

ORIGINAL ARTICLE

Asthma and Lower Airway Disease

Eosinophil extracellular traps activate type 2 innate lymphoid cells through stimulating airway epithelium in severe asthma

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Abstract

Background: Activated eosinophils release extracellular traps (EETs), which contribute to airway inflammation in severe asthma (SA). However, the role of EETs in innate immunity has not yet been completely determined. The present study aimed to demonstrate the mechanism of airway inflammation in SA mediated by EETs.

Methods: Peripheral counts of EET⁺ eosinophils and type 2 innate lymphoid cells (ILC2s) were evaluated in patients with SA (n = 13), nonsevere asthma (NSA, n = 17), and healthy control subjects (HC, n = 8). To confirm the effect of EETs, airway hyper-responsiveness (AHR) and adapted/innate immune responses were assessed in mice. Furthermore, the effects of anti-IL-33/TSLP antibody were tested.

Results: The numbers of EET⁺ eosinophils and ILC2s were significantly elevated in SA, with a positive correlation between these two cells ($r = .539$, $P < .001$). When mice were injected with EETs, we observed significant increases in epithelium-derived cytokines (IL-1 α , IL-1 β , CXCL-1, CCL24, IL-33, and TSLP) and eosinophil/neutrophil count in bronchoalveolar lavage fluid (BALF) as well as an increased proportion of IL-5- or IL-13-producing ILC2s in the lungs. When Rag1^{-/-} mice receiving ILC2s were treated with EETs, increased AHR and IL-5/IL-13 levels in BALF were noted, which were effectively suppressed by anti-IL-33 or anti-TSLP antibody.

Conclusion: EETs could enhance innate and type 2 immune responses in SA, in which epithelium-targeting biologics (anti-IL-33/TSLP antibody) may have a potential benefit.

KEYWORDS

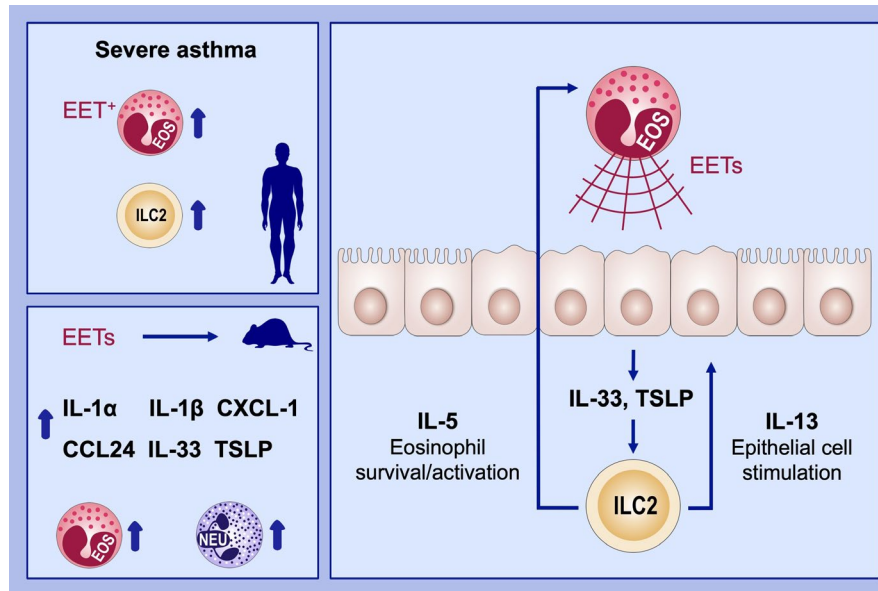
eosinophils, epithelium, extracellular traps, innate lymphoid cells, severe asthma

1 | INTRODUCTION

Asthma is not a single disease entity, but has various phenotypes with different underlying pathophysiologic mechanisms.¹⁻³ However, it has long been regarded as eosinophilic inflammation in the majority of adult asthmatic patients, especially in severe asthma (SA).⁴ Therefore, eosinophils are the major effector cells to induce type

2 immune responses in the upper and lower airways of asthmatic patients.⁵ Patients with high airway/blood eosinophilia experience frequent asthma exacerbations.

Previous studies demonstrated that activated eosinophils release extracellular traps (EETs; a meshwork of DNA fibers and granule proteins), which contribute to allergic diseases in association with blood and tissue eosinophilia.⁶⁻¹¹ Our recent study showed significantly



GRAPHICAL ABSTRACT

The numbers of EET⁺ eosinophils and type 2 innate lymphoid cells (ILC2s) are significantly elevated in severe asthma. EETs treatment in mice induces significant increase of epithelium-derived cytokines (IL-1 α , IL-1 β , CXCL-1, CCL24, IL-33, and TSLP) and eosinophil/neutrophil count in bronchoalveolar lavage fluid. EETs activate IL-5 or IL-13-producing ILC2s through stimulating airway epithelium.

Abbreviations: EETs, Eosinophil extracellular traps; TSLP, Thymic stromal lymphopoietin

increased levels of EETs in patients with SA that had inflammatory effects on airway epithelial cells and neighboring eosinophils *in vitro*.¹² The function of EETs in asthmatic airways has not yet been completely understood; however, it is suggested that their unique biologic activities may play a role in eosinophilic inflammation in SA.

Severe asthma remains incompletely understood, but it is certain that eosinophils and their association with other inflammatory and structural cells are closely interactive. Airway epithelial cells, the first line of the epithelial barrier, produce several cytokines such as interleukin (IL)-33, IL-25, and thymic stromal lymphopoietin (TSLP), which are key cytokines for inducing eosinophilic inflammation via innate immune responses. These cytokines further activate type 2 innate lymphoid cells (ILC2s; producing type 2 cytokines such as IL-5 and IL-13), ultimately leading to persistent eosinophilia and steroid resistance.¹³⁻¹⁹ However, upstream mechanisms involved in the ongoing production of IL-4, IL-5, and IL-13 in asthmatic airways have not yet been fully determined.

We hypothesized that EETs might enhance airway inflammation through stimulating airway epithelium. This study aimed to investigate (a) the clinical significance of EET⁺ eosinophils and ILC2s in SA; (b) the effect of EETs on airway inflammation and steroid resistance in mice with allergic asthma; and (c) therapeutic intervention for controlling type 2 immune responses in asthmatic airways of SA.

