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IL33 contributes to diesel pollution-mediated increase in experimental asthma severity

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Abstract

Background: Exposure to traffic pollution, notably diesel exhaust particles (DEP), increases risk for asthma and asthma exacerbations. The contribution of cytokines generated by stressed lung epithelial cells (IL25, IL33, TSLP) to DEP-induced asthma severity remains poorly understood.

Methods: BALB/c mice were exposed intratracheally once to DEP or 9 times over 3-weeks to either saline, DEP, and/or house dust mite extract (HDM). Airway hyper-responsiveness (AHR), pulmonary inflammation, and T-cell subsets were assessed 24 hours after the last exposure in mice sufficient and deficient for the IL33 receptor ST2.

Results: DEP exposure induces oxidative stress, IL6, neutrophils and pulmonary accumulation of IL33, but not IL25 or TSLP or other features of allergic disease. When mice are co-exposed to DEP and low doses of HDM, DEP increases IL33 lung levels and Th2 responses. ST2 deficiency partially protected mice from HDM + DEP induced AHR in association with decreased type 2 inflammation and lung levels of IL5⁺IL17A⁺ co-producing T-cells. Upon in vitro HDM challenge of lung cells from HDM ± DEP exposed ST2^{-/-} mice, secretion of IL5, IL13, IL6 and IL17A was abrogated by a mechanism involving IL33 signaling in both dendritic cells and T-cells. HDM + DEP exposed bone marrow derived dendritic cells and IL33 pulsed BMDC promote a mixed Th2/Th17 response that was dependent on ST2 expression by CD4⁺ T-cells.

Conclusion: IL33 contributes to DEP mediated increase in allergen-induced Th2 inflammation and AHR in a mouse model of severe steroid resistant asthma, potentially through the accumulation of pathogenic IL5⁺IL17A⁺ CD4⁺ effector T-cells.

KEYWORDS

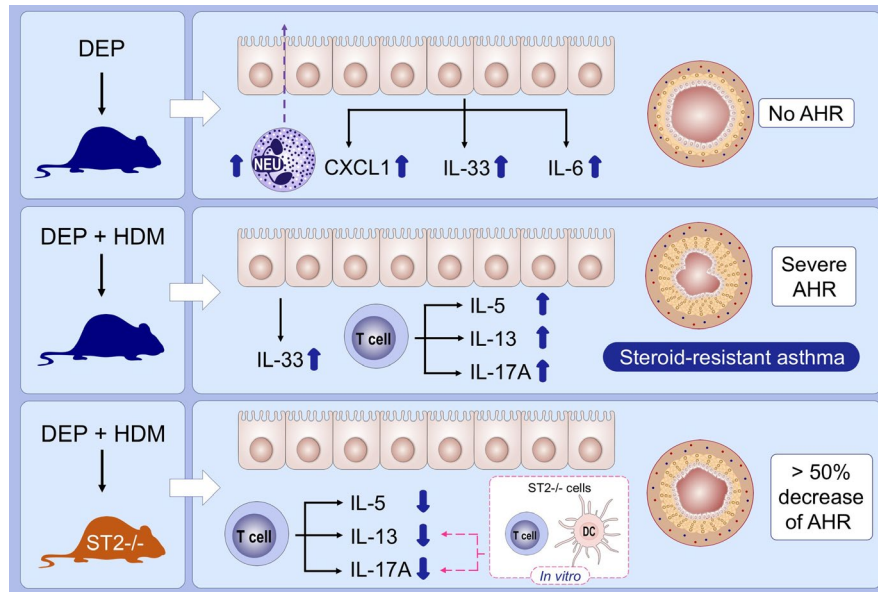
diesel exhaust particles, house dust mite, IL33, steroid resistant asthma, traffic pollution

1 | INTRODUCTION

Asthma is a heterogeneous disease affecting a growing number of children worldwide. A large proportion of the costs associated with treating asthma is generated by patients suffering from severe asthma, resulting from poorly controlled symptoms requiring recurring visits to the emergency room. Severe asthma is commonly treated with high doses of inhaled or oral corticosteroids in

combination with a second controller such as long-acting beta-agonist in order to alleviate life-threatening disease exacerbations.

The rising incidence of allergic asthma points to the contribution of environmental factors. Indeed, exposure to traffic related air pollution, most notably diesel exhaust particles (DEP), a major component of particulate matter with diameter smaller than 2.5 µm (PM_{2.5}), has been implicated in the development of asthma and severity of asthma symptoms.^{1,2} We have previously shown that in



GRAPHICAL ABSTRACT

- Diesel exhaust particles (DEP) induce IL-33 but not allergic disease, whereas house dust mite (HDM) and DEP co-exposure promote a mixed Th2/Th17 response and elevated airway hyper-responsiveness (AHR).
- IL-33 receptor deficient ($ST2^{-/-}$) mice had milder allergic airway disease with decreased IL-13, and fewer $\gamma\delta$ T-cells and pathogenic $IL5^{+}IL17A^{+}$ co-producing T-cells.
- IL-33 signaling in T-cells and dendritic cells both contribute to allergen-induced Th2/Th17 responses.

children with allergic asthma, exposure to traffic related air pollution was associated with more frequent asthma symptoms and increased IL17A blood levels.³ Indeed, we found that co-exposure to DEP and a common aero-allergen, house dust mite (HDM), was associated with increased asthma severity in mice and that elevated airway hyper-responsiveness (AHR) to methacholine in these HDM + DEP exposed mice was partially mediated by IL17A.³ Treatment with an IL17A neutralization antibody in combination with dexamethasone significantly decreased AHR compared to steroid treatment alone,⁴ suggesting that anti-IL17A treatment could benefit poor steroid responders with elevated IL17A levels and reported exposure to high levels of traffic related diesel pollution.

