaling for Alt-Ext-induced IL-33 release. First, we checked the time course of IL-33 and TSLP in the BALF and lung homogenates after one Alt-Ext-challenge. IL-33 protein in BALF peaked 1 hour after the Alt-Ext-challenge, but TSLP

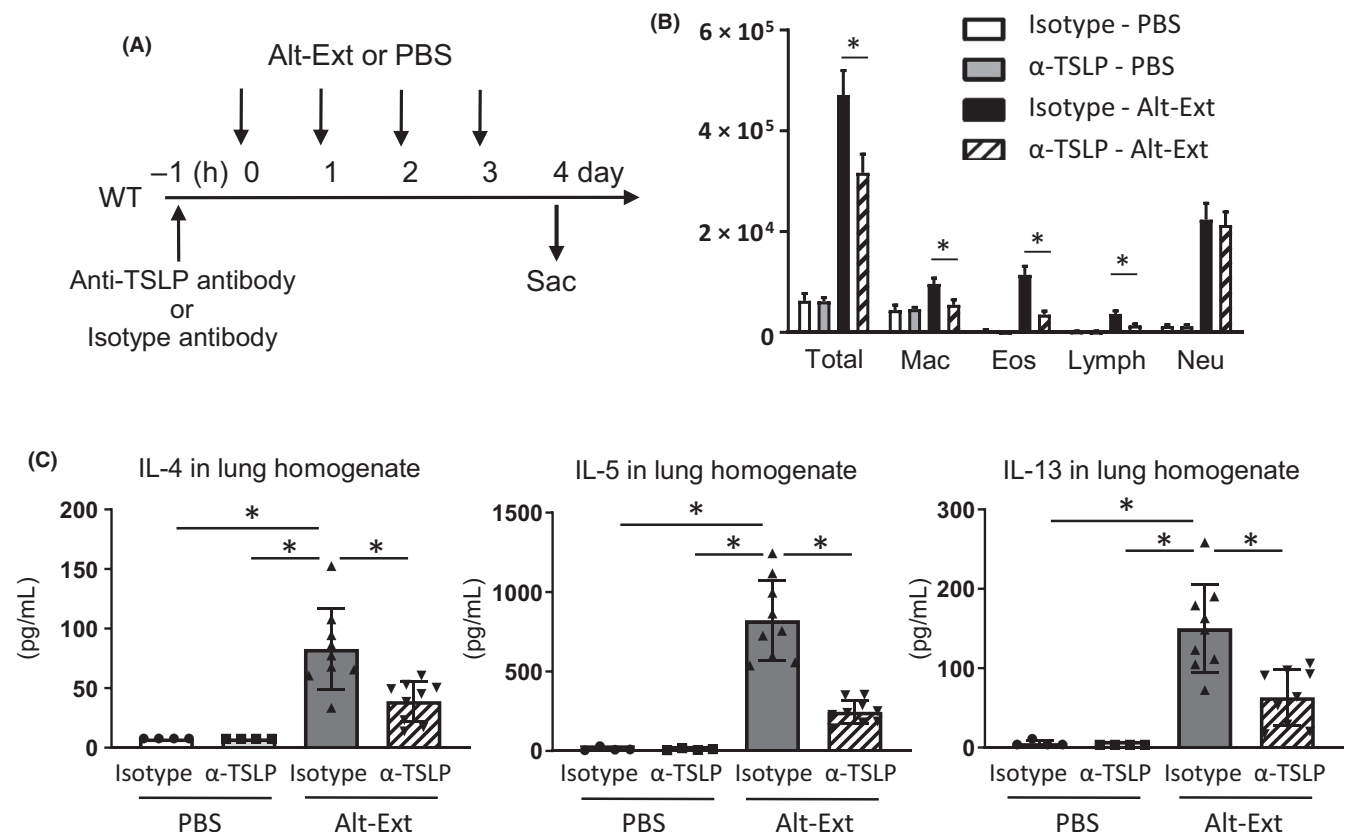


FIGURE 2 Endogenous TSLP neutralization decreased *Alternaria* extract (Alt-Ext)-induced type-2 airway inflammation. A, Anti-TSLP antibody (α -TSLP) or the isotype antibody was administered subcutaneously 1 h prior to first Alt-Ext-challenge. Bronchoalveolar lavage fluid (BALF) and lung were harvested 24 h after the 4th Alt-Ext-challenge. B, Cell differentials in BALF. C, The protein level of IL-4, IL-5, and IL-13 in the lung homogenates. The data are a combination of 2 independent experiments and shown as mean $\pm$ SD (n = 4-9). *P < .05

was not detected in the BALF (Figure 3A). Instead of BALF, TSLP protein peaked 6 hour after Alt-Ext-challenge in the lung homogenates (Figure 3A). These results are same as previous studies.^{21,22} To determine the effect of exogenous TSLP on IL-33 release into the BALF, mouse recombinant TSLP (rTSLP) was administered intranasally to WT mice 6 hour prior to Alt-Ext-challenge, and then, the BALF was harvested 1 hour after the Alt-Ext-challenge (Figure 3B). We found that rTSLP pretreatment enhanced the protein level of IL-33 in the BALF 1 hour after the Alt-Ext-challenge compared with

vehicle treatment (Figure 3C). The enhanced IL-33 release by rTSLP pretreatment was abolished in TSLPR^{-/-} mice (Figure S2).

To determine whether rTSLP treatment increases IL-33 expression to enhance Alt-Ext-induced IL-33 release, we measured IL-33/citrine expression in the lung cells from IL-33^{Citrine/+} reporter mice treated with rTSLP or vehicle for 6 hour. Although a portion of lung ECs (CD45⁻ EpCAM⁺) expresses IL-33/citrine (Figure S3A), there was no difference in the mean fluorescence intensity (MFI) of IL-33/citrine between rTSLP treatment and vehicle treatment (Figure S3B,C). In contrast,

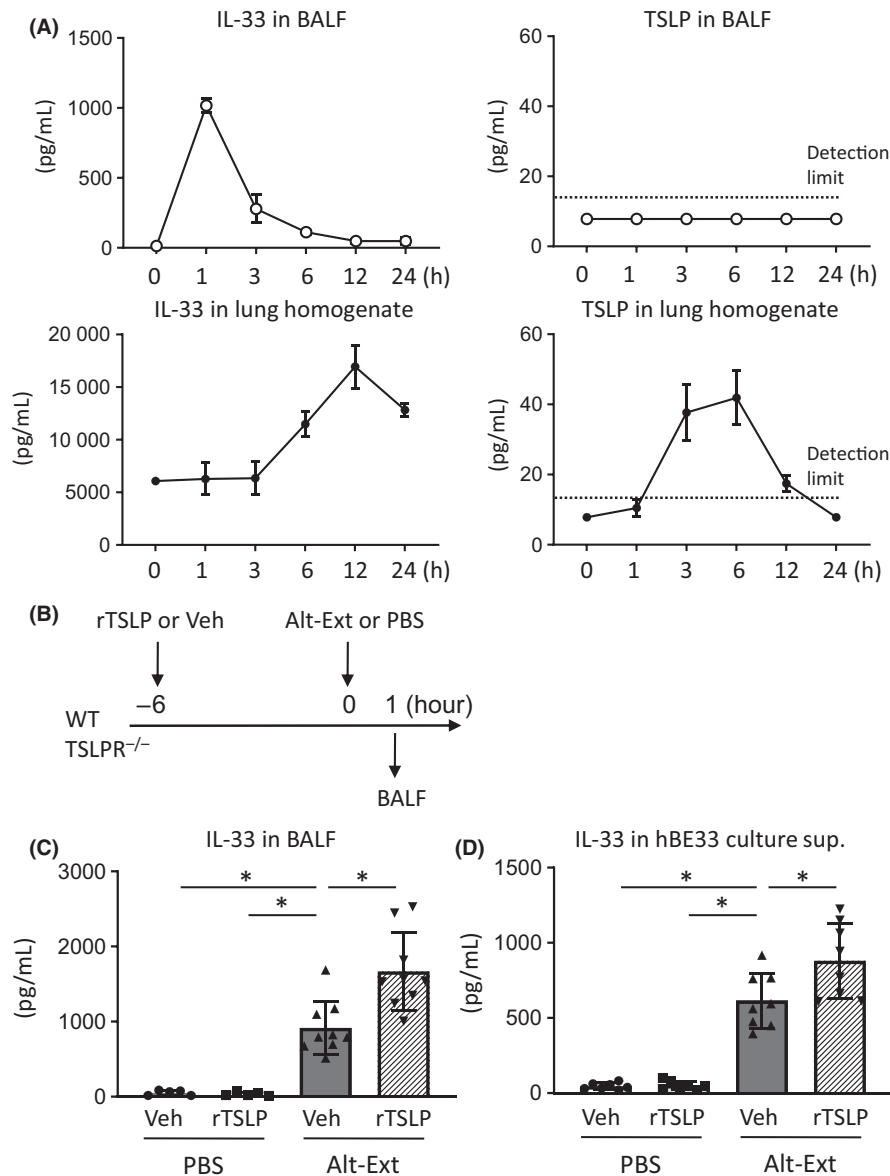


FIGURE 3 TSLPR signaling increased IL-33 release and IL-33 receptor (ST2) signaling increased TSLP expression in the lung. A, The time course of TSLP and IL-33 in bronchoalveolar lavage fluid (BALF) and lung homogenates after one *Alternaria* extract (Alt-Ext)-challenge. B, E, G, I, Diagrams of experimental design using mouse model to detect IL-33 in BALF and TSLP in the lung. C, IL-33 protein level in the BALF from wild-type (WT) mice challenged with Alt-Ext or PBS in the presence or absence of recombinant TSLP (rTSLP) treatment. D, IL-33 protein level in cell-free culture supernatant (sup) from human bronchial epithelial cells stably expressing IL-33 (hBE33) challenged with Alt-Ext or PBS in the presence or absence of human recombinant TSLP treatment. F, IL-33 protein level in the BALF from WT and TSLPR^{-/-} mice challenged with Alt-Ext or PBS. H, IL-33 protein level in the BALF from WT mice challenged with Alt-Ext or PBS in the presence or absence of anti-TSLP antibody (α -TSLP) treatment. J, TSLP protein level in the lung homogenates from WT and ST2^{-/-} mice challenged with Alt-Ext or PBS. The data are a combination of 2 independent experiments and shown as mean $\pm$ SD (n = 3-9). *P < .05

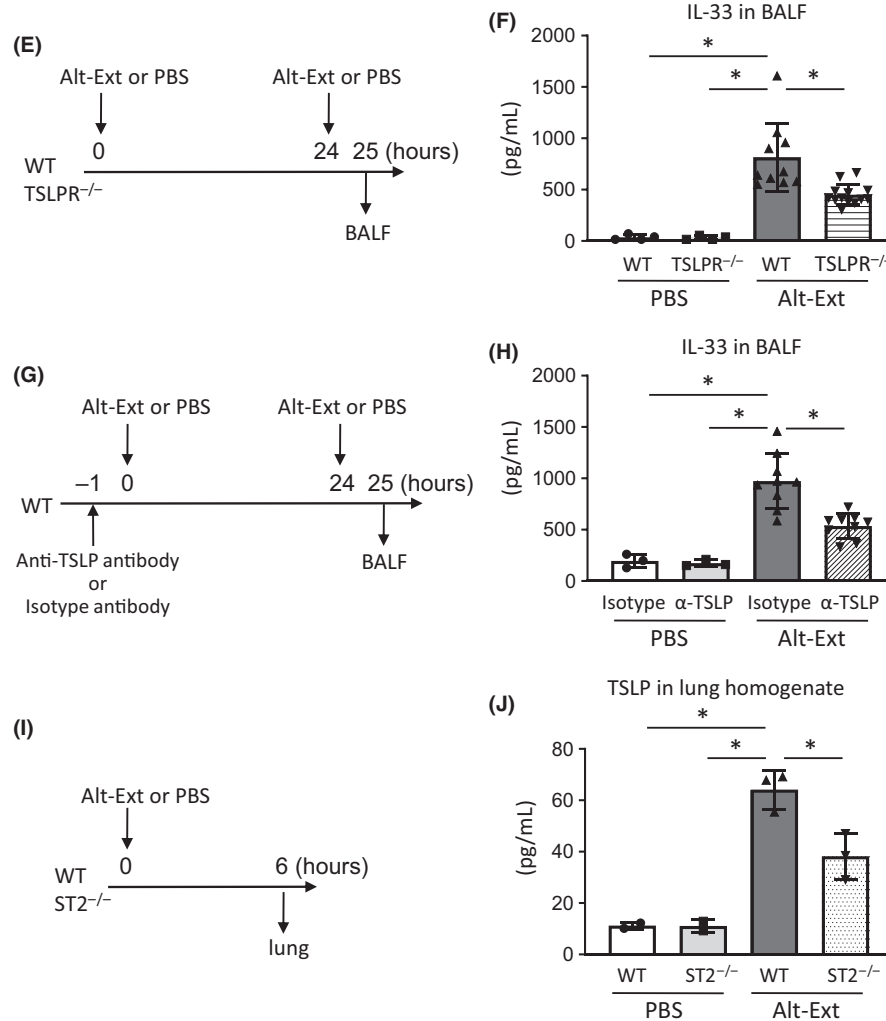


FIGURE 3 (Continued)

IL-33/citrine was not detected on lung T cells, B cells, neutrophils, eosinophils, CD11c⁺ macrophages, and dendritic cells from IL-33^{Citrine/+} reporter mice treated with rTSLP or vehicle (Figure S3D-G).