2 | METHODS

2.1 | Patient recruitment and clinical characteristics

The study was approved by the Institutional Review Board of Ajou University Hospital (AJIRB-GEN-SMP-13-108). Asthmatic patients

were diagnosed by recurrent episodes of wheezing, dyspnea, cough, and sputum production as well as evidence of either airway hyper-responsiveness to methacholine or reversible airway obstruction improved by a short-acting β_2 agonist.²⁰ Atopy status was evaluated by at least 1 positive result on skin prick tests with common inhaled allergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat, dog, cockroach, tree and grass pollen mixtures, mugwort, ragweed, Japanese hops, *Aspergillus*, and *Alternaria*; Bencard). Serum total IgE was measured using the ImmunoCAP system (ThermoFisher Scientific). Among patients with eosinophilic asthma enrolled, SA was defined according to the definition by The International European Respiratory Society/American Thoracic Society guidelines.²¹ In addition, all patients who are current smokers or comorbid with chronic obstructive pulmonary disease or other chronic diseases affecting asthma outcomes were excluded. As a control group, healthy control subjects (HCs) were recruited. All the study subjects submitted informed consent. Plasma samples from the patients were collected simultaneously during the collection process of peripheral eosinophils. Levels of plasma IL-13, IL-33, and TSLP (R&D systems) were measured using ELISA kits according to the manufacturer's instructions.

2.2 | EET isolation and detection

Blood samples were collected in BD Vacutainer[®] tubes containing acid citrate dextrose solution (BD Biosciences). These samples were layered on a Lymphoprep[™] Solution (Axis-Shield), followed by centrifugation at 879 g at 20°C for 25 minutes. The layer containing granulocytes was separated and placed in Hank's balanced salt solution buffer, with 2 mmol/L ethylenediaminetetraacetic

acid (EDTA) and 2% dextran, for 20 minutes. After removing red blood cells by hypotonic lysis, eosinophils were isolated from the fraction containing granulocytes using the Eosinophil Isolation Kit (Miltenyi Biotec Inc) according to the manufacturer's instructions. To induce EET formation, eosinophils (1×10^6) were primed with 25 ng/mL human recombinant IL-5 (Sigma-Aldrich) for 20 minutes and stimulated with 300 ng/mL LPS (Sigma-Aldrich) for 4 hours in serum-free RPMI-1640 medium (Sigma-Aldrich). After stimulation, medium was removed and its sticky layer was washed out with phosphate-buffered saline (PBS). Finally, 30 minutes after treatment with 1 U/mL MNase (Thermo Fisher Scientific), the supernatant was harvested by centrifugation at 2500 g for 5 minutes. EET-forming eosinophils stained with DAPI (Sigma-Aldrich) and anti-EPO antibody (Cell Signaling) were observed using a Zeiss LSM710 confocal microscope (Carl Zeiss AG). Components of granule proteins in EETs were also confirmed by Western blotting using anti-MBP antibody (Abcam), anti-ECP antibody (Santa Cruz), or anti-EPO antibody (Santa Cruz). For a transmission electron microscopy, EETs stained with 2% uranyl acetate were further investigated using a JEM1011 microscope (JEOL) at accelerating voltage of 100 kV.

2.3 | Mouse models

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Ajou University (IACUC-2017-0067). Female 6-week-old BALB/c wild-type mice (Jackson Laboratory) were maintained under specific pathogen-free conditions. In this study, recombinant mouse IL-5/IL-13 (R&D systems) and monoclonal anti-IL-33/TSLP antibodies (R&D systems) were used. The flexiVent System (SCIREQ) was used to measure AHR. Each mouse was injected with aerosol methacholine (Sigma-Aldrich). The types of inflammatory cells in bronchoalveolar lavage fluid (BALF) were determined by Diff-quick staining (Dade Behring), which were classified as macrophages, eosinophils, neutrophils, or lymphocytes. Levels of epithelial cytokines, including IL-1 α , IL-1 β , CXCL-1, CCL24, IL-33, TSLP, IL-5, and IL-13 (R&D Systems), were measured using ELISA kits according to the manufacturer's instructions. For histology, lungs were observed using ImageJ (National Institutes of Health).

2.4 | EET trafficking

Eosinophils release extracellular traps were labeled with Cy7 mono NHS ester (GE Healthcare) for 1-hour incubation at 37°C. Then, mice were intranasally treated with Cy7-labeled EETs (50 μ g in total protein). Cy7 signals were measured using the IVIS Spectrum (Caliper Life Sciences).

2.5 | Flow cytometry

For the isolation of airway epithelial cells, fluorochrome-conjugated anti-TER119 (eBioscience), anti-EpCAM (eBioscience), anti-CD45 (eBioscience), and anti-CD31 (BioLegend) antibodies were used.

For the detection of lung ILC2s, anti-lineage marker cocktail (BD Biosciences), anti-CD45 (BioLegend), anti-CD25 (eBioscience), anti-CD90.2 (eBioscience), anti-ST2 (eBioscience), anti-IL-5 (eBioscience), and anti-IL-13 (eBioscience) antibodies were used. Cells were analyzed by FACS Aria III (BD Biosciences). Graphs were produced using FlowJo software (Tree Star).

2.6 | qRT-PCR

Total RNA was isolated from airway epithelial cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The following primers (Bioneer) were used: mouse L32 (forward: 5'-GAAACTGGCGGAAACCCA; reverse: 5'-GGATCTGGCCCTTGAACCTT), mouse IL-33 (forward: 5'-TGAGACTCCGTTCTGGCCTC-3'; reverse: 5'-CTCTTCATGCTTGGTACCCGAT-3'), and mouse TSLP (forward: 5'-CGACAGCATGGTTCTTCTCA-3'; reverse: 5'-CGATTGCTCGAACTTAGCC-3').

2.7 | Adoptive cell transfer

Rag1^{-/-} mice (no mature T and B lymphocytes) experiments were performed in accordance with animal guidelines from animal research committee of POSTECH (POSTECH-2016-0079-R2). Isolated lung ILC2s (1×10^5 cells/mouse) from wild-type mice were adoptively transferred to recipient Rag1^{-/-} mice by intravenous injection. After the transfer, mice were intranasally administered 1 μ g of EETs with or without 10 μ g of anti-IL-33 or anti-TSLP antibody for 3 days.

2.8 | Statistical analysis

All statistical analyses were performed using IBM SPSS software, version 22.0 (IBM Corp.). *P* values of <.05 were considered statistically significant. GraphPad Prism 5.0 software (GraphPad Inc) was used to create graphs.

