

ORIGINAL ARTICLE

Basic Mechanisms in Allergic Disease

IL-33 signalling contributes to pollutant-induced allergic airway inflammation

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Summary

Background: Clinical and experimental studies have identified a crucial role for IL-33 and its receptor ST2 in allergic asthma. Inhalation of traffic-related pollutants, such as diesel exhaust particles (DEP), facilitates the development of asthma and can cause exacerbations of asthma. However, it is unknown whether IL-33/ST2 signalling contributes to the enhancing effects of air pollutants on allergic airway responses.

Objective: We aim to investigate the functional role of IL-33/ST2 signalling in DEP-enhanced allergic airway responses, using an established murine model.

Methods: C57BL/6J mice were exposed to saline, DEP alone, house dust mite (HDM) alone or combined DEP+HDM. To inhibit IL-33 signalling, recombinant soluble ST2 (r-sST2) was given prophylactically (ie, during the whole experimental protocol) or therapeutically (ie, at the end of the experimental protocol). Airway hyperresponsiveness and the airway inflammatory responses were assessed in bronchoalveolar lavage fluid (BALF) and lung.

Results: Combined exposure to DEP+HDM increased IL-33 and ST2 expression in lung, elevated inflammatory responses and bronchial hyperresponsiveness compared to saline, sole DEP or sole HDM exposure. Prophylactic interference with the IL-33/ST2 signalling pathway impaired the DEP-enhanced allergic airway inflammation in the BALF, whereas effects on lung inflammation and airway hyperresponsiveness were minimal. Treatment with r-sST2 at the end of the experimental protocol did not modulate the DEP-enhanced allergic airway responses.

Conclusion: Our data suggest that the IL-33/ST2 pathway contributes to the onset of DEP-enhanced allergic airway inflammation.

KEYWORDS

Asthma, Air pollution, Diesel Exhaust Particles, House Dust Mite, Airway epithelium, IL-33 and ST2

1 | INTRODUCTION

Asthma is one of the most common chronic airway diseases and is a serious global health problem.^{1,2} It is generally characterized by a

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chronic airway inflammation of the conducting airways, causing reversible airway obstruction, excessive mucus production, airway hyperresponsiveness (AHR) and remodelling. Asthma has been defined as a heterogeneous disease, with multiple phenotypes that are distinguished based on the patient's clinical characteristics and inflammatory profile.³ The best characterized phenotype is allergic asthma, a type 2-mediated immune disorder caused by exposure to common inhaled allergens, such as house dust mite (HDM).^{3,4}

The airway epithelium is continuously exposed to inhaled environmental triggers. It acts not only as a passive barrier but is also actively involved in respiratory defence through pro-inflammatory cytokine production.^{5,6} Interleukin (IL)-33 plays a key role in bridging innate and adaptive immune responses and contributes to the regulation of tissue homeostasis, injury and repair. IL-33 has multiple bioactive forms: a full-length and several more potent cleaved forms. Both forms can bind the ST2 transmembrane receptor that is expressed on various immune cells, including mast cells, basophils, dendritic cells (DC), macrophages, eosinophils, iNKT cells, innate lymphoid cells type 2 (ILC2), CD4⁺ T cells and CD8⁺ T cells. Besides the transmembrane form, ST2 also exists as a soluble form, sST2, which acts as a decoy receptor to prevent IL-33-mediated cellular activation.⁷⁻⁹

Several genome-wide association studies have shown that IL-33 and its receptor ST2 are associated with asthma susceptibility.¹⁰⁻¹² In patients with asthma, elevated IL-33 protein levels were observed in serum, induced sputum, bronchoalveolar lavage fluid (BALF) and bronchial epithelial cells, which all correlated positively with the severity of the disease.¹³⁻¹⁶ Moreover, most murine studies using IL-33 or ST2 knockout (KO) mice, anti-IL-33 or recombinant sST2 (r-sST2) showed a critical role for IL-33/ST2 signalling in allergen-induced airway inflammation.¹⁷⁻²⁴ Targeting IL-33 could therefore be a new promising therapeutic approach for asthma.

Over the years, substantial epidemiological evidence has linked exposure to traffic-related particulate matter, such as diesel exhaust particles (DEP), with the onset, progression and exacerbation of asthma.²⁵ Studies with controlled human exposures to DEP + allergen demonstrated augmented allergen-specific responses, including increased airway eosinophilia, type 2 cytokine production and allergen-specific immunoglobulin (Ig) E levels.^{26,27} The underlying mechanisms are however incompletely known. To elucidate these, we and others developed and characterized murine models that mimic DEP-enhanced allergic airway responses.²⁸⁻³⁰ We demonstrated that combined exposure to DEP+HDM was associated with AHR, increased inflammatory cells in the airways, type 2 cytokine responses, increased immunoglobulin production and mucus metaplasia.^{29,30} Moreover, we observed elevated IL-33 protein levels in lungs of mice that received combined DEP+HDM.²⁹ An increased IL-33 production was also observed in epithelial cells derived from severe asthmatics that were treated with particulate matter.³¹ However, whether IL-33 signalling contributes to the aggravating effect of DEP on allergic airway inflammation remains unknown.

Here, we aim to investigate the role of IL-33/ST2 signalling in DEP-enhanced allergic airway inflammation. For that, r-sST2, which neutralizes IL-33 activity, was administered in our established

DEP+HDM mouse model. We demonstrate that interfering with the IL-33/ST2 pathway prophylactically (ie, during the whole experimental protocol) results in an impaired DEP-enhanced allergic airway inflammation, whereas r-sST2 administration at the end of the exposure protocol does not modulate the DEP-enhanced allergic airway responses.

2 | MATERIALS AND METHODS

2.1 | Mice

Female 6- to 8-week-old wild-type (WT) C57BL/6J mice (4-8 mice/group) were obtained from the Jackson Laboratory. All in vivo manipulations were approved by the Animal Ethical Committee of the Faculty of Medicine and Health Sciences of Ghent University (approval number ECD 12/59).

2.2 | Exposure protocol

DEP (SRM 2975) was obtained from the National Institute for Standards and Technology. HDM (*Dermatophagoides pteronyssinus*) was obtained from Greer Laboratories. On days 1, 8 and 15, isoflurane-anesthetized mice were intranasally instilled with saline, 25 µg DEP suspended in saline + 0.05% Tween 80, 1 µg HDM in saline or the combination of DEP and HDM as described previously (Figure 1A).^{29,30} On day 17, the animals were sacrificed by a lethal dose of pentobarbital intraperitoneal. Additionally, mice were exposed to saline, DEP, HDM or DEP+HDM on days 1 and 8, and sacrificed at day 14 (determination time-point therapeutic treatment).

For the prophylactic inhibition of IL-33, mice received PBS or 25 µg r-sST2³² in PBS intranasally on days 1, 8 and 15. This r-sST2 showed neutralizing activity towards murine IL-33 in a biological assay measuring IL-33-induced NF-κB receptor activation in ST2+IL1RAcP-transfected HEK293T cells.³² Upon increasing concentration of r-sST2, the IL-33-induced NF-κB luciferase activity decreased (Figure S1). The r-sST2 instillation was performed 8 min, previously optimized and published by our co-authors, after the exposure to saline, DEP, HDM or DEP+HDM (Figure 2A).³³ For the therapeutic approach, mice were treated with PBS or 25 µg r-sST2 in PBS on days 14, 15 and 16 (Figure 4A).

2.3 | BALF

A tracheal cannula was inserted, and BALF was recuperated by instillation of 3 × 300 µL 1% HBSS supplemented with 1% BSA and 6 × 500 µL HBSS supplemented with EDTA. The lavage fractions were pooled, and total cell counts were measured using a Bürker chamber.

2.4 | Lung and mediastinal lymph node (MLN) single-cell suspensions

The pulmonary circulation was rinsed with saline supplemented with EDTA to remove the intravascular pool of cells. Lungs and

MLN were minced and incubated for 45 min in digestion medium (Roswell Park Memorial Institute medium 1640 supplemented with 5% fetal calf serum, 2 mM L-glutamine, 0.05 mM 2-mercaptomethanol, 100 U/mL penicillin, 100 mg/mL streptomycin, 1 mg/mL collagenase type 2 and 0.02 mg/mL DNase I) at 37°C and 5% CO₂. Red blood cells were lysed using ammonium chloride buffer. Total cell counts were performed with a Z2 Coulter Counter.

2.5 | MLN cell culture

MLN single cells were cultured in culture medium alone or supplemented with 3.75 µg/well HDM in round bottom, 96-well plates and incubated in a humidified 37°C incubator with 5% CO₂. After 5 days, supernatants were harvested for cytokine measurements.

2.6 | Flow cytometry and cell sorting

To minimize non-specific binding, BALF cells and lung single-cell suspensions were incubated with anti-CD16/CD32 (clone 2.4G2). Cells were further labelled with a combination of anti-mouse fluorochrome-conjugated monoclonal antibodies against CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), Ly6C (AL-21), Ly6G (1A8), MHCII (2G9), Siglec-F (E50-2440), CD3 (145-2C11), CD90.2 (30.H12), CD5 (53-7.3), CD11c (N418), CD25 (PC61.5), CD45R (RA3-6B2), NK1.1 (PK136), TCRβ (H57-597) and ST2 (DJ8). For cytoplasmatic IL-13 (eBio13A) or matched isotype staining, cells were stimulated with ionomycin and phorbol 12-myristate 13-acetate, supplemented with brefeldin A and monensin at 37°C for 4 h. Data acquisition was performed on a LSRFortessa cytometer. Cell subsets were analysed using FlowJo software (FlowJo LLC, Ashland, Oregon, USA).

CD45⁺ and CD45⁻ lung cells were sorted using an OctoMACS separator and CD45 microbeads following the manufacturer's instructions (Miltenyi, Bergisch Gladbach, Germany).

2.7 | Histology

The left lung was fixated with 4% PFA and embedded in paraffin. Transversal sections (3 µM) were cut and treated with Ultra V Block. To identify eosinophils and goblet cells, lung sections were stained with Congo red or periodic acid-Schiff staining, respectively. Quantitative measurements were performed on an Axioimager running Axiovision software.