Functional IL-33 is released from damaged or necrotic epithelial cells.¹⁰ Therefore, we measured IL-33 release in human bronchial epithelial cells stably expressing IL-33 (hBE33) culture supernatant to confirm whether human recombinant TSLP treatment increases IL-33 release after Alt-Ext-challenge in an in vitro model. Alt-Ext stimulation induced IL-33 release from hBE33 into the culture supernatant (Figure 3D). Human recombinant TSLP treatment significantly increased Alt-Ext-induced IL-33 release compared with vehicle treatment (Figure 3D). To determine whether rTSLP treatment increases IL-33 expression in hBE33 to enhance IL-33 release, we measured GFP co-expressed with IL-33 in hBE33²³ after rTSLP or vehicle treatment. There was no difference in the MFI of IL-33/GFP between rTSLP-treated cells and vehicle-treated cells (Figure S4A,B). Further, we tested treatment with the signal transducer and activator of transcription 5 (STAT5) inhibitor pimozide with rTSLP to investigate the effect of TSLPR-STAT5 cascade for IL-33 release from hBE33. Pimozide + rTSLP treatment significantly decreased the Alt-Ext-induced IL-33 release compared with rTSLP treatment alone (Figure S4C). In addition, we

found that rTSLP treatment increased lactate dehydrogenase (LDH) in the culture supernatant compared with vehicle treatment (Figure S4D). Pimozide + rTSLP treatment significantly decreased LDH compared with rTSLP treatment alone (Figure S4D). These in vivo and in vitro results suggested that exogenous TSLP enhanced Alt-Ext-induced IL-33 release from lung epithelial cells by promoting the process of necrosis.

Next, we tested the effect of endogenous TSLP on IL-33 release. Since there is no endogenous TSLP in the lung 1 hour after the first Alt-Ext-challenge (Figure 3A), a second Alt-Ext-challenge is necessary to induce TSLP expression in order to determine the effect of endogenous TSLP on IL-33 release. Therefore, we performed the second Alt-Ext-challenge 24 hour after the first challenge to allow the TSLP expression after the first challenge to exert its biological effect, and then, we harvested the BALF from WT and TSLPR^{-/-} mice 1 hour after second Alt-Ext-challenge (Figure 3E). The second Alt-Ext-challenge significantly induced IL-33 protein in BALF compared with PBS challenge, and the Alt-Ext-induced IL-33 was decreased in TSLPR^{-/-} mice compared with WT mice (Figure 3F). To confirm the effect of endogenous TSLP, WT mice were treated with anti-TSLP ab or isotype ab 1 hour prior to first Alt-Ext-challenge to neutralize the Alt-Ext-induced endogenous TSLP. We then

harvested BALF 1 hour after a second Alt-Ext-challenge (Figure 3G) to evaluate whether endogenous TSLP neutralization suppresses the Alt-Ext-induced IL-33 release. Anti-TSLP ab treatment significantly decreased the second Alt-Ext-challenge-induced IL-33 release in the BALF compared with isotype ab treatment (Figure 3H).

In addition, to evaluate an effect of endogenous ST2 signaling for Alt-Ext-induced TSLP expression in the lung, WT and ST2^{-/-} mice were challenged with Alt-Ext once, and 6 hour later, the lungs were harvested to detect TSLP protein (Figure 3I). Alt-Ext-challenge significantly induced TSLP protein in the lung homogenates compared with PBS challenge. The Alt-Ext-induced TSLP was significantly decreased in ST2^{-/-} mice compared with WT mice (Figure 3J). To confirm the TSLP expression on lung ECs, we isolated CD45⁻ EpCAM⁺ cells from naïve WT mice to detect mRNA expression of TSLP. Flow cytometry analysis indicated that CD45⁻ EpCAM⁺ cell fraction by magnetic isolation had 85.1% purity (Figure S5A). TSLP mRNA expression was detected in the isolated CD45⁻ EpCAM⁺ cells (Figure S5B). These results revealed that TSLPR signaling and ST2 signaling reciprocally induced the expression of each other's receptor cytokine after Alt-Ext challenge in the lung.

3.4 | rTSLP treatment does not induce PAR-2 expression on IL-33⁺ lung ECs

A previous study reported that the Alt-Ext-induced IL-33 release from lung ECs was mediated by protease-activated receptor 2 (PAR-2) signaling.²² Therefore, to determine whether TSLPR signaling increases PAR-2 expression, we measured PAR-2 expression on lung ECs expressing IL-33 using IL-33^{Citrine/+} mice following intranasal rTSLP treatment for 6 hour. The ECs expressing IL-33 were identified as CD45⁻ EpCAM⁺ citrine⁺ cells (Figure S6A). TSLPR and PAR-2 were expressed on the lung ECs expressing IL-33 (Figure S6B); however, rTSLP treatment did not change the MFI of PAR-2 (Figure S6C).

3.5 | Alt-Ext-induced IL-33 does not increase TSLPR expression on lung ECs

To determine whether Alt-Ext-induced IL-33 enhances TSLPR expression on lung ECs, we performed flow cytometry using WT and ST2^{-/-} mice challenged with Alt-Ext or PBS for 24 hour. The gating strategy of lung ECs is shown in Figure S7A. TSLPR was expressed on lung ECs from both WT and ST2^{-/-} mice; however, there was no difference in the MFI of TSLPR between WT and ST2^{-/-} mice challenged with PBS (Figure S7B,C). Alt-Ext-challenge did not alter the MFI of TSLPR on the lung ECs from both WT and ST2^{-/-} mice (Figure S7B,C).

3.6 | rTSLP increases ST2, and rIL-33 increases TSLPR on lung ILC2 in vivo and in vitro

Thymic stromal lymphopoietin synergistically activates ILC2 with IL-33-ST2 signaling.¹⁴ However, the mechanism of the synergistic effect of TSLP and IL-33 is still unknown. To determine the effect of TSLPR and

ST2 signaling on the expression of each other's receptor on ILC2, we first measured ST2 and TSLPR expression on lung ILC2 in vivo. WT mice were treated intranasally with rTSLP, mouse recombinant IL-33 (rIL-33), or vehicle, and then 18 hour later, ST2 and TSLPR expression on lung ILC2 was measured by flow cytometry. Lung ILC2 were identified as lineage⁻ CD45⁺ CD3⁻ CD25⁺ CD127⁺ (Figure S8). We found that rTSLP treatment increased the MFI of ST2 on lung ILC2 compared with vehicle treatment (Figure 4A,4). However, rTSLP treatment decreased the MFI of TSLPR compared with vehicle treatment (Figure 4C,4). Meanwhile, rIL-33 treatment significantly increased the MFI of both TSLPR and ST2 on lung ILC2 compared with vehicle treatment (Figure 4A-D). To confirm the results in this in vivo model, we performed ILC2 treatment in an in vitro model. Isolated lung ILC2 were treated with rTSLP, rIL-33, rTSLP + rIL-33, or vehicle for 40 hour, and then, ST2 and TSLPR expression on the treated ILC2 was measured by flow cytometry. We found that rTSLP treatment increased the MFI of ST2, but decreased the MFI of TSLPR on the isolated lung ILC2 compared with vehicle treatment (Figure 4E-H). rIL-33 treatment significantly increased the MFI of TSLPR and ST2 on the isolated lung ILC2 compared with vehicle treatment (Figure 4E-H). rTSLP + rIL-33 treatment further enhanced the MFI of ST2 compared with rTSLP or rIL-33 treatment alone (Figure 4E,4).

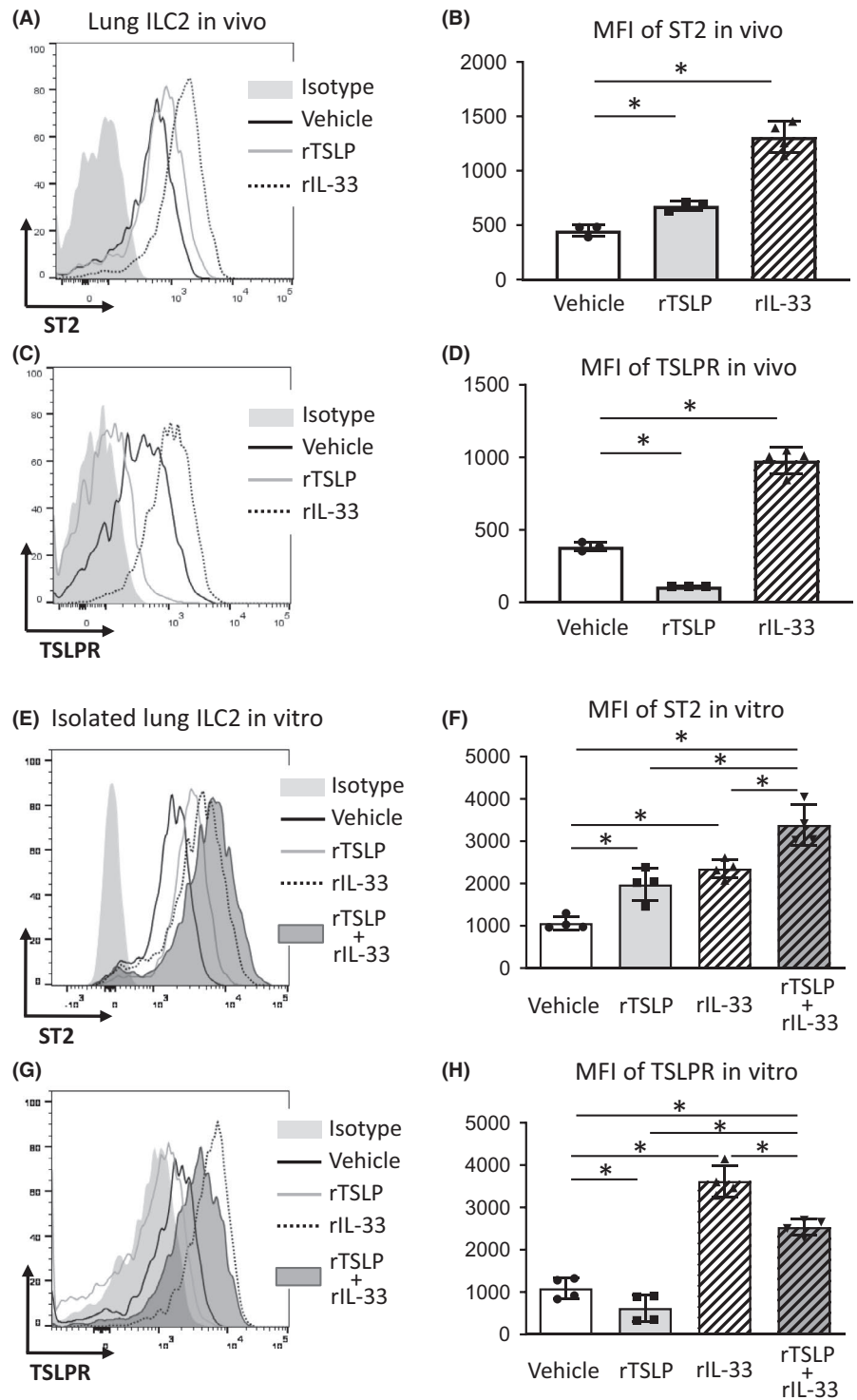
Based on our results that TSLP augmented ST2 expression on ILC2 and IL-33 increased TSLPR expression on ILC2, we measured the protein levels of IL-5 and IL-13 in the isolated ILC2 culture supernatant to confirm the synergistic effect of rTSLP and rIL-33. rTSLP treatment did not statistically induce IL-5 and IL-13 compared with vehicle treatment (Figure S5). rIL-33 treatment significantly induced IL-5 and IL-13 compared with vehicle treatment. Further, rTSLP + rIL-33 treatment synergistically increased IL-5 and IL-13 at, respectively, 10-fold and 8-fold greater than the treatment of rIL-33 alone (Figure S9).

To confirm the synergistic effect of TSLPR and ST2 signaling for ILC2 activation, we isolated lung ILC2 from WT, TSLPR^{-/-}, and ST2^{-/-} mice. The cells were treated with rTSLP, rIL-33, rTSLP + rIL-33, or vehicle for 40 hour. rIL-33 treatment increased TSLPR expression on WT lung ILC2, and the rIL-33-enhanced TSLPR expression was abolished on ST2^{-/-} lung ILC2 (Figure S10A). rTSLP treatment decreased TSLPR expression on ST2^{-/-} lung ILC2 as well as WT lung ILC2 (Figure S10A). rTSLP + rIL-33 treatment synergistically increased ST2 expression on WT lung ILC2, and the synergistic enhanced-ST2 expression was abolished on TSLPR^{-/-} lung ILC2 (Figure S10B). Further, the synergistic increase of IL-5 and IL-13 in WT lung ILC2 by rTSLP + rIL-33 treatment was abolished in TSLPR^{-/-} lung ILC2 (Figure S10C). Meanwhile, there was no cytokine detection in all of ST2^{-/-} lung ILC2 (Figure S10C). These findings suggest that TSLP and IL-33 reciprocally enhance the expression of receptors, and this may be one of the mechanisms by which these cytokines induce synergistic ILC2 activation.