3 | RESULTS

3.1 | The numbers of EET⁺ eosinophils and ILC2s are elevated in SA

Demographic data from asthmatic patients are summarized in Table 1. Baseline FEV₁ (%) and PC₂₀ methacholine values were significantly lower, but sputum eosinophil counts (cells/ μ L) were higher in patients with SA than in those with nonsevere asthma (NSA; *P* < .001, *P* = .006 and *P* = .018, respectively). To induce EET formation, peripheral blood eosinophils were stimulated with IL-5 and LPS (Figure S1A). Activated eosinophils released condensed DNA of spherical shape (Figure S1B,C). Several eosinophil granule proteins were co-localized in the DNA (Figure S1D). Moreover, the present study found significantly elevated EET⁺ eosinophil counts (total eosinophil count $\times$ percentage of EET-forming eosinophils) in patients with SA compared to those with NSA or HCs (*P* < .05 for each; Figure 1A). The number of peripheral ILC2 was also markedly

Variables	NSA (n = 17)	SA (n = 13)	HC (n = 8)	P value (NSA vs SA)
Age (y)	56.9 ± 13.1	54.8 ± 9.5	31.9 ± 3.4	.652
Female sex (%)	52.9	69.2	62.5	.465
Atopy (%)	70.6	61.5	50.0	.705
Smoking history (%)	23.5	15.4	12.5	.672
Chronic rhinosinusitis (%)	29.4	61.5	ND	.138
Nasal polyps (%)	11.8	30.8	ND	.360
Baseline FEV ₁ (%)	99.0 ± 11.7	67.3 ± 13.7	ND	<.001
PC ₂₀ (mg/mL)	20.5 ± 8.3	3.5 ± 2.0	ND	.006
Total IgE (IU/mL)	112.4 ± 101.6	178.2 ± 131.5	70.6 ± 56.2	.227
Sputum eosinophils (%)	7.1 ± 19.7	43.0 ± 37.4	ND	.018
Sputum neutrophils (%)	68.5 ± 33.2	42.7 ± 35.4	ND	.110
Total eosinophil count (/μL)	625.2 ± 268.7	796.3 ± 277.8	160.1 ± 45.6	.098
EET-forming eosinophils (%)	20.1 ± 9.9	29.6 ± 11.8	19.3 ± 6.1	.037

Note: Values are given as n (%) for categorical variables and as mean ± SD for continuous variables. P values were applied by the Pearson chi-square test for categorical variables and Student's *t* test for continuous variables.

Abbreviations: EETs, eosinophils release extracellular traps; FEV₁, forced exhaled volume at 1 s; IgE, immunoglobulin E; ND, no data; NSA, nonsevere asthma; PC₂₀, concentration of methacholine to induce a 20% decline in FEV₁; SA, severe asthma.

TABLE 1 Clinical characteristics of the study subjects

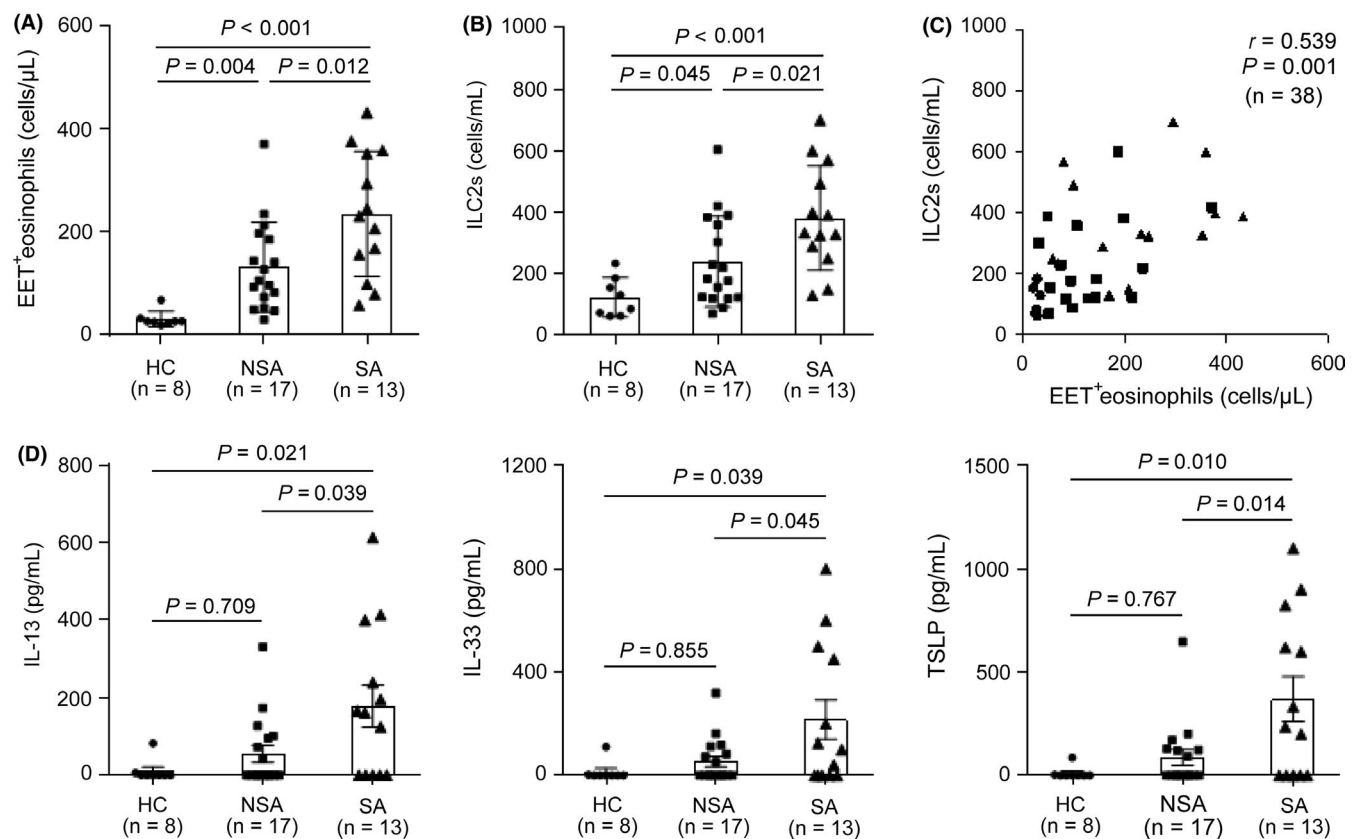
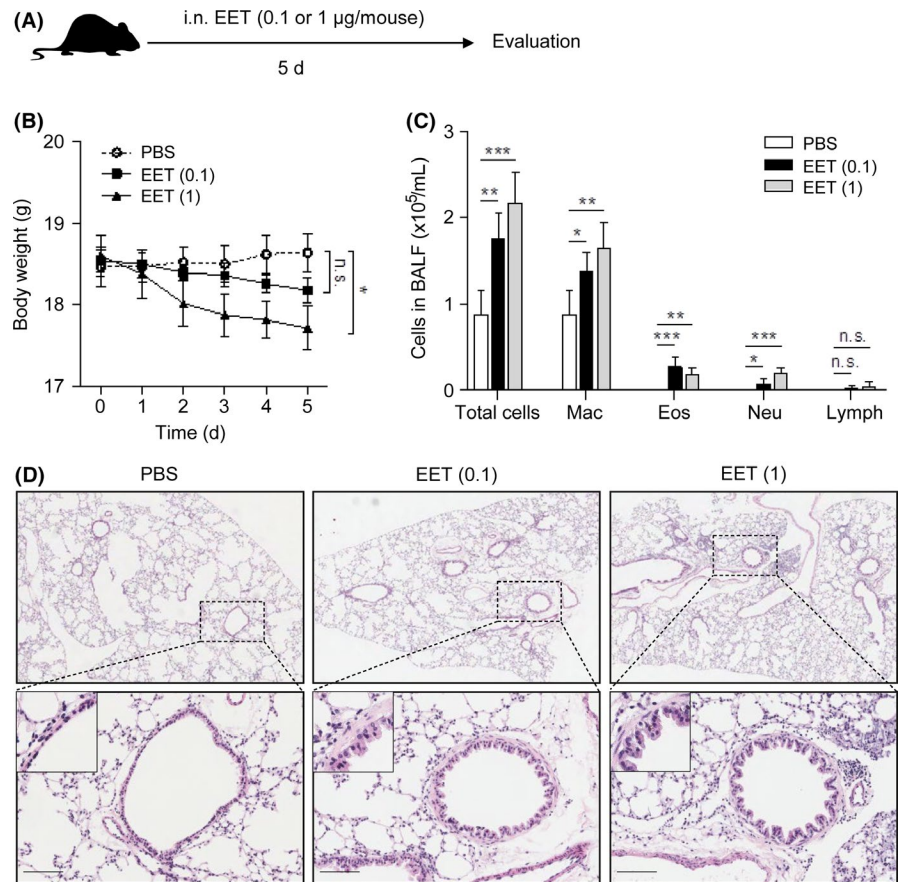