2.8 | Lung homogenates

The lung was homogenized in T-PER tissue protein extraction reagent containing Halt Protease Inhibitor Cocktail Kit using a TissueRuptor. The homogenates were centrifuged, and the middle layer was isolated. Total protein concentration was measured using the Pierce BCA Protein Assay Kit.

2.9 | Protein measurements

IL-13 levels in BALF and MLN and IL-1β in lung homogenate were measured using commercially available ELISA kits (R&D systems Abingdon, United Kingdom).

HDM-specific IgG1 was determined on serum that was collected by retro-orbital bleeding. Briefly, plates were coated with HDM extract. Serum was added, followed by a horseradish-conjugated polyclonal goat anti-mouse IgG1 Ab. IgG1 values were reported in optical density (ODs).

2.10 | Western blotting

Protein extracts of lung tissue homogenates were loaded on a 15% Tris/HCl gel (25 µg/slot), separated by SDS PAGE and blotted onto a nitrocellulose membrane. Murine IL-33 was detected by mouse IL-33 antigen affinity-purified polyclonal goat IgG antibody in combination with the donkey anti-goat horseradish peroxidase (HRP) antibody. As a loading control, rabbit anti-glyceraldehyde-3-phosphate dehydrogenase antibody in combination with donkey anti-rabbit HRP antibody was used. Positive bands were visualized with Immobilon Western Chemiluminescent HRP Substrate and detected by ChemiDoc Touch Imaging System. Semi-quantitative analysis of band intensities was performed by measuring the area under the peak of plotted lanes with ImageJ software.

2.11 | qRT-PCR

RNA from total lung tissue or from CD45^{+/−} sorted lung cells was extracted using the miRNeasy mini kit (Qiagen), and cDNA synthesis (Transcriptor Universal cDNA Master, Roche) was performed following the manufacturer's instructions. IL-33 and ST2 expression was analysed with TaqMan Gene Expressions assays using a LightCycler 96 system. IL-33 and ST2 expression was normalized based on the expression of the HPRT reference gene.

2.12 | Airway hyperresponsiveness

Airway hyperresponsiveness (AHR) in response to increasing doses carbachol (0, 20, 40, 80, 160, 320 and 640 µg/kg) was measured 48 h after the last intranasal instillation of DEP and/or HDM using the forced oscillation technique (FlexiVent System). Mice were anaesthetized with a 1/10 dilution pentobarbital, and neuromuscular blockade was induced by injecting 1 mg/kg pancuronium bromide intravenously. A "snapshot perturbation" manoeuvre was imposed to measure the resistance (R) of the whole respiratory system (airways, lung and chest wall).

2.13 | Statistical analysis

Statistical analysis was performed with SPSS, version 24.0 (Chicago, Illinois, USA). Non-parametric tests (Kruskal-Wallis and Mann-Whitney *U* test) were used to compare different groups, according to the standard statistical criteria. Values were reported as mean ± SEM. *P*-values < 0.05 (*) were considered as significant.

3 | RESULTS

3.1 | Combined DEP+HDM exposure increases IL-33 and ST2 expression in the murine lung

C57BL/6J mice were exposed to saline, DEP alone, HDM alone or combined DEP+HDM (Figure 1A). To optimally investigate the enhancing capacity of DEP on HDM-induced allergic airway responses, we have previously optimized doses of both DEP and HDM that elicit limited inflammatory responses on their own.^{29,30}

Compared to the three control groups (ie, saline, DEP alone and HDM alone), combined exposure to DEP+HDM increased the mRNA expression of IL-33 and ST2 in total lung tissue (Figure 1B, C). To identify their origin, we sorted hematopoietic (CD45⁺) versus non-hematopoietic (CD45⁻) cells from lung tissue. IL-33 mRNA was predominantly expressed in CD45⁻ non-hematopoietic cells, while it was barely detectable in CD45⁺ hematopoietic cells (Figure 1D). Contrary to this, ST2 mRNA was preferentially expressed in the CD45⁺ cell population, whereas it was minimally expressed in the CD45⁻ cell fraction (Figure 1E). Similar to the data from total lung tissue, combined exposure to DEP+HDM increased expression of IL-33 in the sorted CD45⁻ cells and ST2 mRNA in the sorted CD45⁺ cells, when compared with the three control groups (Figure 1D,E).

We previously showed that IL-33 protein levels in lung homogenates increased upon combined DEP+HDM exposure compared to saline, DEP and HDM exposure.²⁹ To identify which protein form of IL-33 was increased, we performed western blot analysis. Both full-length IL-33 (~30 kDa) and cleaved IL-33 (~18 kDa) were significantly increased in WT mice that received combined DEP+HDM in comparison with the three control groups (Figure 1F-I).

3.2 | Prophylactic administration of recombinant soluble ST2 attenuates DEP-enhanced allergic airway inflammation in BALF

To evaluate the functional role of the IL-33/ST2 axis in the model of DEP-enhanced allergic airway inflammation, we inhibited IL-33/ST2 signalling by administering r-sST2 or PBS prophylactically (ie, from the start of the exposure protocol) to C57BL/6J mice (Figure 2A).

Combined exposure to DEP+HDM in PBS-treated mice vastly increased the numbers of DC, neutrophils, eosinophils, ILC2 and CD4⁺ T cells in the BALF (Figure 2B-F), whereas exposure to sole DEP or sole HDM elicited minimal inflammation. Moreover, elevated IL-13 protein levels and increased numbers of IL-13⁺ ILC2 and IL-13⁺ CD4⁺ T cells were observed in the BALF of combined DEP+HDM-exposed mice, when compared to saline-, DEP- or HDM-exposed mice (Figure 2G-I). Concomitant exposure to DEP+HDM modestly increased the IL-1 β levels compared with the controls (Figure 2J).

Upon prophylactic administration of r-sST2, the DEP-enhanced allergic airway inflammation was significantly reduced. r-sST2-treated mice had reduced numbers of DC, neutrophils, eosinophils, ILC2 and CD4⁺ T cells in BALF on exposure to combined

DEP+HDM, when compared with the PBS-treated mice that received DEP+ HDM (Figure 2B-F). In addition, IL-13 protein levels as well as IL-13⁺ ILC2 and IL-13⁺ CD4⁺ T cells in BALF were decreased in DEP+HDM-exposed mice that were treated with r-sST2, in comparison with the PBS-treated DEP+HDM group (Figure 2G-I). Moreover, administration of r-sST2 lowered the IL-1 β levels in the DEP+HDM-exposed mice compared with the PBS-treated DEP+HDM controls (Figure 2J). It has to be acknowledged however that r-sST2 treatment did not completely abolish the allergic airway inflammation, since combined DEP+HDM-exposed mice treated with r-sST2 still had higher inflammatory responses in the BALF when compared to saline-, DEP- or HDM-exposed mice treated with r-sST2 (Figure 2B-I). Of interest, mice that were exposed to sole HDM, prophylactic treatment with r-sST2 also reduced the number of ILC2 and IL-13⁺ ILC2 in the BALF when compared to the PBS-treated HDM group (Figure 2E,H).

3.3 | Prophylactic r-sST2 administration does not prevent tissue inflammation, type 2 sensitization and airway hyperresponsiveness upon combined DEP+HDM exposure

Combined DEP+HDM-exposed mice that were treated with PBS showed a significantly higher amount of peribronchial eosinophils and goblet cell metaplasia in lung tissue, compared to saline-, sole DEP- or sole HDM-exposed mice (Figure 3A,B, Figure S2A,B for additional images). Moreover, concomitant DEP+HDM exposure in PBS-treated animals was associated with increased IL-13 production in the mediastinal lymph nodes (MLN) and elevated serum HDM-specific IgG1 levels (Figure 3C,D).

Prophylactically interfering with the IL-33/ST2 pathways had however no significant effect on the peribronchial eosinophilic inflammation, goblet cell metaplasia, IL-13 levels in MLN and HDM-specific IgG1 levels in serum since similar levels were observed in DEP+HDM-exposed mice that were treated with r-sST2 compared to the DEP+HDM group that was treated with PBS (Figure 3A-D). Of note, although r-sST2 lowered the amount of peribronchial eosinophils and goblet cells in HDM-exposed mice compared to their PBS controls, this was not statistically significant (Figure 3A,B).

Interestingly, mice that were concomitantly exposed to DEP+HDM showed airway hyperresponsiveness (AHR) compared to sole HDM-exposed mice. Prophylactically administering r-sST2 to DEP+HDM-exposed mice led to a slight reduction—although not significantly—in AHR (Figure 3E).

3.4 | Therapeutic r-sST2 administration does not modulate the DEP-enhanced allergic airway inflammation

To investigate the therapeutic potential of r-sST2 in DEP-enhanced allergic airway inflammation, we administered r-sST2 only at the end of the exposure protocol (Figure 4A). At the time-point of the