We have also demonstrated that co-exposure to HDM and DEP significantly enhances pulmonary accumulation of effector Th2 cells, Th17 cells and IL17A and IL13 double producing T-cells compared to mice exposed to HDM alone.^{3,5} Asthma patients have a higher frequency of dual Th2/Th17 cells in their BALF compared to healthy controls and these cells are more resistant to steroids *in vitro*.⁶ Additionally, asthmatics with a predominant Th2/Th17 phenotype had more severe asthma.⁶ In mice, *in vitro* generated double producing Th2/Th17 cells have been shown to be more pathogenic than classic Th2 cells and exacerbate chronic allergic asthma upon transfer.⁷ Similarly, a recent study demonstrated that in mice exposed to either IL33 or the papain protease, lung innate lymphoid cells produce not only IL5 and IL13 but also IL17A and that these IL17A + ILC2 are more pathogenic than classical ILC2s.⁸

While we have demonstrated that exposure to DEP derived from diesel exhaust is associated with elevated lung levels of IL6, IL17A, and neutrophilia in mice, these findings don't explain the large synergistic increase in allergen-induced Th2 responses observed in mice co-exposed to HDM and DEP. One likely mechanism could involve the production by stressed epithelial cells of innate cytokines like TSLP, IL25, and IL33, which promote and amplify Th2 responses via innate cells (ILC2, basophils, mast cells, eosinophils), skew dendritic cells toward a pro-Th2 phenotype,^{9,10} or directly act on Th2 cells¹¹ and memory Th2 cells *in vivo*.^{12,13} Indeed, DEP has been reported to induce TSLP, IL-25, and IL-33 secretion by bronchial epithelial cells.^{14,15} Furthermore, elevated levels of cord blood IL33 and TSLP were associated with maternal allergy and self-reported exposure to heavy street traffic.¹⁶ A recent study has proposed a role for IL33 in mouse models of particulate matter (PM2.5)-induced asthma exacerbation, albeit without assessing AHR or Th2 responses.¹⁷

In the present study, we demonstrate that DEP exposure induces IL33 rather than IL25 or TSLP. Using mice deficient for the IL33 receptor ST2, we demonstrate the central role played by DEP-induced IL33 in enhancing the severity of allergic AHR and assessed the impact of ST2 deficiency on innate cells ($\gamma\delta$ T-cells and ILCs) and on effector T-cells, highlighting a population of pathogenic $CD4^{+}$ T-cells that express both IL17A and IL5. Finally, we demonstrated that IL33 signaling in T-cells and dendritic cells contributes to allergen-induced Th2/Th17 responses.

2 | METHODS

2.1 | Mouse asthma model

Wild type 5-7 weeks old BALB/cJ mice were purchased from Jackson Labs (Bar Harbor, ME). *St2*^{-/-} mice, on BALB/c background, are a generous gift obtained from Andrew McKenzie's laboratory (Cambridge University, Cambridge, United Kingdom). House dust mite extract (*Dermatophagoides pteronyssinus*) was purchased from Greer Laboratories (Lenoir, NC). DEP was generated from a 4-cylinder Deutz diesel engine at the EPA (Research Triangle Park, NC); detailed characterization of this compressor DEP has been described elsewhere.¹⁸ In a first set of experiments, mice were challenged once intratracheally with either saline or DEP (150 µg in 50 µL). In our model of DEP mediated severe asthma, mice received 3 intratracheal challenges times a week for 3 weeks with either 50 µL of saline, 100 µg of DEP, 10 µg of HDM extract (representing 2.2 µg of protein; 0.1 µg of Der p1; 0.3 EU of endotoxin) or both and were sacrificed 1 day after the last exposure (Figure 2A). The mice were maintained and handled under Institutional Animal Care and Use Committee approved procedures (Cincinnati Children's Hospital Medical Center) and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council).

2.2 | Airway hyperresponsiveness

Mice were anesthetized with ketamine, xylazine, and acepromazine (100, 20 and 10 mg/mL respectively mixed at a ratio of 4:1:1). Invasive measurements of AHR were made using the FlexiVent apparatus (Scireq, Montreal, Canada). Mouse tracheas were cannulated with a 20-gauge blunt needle and the mice were ventilated at 150 breaths/min, 3.0 cm water positive end expiratory pressure. Two total lung capacity perturbations were then performed for airway recruitment before baseline measurement and subsequent methacholine challenges were performed. Dynamic resistance (R) was assessed following exposure to increasing concentrations of aerosolized methacholine (0, 6.25, 12.5, 25, 50 mg/mL). The average of the 3 highest R values with a coefficient of determination of 0.9 or greater (as determined by the FlexiVent software) was used to determine the dose-response curve.

2.3 | BALF collection and differential counts

Bronchoalveolar lavage was performed by cannulation of the trachea. The lungs were lavaged with 1 ml PBS + 0.5% BSA + 2 mM EDTA. The collected BALF was centrifuged and the total cell numbers counted with a hemacytometer. Cells were spun onto frosted slides (Fisher Scientific, Pittsburgh, PA) and stained with the HEMA3 stain set (Fisher Scientific, Kalamazoo, MI). A minimum 200 cells were counted per slide by an investigator blinded

to treatment and genotype before the total number of each cell type was calculated.

2.4 | ELISA on supernatants and lung homogenates

For each group, the right lung middle lobe was collected in a 2 mL tube and frozen. The lungs were thawed in 500 µL of RIPA buffer with HALT protease inhibitors (ThermoFisher Scientific, Rockford, IL), homogenized on ice, centrifuged at 10 000 g for 5 minutes, and the supernatant assessed for cytokines. Total IL13 levels (free and IL13Rα2-bound) were measured as previously described.¹⁹ IL5, IL6, TNFα and IL17A levels in supernatants and lung homogenates were assessed by ELISA according to manufacturer's instructions (BioLegend, San Diego, CA).

2.5 | Histology and immunohistochemistry

The left lobe of the lung was fixed in formalin, paraffin embedded and cut into 5µm sections. Sections were stained with Periodic Acid Schiff Reaction (PAS) according to the manufacturer's recommendations (PolyScientific R&D Corp, Bay Shore, NY).