3.7 | IL-33 treatment enhanced TSLP-induced pSTAT5

To confirm the effect of enhanced TSLPR expression on lung ILC2 by IL-33 treatment, we measured phosphorylated STAT5 (pSTAT5)

FIGURE 4 Recombinant TSLP (rTSLP) and recombinant IL-33 (rIL-33) treatment reciprocally increased IL-33 receptor (ST2) and TSLPR on lung group 2 innate lymphoid cells (ILC2). A, C, Representative histograms of ST2 and TSLPR expression on lung ILC2 from wild-type (WT) mice treated with rTSLP, rIL-33 or the vehicle in an in vivo model. B, D, Mean fluorescence intensity (MFI) of ST2 and TSLPR on lung ILC2 in vivo. E, G, Representative histograms of ST2 and TSLPR expression on isolated lung ILC2 treated with rTSLP, rIL-33, rTSLP + rIL-33, or the vehicle in an in vitro model. F, H, MFI of ST2 and TSLPR on isolated lung ILC2 in vitro. The data are a combination of 2 independent experiments and shown as mean $\pm$ SD. (n = 3-4). *P < .05



which is down stream of TSLPR signaling.²⁴ Isolated lung ILC2 were treated with IL-33 or vehicle for 24 hour, and then, the cells were treated with rTSLP or vehicle for 1 hour to detect pSTAT5 protein by Western blotting. There was no pSTAT5 in the ILC2 treated with vehicle or IL-33. In contrast, pSTAT5 was detected in the ILC2 treated with rTSLP. rIL-33 pretreatment increased rTSLP-induced pSTAT5 (Figure 5A,5). To confirm the synergistic effect of ILC2 activation mediated by STAT5 cascade, the isolated lung ILC2 was treated

with rIL-33, rTSLP + rIL-33, or vehicle in the presence or absence of 5 μ mol/L pimozide for 40 hour. Pimozide significantly decreased IL-5 and IL-13 from ILC2 treated with rTSLP + rIL-33 compared with vehicle treatment (Figure 5C). In contrast, pimozide slightly enhanced rIL-33 treatment-induced IL-5 and IL-13 from ILC2 (Figure 5C). These results revealed that IL-33 treatment augmented TSLP-induced phosphorylation of STAT5 in lung ILC2, and the STAT5 cascade elicited a synergistic effect in ILC2 activation.

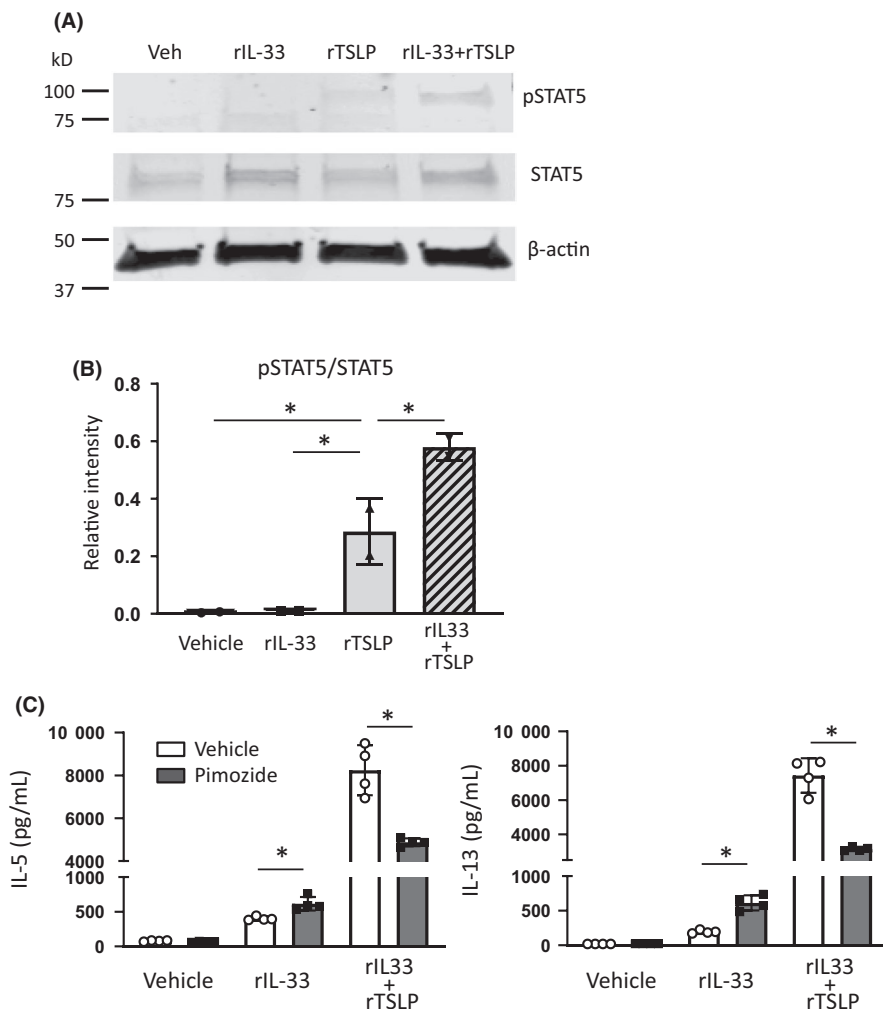


FIGURE 5 Recombinant IL-33 (rIL-33) treatment enhanced TSLP-induced signal transducer and activator of transcription 5 (STAT5) signaling. A, Representative image of Western blotting. Isolated lung group 2 innate lymphoid cells (ILC2) were stimulated for 24 h with rIL-33 (10 ng/mL) or vehicle; then, the cells were stimulated for 1 h with recombinant TSLP (rTSLP) (10 ng/mL) or vehicle. Cell lysates were separated by SDS-PAGE and analyzed by Western blotting with antibodies to phosphorylated STAT5 (pSTAT5), STAT5, and β-actin. B, Relative intensity of pSTAT5/STAT5 was analyzed by densitometry. C, Isolated lung ILC2 were stimulated with rIL-33, rTSLP + rIL-33, or vehicle in presence or absence of pimozide (5 μmol/L) for 40 h. IL-5 and IL-13 protein in the ILC2 culture supernatant were measured by ELISA. The data are a combination of 2 independent experiments. * $P < .05$

4 | DISCUSSION

Previous studies reported that TSLPR and ST2 signaling promotes adaptive immunity-driven allergic inflammation, and as a result, TSLP and IL-33 have been identified as therapeutic targets for allergic disorders.^{3,25,26} This is the first report of the contribution of TSLPR signaling to the T cell-independent innate immunity-driven allergic inflammatory response. We found that TSLPR deficiency significantly decreased the number of lung ILC2 expressing IL-5 and IL-13, airway eosinophils and lymphocytes, and mucus production compared with WT mice in an innate allergic inflammation model by which the mice were challenged with Alt-Ext for 4 consecutive days. Neutralization of endogenous TSLP also decreased the Alt-Ext induced eosinophilia, IL-5 and IL-13 expression in the lung. Regarding the mechanisms of lung ILC2 activation during Alt-Ext-challenge, we found that TSLPR and ST2 signaling reciprocally enhanced Alt-Ext-induced IL-33 release and TSLP expression in the lung. Further, rTSLP and rIL-33 stimulation reciprocally induced each other's receptor expression on lung ILC2 both in vivo and in vitro.

In this mouse model, 4 consecutive days of Alt-Ext-challenge induced allergic inflammation without CD4 T-cell development, total IgE, and the *Alternaria* antigen-specific IgG1 induction.²⁷ However,

the number of lung ILC2 expressing IL-5 and IL-13 was significantly greater than lung CD4 T cells expressing IL-5 and IL-13.^{27,28} Based on these reports, ILC2 are a primary driver of the innate type-2 inflammation induced by Alt-Ext-challenge. We detected LPS contamination as 0.03 EU, approximately 3 pg in 5 μg protein amount of Alt-Ext. *Alternaria alternata* is a genus of ascomycete fungi, and the Alt-Ext includes large amount of fungus components, such as lipoprotein, β-glucan, chitin, and nucleotides to stimulate TLR2/TLR6, TLR3, TLR7, and TLR9 for inflammatory responses.^{29,30} Therefore, we did not distinguish between effects of contaminated LPS and effects of fungal pathogens.

We found that TSLPR and PAR-2 were expressed on lung ECs expressing IL-33. While rTSLP treatment increased Alt-Ext-induced IL-33 release, PAR-2 expression was not changed by rTSLP treatment on lung ECs. Previous studies reported that *Alternaria*-induced rapid IL-33 release from normal human bronchial epithelial cells (NHBE) was mediated by ATP autocrine and consequent Ca^{2+} influx through P2Y₂ or P2X₇ receptor,²¹ and the consequent NADPH oxidase dual oxidase 1 (DUOX1) activation through the Ca^{2+} influx.³¹ Further, Wilson et al reported that TSLP treatment of dorsal root ganglia neurons resulted in Ca^{2+} influx to induce itch.³² These previous studies may suggest the possibility that TSLPR signaling has

an influence on internal signaling, but not PAR-2 expression on lung epithelial cells. While intracellular caspase during apoptosis cleaved IL-33 to inhibit biological activity,³³ full length of IL-33 molecule is released from damaged or necrotic epithelial cells.¹⁰ Although we could not find an increase of IL-33 expression in lung ECs by rTSLP treatment in both in vivo and in vitro, the rTSLP treatment enhanced Alt-Ext-induced IL-33 and LDH release from hBE33. This suggests that TSLPR-STAT5 cascade may participate in the cell damage that leads to IL-33 release.

The mechanisms of the synergistic activation of ILC2 by IL-33 and TSLP are not fully understood. Previous studies reported that ST2 expression level was significantly increased in the bone marrow ILC2 by IL-33 treatment,^{34,35} and it suggests a positive feedback loop amplifying ILC2 activation by IL-33. In this study, we newly found that TSLP and IL-33 treatment reciprocally enhanced the expression of each other's receptor on lung ILC2. Further, IL-33 treatment enhanced phosphorylation of STAT5 following TSLPR signaling. Although pimozone, STAT5 inhibitor treatment significantly decreased the rTSLP + rIL-33 enhanced IL-5 and IL-13, but slightly increased rIL-33-induced IL-5 and IL-13. These findings indicate one of mechanisms of the synergistic ILC2 activation by which IL-33-ST2 signaling increased TSLPR expression and augmented consequent STAT5 phosphorylation and the signal transduction.

Development of human monoclonal antibodies is progressing as promising biological therapeutic agents for allergic diseases.³⁶ Tezepelumab (AMG 157/MEDI9929), an anti-human TSLP antibody, attenuated allergen-induced bronchoconstriction in both early and late asthmatic responses and decreased in levels of blood and sputum eosinophils.³⁷ Further, Tezepelumab reduced the rate of asthma exacerbations in adult patients whose asthma remained uncontrolled despite treatment with medium-to-high doses of inhaled glucocorticoids.³⁸ In addition, anti-IL-33 monoclonal antibodies (ANB020, AMG282, and REGN3500) and an anti-ST2 monoclonal antibody (GSK3772847) are currently in ongoing clinical trials.³⁹ Our findings highlight a synergistic suppressive effect by TSLP deficiency for allergic inflammation mediated by down-regulation of IL-33 release and consequent ILC2 activation. Therefore, this study suggests that therapeutic treatments that inhibit TSLP signaling may have the added bonus of inhibiting IL-33-mediated inflammation, while IL-33 antagonism may also reduce TSLP-mediated allergic responses. Thus, targeting the antagonism of one of these cytokines may be an effective and efficient method to decrease allergic inflammation mediated by both cytokines in allergic diseases such as asthma.

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CONFLICT OF INTEREST

The authors that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

ST designed and performed experiments, analyzed data, and wrote the manuscript. KG and JZ contributed to the acquisition of data. WZ and DCN advised and contributed to the overall development of this study. BZ contributed to TSLPR^{-/-} mice study. HK contributed to hBE33 in vitro study. KLB contributed to the analysis interpretation of histopathology data. RSP designed and supervised the research and edited the manuscript.