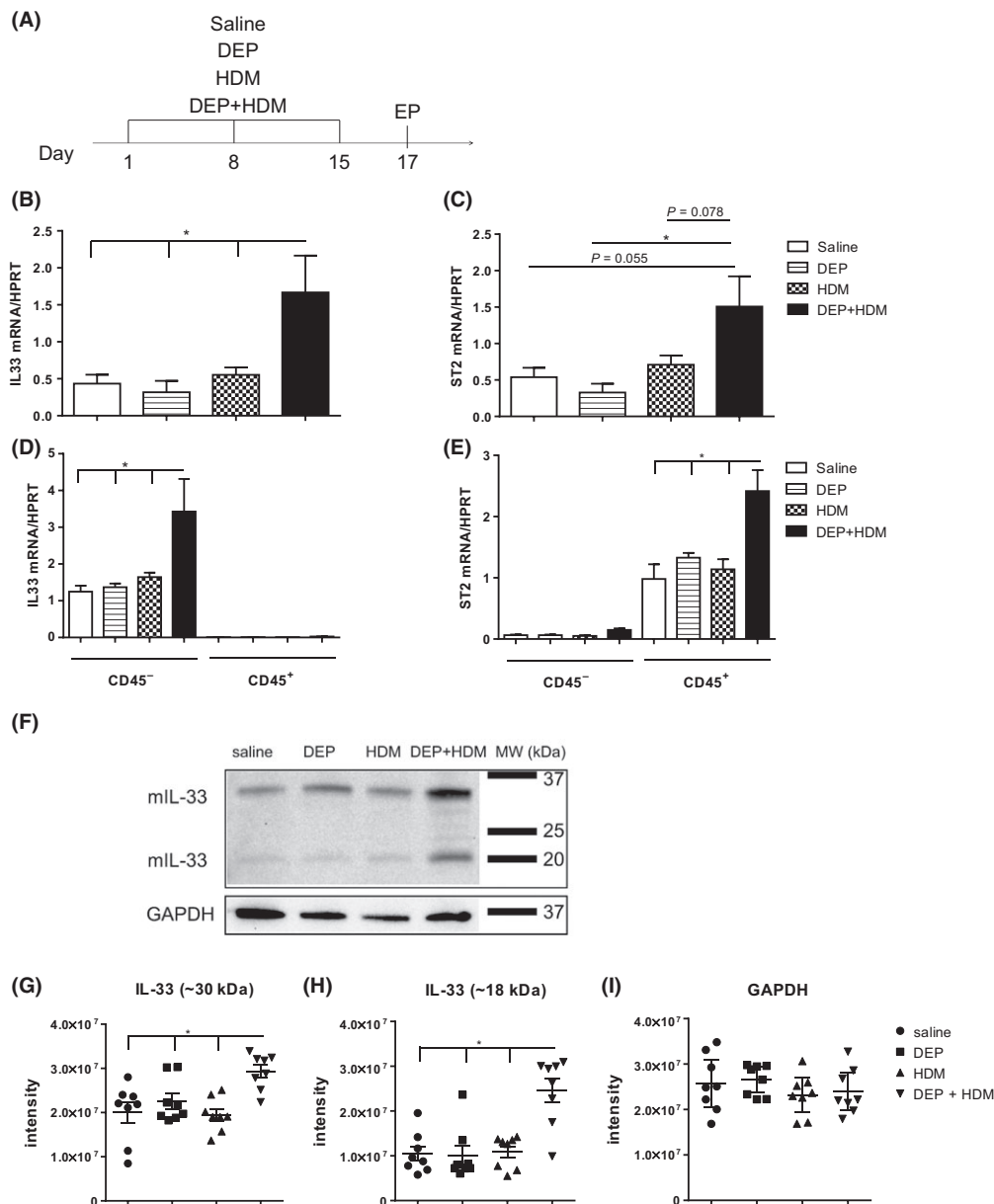


FIGURE 1 Combined DEP+HDM exposure increases IL-33 and ST2 expression in the murine lung. (A) C57BL/6J mice were intranasally exposed to saline (white bars), 25 μ g DEP (striped bars), 1 μ g HDM (checked bars) or combined DEP+HDM (black bars) on days 1, 8 and 15. Forty-eight hours after the last exposure, end-points were performed. (B, C), IL-33 (B) and ST2 (C) mRNA expression in total lung tissue was determined using qRT-PCR. (D, E), IL-33 (D) and ST2 (E) mRNA expression in sorted non-hematopoietic (CD45⁻) and hematopoietic (CD45⁺) lung cells was determined with qRT-PCR. (F-I), Full-length, cleaved murine IL-33 and GAPDH in lung homogenates were examined by western blot, and band intensities were measured. Results are expressed as mean $\pm$ SEM. $n = 4-8$ mice per group. * $P < 0.05$

first r-sST2 treatment (day 14), the allergic airway inflammation characterized by increased DC, neutrophils, eosinophils, ILC2 and T cells was already established (Figure S3), although less pronounced than the original DEP+HDM combination model (Figure 2).

Combined DEP+HDM-exposed mice that were therapeutically treated with r-sST2 had a similar increase in BALF DC, neutrophils, eosinophils, ILC2 and CD4⁺ T cells, compared with the PBS-treated DEP+HDM group (Figure 4B-F). Moreover, the increased IL-13 protein levels and total number of IL-13⁺ ILC2 and CD4⁺ T cells in the

BALF did not differ between DEP+HDM-exposed mice that were treated with PBS or r-sST2 (Figure 4G-I). Although total ILC2 numbers did not differ after the therapeutic r-sST2 intervention, the percentage IL-13-producing ILC2 in BALF did reduce upon r-sST2 treatment (Figure S4). A similar increase in IL-1 β levels was observed in DEP+HDM-exposed mice that were treated with PBS or r-sST2 (Figure 4J).

Histological analyses further revealed a comparable peribronchial eosinophilia and goblet cell metaplasia in the lungs of DEP+HDM-exposed mice that were treated with PBS or r-sST2. Despite the fact

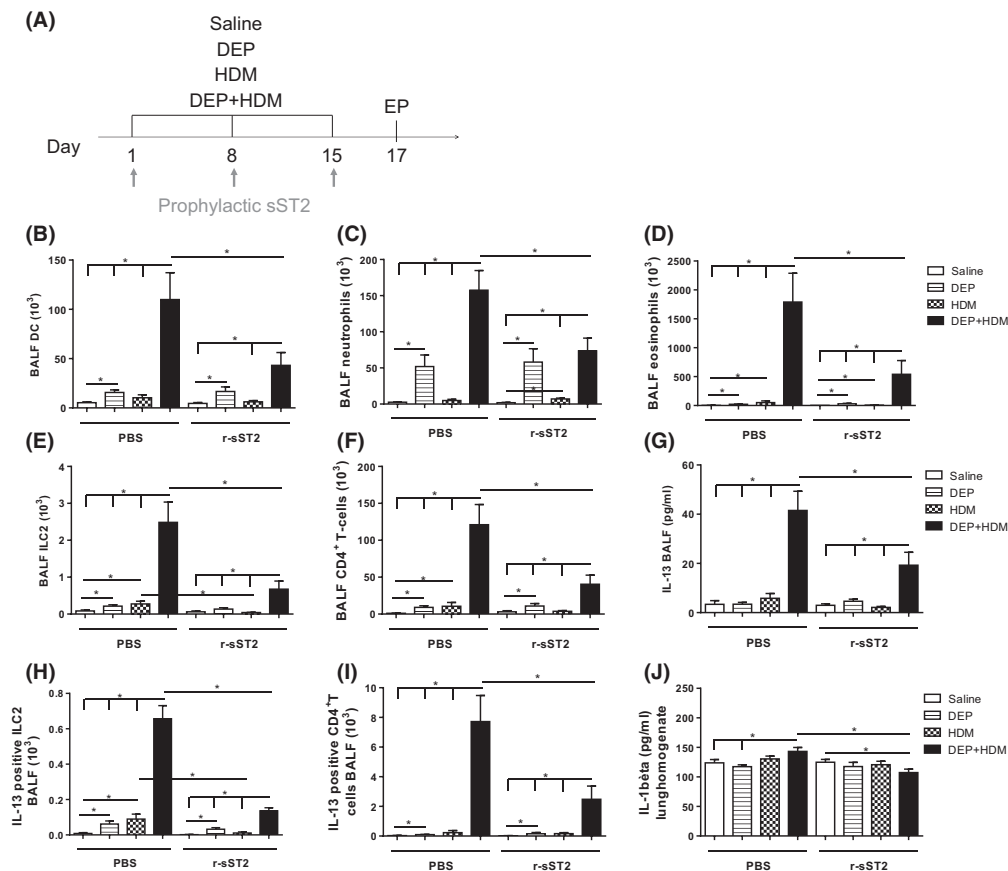


FIGURE 2 Prophyllactic administration of recombinant soluble ST2 attenuates DEP-enhanced allergic airway inflammation in BALF. (A) To prophylactically inhibit IL-33, mice received PBS or 25 μ g r-sST2 intranasally 8 min after the exposure to saline (white bars), DEP (striped bars), HDM (checked bars) or DEP+HDM (black bars). (B–F), DC (CD45⁺, CD11c⁺, CD11c^{high} and MHCII⁺) (B), neutrophils (CD45⁺, CD11c⁺, CD11b⁺, Ly6G⁺ and Ly6C⁺) (C), eosinophils (CD45⁺, CD11c⁺, CD11b⁺ and Siglec-F⁺) (D), ILC2 (CD45⁺, Lin[−] (CD3[−], CD5[−], NK1.1[−], TCR β [−], CD11c[−], CD11b[−] and CD45R[−]), CD25⁺, CD90⁺ and ST2⁺) (E) and CD4⁺ T cells (CD45⁺, CD3⁺, CD4⁺ and CD8[−]) (F) in BALF were determined by flow cytometry. (G), IL-13 protein levels in BALF were determined by ELISA. (H, I), BALF cells were stimulated for 4 h with PMA/ionomycin + protein transport inhibitors, intracellularly labelled and analysed using flow cytometry. Number of IL-13-expressing ILC2 (CD45⁺, Lin[−], CD25⁺ and CD90⁺) (H) or CD4⁺ T cells (CD45⁺, CD3⁺ and CD4⁺) (I). (J), IL-1 β levels in lung homogenate were determined by ELISA. Results are expressed as mean $\pm$ SEM. n = 8 mice per group. *P < 0.05

that r-sST2 appeared to lower the amount of peribronchial eosinophils and goblet cells in HDM-exposed mice compared to their PBS controls, this could not be statistically confirmed (Figure 5A,B, Figure S2C,D for additional images).

4 | DISCUSSION

In this paper, we focused on the functional role of IL-33/ST2 signalling in the aggravating effect of DEP on HDM-induced airway responses. We demonstrate that prophylactic neutralization of IL-33 activity with r-sST2 attenuates allergic airway inflammation upon combined DEP+HDM exposure. In contrast, in a therapeutic setting, in which r-sST2 is given only at the end of the exposure protocol, the DEP-enhanced allergic airway responses are unaffected.