2.6 | Isolation of lung cells and staining for flow cytometry

Lungs were removed and the two largest right lobes were minced and incubated 37°C for 30 minutes in 2 mL of RPMI 1640 containing Liberase TL (0.5 mg/mL; Roche Diagnostics, Indianapolis, IN) and DNase I (0.5 mg/mL; Sigma, St Louis, MO). Lung cells were passed through a 70 µm cell strainer with a syringe rubber and the strainer washed with 5ml of RPMI + DNase I media. Cells were centrifuged and resuspended in 1 mL PBS + 0.5% BSA + 2 mM EDTA before being counted. Between 5×10^5 - 10^6 lung cells were transferred into a 96 well plate with V shaped wells on ice, centrifuged and resuspended in PBS containing FcBlock (2.4G2 mAb; BioLegend). ILCs were stained for CD45, CD90, CD25 and were negative for CD4 and other lineage positive cells (antibodies used listed in Table S1). Lung γδT-cells, were characterized as CD3⁺ CD4⁻ and γδTCR⁺ whereas effector T-cells were CD3⁺CD4⁺CD44⁺. All cells were labeled with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit according to manufacturer's instructions (Invitrogen by Life Technologies, Carlsbad, CA). Intracellular staining for IL5, IFNγ, and IL17A were conducted according to manufacturer's instructions (eBioscience, San Diego, CA) following ex vivo stimulation with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL; Sigma) and ionomycin (500 ng/mL; Sigma) for 4 hours, the last 3 hours in the presence of Brefeldin-A (BioLegend). Flow cytometry experiments were acquired on a Special Order BD FACSCanto with an additional 405 nm laser using BD FACSDiva Software version 8.0 (BD Biosciences, San Jose, CA, USA) and all data analyzed with FlowJo v10 software (Tree Star, Ashland, OR).

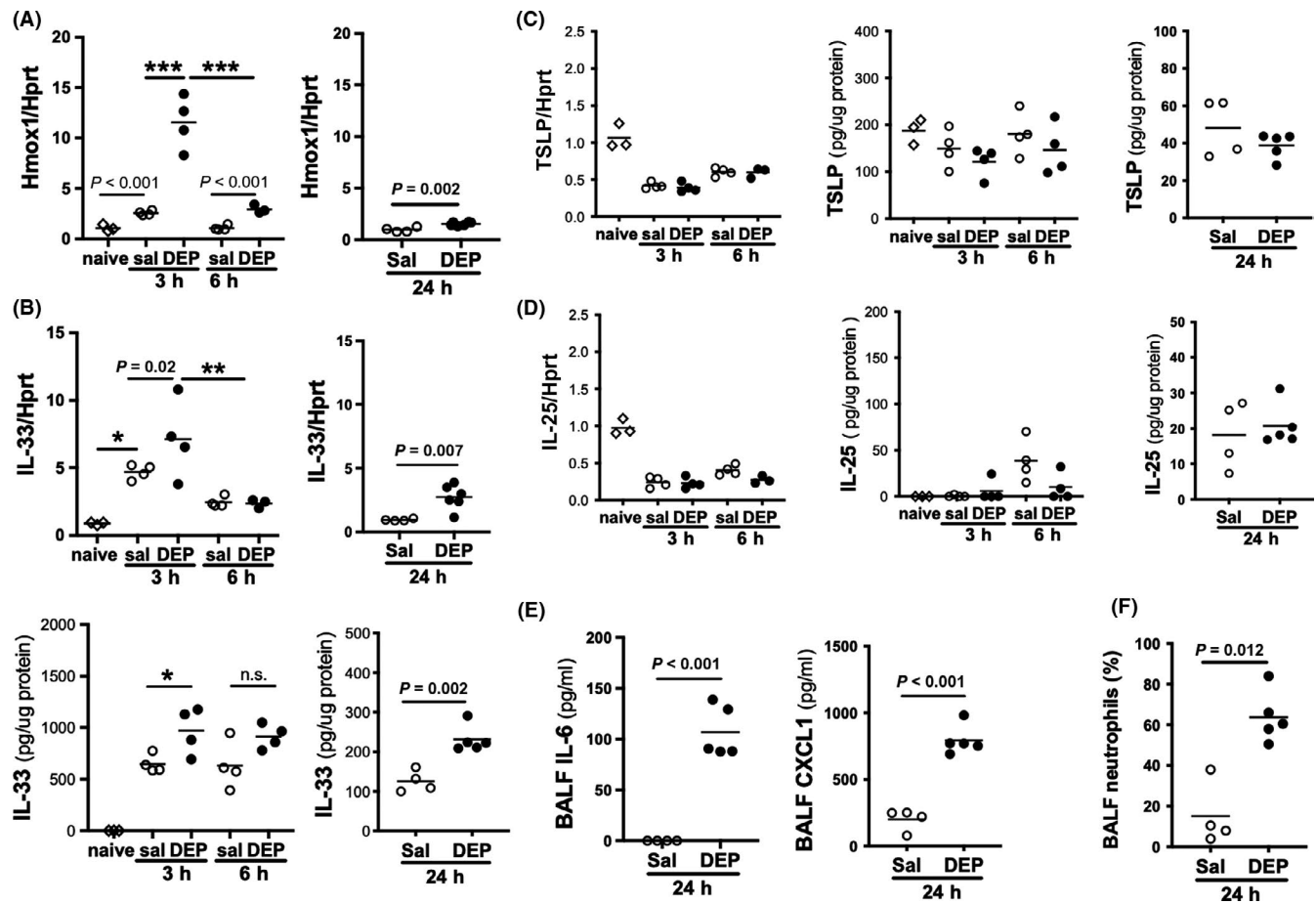


FIGURE 1 Exposure to diesel exhaust particles induces oxidative stress, neutrophilia, IL6, and IL33. BALB/c mice were exposed to a single bolus of DEP and lung mRNA levels of (A) the anti-oxidant gene Hmox-1 and (B) the alarmin IL33, (C) IL25, and (D) TSLP were assessed by real time quantitative PCR and expressed as ratio over the house keeping gene Hprt. IL33, IL25, and TSLP protein levels (B-D) were measured by ELISA in lung lobe homogenates and expressed as a ratio over total protein levels. E, BALF IL6 and CXCL1 levels were measured by ELISA and (F) BALF neutrophils by differential counts. Statistical significance was assessed by t-test (P value) or 1-way ANOVA with post hoc analysis (* $P < .05$, ** $P < .01$, *** $P < .001$)