FIGURE 1 An association between EET⁺ eosinophils and ILC2s in severe asthma. The numbers of (A) EET⁺ eosinophils and (B) ILC2s in the study subjects. Data are represented as means ± SD. P values were obtained by one-way ANOVA with Bonferroni's post hoc test. (C) A positive correlation between the numbers of EET⁺ eosinophils and ILC2s. Data are presented as Pearson correlation coefficient r (P value). (D) Levels of plasma cytokines. Data are represented as means ± SEM. P values were obtained by one-way ANOVA with the Tukey HSD test.

FIGURE 2 Effect of eosinophils release extracellular traps (EETs) on airway inflammation in the mouse lung tissues. (A) Experimental schedule for EET treatment. (B) Changes in body weight. (C) Differential cell count. Data are represented as means $\pm$ SD, $n = 5$. * $P < .05$, ** $P < .01$, and *** $P < .001$ values were obtained by one-way ANOVA with Bonferroni's post hoc test. n.s., not significant. (D) Lung tissues stained with hematoxylin and eosin. Scale bar, 50 μ m



higher in the SA group than in NSA or HC groups ($P < .05$ for each; Figure 1B). A significant positive correlation between EET⁺ eosinophils and ILC2s was noted ($r = .539$, $P = .001$; Figure 1C). In addition to cellular profiles, especially levels of plasma IL-13, IL-33, and TSLP were enhanced (Figure 1D). Therefore, elevated EET⁺ eosinophils in relation to the number of ILC2s as well as type 2 cytokine levels in plasma have raised the question of how EETs contribute to type 2 immune responses with eosinophilia.

3.2 | EETs stimulate airway epithelium to induce type 2 immune responses

To confirm the potential impact of EETs, wild-type BALB/c mice were intranasally treated with EETs for 5 days (Figure 2A). Once mice were injected with fluorescent-labeled EETs, the molecules were specifically found in the lung tissues, but not in other tissues (Figure S2). Mouse body weight decreased following continuous EET treatment (Figure 2B), indicating that EETs are harmful in excess. In addition, significantly elevated numbers of eosinophils and neutrophils were noted in bronchoalveolar lavage fluid (BALF; Figure 2C). Lung tissues stained with hematoxylin and eosin showed increased immune cell infiltration and epithelium thickness (Figure 2D). Moreover, expression of tight junction proteins, such as ZO-1, occludin, and claudin-1, was changed (Figure S3). By measuring cytokines in BALF, higher levels of epithelium-derived cytokines, such as IL-1 α , IL-1 β , CXCL-1, CCL24, IL-33, and TSLP,

were found (Figure 3). Significantly elevated levels of *IL33* and *TSLP* mRNA were observed in isolated airway epithelial cells (Figure S4). Furthermore, airway epithelial cell death was induced by EET treatment in vitro (data not shown). These data suggest that EETs induce type 2 immune responses through affecting airway epithelium in the lungs.

3.3 | IL-5- or IL-13-producing ILC2s are related to EET-mediated airway inflammation

As IL-33 and TSLP are known as strong ILC2 stimulators, lung ILC2s were isolated/analyzed using flow cytometry (Figure 4A). As a result, the proportion of IL-5- or IL-13-producing lung ILC2s were elevated when mice were treated with EETs (Figure 4B). The present study further evaluated the function of IL-5 and IL-13 in the lungs (Figure S5A). When mice were injected with these 2 cytokines, mice developed AHR with eosinophilia in BALF (Figure S5B,C); however, IL-33 and TSLP levels derived from the epithelium were significantly enhanced by IL-13, but not by IL-5 (Figure S5D). Taken together, ILC2s are an important cell involved in airway inflammation by releasing type 2 cytokines.