IL-33 is an important epithelial-derived cytokine that has been implicated in the pathogenesis of asthma. Indeed, increased IL-33 expression was already demonstrated in asthmatic patients which

correlated with disease severity.¹⁴ Also in murine models of asthma, a rapid IL-33 increase in the BALF and lung tissue was observed upon allergen exposure.^{20,22,34} Although in our model no increase in lung IL-33 was observed in response to suboptimal HDM doses, concomitant DEP+HDM exposure vastly increased the pulmonary IL-33 mRNA and protein levels. Accordingly, in vitro exposure of human bronchial epithelial cells from severe asthmatics to PM resulted in increased IL-33 production.³¹ Although under inflammatory conditions IL-33 can be induced in immune cells,⁸ we identified non-hematopoietic cells as the major IL-33 producers in our model of DEP-enhanced allergic airway inflammation, whereas ST2 was predominantly expressed on the hematopoietic cells. Moreover, although this should be further examined, the increase in cleaved IL-33 that was seen upon combined exposure to DEP+HDM could have important implications, as cleaved IL-33 can be up to 30-fold more potent than the full-length IL-33 form.^{7,9}

To assess the functional role of the IL-33/ST2 pathway in DEP-enhanced allergic airway responses, r-sST2 was intranasally

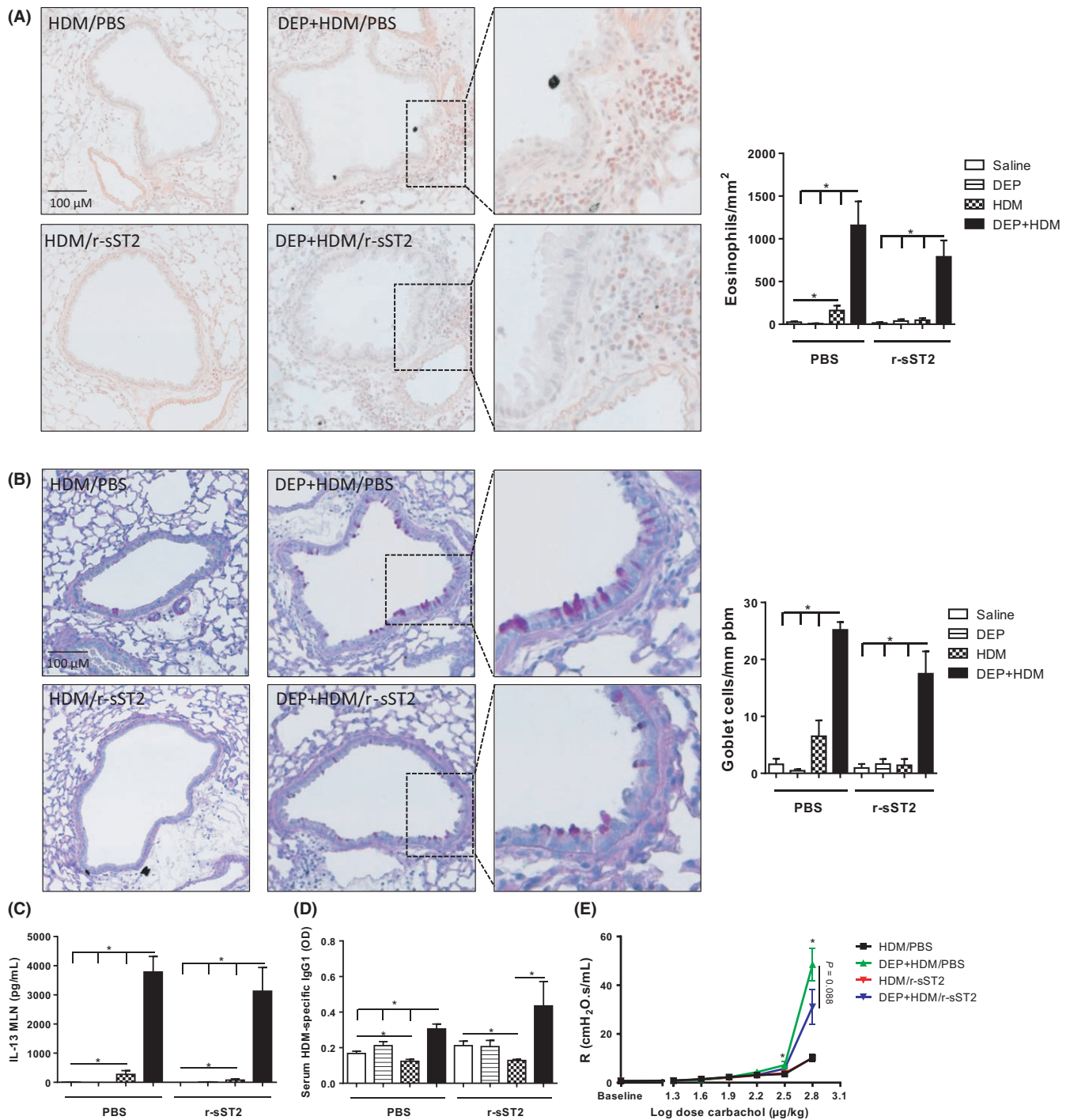


FIGURE 3 Prophylactic r-sST2 administration does not prevent tissue inflammation, type 2 sensitization and airway hyperresponsiveness upon combined DEP+HDM exposure. C57BL/6J mice were intranasally exposed to saline (white bars), 25 μg DEP (striped bar), 1 μg HDM (checked bar) or combined DEP+HDM (black bar). Eight minutes after each exposure, the mice were treated with PBS or 25 μg r-sST2. (A, B), Quantification and representative photomicrographs (magnification 20×) of Congo red-stained peribronchial eosinophils (A) or periodic acid-Schiff-stained mucus-producing goblet cells (B) in HDM and DEP+HDM-exposed mice. (C, D), IL-13 levels in the supernatants of MLN restimulated with HDM (C) and HDM-specific IgG1 levels in serum (D) were determined by ELISA. E, Airway resistance (R) of HDM-exposed mice treated with PBS (black line), combined DEP+HDM-exposed mice treated with PBS (green line), HDM-exposed mice treated with r-sST2 (red line) and DEP+HDM-exposed mice treated with r-sST2 (blue line) was measured in response to increasing doses of carbachol. Results are expressed as mean ± SEM. n = 5–8 mice per group. *P < 0.05

administered from the start of the exposure protocol. We demonstrated that prophylactic administration of r-sST2 in DEP+HDM-exposed mice decreased the numbers of eosinophils, T_H2 cells, ILC2

and IL-13 levels in the BALF. In contrast, r-sST2 had no significant effect on the DEP+HDM-induced responses in lung tissue, HDM-specific IgG1 levels and AHR. Considering that only a local response