2.7 | RNA isolation and real time quantitative PCR

Total RNA was isolated from each mouse's homogenized accessory lung lobe using RiboZol according to manufacturer's instructions (VWR, Radnor, PA) and DNase treated (Qiagen, Valencia, CA), before being reverse transcribed with First Strand Superscript Synthesis IV kit (Invitrogen). cDNA was amplified using LightCycler FastStart DNA master SYBR green I and the primers listed in Table S2. Quantitative real-time PCR was done and analyzed on a LC480 (Roche) and gene expression was normalized to the house keeping gene Hprt.

2.8 | In vitro restimulation

Lung cells were isolated as described above. After red blood cell lysis, cells were counted and plated in round bottom 96 well plates at a density of 100 000 cells/well in triplicate. The cells were then cultured in the presence of either HDM (25 $\mu\text{g/mL}$) or soluble anti-CD3

(1 $\mu\text{g/mL}$) for 6-7 days. Supernatants were collected and replicates were pooled before being stored at -20°C .

2.9 | Generation of bone marrow derived DC and co-culture with CD4⁺ T-cells

BMDC were generated as previously described.²⁰ Briefly, bone marrow cells were cultured in the presence of GM-CSF (20 ng/mL) for 3 days in a T75 flask. After 3 days, fresh media + GM-CSF was added for another 3 days of culture. Cells were harvested, counted and transferred into a flat bottom 96 well plate at 50 000 cells/well in the presence of GM-CSF. The next day, BMDC were exposed overnight to IL33 (10 ng/mL), HDM (20 $\mu\text{g/mL}$) in the presence or absence of DEP (5 $\mu\text{g/cm}^2$) before being washed twice with PBS. CD4⁺ T-cells were isolated from the lungs or spleen of saline, DEP, HDM and HDM + DEP exposed mice and pooled before using CD4⁺ T-cells isolation kit II followed by negative selection using LS columns according to the manufacturer's instructions (Miltenyi Biotec, Auburn,

CA). CD4⁺ T cells were then added to BMDC at a concentration of 200 000 cells/well and cultured for a week in media without GM-CSF. Supernatants were harvested before being stored at -20°C.

2.10 | Statistical analysis

Statistical analyses were done using PRISM software version 8.2 (GraphPad Software Inc, La Jolla, CA). Statistical significance between groups was assessed using either one-way ANOVA followed by respectively Sidak post-hoc testing between relevant preselected pairs of columns or two-way ANOVA followed by Tukey post-hoc test. To compare two groups t-tests were used and the p value stated in the figure. A P value <0.05 was considered significant.

3 | RESULTS

3.1 | Exposure to DEP induces IL33 but not airway hyperresponsiveness

Mice exposed to a single bolus of sonicated DEPs resuspended in saline demonstrated increased lung levels of Hmox-1, an anti-oxidant gene induced by oxidative stress, which peaked around 3 hours after exposure before returning to baseline by the next day (Figure 1A). Stress-induced by pulmonary exposure to saline is sufficient to enhance lung IL33 mRNA and protein levels (Figure 1B). DEP exposure further increases IL33 levels, which remain significantly elevated 24 hours after exposure (Figure 1B). In contrast, IL25 and TSLP lung mRNA levels were decreased following intratracheal challenge and protein levels remained unchanged (Figure 1C,D). IL5 and IL13 lung levels were also not affected and no eosinophils were detected in the BALF (data not shown). Exposure to DEP promoted a dramatic increase in BALF levels of IL-6 and the neutrophil chemokine CXCL1 (Figure 1E) in association with BALF neutrophilia (Figure 1F).

Repeated exposure to DEP over the course of 3 weeks (Figure 2A) did result in pulmonary accumulation of IL33 (Figure 2B) but not TSLP, IL25, IL5, or IL13 (Figure 2C,D). Importantly, repeated exposure to DEP did not generate AHR and hardly any mucus production (Figure 2E,F). Taken together, these results suggest that despite promoting IL33 generation by epithelial cells, DEP does not by itself generate experimental asthma in mice but rather acts as an adjuvant to promote more severe allergen-induced responses.

3.2 | Co-exposure to HDM + DEP increases IL33 and promotes steroid resistant experimental asthma

In accordance with our previous published findings,^{3,5} nine exposures in wild type BALB/c mice to HDM alone was associated with increased AHR, elevated IL-13, and mucus production, and these

were abrogated by treatment with dexamethasone (Figure 2D-F). IL33 was undetectable in the BALF, we therefore measured total IL33, IL5 and IL13 levels in homogenized right lung lobes. The low dose of HDM (10 µg of extract or about 2.2 µg of protein) was selected to ensure that HDM exposure would generate features classically associate with mild asthma but also allow us to demonstrate a synergic increase in AHR and Th2 associated cytokines following co-exposures to HDM and DEP. Indeed, co-exposure to HDM and DEP resulted in a synergistic increase in IL33, IL5, and IL13 levels in the lungs compared to exposure to HDM alone (Figure 2B,D). HDM + DEP induced AHR and mucus production were resistant to treatment with dexamethasone (Figure 2E,F), despite significantly reduced IL13 pulmonary levels (Figure 2D).