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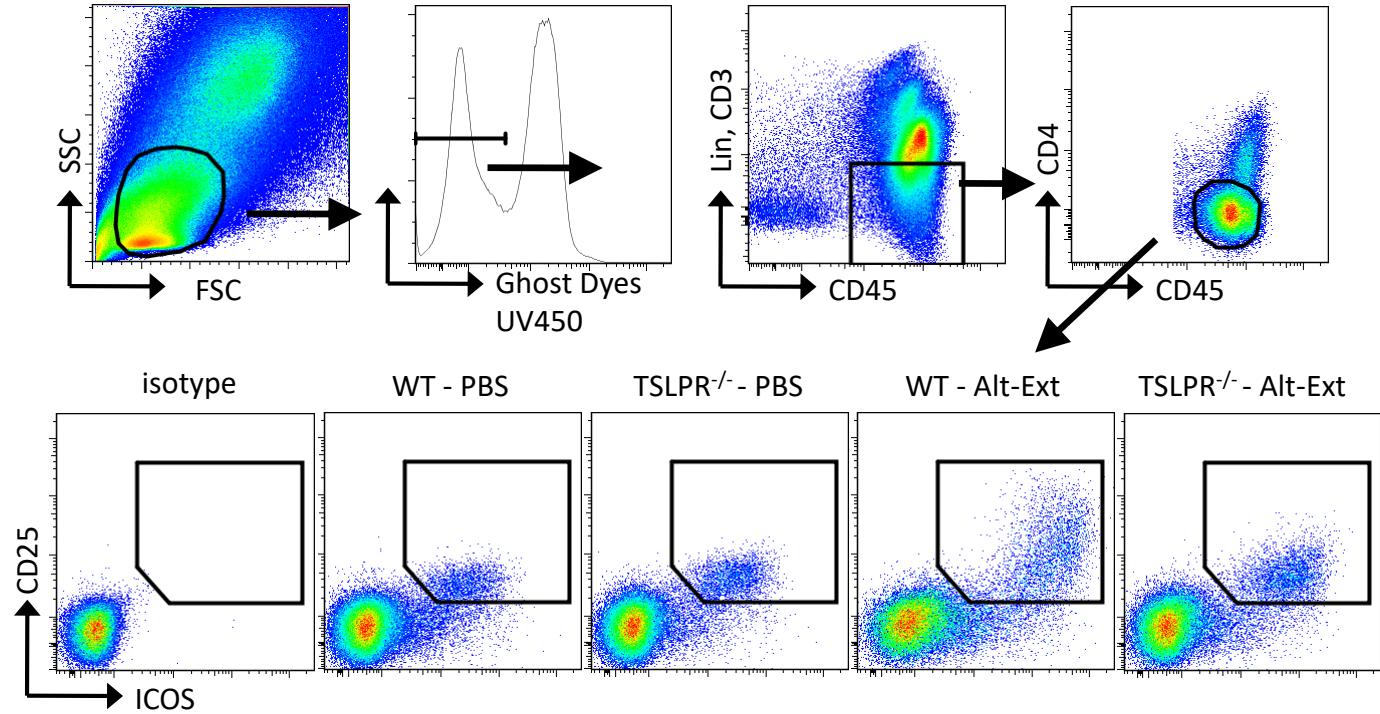
SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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FIGURE S1.

(A)



(B)

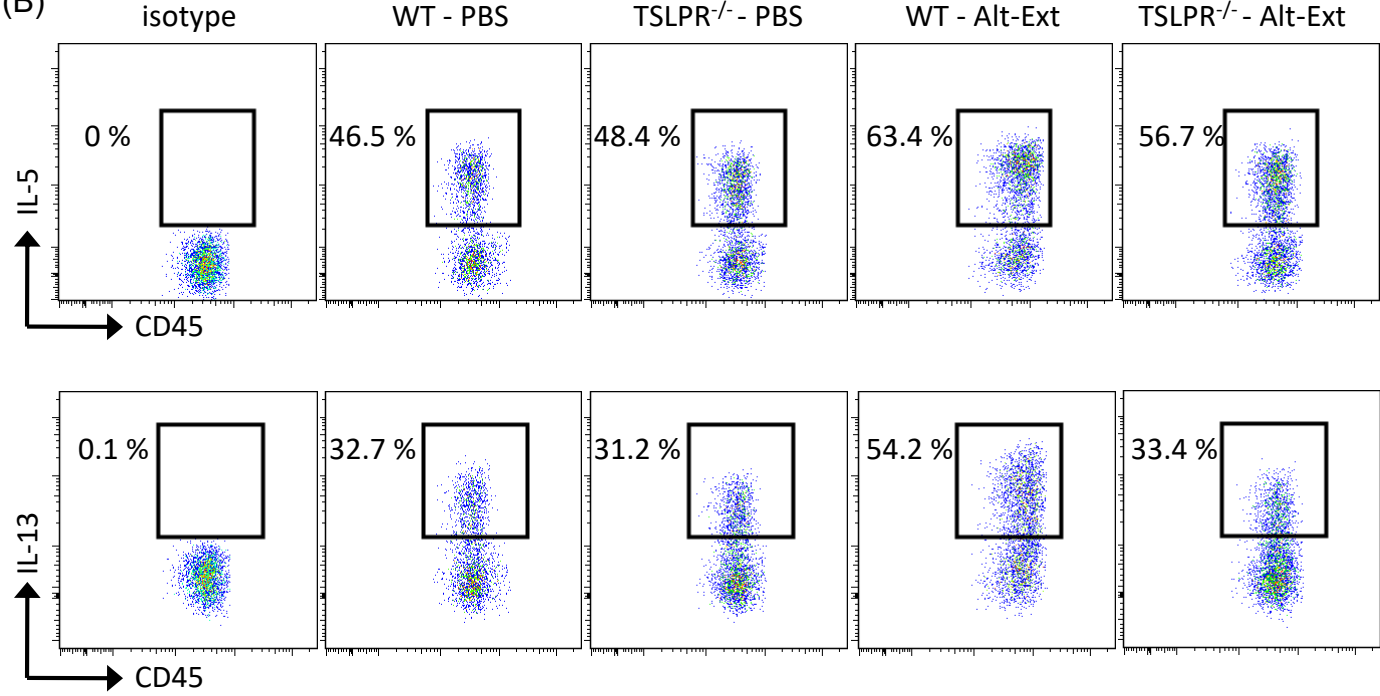


FIGURE S2.

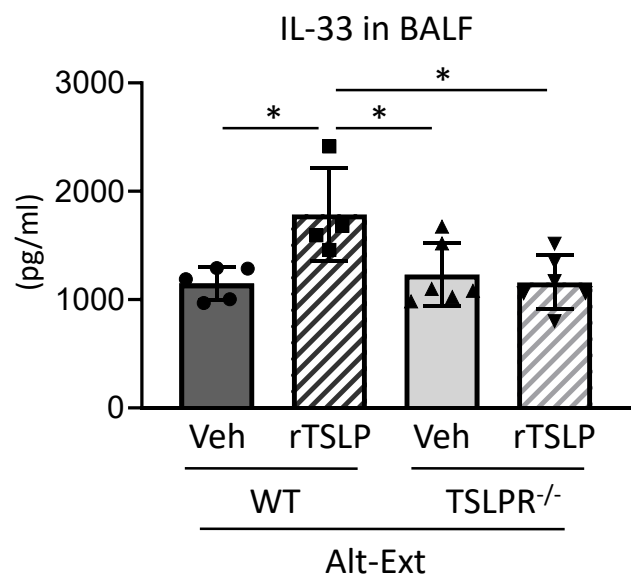
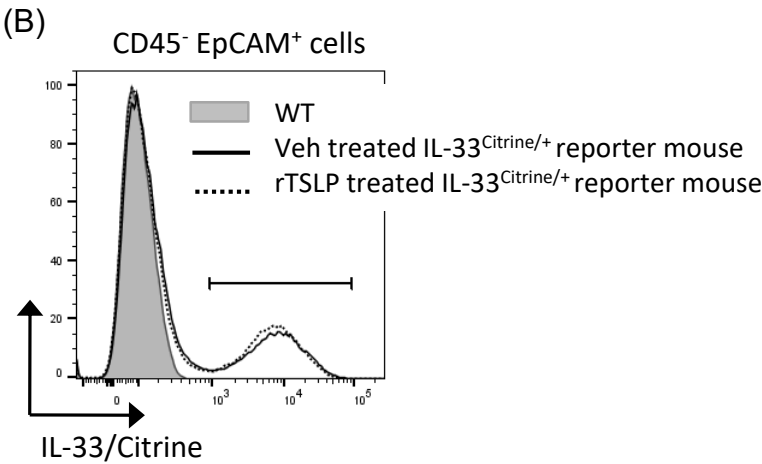
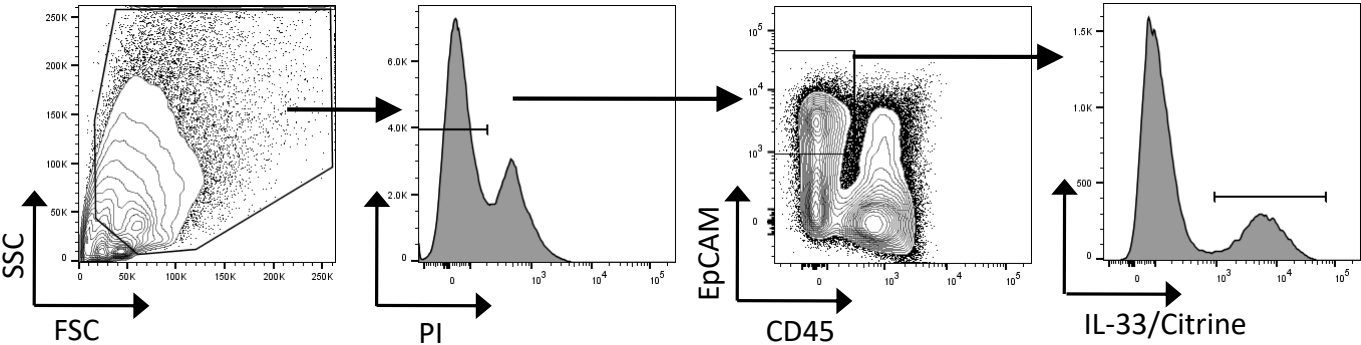


FIGURE S3

(A) Gating strategy of IL-33⁺ lung epithelial cells



(C) MFI of IL-33 on IL-33⁺ ECs

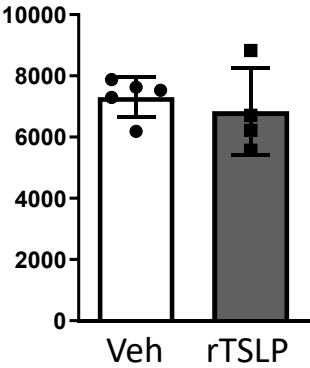
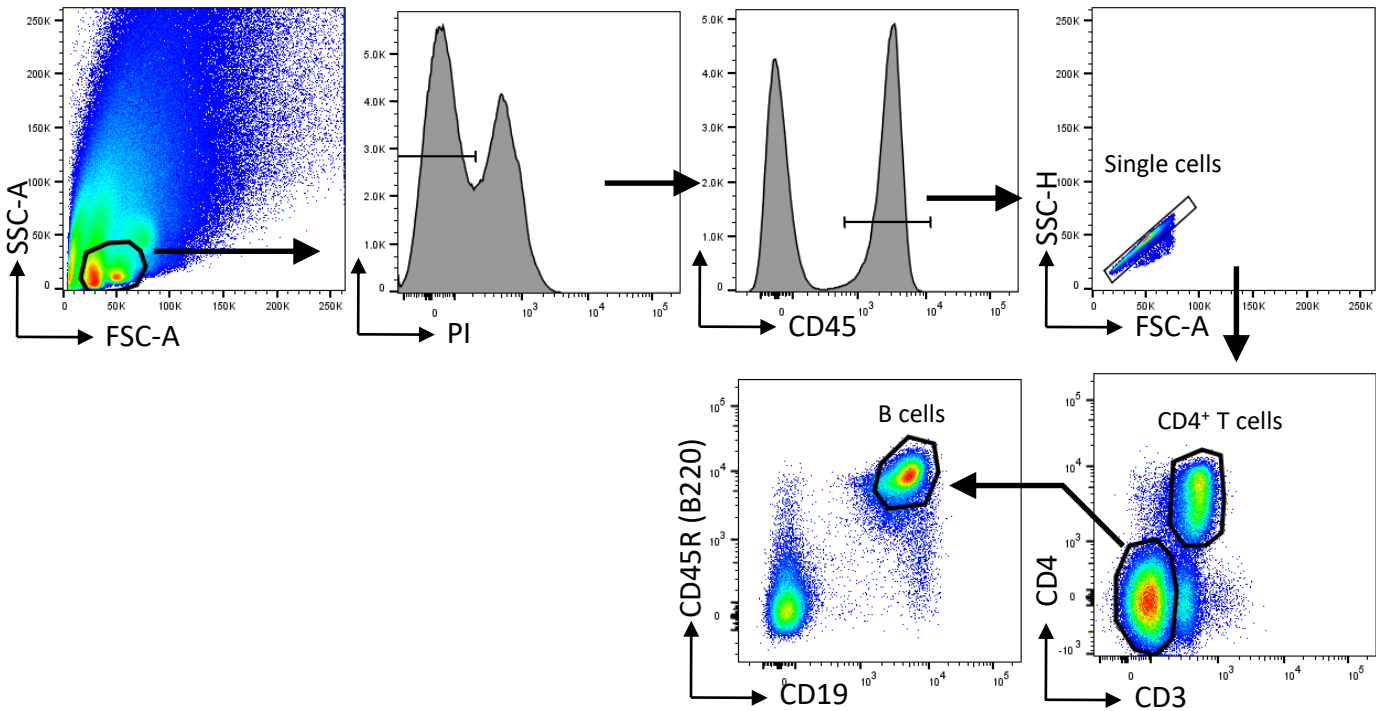