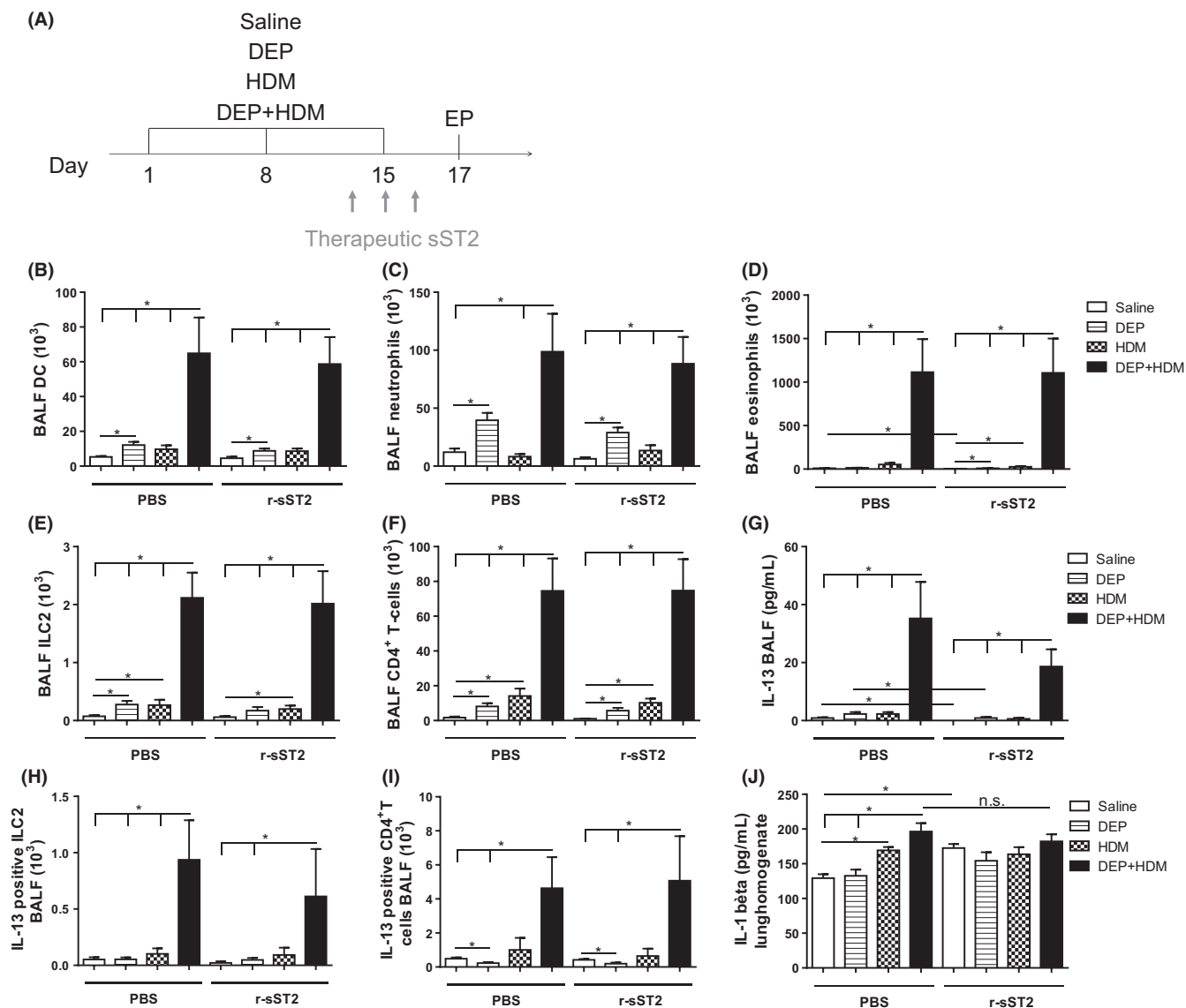


FIGURE 4 Therapeutic r-sST2 administration does not modulate the DEP-enhanced allergic airway inflammation in the BALF. (A) C57BL/6J mice were intranasally exposed to saline (white bars), 25 μ g DEP (striped bar), 1 μ g HDM (checked bar) or combined DEP+HDM (black bar). For the therapeutic approach, mice were treated with PBS or 25 μ g r-sST2 on days 14, 15 and 16. (B–F), DC (CD45⁺, CD11c^{high} and MHCII⁺) (B), neutrophils (CD45⁺, CD11c⁺, CD11b⁺, Ly6G⁺ and Ly6C⁺) (C), eosinophils (CD45⁺, CD11c⁺, CD11b⁺ and Siglec-F⁺) (D), ILC2 (CD45⁺, Lin⁺ (CD3⁺, CD5⁺, NK1.1⁺, TCR β ⁺, CD11c⁺, CD11b⁺ and CD45R⁺), CD25⁺, CD90⁺ and ST2⁺) (E) and CD4⁺ T cells (CD45⁺, CD3⁺, CD4⁺ and CD8⁺) (F) in BALF were determined by flow cytometry. (G), IL-13 protein levels in BALF were determined by ELISA. (H, I), BALF cells were stimulated for 4 h with PMA/ionomycin + protein transport inhibitors, intracellularly labelled and analysed using flow cytometry. Number of IL-13-expressing ILC2 (CD45⁺, Lin⁺, CD25⁺ and CD90⁺) (H) or CD4⁺ T cells (CD45⁺, CD3⁺ and CD4⁺) (I). (J), IL-1 β levels in lung homogenate were determined by ELISA. Results are expressed as mean $\pm$ SEM. n = 4–8 mice per group. *P < 0.05

towards r-sST2 was observed, whereas the effects in lung tissue and draining LN were limited, it could be that the half-life of r-sST2 was too short or dosing was suboptimal due to the intranasal administration route. In a model of SplD-induced asthma, however, intratracheal administration of r-sST2 also minimally affected the inflammatory responses in the lung tissue.³³ Furthermore, an i.p. injection of r-sST2 at the time of HDM sensitization showed also no apparent differences in type 2 immune responses in the lymph nodes and amount of goblet cells.³⁴ Finally, it should be mentioned that—at least in murine models of asthma—discrepant results have

been reported concerning the functional role of IL-33 signalling. Although a reduction in the HDM- or OVA-induced allergic airway inflammation and AHR was shown in most studies with IL-33 KO mice, ST2 KO mice or mice that were treated with anti-IL-33 or r-sST2,^{17,20,23,24,35} some studies found no differences.^{36,37} Of note, with exception of the total number and cytokine-producing ILC2 in the BALF, also in our model, r-sST2 did not affect allergic airway responses towards the suboptimal doses of sole HDM.

As it was previously demonstrated that anti-IL-33 treatment could decrease a PM-induced asthma exacerbation in mice,³⁸ we

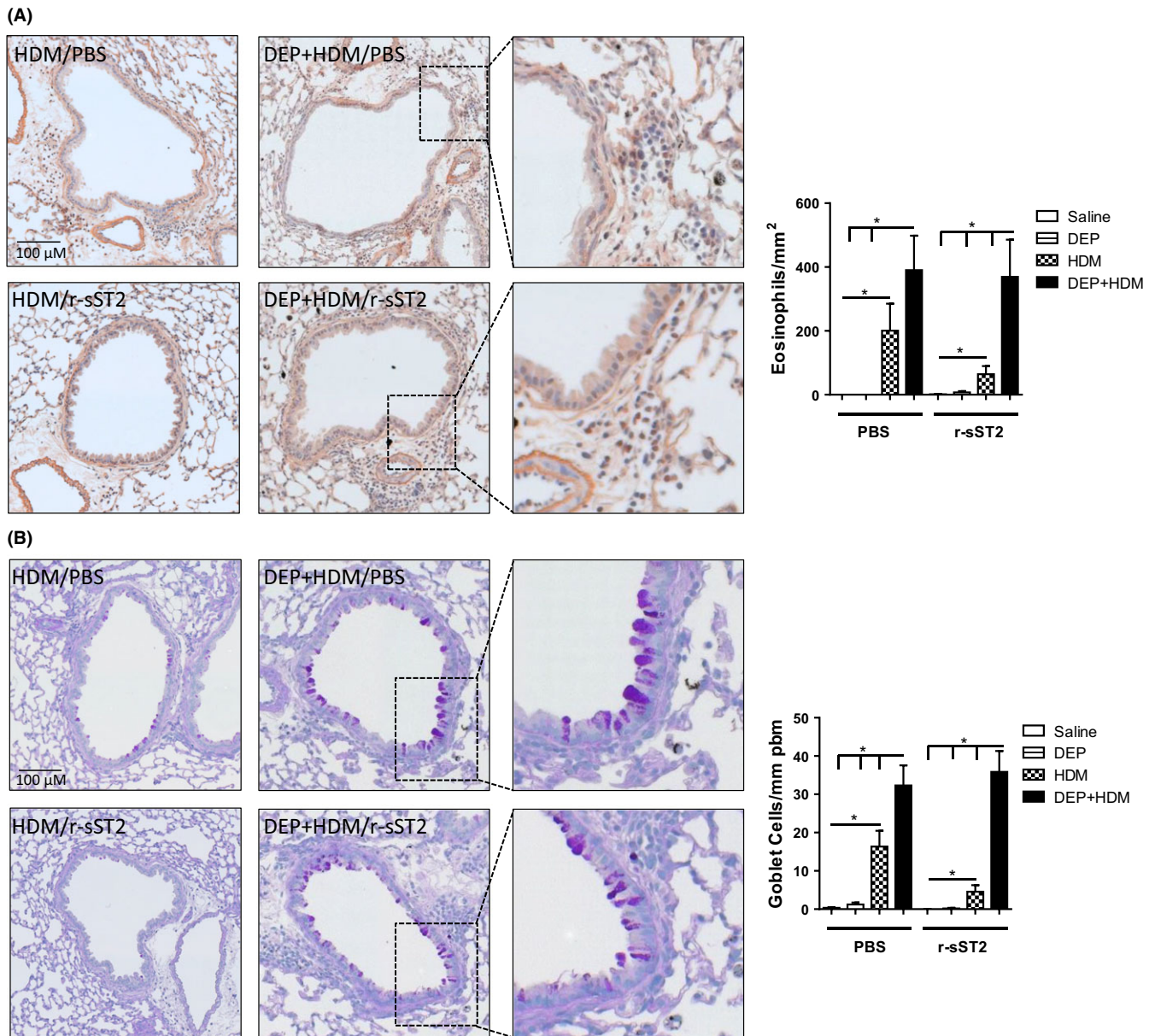


FIGURE 5 Therapeutic r-sST2 administration does not modulate the DEP-enhanced allergic lung inflammation. C57BL/6J mice were intranasally exposed to saline (white bars), 25 μ g DEP (striped bar), 1 μ g HDM (checked bar) or combined DEP+HDM (black bar). On days 14, 15 and 16, mice were treated with PBS or 25 μ g r-sST2. (A, B), Quantification and representative photomicrographs (magnification 20 $\times$) of Congo red-stained peribronchial eosinophils (A) and periodic acid-Schiff-stained mucus-producing goblet cells (B) in HDM and DEP+HDM-exposed mice. Results are expressed as mean $\pm$ SEM. $n = 7$ -8 mice per group. * $P < 0.05$

were interested whether r-sST2 could be therapeutically beneficial during the aggravating effects of DEP on allergic airway inflammation. When r-sST2 was administered only at the end of the exposure protocol, no differences in airway immune responses could be observed, suggesting that signalling via the IL-33/ST2 pathway is more important during the onset of DEP-enhanced allergic airway inflammation. Our observations are in line with data from mouse models of HDM-induced asthma, in which it was shown that administration of r-sST2 during the challenge phase had no effect on airway eosinophilia,³⁴ whereas IL-33 neutralization at the time of sensitization attenuated the HDM-induced eosinophil and

lymphocytic inflammation in the BALF.^{34,39} We can however not exclude that the lack of efficacy in this therapeutic setting could be due to the half-life of r-sST2 or intranasal application route. A limitation of our study is that we could not demonstrate that r-sST2 reduced the in vivo availability of IL-33. To date, commercially available ELISA kits are not able to discriminate between free IL-33 and IL-33 bound to r-sST2 (data not shown). In addition, co-immunoprecipitation experiments of sST2/IL-33 from lung homogenates were not sensitive enough to demonstrate their in vivo interaction. Of interest, we showed that in both experimental set-ups, the percentage of IL-13-producing ILC2 reduced upon r-sST2 treatment,

indicative that r-sST2 did influence the in vivo availability of IL-33. In this regard, other approaches could give additional insights concerning the potential therapeutic effect of IL-33 blockage.