3.3 | IL33 contributes to DEP-induced increase in experimental asthma severity

In order to directly assess the role of IL33 in DEP-mediated increased asthma severity, we used mice deficient for the IL33 receptor (ST2). In ST2-deficient mice exposed to HDM alone, AHR was almost completely abrogated (Figure 3A and S1), consistent with absent mucus production and ablated BALF eosinophilia (Figure 3B-D and S2C). In contrast, AHR, BALF eosinophil and T-cell levels were only partially decreased in HDM + DEP co-exposed ST2-deficient mice compared to wild type HDM + DEP exposed mice (Figure 3A-C) and the extent of mucus production in the large airways following HDM + DEP co-exposure appeared similar between ST2 deficient and sufficient mice (Figure 3D and S2C). Taken together, these findings suggest that the observed increased in asthma severity in mice co-exposed to HDM and DEP compared to mice exposed to HDM alone is partially mediated by IL33 signaling.

3.4 | Th2 cytokine production is decreased in ST2 deficient mice co-exposed to HDM + DEP

Since BALF T-cell numbers were significantly lower in ST2^{-/-} mice compared to wild type mice (Figure 3C and S2A), we assessed Th2 cytokines in the BALF of HDM and HDM + DEP exposed wild type and ST2^{-/-} mice. BALF levels of IL5 and IL13 were significantly increased in HDM + DEP exposed mice compared to mice exposed only to HDM (Figure 3E and S2B). A significant decrease was observed in BALF IL13 levels (Figure 3E) as well as in lung IL13 mRNA levels in HDM + DEP exposed ST2^{-/-} mice compared to ST2^{+/+} mice (Figure 3F).

3.5 | Lung accumulation of pathogenic Th2/Th17 effector cells is impaired in ST2^{-/-} mice

We next assessed the nature of the of IL5, IL13 and IL17A producing cells in the lungs by flow cytometry. In HDM-exposed mice,

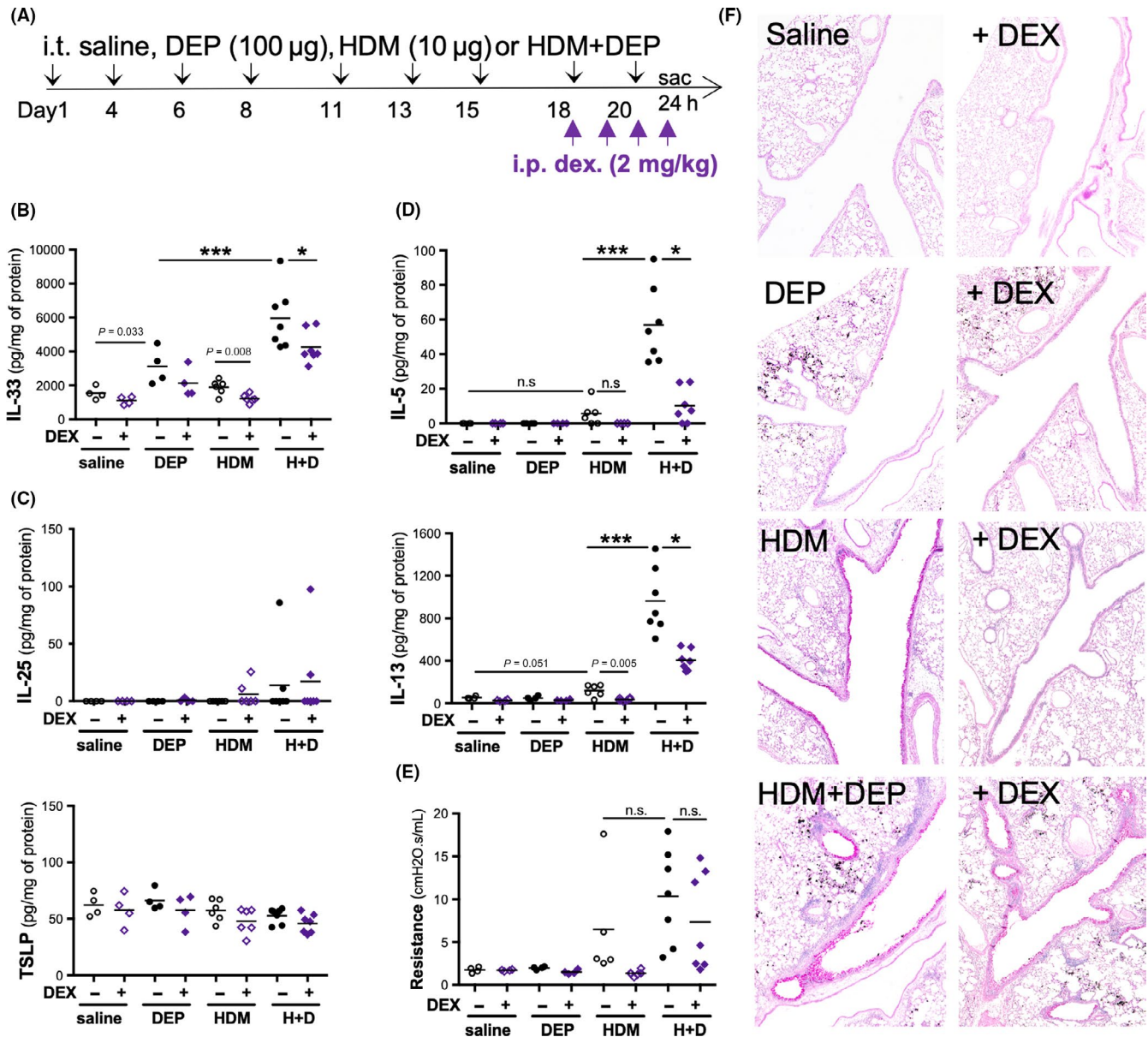


FIGURE 2 Co-exposure to house dust mite extract (HDM) and DEP increases IL33 and promotes steroid resistant experimental asthma. A, Experimental protocol. Mice were challenged intratracheally (i.t.) with either 50 µL of saline, DEP, HDM or HDM + DEP. Dexamethasone (dex) was administered intraperitoneally (i.p.) during the last 4 days. IL33, IL25, TSLP, IL5, and IL13 (B-D) protein levels were measured by ELISA in lung homogenates and expressed as a ratio over total protein. E, Airway resistance was measured a day after the last challenge using FlexiVent (n = 4-7 mice/group; t-test (P = value) or 1-way ANOVA, *P < .05, ***P < .001 n.s., not significant) (F) Representative photomicrographs of PAS-stained large airway sections