FIGURE S3

(D) Gating strategy of lung T cells and B cells



(E) Gating strategy of lung neutrophils and eosinophils

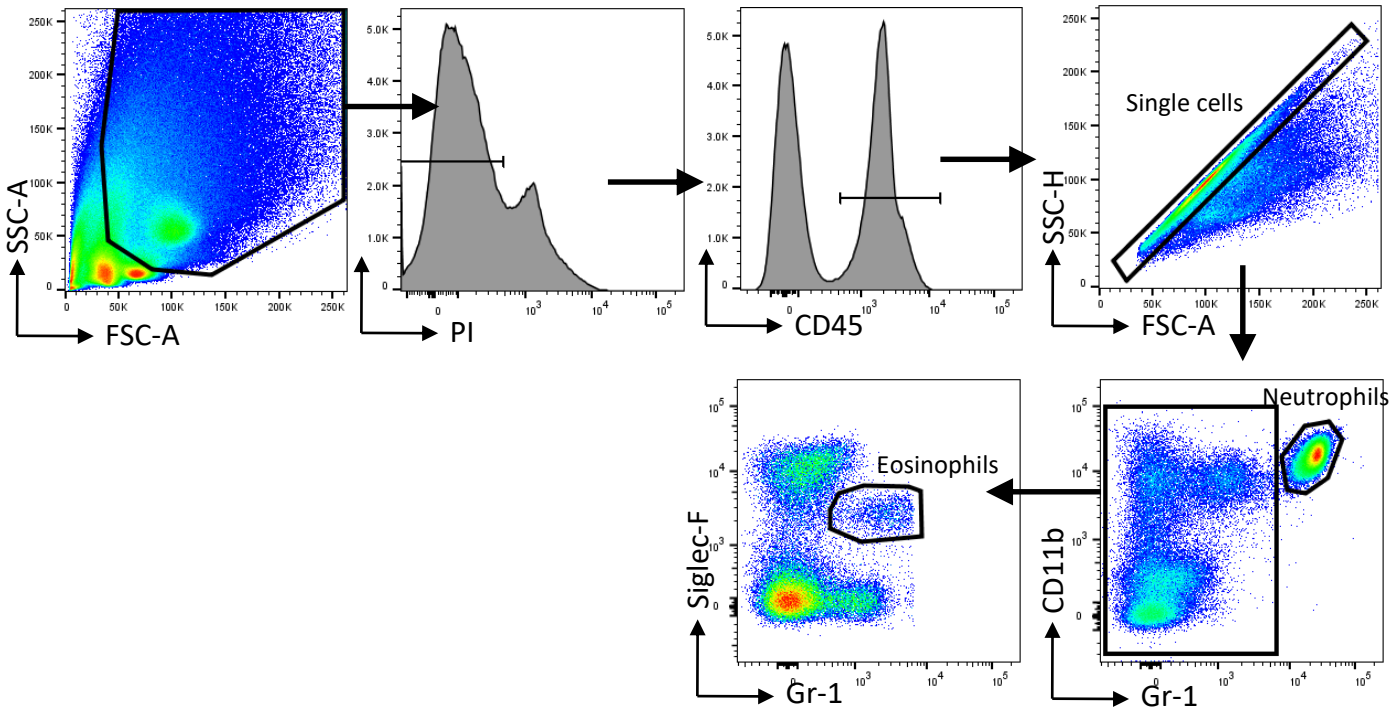
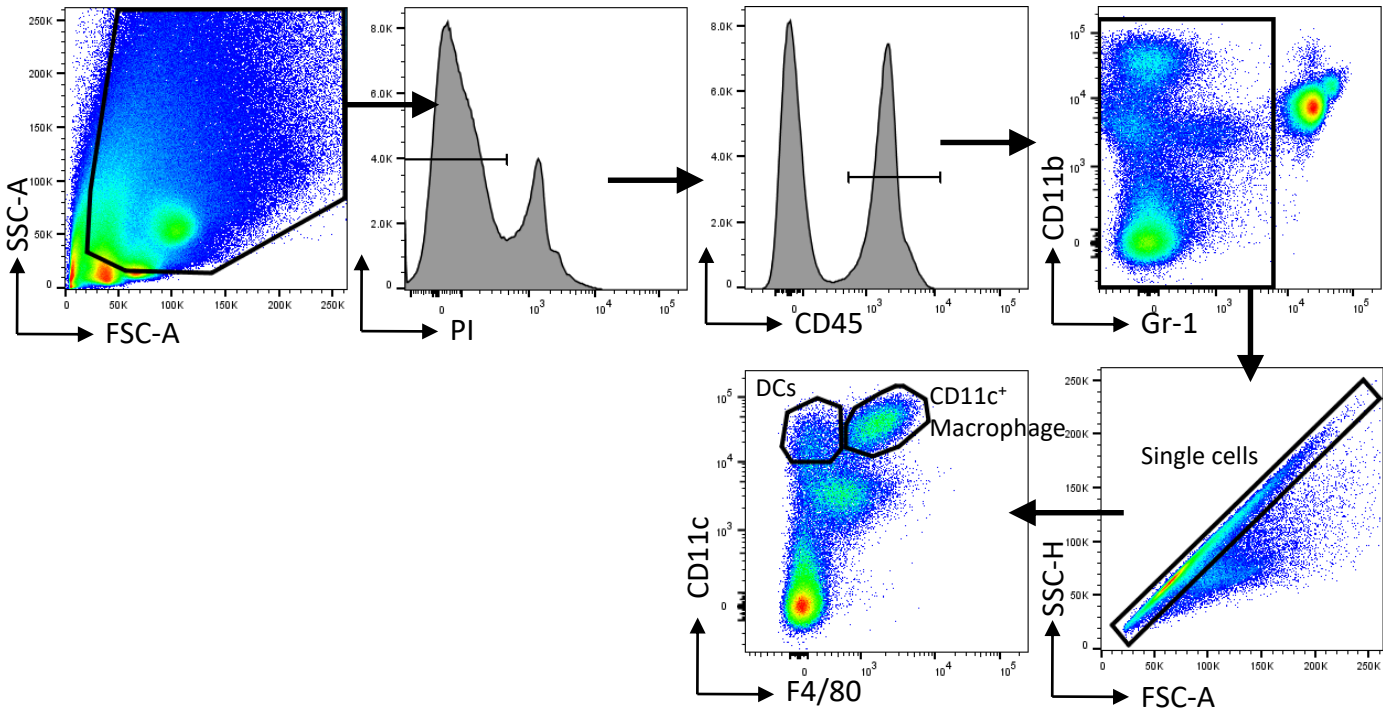


FIGURE S3

(F) Gating strategy of lung CD11c⁺ macrophages and dendritic cells



(G)

■ WT
— Veh treated IL-33^{Citrine/+} reporter mouse
..... rTSLP treated IL-33^{Citrine/+} reporter mouse

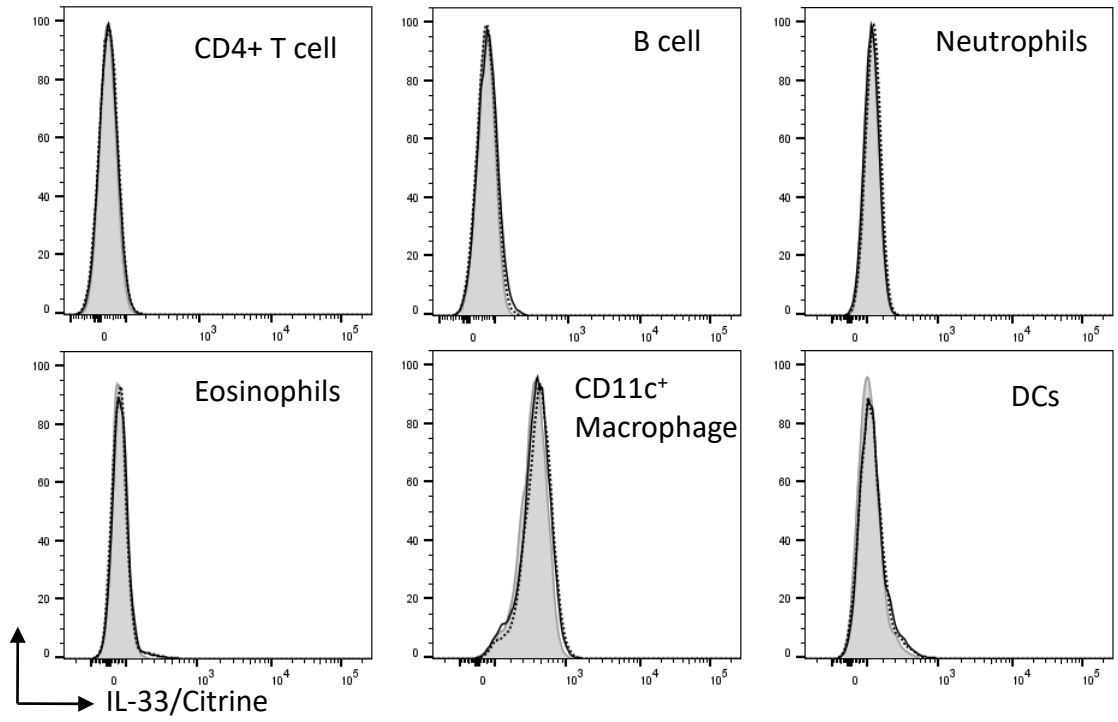


FIGURE S4

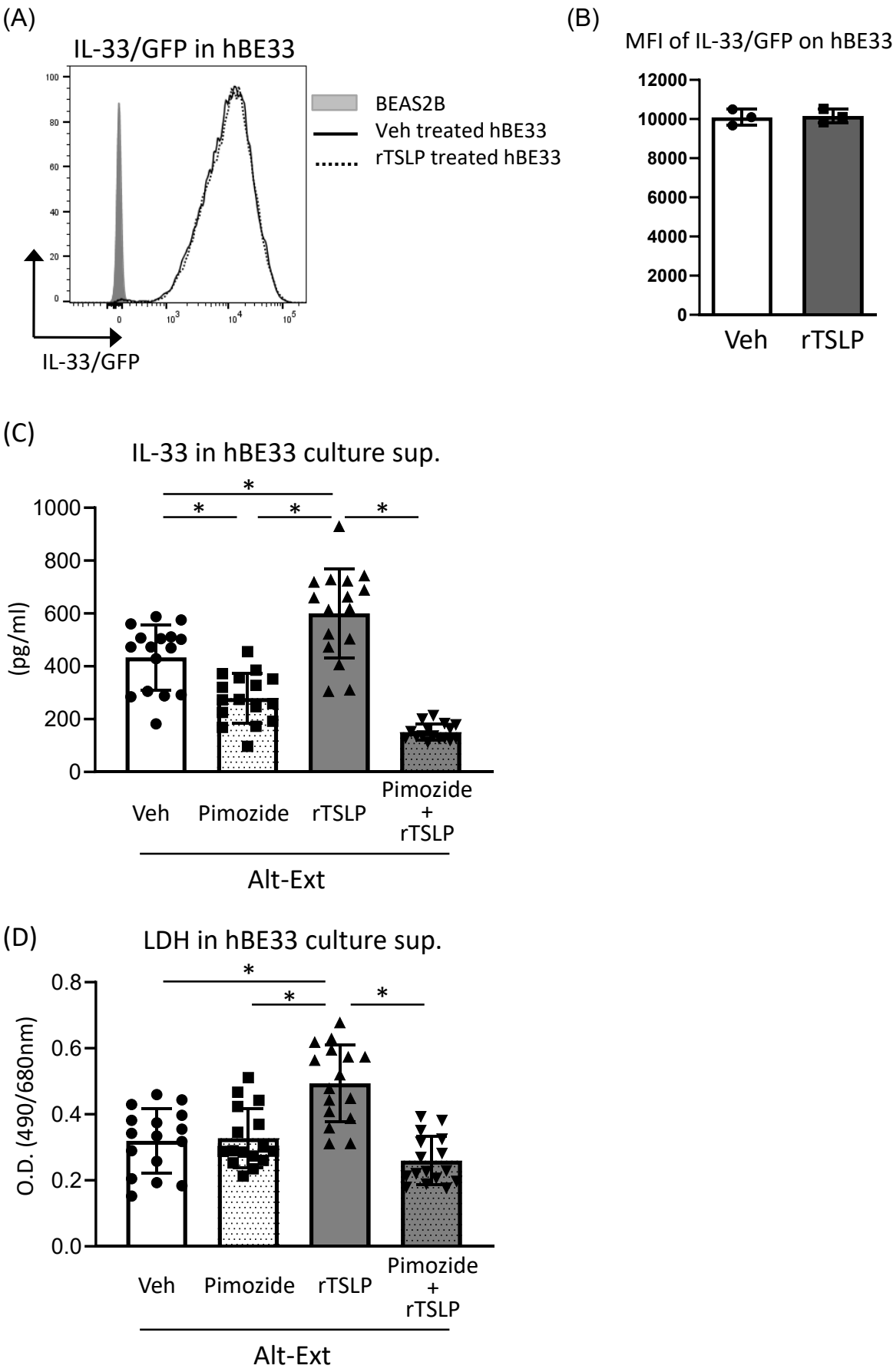
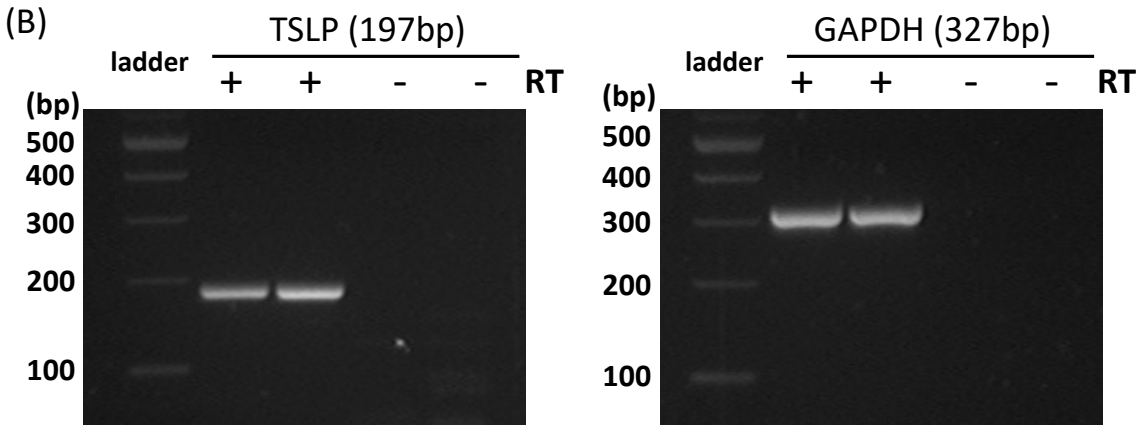
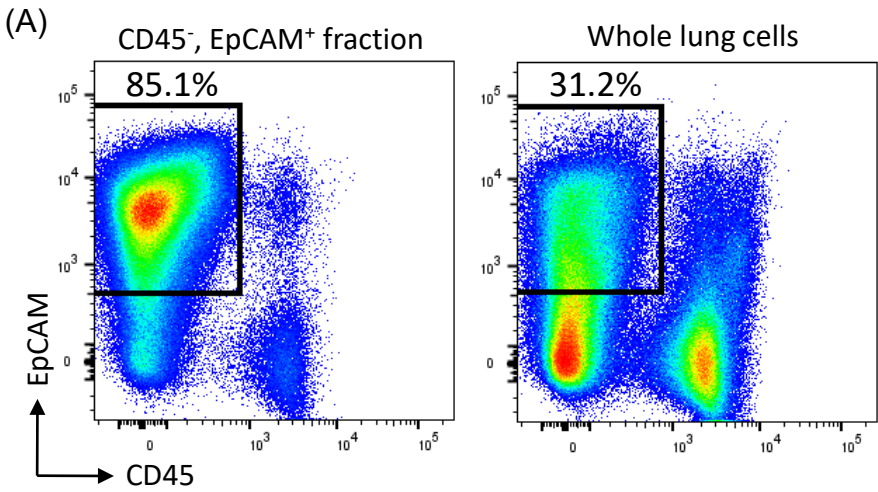