Importantly, the mechanisms underlying the discrepant results of IL-33 during the prophylactic and therapeutic setting should be further investigated. As decreased IL-1 β levels were observed in DEP+HDM-exposed mice that were prophylactically treated with r-sST2, but not therapeutically, this could be a potential mechanism of the observed discrepancy in inflammatory airway responses since IL-1 β was previously found to be involved in type 2 promoting responses.^{40,41}

At the moment, human studies with regard to anti-IL-33 are ongoing^{42,43} and still have to prove their beneficial effect in asthmatic patients. Moreover, it would be interesting to examine the functional role of other epithelial type 2-promoting cytokines, for example, IL-25 and TSLP, during DEP-enhanced allergic airway inflammation, as it was suggested that combinatorial blockage of these cytokines could be necessary to effectively inhibit lung inflammation.⁴⁴

In conclusion, our preclinical data suggest that signalling through the IL-33/ST2 pathway has a functional role during the onset of DEP-enhanced allergic airway inflammation, whereas interference with IL-33 signalling in established DEP+HDM-induced inflammation is ineffective.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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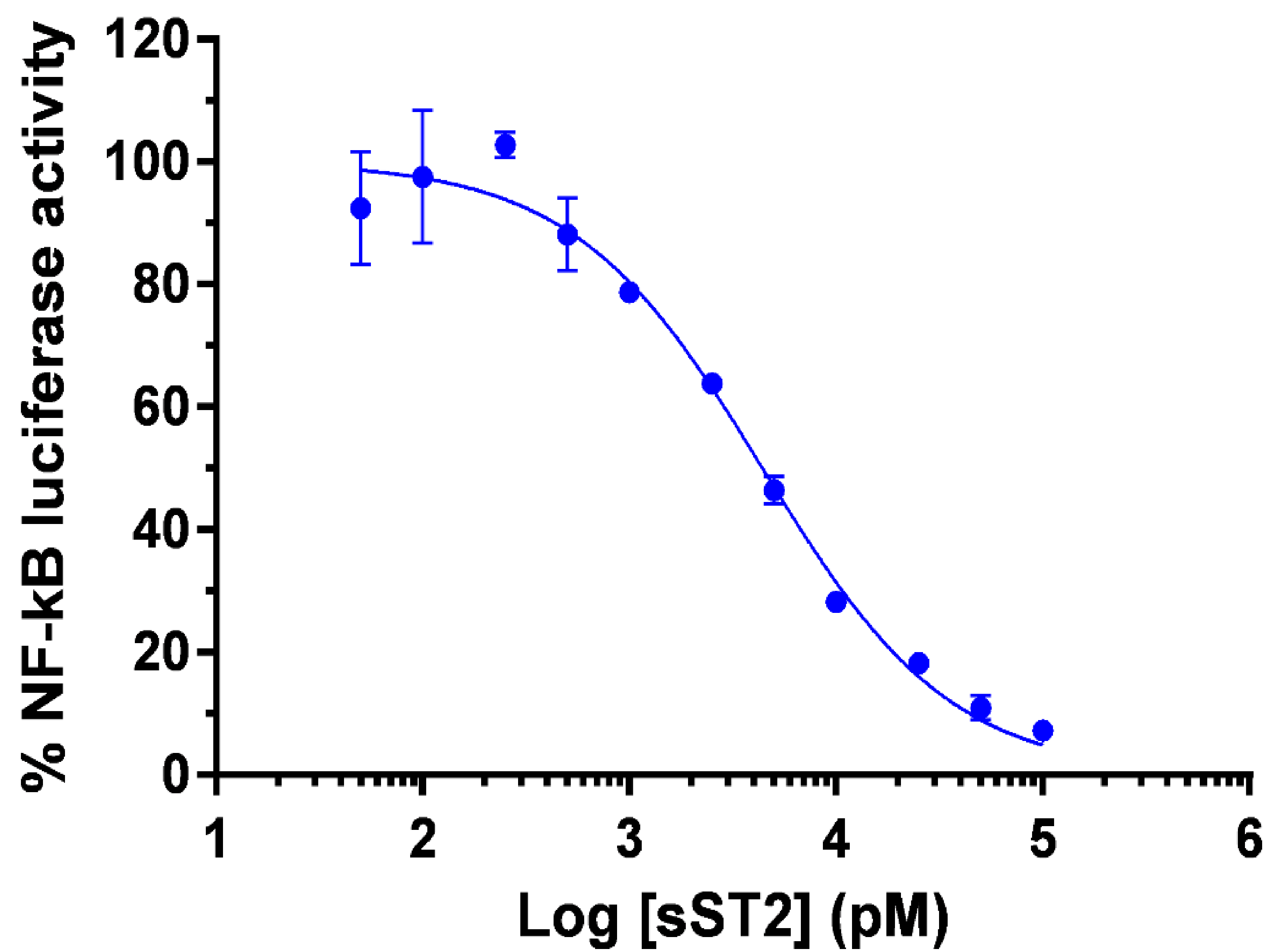
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SUPPORTING INFORMATION

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

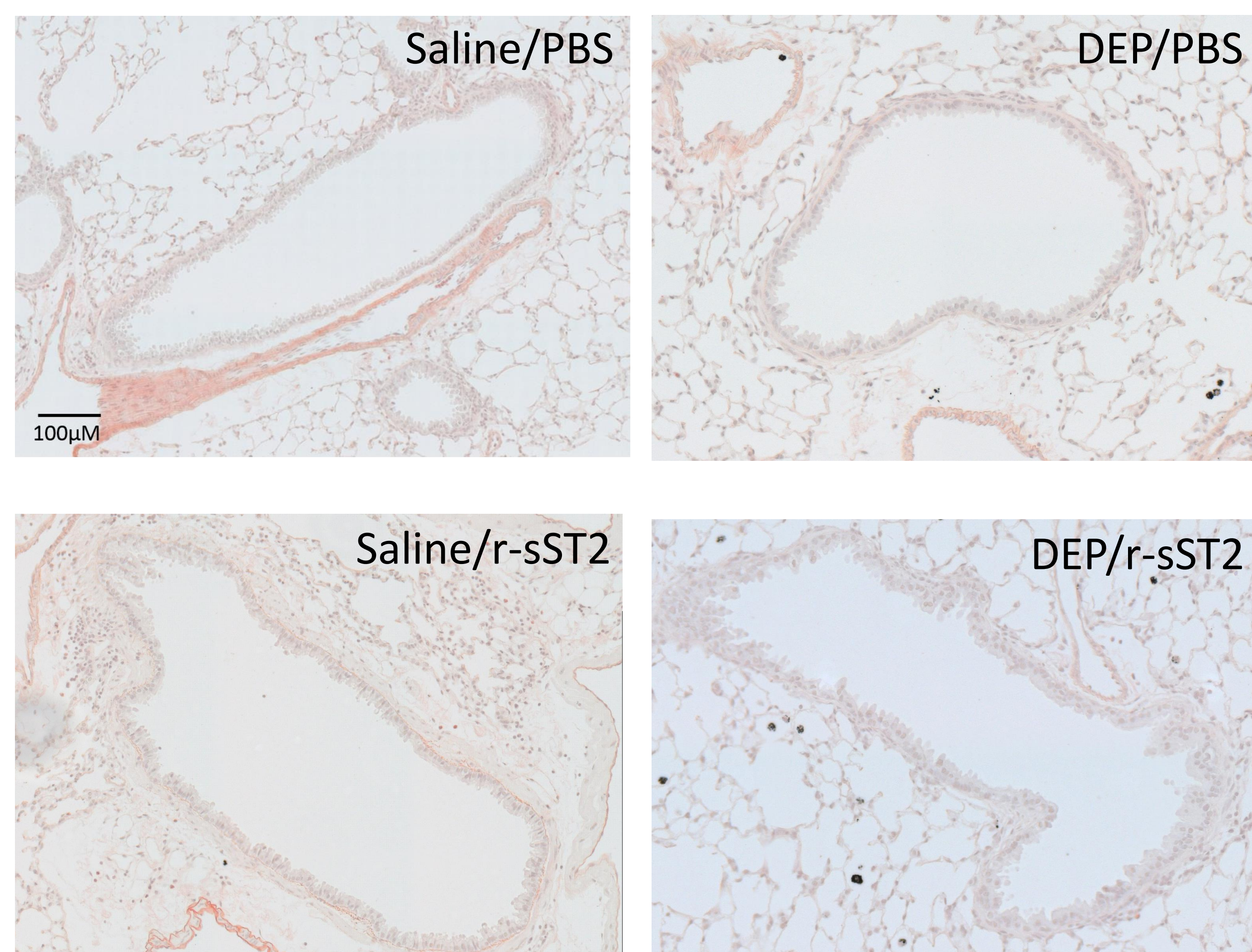
How to cite this article: De Grove KC, Provoost S, Braun H, et al. IL-33 signalling contributes to pollutant-induced allergic airway inflammation. *Clin Exp Allergy*. 2018;48:1665–1675. <https://doi.org/10.1111/cea.13261>