an increased proportion of IL13 producing effector cells was observed among wild type CD4⁺ T-cells but not in ST2 deficient mice (Figure 4A). Co-exposure to HDM + DEP further increased the proportion of IL13⁺ T-cells, and this increase was significantly blunted in ST2^{-/-} mice (Figure 4A). DEP exposure promotes the accumulation of IL17A⁺ effector T-cells and γδ T-cells (Figure 4B,C), another major source of IL17A in mouse lungs. In ST2^{-/-} mice exposed to HDM ± DEP, the proportion of γδ T-cells decreased significantly (Figure 4C), and IL17A⁺ effector T-cells trended lower (Figure 4B). These observed decreases in Th2 and Th17 cells were mainly driven by an overall decrease in CD44⁺ effector T-cells (Figure 4D). Indeed,

when the proportion of IL5 or IL17A producing cells among CD44⁺ effector T-cells were assessed, ST2 deficiency did not significantly alter the proportion of IL5⁺ (or IL13⁺) Th2 cells or IL17A⁺ Th17 cells among effector T-cells (Figure 4E and data not shown). However, the proportion of pathogenic effector T-cells expressing both IL5 and IL17A was significantly decreased in the lungs of ST2-deficient mice compared to wild type mice exposed to either HDM or co-exposed to HDM and DEP (Figure 4E).

Another important source of Th2 cytokine in the mouse lungs are type 2 innate lymphoid cells (ILC2). The number of ILC2 cells is significantly different between male and female mice²¹ and our

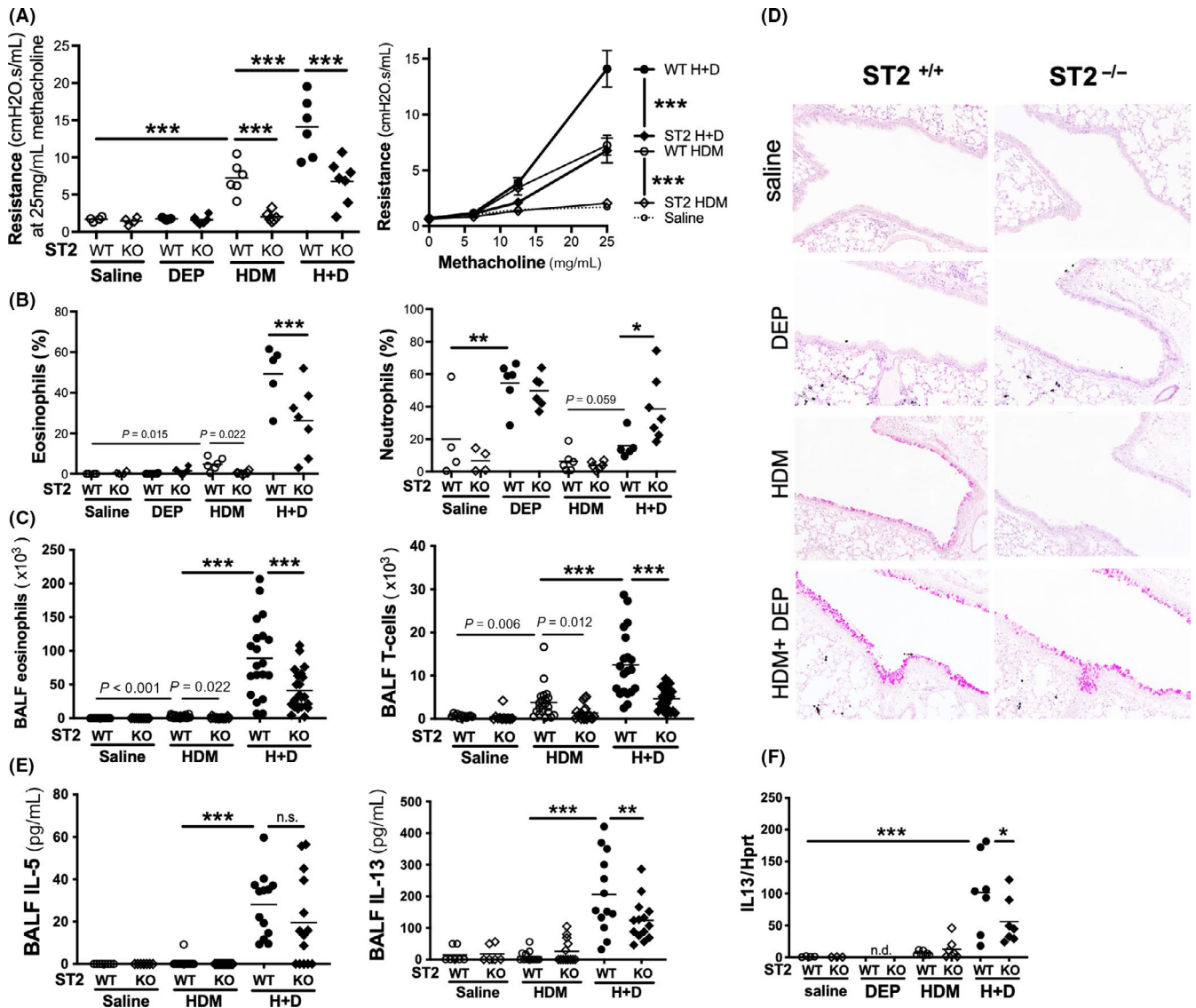


FIGURE 3 Decreased Th2 airway responses in ST2 deficient mice exposed to HDM and DEP. A, Airway resistance was measured a day after the last challenge ($n = 4-7$ mice/group; 1 or 2-way ANOVA, $***P < .001$) (B) Proportions of BALF eosinophil and neutrophils ($n = 4-7$ mice/group). C, Total BALF eosinophil and T-cell counts compiled from 3 separate experiments ($n = 11-22$ mice/group). D, Representative photomicrographs of PAS-stained large airway sections. E, BALF IL5 and IL13 levels were assessed by ELISA ($n = 7-15$ mice/group from 2 separate experiments). F, IL13 lung mRNA levels were assessed by real time PCR and expressed as ratio over Hprt ($n = 4-7$ mice/group). Statistical significance was assessed by t-test ($P = \text{value}$) or 1-way ANOVA; $*P < .05$, $**P < .01$, $***P < .001$, n.s., not significant)