FIGURE S5



GAPDH (327bp)

ladder

+

+

-

-

RT

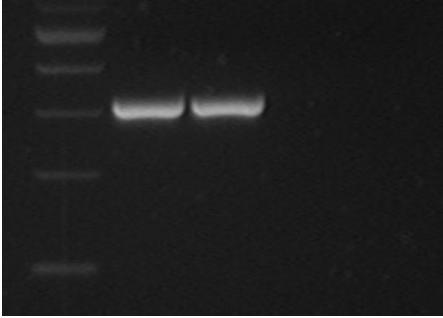


FIGURE S6

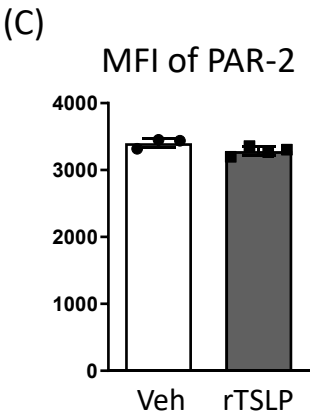
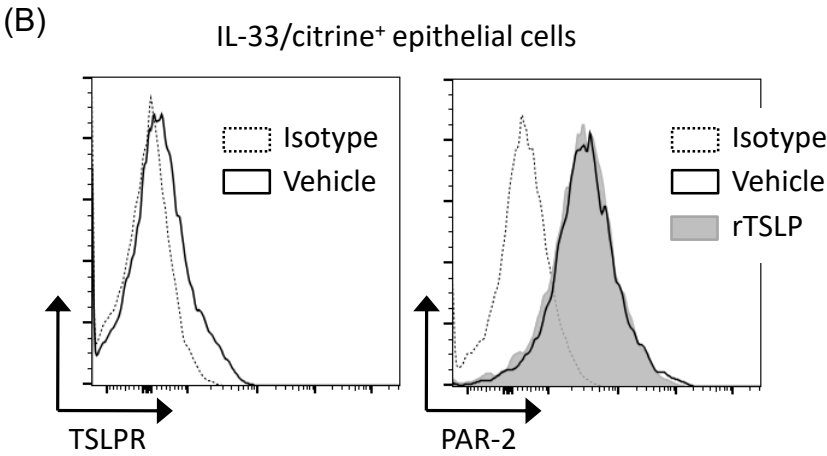
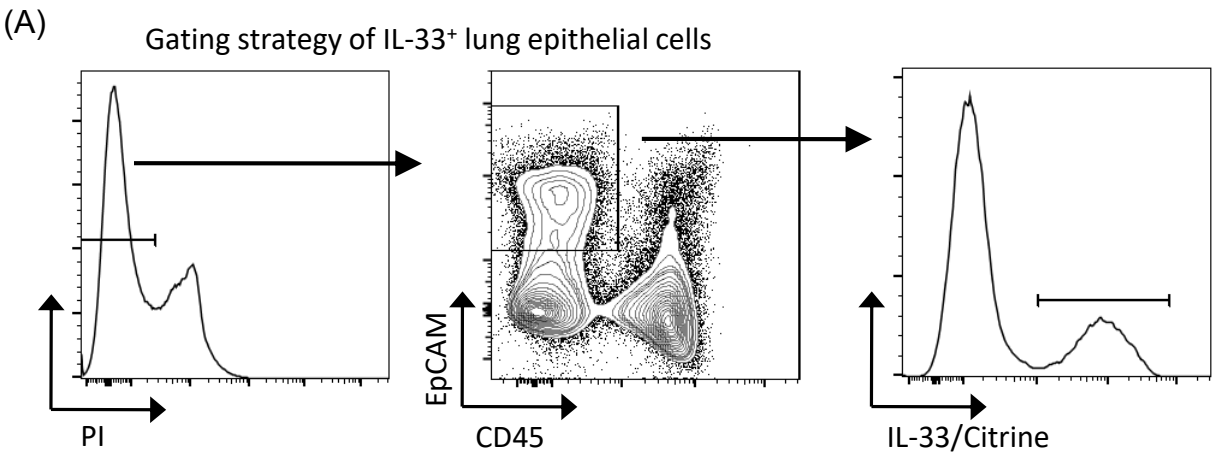
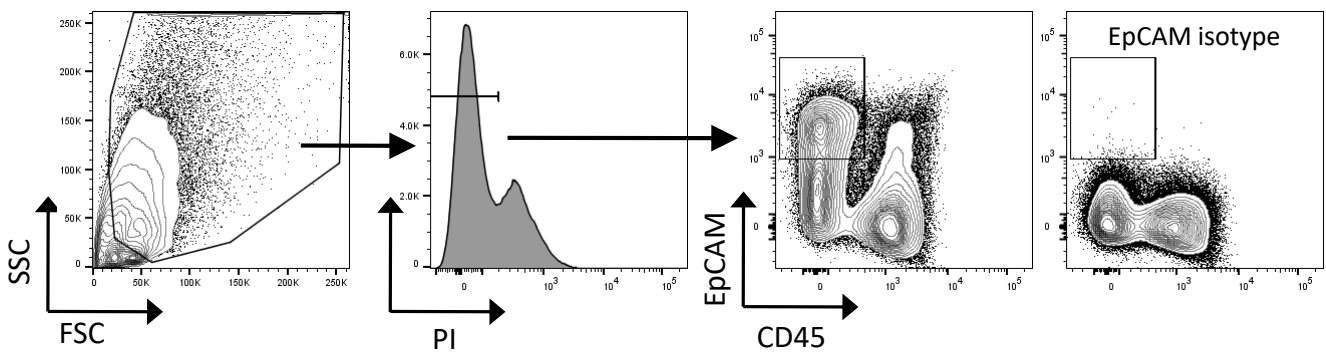


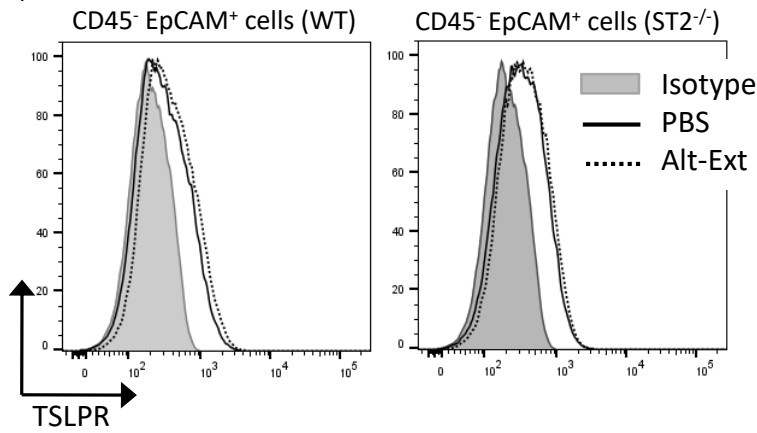
FIGURE S7

(A)

Gating strategy of lung epithelial cells



(B)



(C)

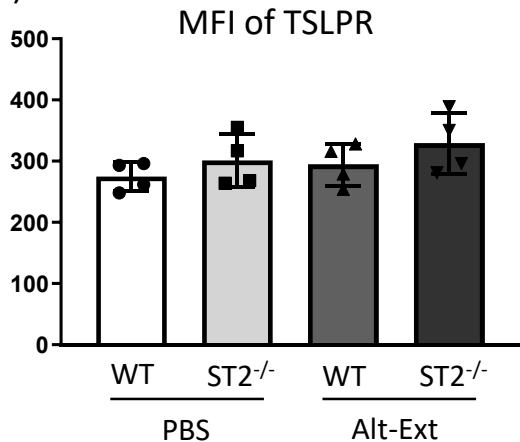
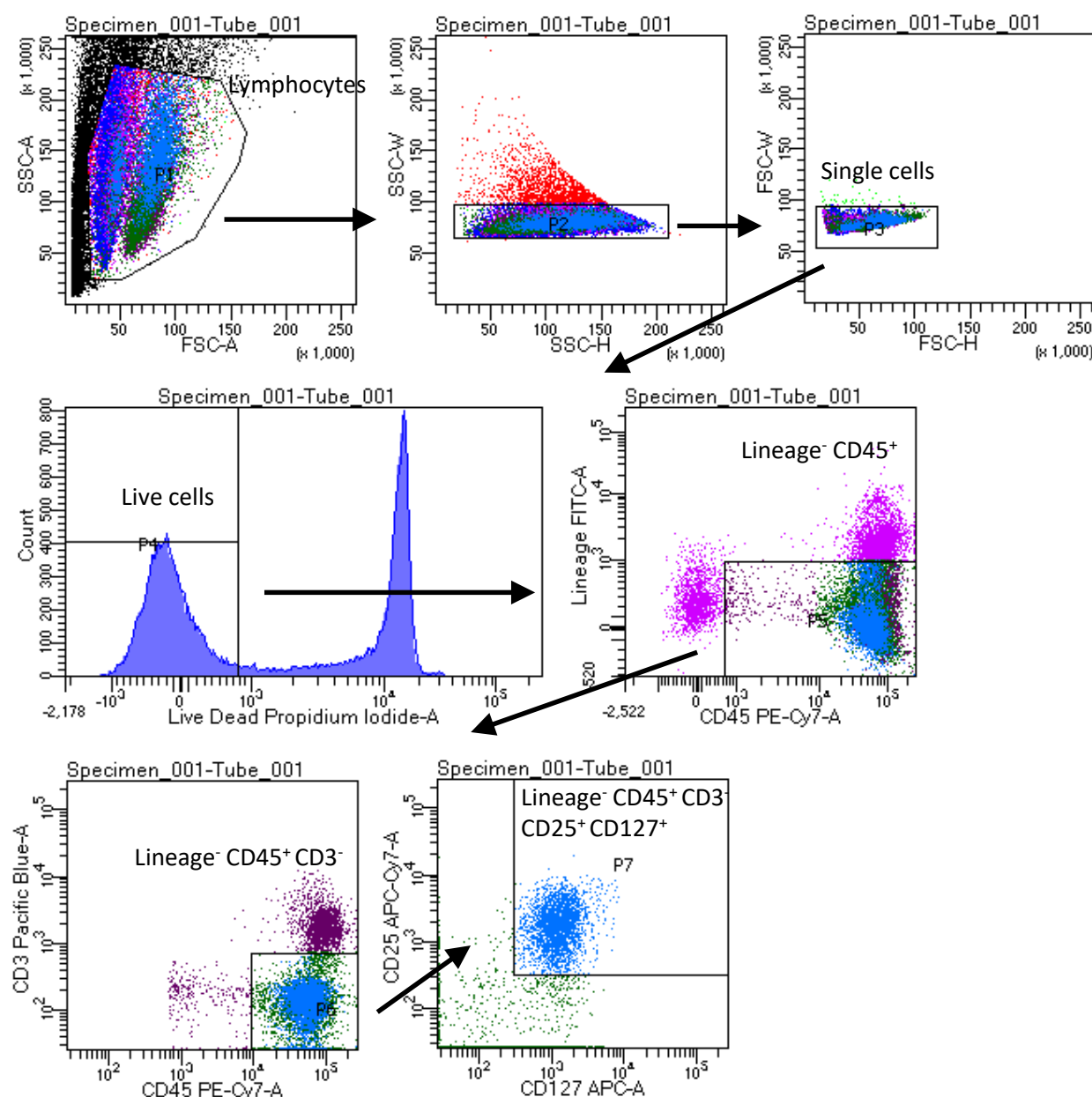