IL-33 (0.5 nM) inhibition
NF- κ B luciferase

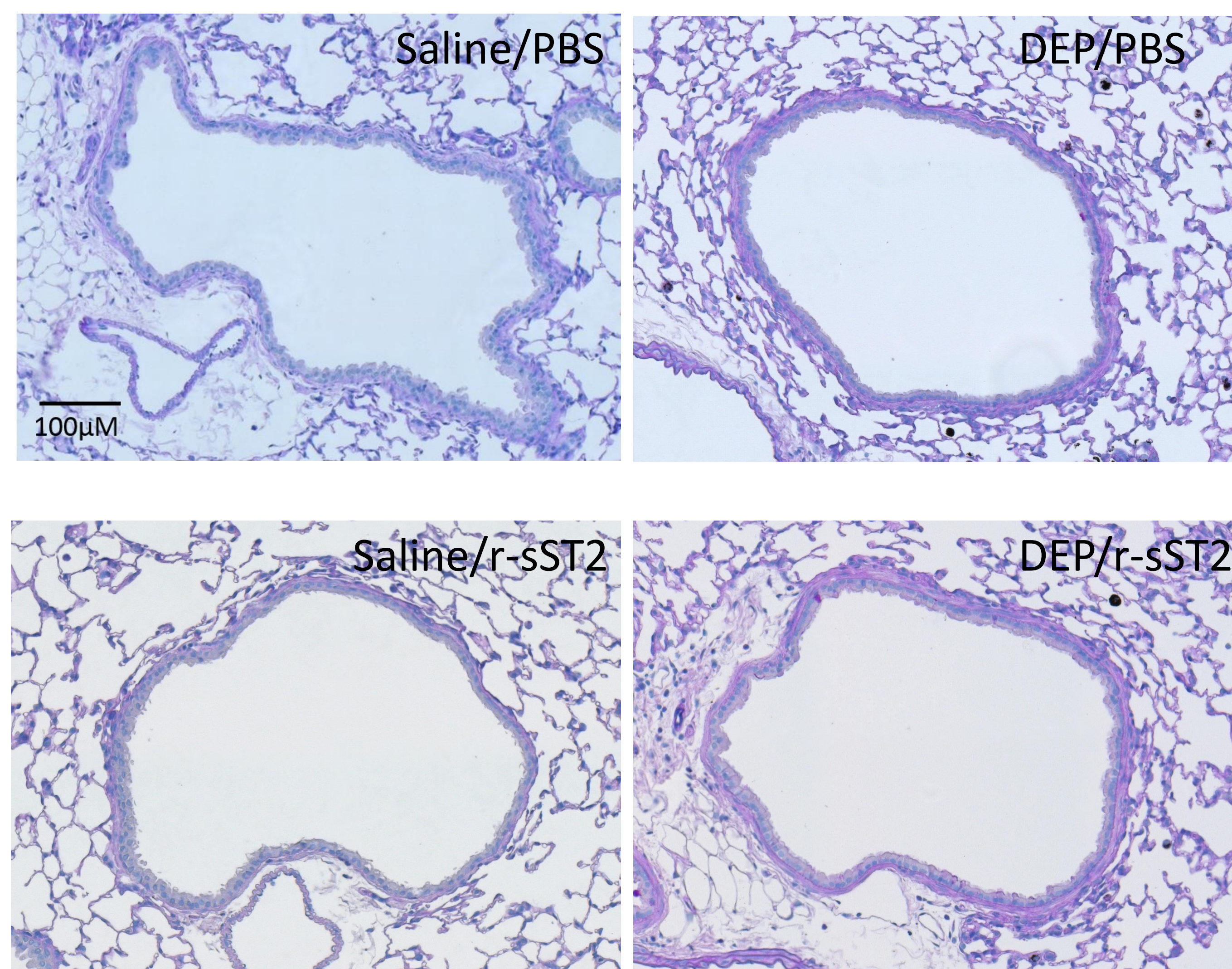


SUPPLEMENTAL FIGURE 2

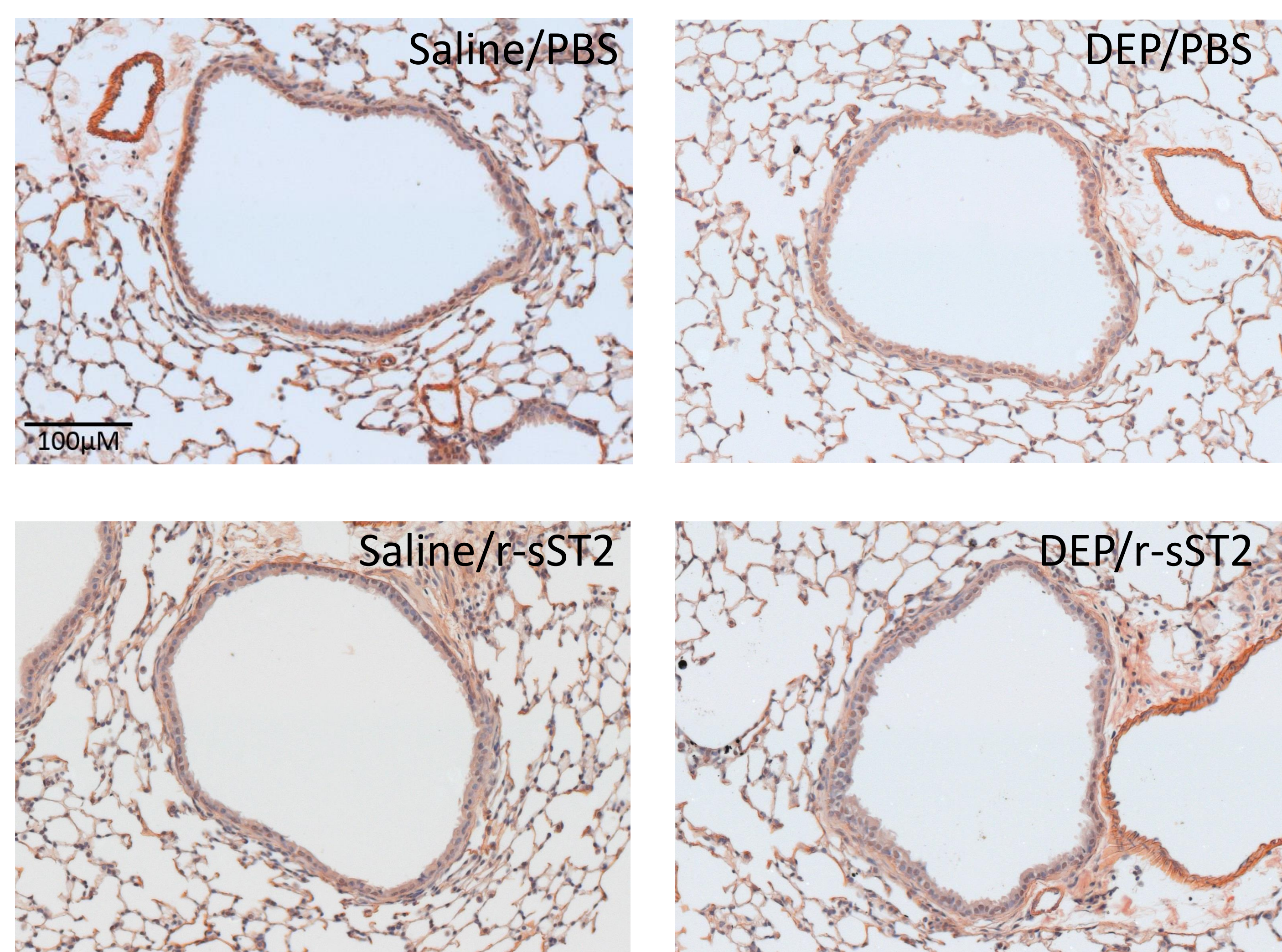
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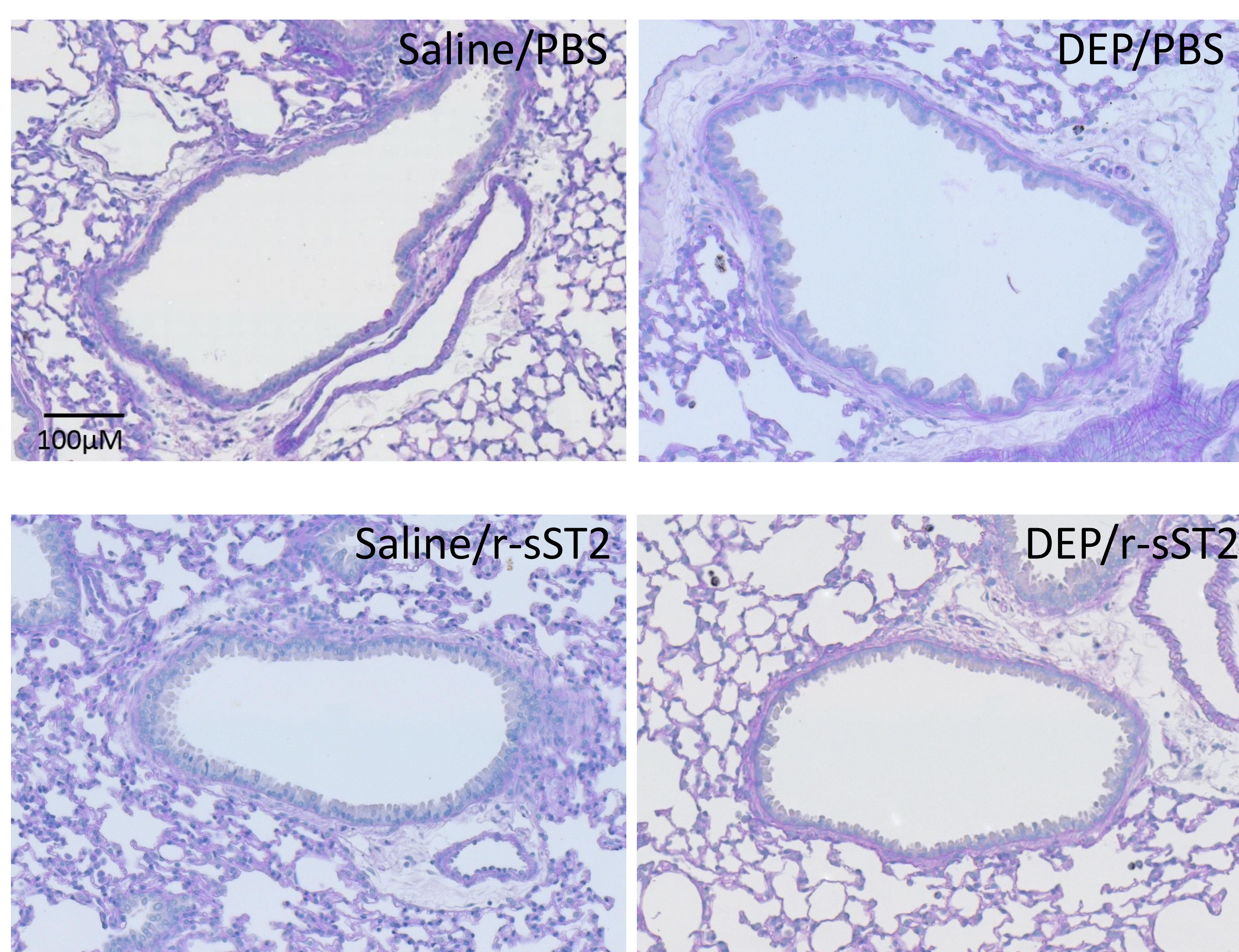
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C.