experiments included a mix of both sexes. The proportion of IL5⁺ ILC2 among lung cells was significantly greater in HDM exposed female mice compared to saline exposed mice and were ablated in HDM-exposed ST2^{-/-} mice (Figure 4F). No significant differences in ILC2 levels were observed in HDM ± DEP exposed male ST2 deficient mice compared to wild type controls (Figure 4F).

3.6 | HDM-induced Th2 and Th17 responses are impaired in ST2 deficient lung cell cultures

While the results above demonstrate the presence effector T-cells and ILCs in the lungs of mice and their potential ability to generate IL5,

IL13 or IL17A, they do not reflect the response of these lung cells to a HDM challenge. We next examined this ex vivo by stimulating lung cells from HDM and HDM + DEP exposed ST2^{+/+} and ST2^{-/-} mice with HDM (Figure 5A). Lung cells from HDM + DEP exposed ST2^{+/+} mice released significantly more IL5 and IL13 than lung cells from HDM, DEP or saline exposed ST2^{+/+} mice (Figure 5A), in accordance with the increased number of Th2 cells present in the lungs of HDM + DEP exposed mice compared to mice exposed to HDM (Figure 4A). However, when lung cells from HDM + DEP co-exposed mice were stimulated ex vivo with HDM, a striking decrease in HDM-induced IL5 and IL13 secretion was observed in culture supernatants from ST2 deficient lung cells (Figure 5A). HDM induced IL6 and IL17A secretion was notably observed in DEP and DEP + HDM exposed mice and largely