FIGURE S8

Gating strategy of lung ILC2 isolation from Lin⁻CD45⁺ cells



Tube: Tube_001

Population	#Events	%Parent	%Total
All Events	123,670	####	100.0
P1	25,393	20.5	20.5
P2	23,711	93.4	19.2
P3	23,673	99.8	19.1
P4	12,225	51.6	9.9
P5	7,971	65.2	6.4
P6	5,786	72.6	4.7
P7	2,771	47.9	2.2

FIGURE S9

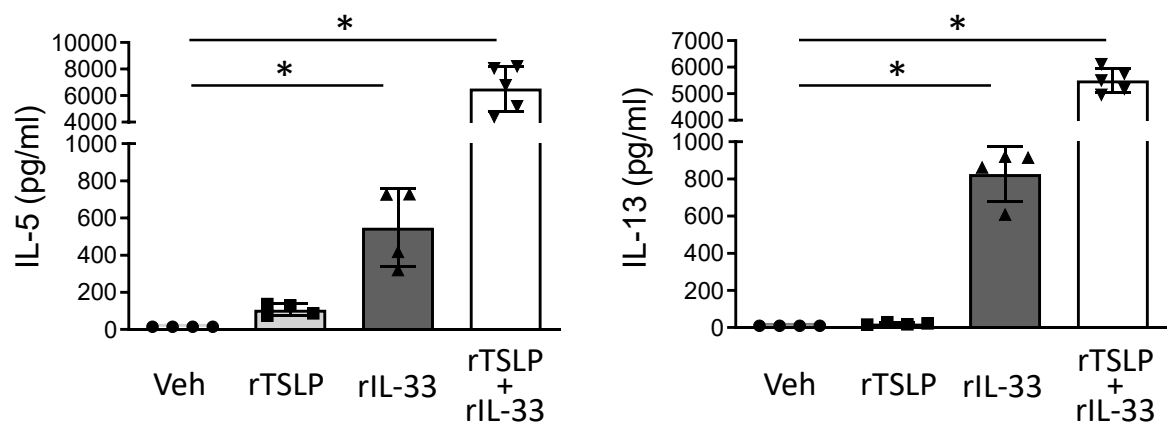
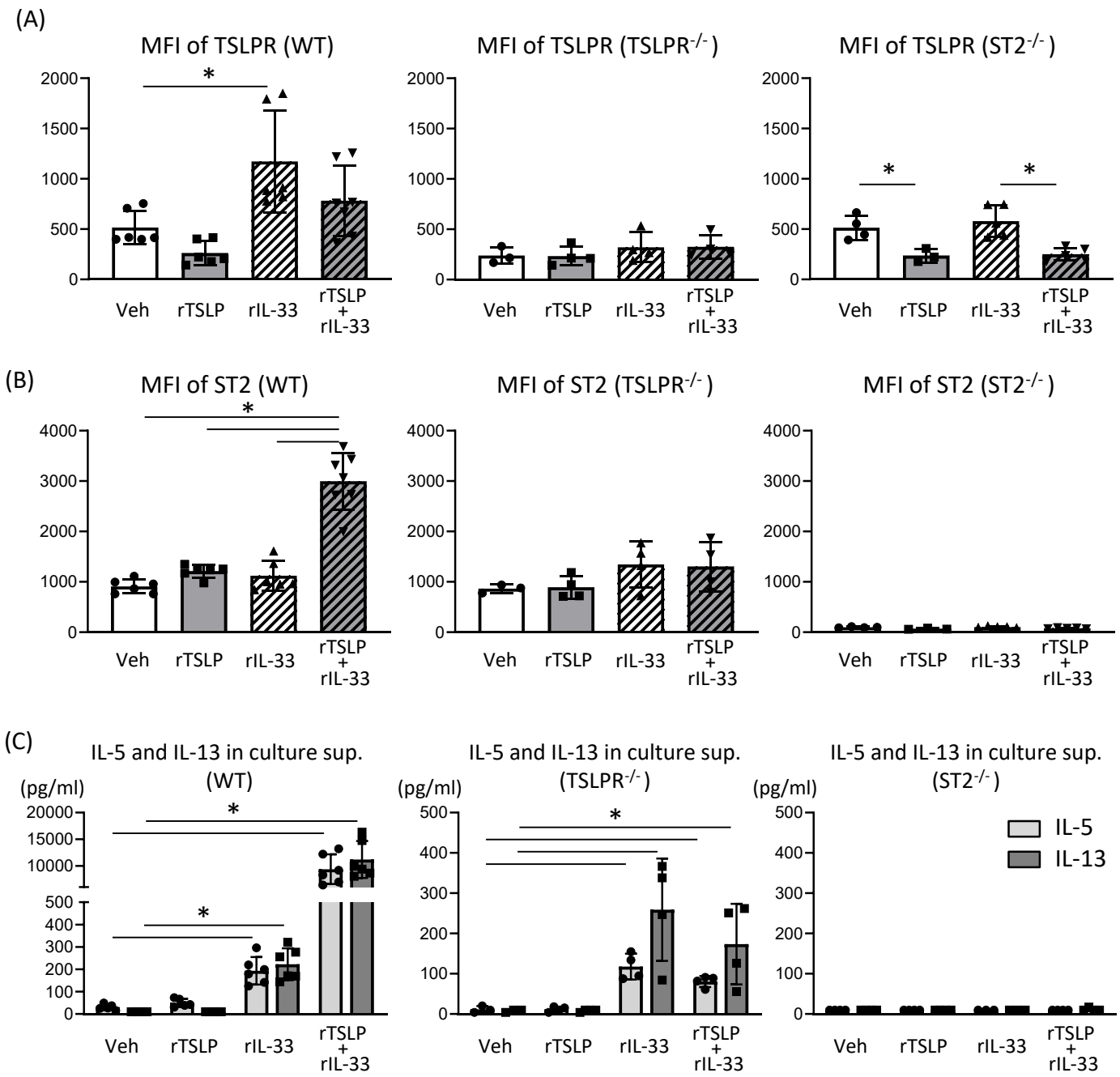


FIGURE S10



1 **Supporting Information**

2 **METHODS**

3 **Time course of Alt-Ext-induced alarmin**

4 Wild type (WT) BALB/c mice were challenged once intranasally with 5 µg (protein amount) of
5 *Alternaria* extract (Alt-Ext) in 100 µl PBS. One, 3, 6, 12, or 24 hours after the challenge, the
6 mice and naïve mice as a 0-hour control were sacrificed, and bronchoalveolar lavage fluid
7 (BALF) and the whole lungs were harvested.

9 **Alt-Ext-challenge model for TSLP and IL-33 induction**

10 Mouse recombinant TSLP (rTSLP) (200 ng) in 100 µl of 0.1% BSA/PBS or 100 µl of 0.1%
11 BSA/PBS as the vehicle was administered intranasally to WT BALB/c mice and TSLPR^{-/-} mice
12 6 h prior to intranasal challenge with either 5 µg (protein amount) of Alt-Ext in 100 µl of PBS or
13 100 µl of PBS as the vehicle. One hour after the Alt-Ext or PBS challenge, the mice were
14 sacrificed and BALF was harvested to measure IL-33 protein by ELISA. WT BALB/c and
15 TSLPR^{-/-} mice were challenged with Alt-Ext or PBS for 2 consecutive days. In the endogenous
16 TSLP neutralization model, anti-TSLP antibody (ab) or isotype ab were injected intraperitoneally
17 1 h prior to the first Alt-Ext or PBS challenge. The mice were sacrificed and BALF was
18 harvested 1 h after the second Alt-Ext or PBS challenge to measure IL-33 protein. WT BALB/c
19 and ST2^{-/-} mice were challenged with Alt-Ext or PBS. Six hours after the challenge, the mice
20 were sacrificed and the whole lungs were harvested to measure TSLP protein.

22 **hBE33 culture and stimulation**

23 Human bronchial epithelial cells stably expressing IL-33 (hBE33) were generated by transfection

methods with immortalized human bronchial epithelial cells from Lonza (Walkersville, MD) (1). The hBE33 were cultured with bronchial epithelial cell growth medium (BEGM) supplemented with growth factors but except retinoic acid (Lonza). The confluent cells were treated with human recombinant TSLP (20 ng/ml) or the vehicle (0.1%BSA/PBS) in the presence or absence of pimozide (2 μ M) for 6 h, and then the cells were stimulated with Alt-Ext (60 μ g/ml) or PBS for 1 h. The culture supernatants were used for ELISA to detect IL-33 and lactate dehydrogenase (LDH) assay.

Exogenous TSLP and IL-33 treatment for lung ILC2 detection in vivo

rTSLP (200 ng in 100 μ l of 0.1% BSA/ PBS), mouse recombinant IL-33 (rIL-33) (500 ng in 100 μ l of 0.1%BSA/PBS), or the vehicle were treated intranasally to WT BALB/c. Eighteen hours after the treatment, the mice were sacrificed and whole lungs were harvested for flow cytometry analysis.

Flow cytometry

The harvested lungs were minced and digested with collagenase IV (1 mg/ml, SIGMA-Aldrich, St. Louis, MO) and DNase I (50 IU/ml, SIGMA-Aldrich) in RPMI1640 with 5% FBS for 45 minutes at 37 °C and a single cell suspension was obtained by grinding and passing the digested lung through a 70 μ m cell strainer. Cell surface staining was performed to identify lung ILC2 as $\text{lin}^- \text{CD3}^- \text{CD45}^+ \text{CD25}^+ \text{ICOS}^+$ cells for TSLPR and ST2 detection. Furthermore, the lung cells were stimulated with PMA (10 ng/ml), ionomycin (1 μ M), and BD GolgiStopTM (BD Biosciences, San Jose, CA) at the manufacturer's recommended concentration for 5 hours, and then both cell surface and intracellular staining were performed to enumerate ILC2 ($\text{lin}^- \text{CD3}^-$

CD45⁺ CD25⁺ ICOS⁺) expressing IL-5 and IL-13. In the other protocol, digested lung cells from IL-33^{Citrine/+} reporter mice were stained with each antibodies to identify epithelial cells (CD45⁻ EpCAM⁺ cells), T cells (CD45⁺ CD3⁺ CD4⁺ cells), B cells (CD45⁺ CD3⁻ CD4⁻ CD19⁺ B220⁺ cells), neutrophils (CD45⁺ CD11b^{high} Gr-1^{high} cells), eosinophils (CD45⁺ Siglec-F⁺ Gr-1^{int} cells), macrophages (CD45⁺ Gr-1⁻ CD11c^{high} F4/80⁺ cells), and dendritic cells (CD45⁺ Gr-1⁻ CD11c⁺ F4/80⁻ cells) for IL-33/Citrine detection. All samples were measured on a BD LSR II Flow Cytometer (BD Biosciences) and analyzed using FlowJo Software.

Isolation and stimulation of lung ILC2

Lin⁻ CD45⁺ lung cells from mice were isolated using lineage cell depletion kit-mouse and CD45 microbeads (Miltenyi Biotech, Auburn, CA) according to the manufacturer's instructions. The cells were further purified by cell sorting (BD FACS Aria) following cell surface staining. To isolate naïve lung ILC2, we used anti-CD127 ab instead of anti-ICOS ab. Since CD127 expression was decreased in activated ILC2 in our pilot experiment (data not shown), CD127 is appropriate to identify the naïve lung ILC2. The purified lin⁻ CD3⁻ CD45⁺ CD25⁺ CD127⁺ cells as defined ILC2 were cultured in 96-well round bottom plate (20,000 cells/well), and stimulated with rTSLP (10 ng/ml), rIL-33 (10 ng/ml), rTSLP+rIL-33, or the vehicle in the presence or absence of pimozide (5 µM) for 40 h. The stimulated ILC2 were used to detect TSLPR and ST2 by flow and the culture media were used to detect the protein of IL-5 and IL-13 by ELISA. For western blotting, the isolated ILC2 were stimulated with rIL-33 (10 ng/ml), or the vehicle for 24 h, and then the cells were treated with rTSLP (10 ng/ml) or the vehicle for 1 h.

Isolation of CD45⁻ EpCAM⁺ lung epithelial cells

Lung epithelial cells (CD45⁻ EpCAM⁺ cells) from digested lungs were isolated using CD45 microbeads and CD326 (EpCAM) Microbeads (Miltenyi Biotech) according to the manufacturer's instructions. The purity of isolated cells was analyzed by Flow cytometry.

RT-PCR

Total RNA was extracted by RNeasy mini kit (Qiagen, Valencia, CA) from isolated lung CD45⁻ EpCAM⁺ cells. cDNA was synthesized using SuperScript IV (Thermo fisher scientific, Waltham, MA) with oligo dT primer according to manufacturer's instructions. RT product from 50 ng of total RNA was amplified in the presence of GoTaq DNA polymerase (Pomona, Madison, WI) with each primer sets. The primers were purchased from Integrated DNA Technologies (Coralville, IA) and include mouse TSLP (forward, 5'-AACAAGAGAAGCCCTCAATG-3'; reverse, 5'-CCAAGATAGAGATTTTCATAGCAG-3'; 197 bp), and mouse GAPDH (forward, 5'-GGCCCCTCTGGAAAGCTGTGG-3'; reverse, 5'-CCCGGCATCGAAGGTGGAAGA-3'; 327 bp). The TSLP PCR cycling conditions were 95°C for 2 min followed by 40 cycles of 94°C for 15 sec, 60°C for 30 sec, and 72°C for 30sec. The GAPDH PCR cycling conditions were 95°C for 2 min followed by 30 cycles of 94°C for 15 sec, 60°C for 30 sec, and 72°C for 30sec. PCR products were separated using 2% agarose gel electrophoresis and visualized by SYBR[®] safe DNA Gel Stain (Thermo fisher scientific).