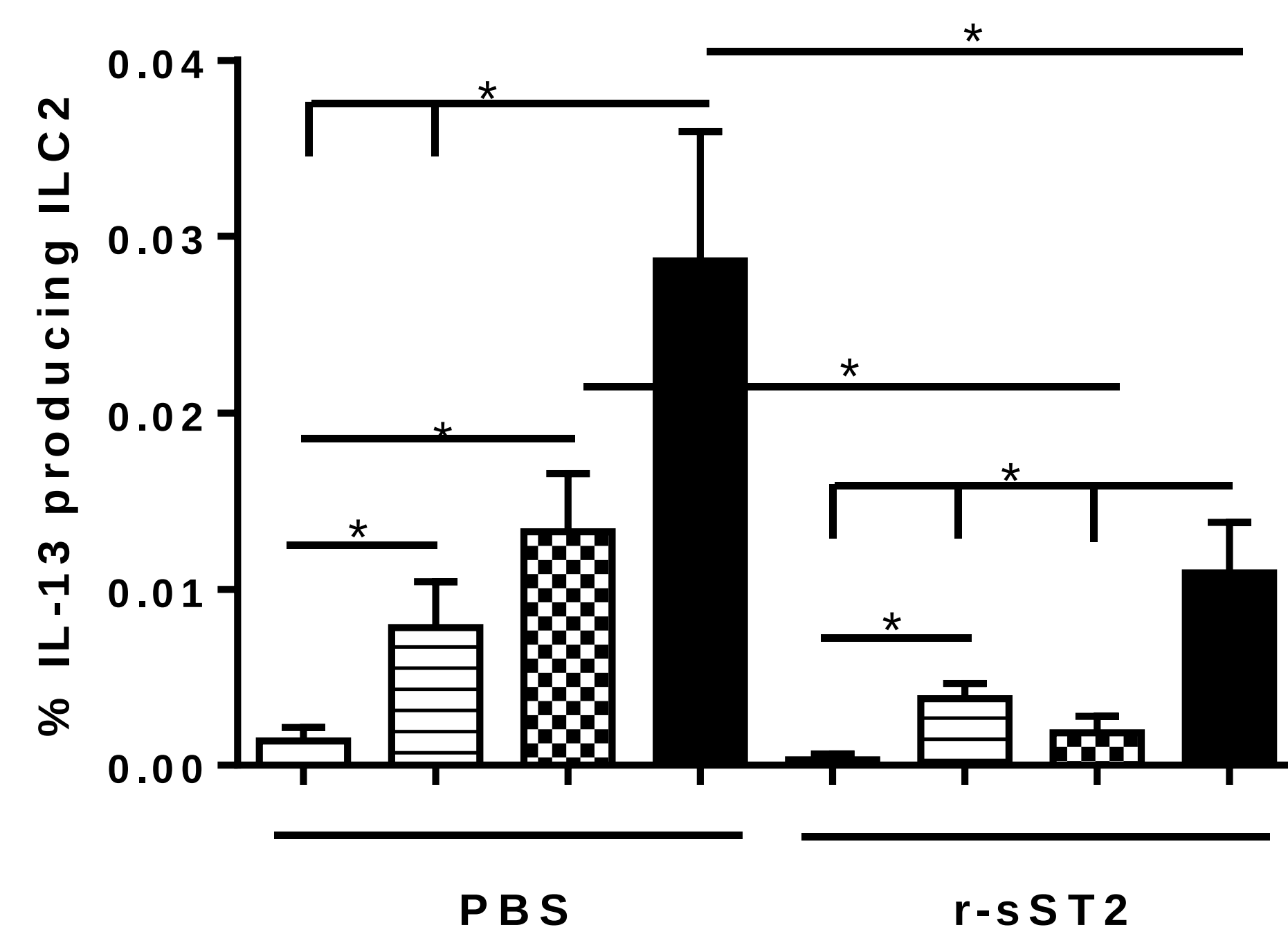
D.



SUPPLEMENTAL FIGURE 4

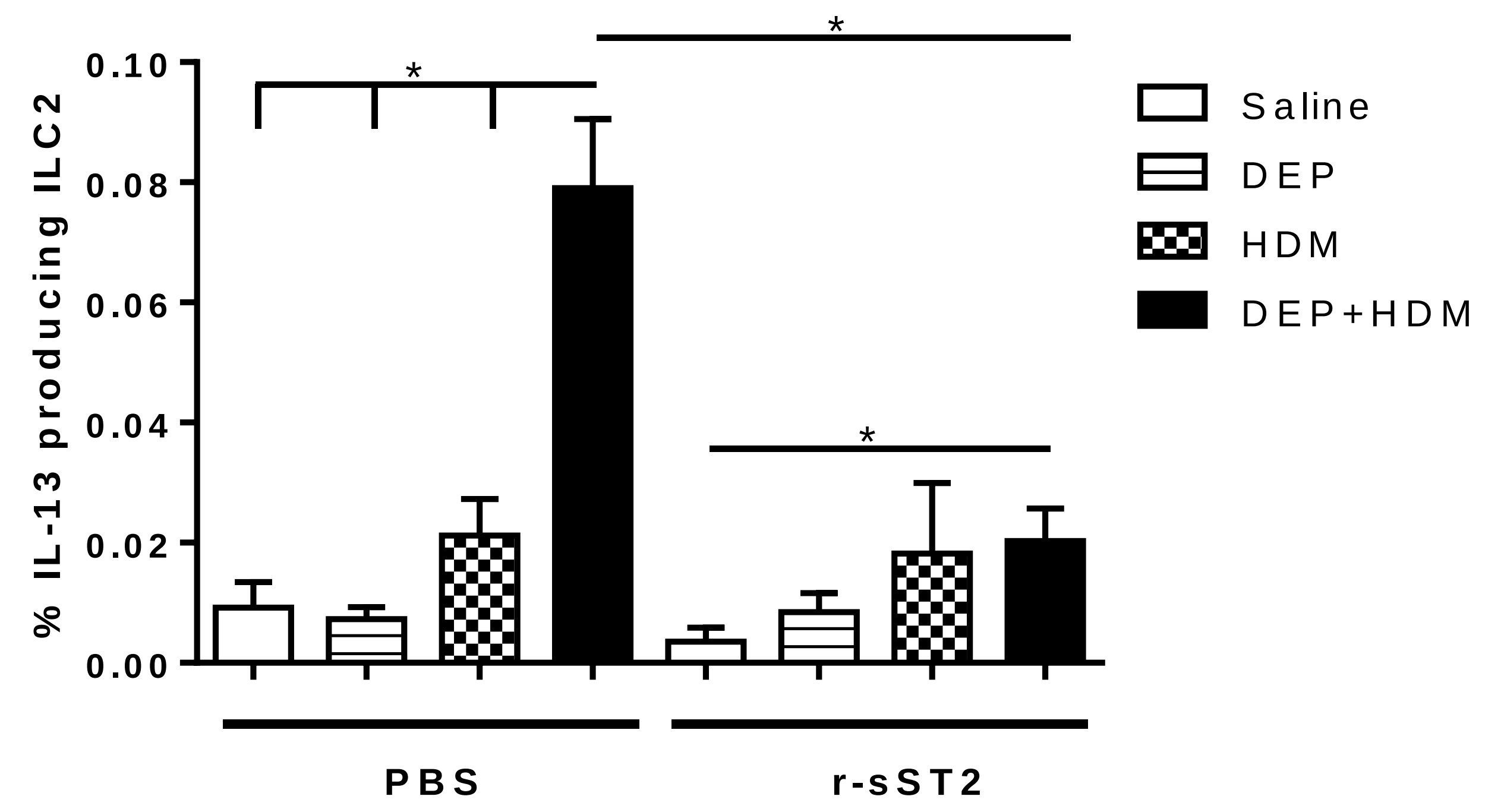
A.

Prophylactic setup



B.

Therapeutic setup



Supplemental Figure Legends

Supplemental Figure 1: Neutralizing activity of recombinant sST2. Mouse IL-33 induced NF- κ B receptor activation was measured in ST2+IL1RAcP transfected HEK293T cells.

Supplemental Figure 2: Peribronchial eosinophils and goblet cells of saline and DEP-exposed mice. C57BL/6J mice were intranasally exposed to saline, DEP, HDM or DEP+HDM on day 1,8 and 15. Endpoints were performed on day 17. **A-B**, Representative photomicrographs (magnification 20x) of congo red-stained peribronchial eosinophils (**A**) and periodic acid-Schiff-stained mucus-producing goblet cells (**B**) of mice that were exposed to saline or PBS and prophylactically treated with r-sST2. **C-D**, Representative photomicrographs (magnification 20x) of congo red-stained peribronchial eosinophils (**C**) and periodic acid-Schiff-stained mucus-producing goblet cells (**D**) of saline or DEP-exposed mice that were treated therapeutically with r-sST2.

Supplemental Figure 3: DEP-enhanced allergic airway inflammation on day 14. **A**, mice were intranasally exposed to saline (white bars), DEP (striped bars), HDM (checked bars) or DEP+HDM (black bars) on day 1 and 8. Endpoint were performed on day 14. **B-F**, DC (CD45⁺, CD11c^{high}, MHCII⁺) (**B**), neutrophils (CD45⁺, CD11c⁻, CD11b⁺, Ly6G⁺, Ly6C⁺) (**C**), eosinophils (CD45⁺, CD11c⁻, CD11b⁺, Siglec-F⁺) (**D**), ILC2 (CD45⁺, Lin⁻ (CD3⁻, CD5⁻, NK1.1⁻, TCR β ⁻, CD11c⁻, CD11b⁻ and CD45R⁻), CD25⁺, CD90⁺, ST2⁺) (**E**) and CD4⁺ T cells (CD45⁺, CD3⁺, CD4⁺, CD8⁻) (**F**) in BALF were determined by flow cytometry. **G-H**, BALF cells were stimulated for 4 hours with PMA/ionomycin + protein transport inhibitors, intracellularly labeled and analyzed using flow cytometry. Number of IL-13 expressing ILC2 (CD45⁺, Lin⁻, CD25⁺, CD90⁺) (**G**) or CD4⁺ T cells (CD45⁺, CD3⁺, CD4⁺) (**H**). Results are expressed as mean $\pm$ SEM. n = 7-8 mice per group. * P < 0.05.

Supplemental Figure 4: Reduced percentage IL-13-producing ILC2 following prophylactic and therapeutic treatment. Mice were intranasally exposed to saline (white bars), DEP (striped bars), HDM (checked bars) or DEP+HDM (black bars) on day 1, 8 and 15. Endpoints were performed on day 17. **A-B**, BALF cells were stimulated for 4 hours with PMA/ionomycin + protein transport inhibitors, intracellularly labeled and analyzed using flow cytometry. Percentage of IL-13 expressing ILC2 (CD45⁺, Lin⁻, CD25⁺, CD90⁺) upon prophylactic treatment (**A**) or therapeutic treatment (**B**). Results are expressed as mean $\pm$ SEM. n = 7-8 mice per group. * P < 0.05.