3.4 | Epithelium-targeting biologics effectively reduce AHR by ILC2 inactivation

To demonstrate a significance of ILC2s contributing to the pathogenesis of asthma, Rag1^{-/-} mice were intravenously administered

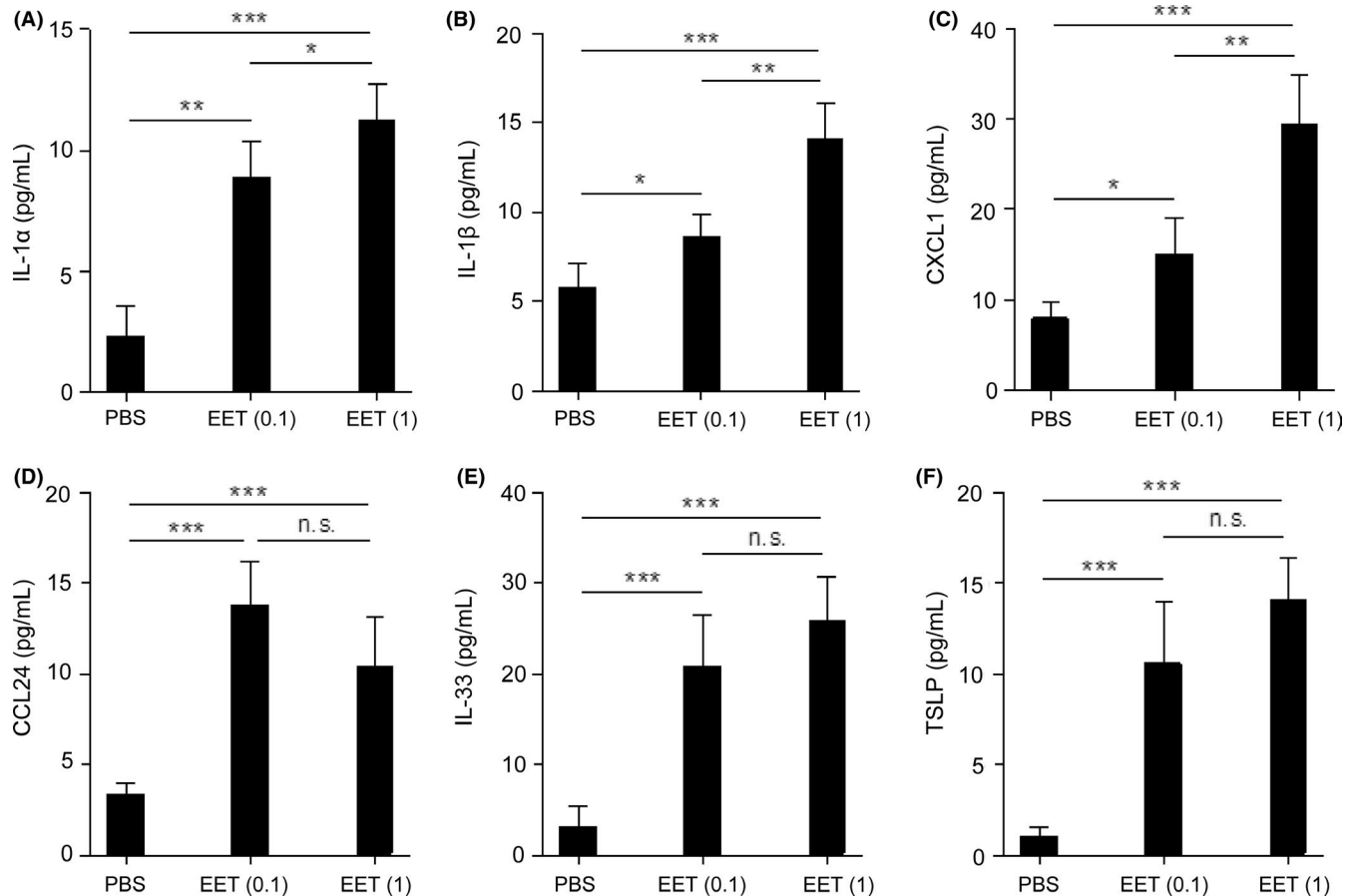


FIGURE 3 Production of airway epithelium-derived cytokines. Levels of (A) IL-1 α , (B) IL-1 β , (C) CXCL-1, (D) CCL24, (E) IL-33, and (F) TSLP in BALF. Data are represented as means $\pm$ SD, $n = 5$. * $P < .05$, ** $P < .01$, and *** $P < .001$ values were obtained by one-way ANOVA with Bonferroni's post hoc test. n.s., not significant

lung ILC2s (Figure 5A). When mice were treated with EETs, AHR as well as levels of type 2 cytokines such as IL-5 and IL-13 in BALF were significantly increased (Figure 5B,C). To manage EET-mediated airway inflammation, the effect of IL-33 or TSLP neutralization was tested. When mice were treated with Anti-IL33 and anti-TSLP antibodies, epithelium-targeting biologics (anti-IL-33/TSLP antibody) significantly reduced AHR (Figure 5B); however, anti-IL-33 antibody, but not anti-TSLP antibody, reduced IL-5 and IL-13 levels in BALF (Figure 5C), indicating that IL-33 derived from airway epithelial cells may be a predominant cytokine involved in ILC2 inactivation.