Cytokine Measurements

DuoSet ELISA kits (TSLP and IL-33), Quantikine[®] ELISA mouse IL-4 from R&D systems (Minneapolis, MN) and Ready-Set-GO![®] ELISA kits (IL-5 and IL-13) from eBioscience (Waltham, MA) were used to measure the concentration of cytokines in BALF and lung

homogenates according to manufacturer's instructions. Quantikine® ELISA Human IL-33 (R&D systems) was used to detect IL-33 in hBE33 culture supernatants. Values below the detection limit were assigned a value at half of the lower limit of detection to allow for statistical analysis.

Lactate dehydrogenase (LDH) assay

Pierce LDH cytotoxicity assay kit (Thermo fisher scientific) was used to evaluate Alt-Ext-induced cell damage associated with IL-33 release from hBE33 according to manufacturer's instructions.

Cell differential counting

Cytospin preparations of BALF cells were performed for each sample. The slides were stained with a Richard-Allan Scientific Three step stain kit (Thermo fisher scientific) to determine cell differentials. Differential counts were based on counting 200 cells per slide.

Histological analyses of lung sections

Forty-eight hours after the last challenge of *Alternaria* extract or PBS, mice were sacrificed and the lung tissue was fixed in 10% formalin solution, paraffin-embedded, cut in 5 µm sections, mounted, and stained with periodic acid–Schiff's (PAS) solution to visualize mucus and mucous producing cells. Slides were scored by a pathologist blinded to the experimental groups. All airways in the section were scored (15–40 airways) according to a five-point scale: 0, no PAS-positive cells; 1, < 5% PAS-positive cells; 2, 5–10% PAS-positive cells; 3, 10–25% PAS-positive cells; and 4, > 25% PAS-positive cells.

Western blotting

After stimulation, isolated lung ILC2 were lysed in RIPA buffer containing protease inhibitor and phosphatase inhibitor. Lysates were denaturized with 4x laemmli sample buffer including DTT on a heating block at 95 °C for 5 min. The lysates were separated by SDS-PAGE using 4-20% Mini-PROTEAN[®] TGX[™] precast gel (Bio-Rad, Hercules, CA). Separated protein was transferred to nitrocellulose membranes using iBlot Transfer stack nitrocellulose mini and iBlot dry blotting system (Thermo fisher scientific). The membrane was blocked with Odyssey Blocking buffer (LI-COR Biosciences, Lincoln NE) for 2 h, incubated overnight at 4 °C with the primary antibodies, and then further incubated for 1 h with the primary antibody-matched secondary antibodies. The stained membrane was scanned and analyzed by Odyssey imaging system (LI-COR Biosciences).

REFERENCES

1. Uchida M, Anderson EL, Squillace DL, Patil N, Maniak PJ, Iijima K, et al. Oxidative stress serves as a key checkpoint for IL-33 release by airway epithelium. *Allergy*. 2017;72:1521-1531.

Figure legends

FIGURE S1 The gating strategy and representative plots of lung group 2 innate lymphoid cells (ILC2) expressing IL-5 and IL-13. A, The gating strategy of lung ILC2. ILC2 were identified by flow cytometry as $\text{lin}^- \text{CD3}^- \text{CD4}^- \text{CD45}^+ \text{CD25}^+ \text{ICOS}^+$ cells. B, Representative plots of IL-5 and IL-13 intracellular staining on lung ILC2 from the wild type (WT) and $\text{TSLPR}^{-/-}$ mice challenged with *Alternaria* extract (Alt-Ext) or PBS.

FIGURE S2 TSLPR signaling enhanced *Alternaria* extract (Alt-Ext)-induced IL-33 release in the airway. Recombinant TSLP (rTSLP) or vehicle was treated intranasally to BALB/c wild type (WT) mice and $\text{TSLPR}^{-/-}$ mice 6 h prior to Alt-Ext-challenge. IL-33 in the bronchoalveolar lavage fluid (BALF) was measured by ELISA. The data are shown as mean $\pm$ SD (n=4-6). * $P < 0.05$.

FIGURE S3 Recombinant TSLP (rTSLP) treatment did not change the IL-33 expression on lung epithelial cells. A, The gating strategy of IL-33⁺ epithelial cells (ECs) in the lungs by flow cytometry. Representative histograms of IL-33/Citrine expression on lung ECs from IL-33^{Citrine/+} reporter mice treated with rTSLP or vehicle for 6 h. C, Mean fluorescence Intensity (MFI) of IL-33/Citrine on the lung IL-33⁺ ECs. The data are a combination of 2 independent experiments and shown as mean $\pm$ SD (n=4-5). D, The gating strategy of CD4⁺ T cells, and B cells in the lungs by flow cytometry. E, The gating strategy of neutrophils, and eosinophils in the lungs. F, The gating strategy of CD11c⁺ macrophages, and dendritic cells in the lungs. G, Representative histograms of IL-33/Citrine expression on lung CD4⁺ T cells, B cells, neutrophils, eosinophils,

CD11c⁺ macrophages, and dendritic cells from rTSLP and vehicle treated IL-33^{Citrine/+} reporter mice. The histograms are representative of 3 different samples.

FIGURE S4 Recombinant TSLP (rTSLP) treatment did not change IL-33/GFP expression on human bronchial epithelial cells stably expressing IL-33 (hBE33), but enhanced *Alternaria* extract (Alt-Ext)-induced IL-33 and lactate dehydrogenase (LDH) release. A, Representative histogram and B, MFI of IL-33/GFP expression on hBE33 treated with rTSLP or vehicle for 6 h. C, hBE33 was cultured with rTSLP (20 ng/ml) or vehicle in presence or absence of pimozide (2 µM) for 6 h. The cell culture supernatant was collected 1 h after Alt-Ext (60 µg/ml) stimulation. IL-33 in the culture supernatant was measured by ELISA. D, LDH was measured by LDH assay kit to evaluate Alt-Ext-induced cell damage. The data are a combination of 4 independent experiments using 2 wells x 2 different passage of cells and shown as mean ± SD (n=16). * $P < 0.05$.

FIGURE S5 Lung epithelial cells (ECs) expressed mRNA of TSLP. A, Flow cytometry analysis of lung ECs (CD45⁻ EpCAM⁺) by magnetic isolation and whole lung cells from naïve wild type (WT) BALB/c mice. B, RT-PCR of TSLP in isolated mouse lung CD45⁻ EpCAM⁺ cells. RT= reverse transcriptase.

FIGURE S6 IL-33⁺ epithelial cells (ECs) expressed protease activated receptor 2 (PAR-2) and TSLPR. A, The gating strategy of IL-33⁺ ECs in the lungs by flow cytometry. B, Representative histograms of PAR-2 and TSLPR expression on IL-33⁺ CD45⁻ EpCAM⁺ ECs in the lungs from IL-33^{Citrine/+} reporter mice treated with vehicle (black solid line) or rTSLP (filled with gray).

Isotype control staining was shown by black dotted line. C, Mean fluorescence Intensity (MFI) of PAR-2 on IL-33⁺ ECs. The data are a combination of 2 independent experiments and shown as mean $\pm$ SD (n=3-4).

FIGURE S7 *Alternaria* extract (Alt-Ext) did not change TSLPR expression on lung epithelial cells (ECs) in both wild type (WT) and IL-33 receptor deficient (ST2^{-/-}) mice. A, The gating strategy of lung ECs by flow cytometry. B, Representative histograms of TSLPR expression on CD45⁻ EpCAM⁺ ECs in the lungs from WT and ST2^{-/-} mice treated with PBS (black solid line) or Alt-Ext (black dotted line). Isotype control staining was shown by filled gray area. C, Mean fluorescence Intensity (MFI) of TSLPR on lung ECs. The data are a combination of 2 independent experiments and shown as mean $\pm$ SD (n=4).

FIGURE S8 The gating strategy of lung group 2 innate lymphoid cells (ILC2) sorting. Lineage (lin)⁻ CD45⁺ cell fraction was separated from whole lung cells by magnetic cell sorting. Lung ILC2 were isolated from the lin⁻ CD45⁺ cell fraction by flow cytometric sorting as lin⁻ CD3⁻ CD45⁺ CD25⁺ CD127⁺ cells.

FIGURE S9 IL-5 and IL-13 protein level in cell-free culture supernatant of isolated lung group 2 innate lymphoid cells (ILC2). The isolated lung ILC2 were treated with recombinant TSLP (rTSLP), recombinant IL-33 (rIL-33), rTSLP+rIL-33, or the vehicle for 40 h. IL-5 and IL-13 protein in the ILC2 culture supernatant were measured by ELISA. The data are shown as mean $\pm$ SD of 3 independent experiments. * $P < 0.05$

FIGURE S10 TSLPR, IL-33 receptor (ST2) expression, and type-2 cytokine production from isolated lung group 2 innate lymphoid cells (ILC2) from WT, TSLP receptor deficient (TSLPR^{-/-}), and IL-33 receptor deficient (ST2^{-/-}) mice. The isolated lung ILC2 from each mice strain were treated with recombinant TSLP (rTSLP), recombinant IL-33 (rIL-33), rTSLP+rIL-33, or the vehicle for 40 h. A, Mean fluorescence Intensity (MFI) of TSLPR on isolated lung ILC2 from each mice strain. B, MFI of ST2 on isolated lung ILC2 from each mice strain. C, IL-5 and IL-13 protein level in culture supernatant of isolated lung ILC2 from each mice strain. The data are shown as mean $\pm$ SD of 4 independent experiments. * $P < 0.05$

Antigen	Fluorochrome	Clone	Supplier
Flow cytometry			
CD3	(biotin)	17A2	TONBO Biosciences (San Diego, CA)
CD3	violetFluor™ 450	17A2	TONBO Biosciences
CD4	PE	GK1.5	BD Pharmingen™ (San Jose, CA)
CD4	APC-Cy7	GK1.5	BioLegend (San Diego, CA)
CD11b	PE	M1/70	BD Pharmingen™
CD11b	PE-Cy7	M1/70	eBioscience™ (Waltham, MA)
CD11c	PE	N418	eBioscience™
CD19	PE	6D5	BioLegend
CD25	Alexa488	PC61	BioLegend
CD45	redFluor™ 710	30-F11	TONBO Biosciences
CD45R (B220)	APC	RA3-6B2	TONBO Biosciences
CD127	APC	A7R34	BioLegend
CD170 (SiglecF)	eFluor 660	1RNM44N	eBioscience™
CD278 (ICOS)	PE	7E.17G9	eBioscience™
CD326 (EpCAM)	APC-Cy7	G8.8	BioLegend
F4/80	APC	BM8	BioLegend
Gr-1 (Ly-6G/Ly-6C)	APC-Cy7	RB6-8C5	BioLegend
IL-5	APC	TRFK5	BD Pharmingen™
IL-13	PE-Cy7	eBio13A	eBioscience™
PAR-2	PE	SAM11	Santa Cruz Biotechnology (Santa Cruz, CA)
TSLPR	PE	(Goat IgG)	R&D Systems (Minneapolis, MN)
ST2	PerCP-Cy5.5	RMST2-2	eBioscience™

Lineage cell detection cocktail-biotin (mouse)	(Biotin)		Miltenyi Biotech Inc. (Auburn, CA)
Streptoavidin-APC-Cy7	APC-Cy7		BioLegend
Ghost Dye TM UV450	UV450		TONBO Biosciences
Propidium iodide			BD Pharmingen™

ILC2 and EpCAM⁺ epithelial cell isolation

Lineage Cell Depletion Kit, mouse			Miltenyi Biotech Inc.
CD45 Micro Beads, mouse			Miltenyi Biotech Inc.
CD326 (EpCAM) Micro Beads, mouse			Miltenyi Biotech Inc.
CD3	Pacific Blue	17A2	TONBO Biosciences
CD25	APC-Cy7	PC61	BioLegend
CD45	PE-Cy7	30-F11	BioLegend
CD127	APC	A7R34	BioLegend
Lineage cell detection cocktail-biotin (mouse)	(Biotin)		Miltenyi Biotech Inc.
Streptoavidin-FITC	FITC		BioLegend
Propidium iodide			BD Pharmingen™

TSLP neutralization

Anti TSLP ab		28F12	Developmental Studies Hybridoma Bank (Iowa City, IA)
Isotype Control ab		RTK2758	BioLegend

Western blotting

Anti-phospho STAT5A/B (Tyr699/694)		8-5-2	Millipore SIGMA (Burlington, MA)
Anti-STAT5		D2O6Y	Cell Signaling Technology (Danvers, MA)

Anti-actin		I-19	Santa Cruz Biotechnology
IRDye® Donkey anti-mouse	680LT		LI-COR Biosciences (Lincoln NE)
IRDye® Goat anti-rabbit	800CW		LI-COR Biosciences
IRDye® Donkey anti-goat	680LT		LI-COR Biosciences