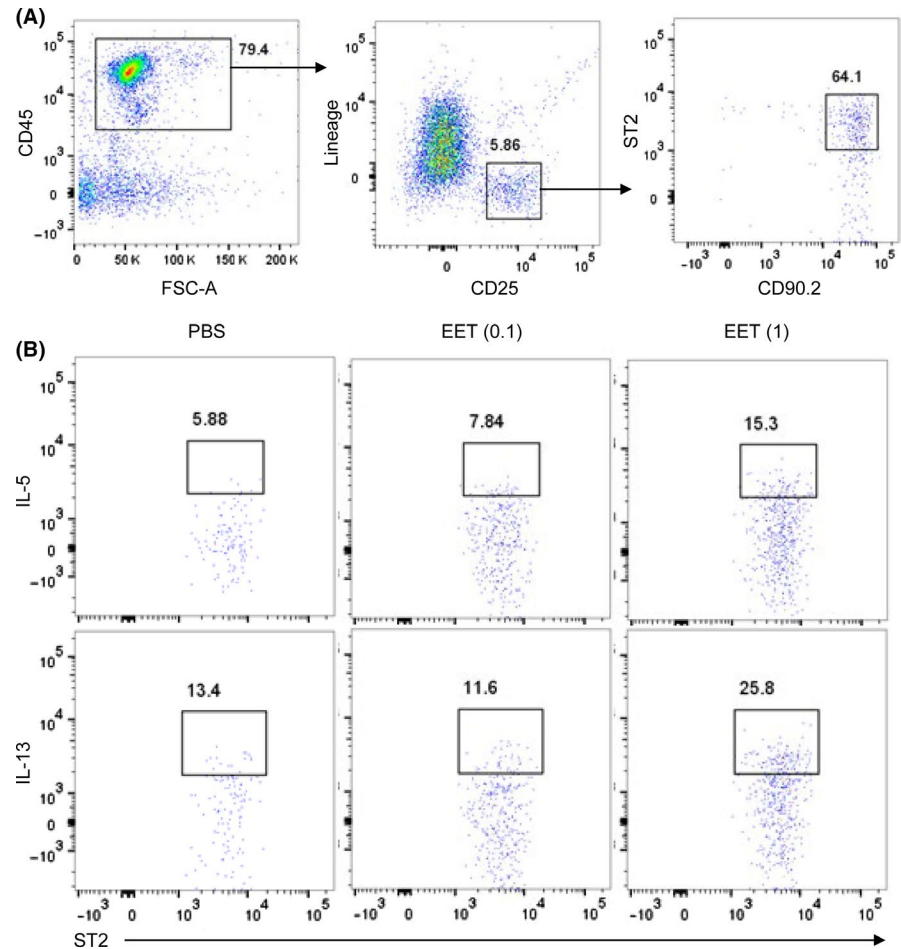
4 | DISCUSSION

This is the first study to show the pathophysiological function of extracellular traps that link eosinophils to ILC2 activation with vicious cycles in the airway inflammation of SA. In addition, we suggest the importance of airway epithelium as a key modulator to enhance major inflammatory cells, leading to type 2 immune responses. It has been known that two major pathways, Th2 and innate/ILC2-mediated responses, are involved in eosinophil activation in the asthmatic airways, in which innate immune responses

are involved in the development of steroid resistance. Causative factors involved in steroid resistance in SA have not yet been fully understood; however, potent toxicity of EETs could be a possible explanation. The present study demonstrated that EETs could activate innate immune responses via close crosstalk between EETs and ILC2s, leading to enhancing type 2 immune responses in the airway inflammation of SA.

Airway eosinophilia in asthma is closely associated with asthma exacerbation. However, the difference in eosinophil function between the SA and NSA groups has not been completely clarified.²² We hypothesized that higher levels of EETs released from eosinophils might contribute to eosinophil activation and asthma exacerbation in SA as suggested in some eosinophilia-associated diseases.¹⁰⁻¹² We aimed to investigate the effect of EETs on airway epithelium, which provides a mechanistic insight into the pathogenesis of severe eosinophilic asthma, as the epithelium is the first barrier that commonly regulates inflammation and permeability in the lungs.^{23,24} It has previously been revealed that eosinophils impaired epithelium through producing granule proteins.²⁵ Because DNA aggregates cytotoxic proteins in EETs, we speculated that EETs might be involved in the pathogenesis of SA. The current study demonstrated that EETs released from SA patients induced epithelium-derived

FIGURE 4 Changes in ILC2 activation status in the mouse lung tissues after eosinophils release extracellular trap (EET) treatment. A, Gating strategy of lung-resident ILC2s. Flow cytometric analysis of ILC2s as defined by lack of lineage markers and expression of CD45, CD25, CD90.2, and ST2. B, Quantification of IL-5- or IL-13-producing ILC2s



cytokines such as IL-1 α , IL-1 β , CXCL-1, CCL24, IL-33, and TSLP. These findings indicate that EETs could elevate the activation status of airway epithelium and innate immune responses.

The present study showed significantly increased ILC2 counts and higher levels of IL-33 and TSLP in the plasma of SA patients. Moreover, a significant positive correlation was noted between peripheral EET⁺ eosinophils and ILC2 counts. Previous studies have also revealed elevated ILC2 count in the airways of the patients with SA and higher levels of IL-33/TSLP in BALF from asthmatic patients.^{26,27} Through *in vivo* experiments in the present study, we further clarified the increased number of lung ILC2s and their activation after EET treatment, leading to type 2 airway inflammation through production of IL-5 and IL-13. As IL-5 is essential for eosinophil survival/activation,²⁸ it could form a vicious cycle to release EETs from eosinophils. IL-13 is known to disrupt airway epithelial barrier integrity by targeting tight junctions.²⁹ The current study also demonstrated that EETs released from SA patients affect tight junction proteins located at the most apical part of neighboring epithelial cells in an *in vivo* model. In addition, this cytokine triggers type 2 inflammation in association with IL-33 and TSLP,^{30,31} indicating that ILC2s may be critical for enhancing type 2 immune responses in the asthmatic airway of SA under increased EET formation.

Asthmatic patients with high eosinophilia in the blood and airways less respond to conventional treatment than nonatopic asthmatic patients (ILC2-mediated eosinophilic inflammation), especially those with late-onset asthma and show resistance to inhaled corticosteroid.^{32,33} Thus, to address therapeutic limitations of steroid treatment, we tested biologics targeting representative epithelium-targeting cytokines, IL-33, and TSLP. As a result, both anti-IL-33 and anti-TSLP antibody treatment attenuated AHR; however, anti-IL-33, but not anti-TSLP antibody, reduced IL-5 and IL-13 levels in BALF, indicating that IL-33 derived from airway epithelial cells may be a predominant cytokine involved in ILC2 inactivation. It has been demonstrated that ILC2 activation is more dependent on IL-33, whereas TSLP is more important for ILC2 survival.³⁴ Although the difference in pathophysiological function between anti-IL-33 and anti-TSLP antibodies has not yet been completely determined, these findings imply that 2 epithelium-targeting biologics under clinical trial may have a potential benefit to suppress type 2 or eosinophilic inflammation associated with the EET-ILC2 axis in patients with SA.

This study has some limitations. First, knockout animal models were not evaluated. Although we confirmed the pathways in Rag1^{-/-} mice, further investigations with IL-33^{-/-} and TSLP^{-/-} mice will strongly support the effect of EETs on airway inflammation through

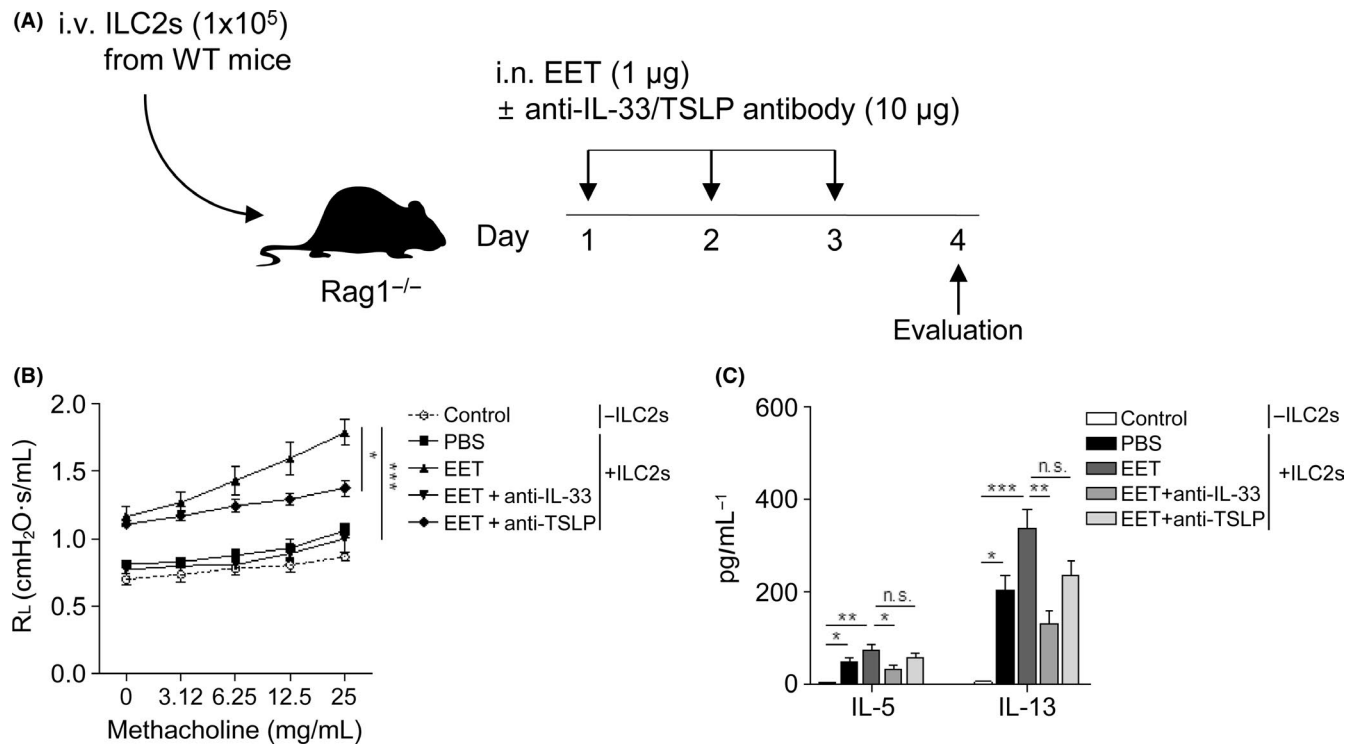


FIGURE 5 Role of eosinophils release extracellular traps (EETs) in airway hyperresponsiveness through stimulation of ILC2. A, Adoptive transfer of ILC2s to Rag1^{-/-} mice. i.n., intranasal injection; iv, intravenous injection. B, Airway hyperresponsiveness. C, Levels of IL-5 and IL-13 in BALF. Data are represented as means $\pm$ SD, $n = 5$. * $P < .05$, ** $P < .01$, and *** $P < .001$ values were obtained by one-way ANOVA with Bonferroni's post hoc test. n.s., not significant

stimulating airway epithelium. Secondly, further clinical translational trials in SA patients according to the results of EET-ILC2 activation status are needed to validate our hypothesis.

In conclusion, we suggest that EETs may play a critical role in the pathogenesis of persistent eosinophilic inflammation in SA through activating airway epithelium and ILC2 cells. Our results provide a rationale for a trial of the 2 epithelium-targeting biologics in severe eosinophilic asthmatics with steroid resistance.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Y. Choi designed the experiments, analyzed the data, and wrote the paper. YM Kim, HR Lee, J. Mun, S. Sim, DH Lee, and DL Pham performed the experiments. SH Kim, YS Shin, and SW Lee helped design

the experiments. HS Park provided overall supervision for the entire study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supporting Information

Eosinophil extracellular traps activate type 2 innate lymphoid cells through stimulating airway epithelium in severe asthma

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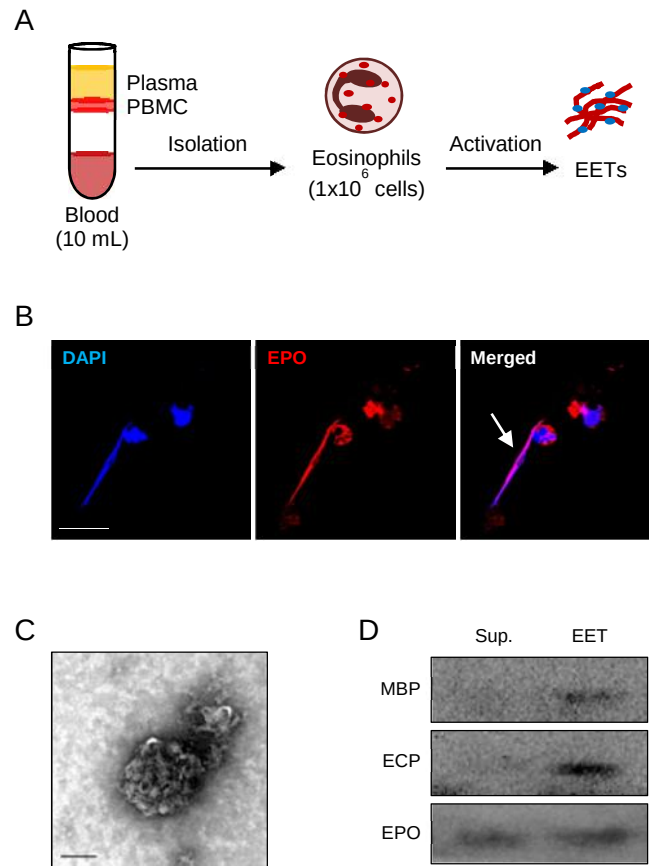


FIGURE S1. Stimulation of peripheral blood eosinophils obtained from asthmatic patients. (A) Schematic picture of EET isolation. (B) Confocal images of releasing EETs (a white arrow). Scale bar, 10 μ m. (C) Transmission electron microscopy. Scale bar, 200 nm. (D) Cytotoxic granule proteins analyzed using Western blot analysis.

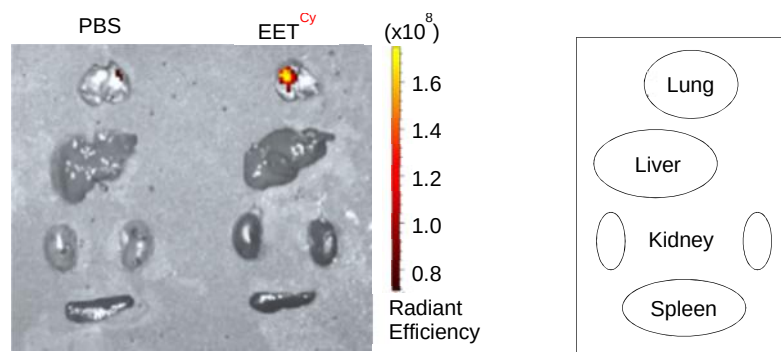


FIGURE S2. Quantification of radiant efficacy of Cy7-labeled EETs in the mouse lung tissues.

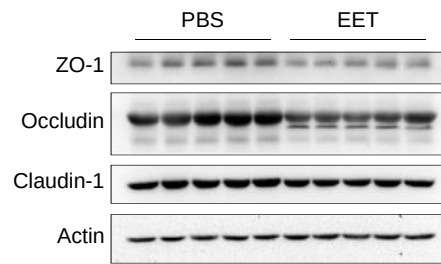


FIGURE S3. Changes in expression of ZO-1, Occludin, and Claudin-1 in the mouse lung tissues.

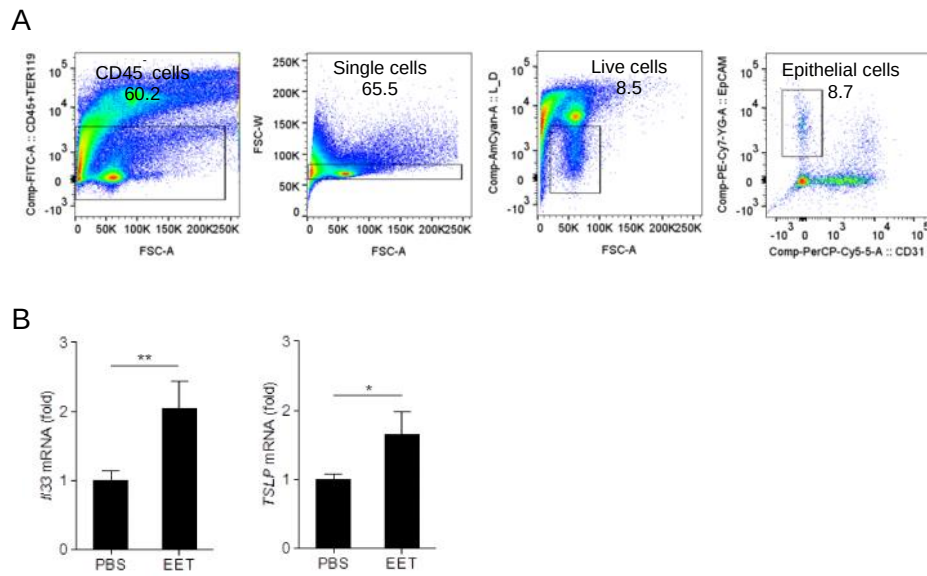


FIGURE S4. Evaluation of IL-33 and TSLP expressions in the mouse lung tissues. (A) Isolation of airway epithelial cells using flow cytometry. (B) Expression of *Il33* and *Tslp* mRNA. Data are represented as means $\pm$ SD, $n = 5$. * $P < .05$ and ** $P < .001$ by the Mann-Whitney U test.

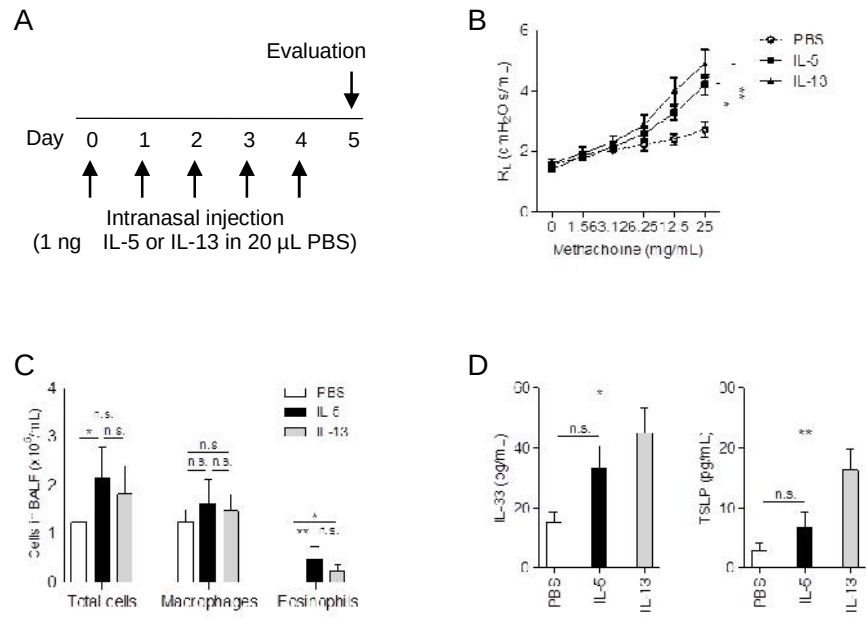


FIGURE S5. Effect of type 2 cytokines on airway inflammation. (A) Experimental schedule. (B) Airway hyperresponsiveness. (C) Differential cell count. (D) Levels of cytokines in BALF. Data are represented as means $\pm$ SD, $n = 5$. $*P < .05$ and $**P < .01$ by one-way ANOVA with Bonferroni's *post hoc* test. n.s., not significant.