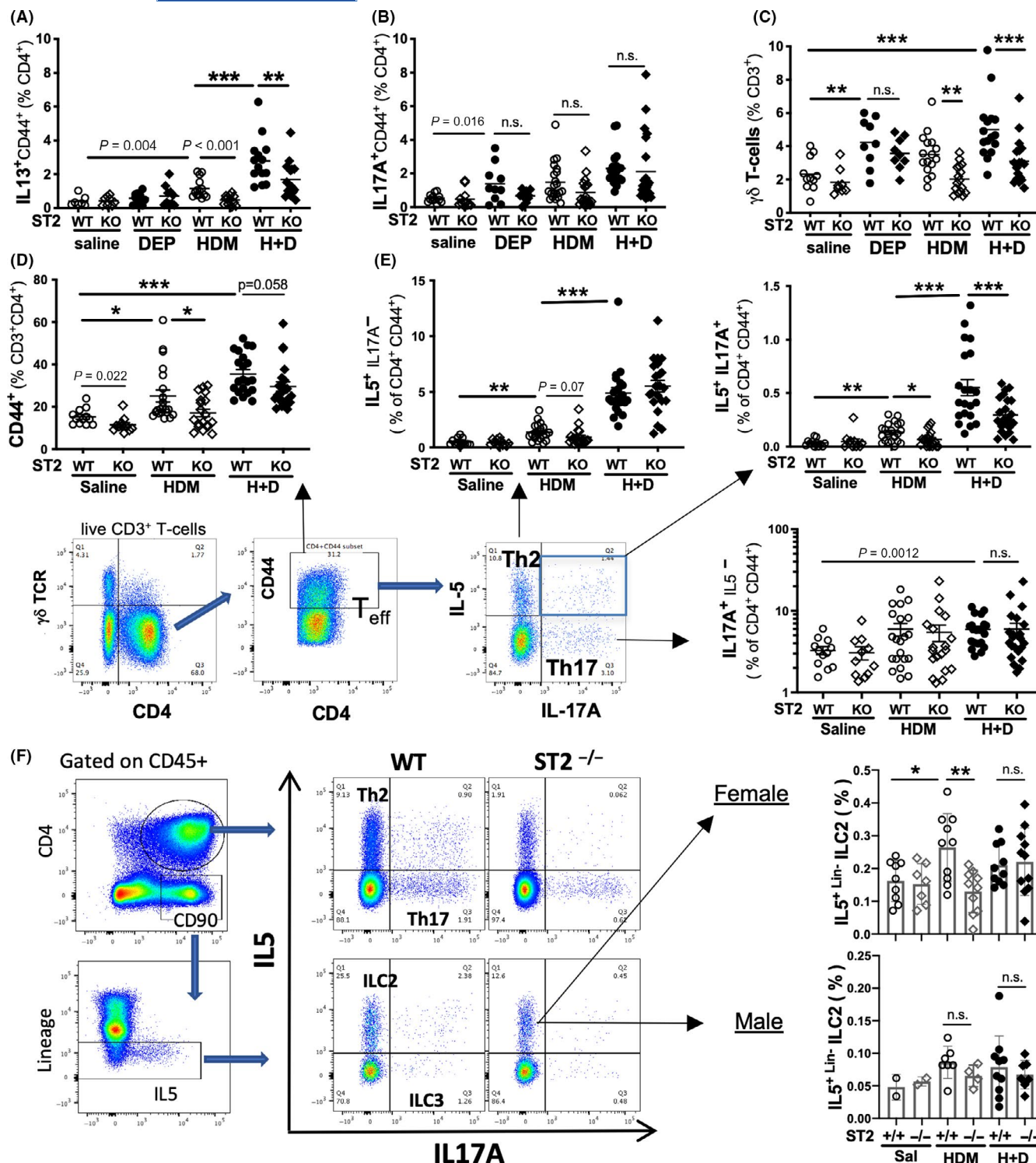


FIGURE 4 Lung accumulation of pathogenic IL5⁺IL17A⁺ T-cells is impaired in HDM + DEP exposed ST2 deficient mice. A, Proportion of IL13⁺, and (B) IL17A⁺ CD44⁺ effector cells among live lung CD4⁺CD3⁺ T-cells. C, Proportion of $\gamma\delta$ TCR⁺ cells among live CD3⁺ T-cells. D, Proportion of CD44⁺ effector T-cells among CD4⁺CD3⁺ T-cells. E, The proportions of lung T-cells expressing IL5, IL17A, or both was assessed by FACS among CD4⁺CD44⁺ effector T-cells cells (Teff; see dot plots) whereas (F) innate lymphoid cells (ILC) expressing IL5, IL17A, or both were identified as Lineage negative CD4⁻CD90⁺ lung cells following 4 hours of ex vivo PMA/ionomycin stimulation in the presence of Brefeldin A. Statistical significance was assessed by t-test (P = value) or 1-way ANOVA: * P < .05, ** P < .01, *** P < .001, n.s., not significant).

abrogated in supernatants of lung cells from ST2^{-/-} mice (Figure 5A). However, HDM induced TNF α levels were similarly elevated in ST2^{+/+} and ST2^{-/-} mice exposed to HDM + DEP (Figure 5A).

Direct stimulation of lung T-cells with anti-CD3 resulted in similar findings (Figure S3B). Thus, despite having similar proportions of lung CD11b⁺ dendritic cells expressing similar levels of OX40L,

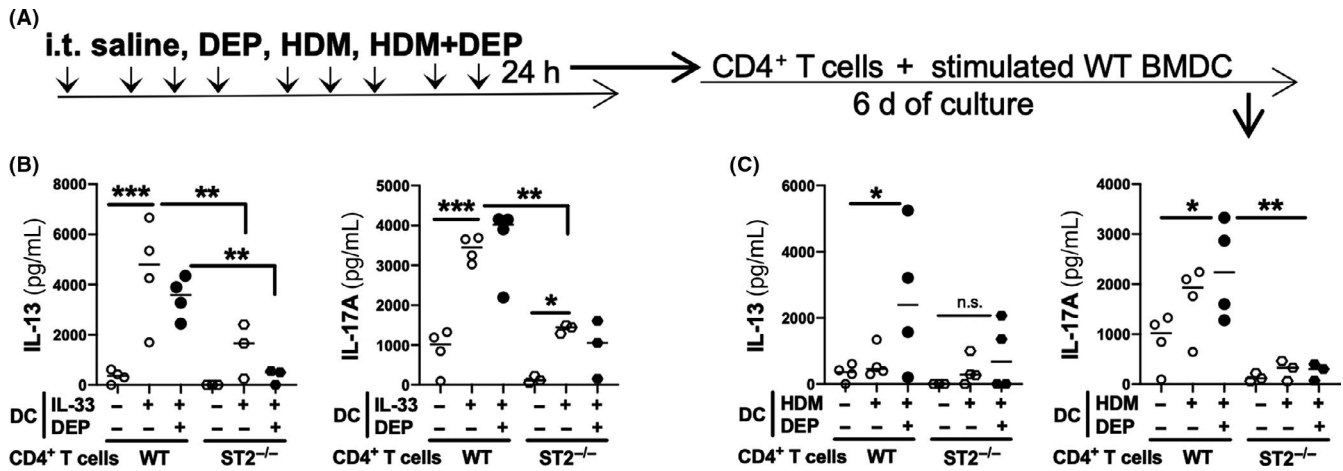


FIGURE 6 IL33 signaling in T-cells is contributing to allergen-induced Th2/Th17 responses. A, Experimental protocol. BMDC were exposed overnight to IL33 or HDM ± DEP, washed and co-cultured for a week with CD4⁺ T-cell isolated from saline, DPE, HDM or HDM + DEP exposed ST2^{+/+} and ST2^{-/-} mice. IL13 and IL17A released in (B) IL33 ± DEP exposed DC co-cultures and (C) HDM ± DEP exposed DC co-cultures were assessed by ELISA (significance was assessed 1-way ANOVA; **P* < .05, ***P* < .01, ****P* < .001, n.s., not significant)

a surface receptor associated with Th2 responses, ST2^{-/-} T-cells release significantly less IL5, IL13 and IL17A upon CD3 stimulation (Figure S3A), suggesting ST2 presence on T cells is required for optimal allergen-specific Th2/Th17 responses in HDM ± DEP exposed mice.

3.7 | Impaired HDM-recall airway responses in ST2 deficient mice is associated with decreased presence of IL17A producing lung cells

We previously demonstrated that repeated co-exposure to HDM + DEP results in larger accumulation of effector/memory Th2 and Th17 cells in the lungs compared to mice exposed to HDM alone,⁵ resulting in increased AHR and Th2/Th17 responses following HDM challenge 7 weeks later compared to mice pre-exposed only to HDM or DEP (Figure S4). In ST2 deficient mice, elevated AHR in HDM ± DEP pre-exposed mice was abrogated following HDM challenge (Figure 5B). This was associated with decreased lung levels of IL6 (Figure 5C), but not IL5 (Figure S5C). Since the primary Th2 response was only modestly decreased in ST2 deficient mice (Figure 4), it is not surprising that the decreased accumulation of effector/memory Th2 cells in the lung of ST2 deficient mice did not reach significance (Figure 5D). IL17A producing T cells, especially pathogenic IL5/IL17A double producing CD4⁺ T-cells (Figure 5E) and γδ T-cells (Figure 5F) were significantly lower in HDM + DEP pre-exposed ST2^{-/-} mice compared WT mice.

3.8 | IL33 signaling in T-cells and dendritic cells both contribute to allergen-induced Th2/Th17 responses

To investigate the respective contributions of dendritic cells and T-cells to ST2 mediated Th2/Th17 cytokine production, bone marrow

derived dendritic cells (BMDC) from WT and ST2^{-/-} mice were cultured in the presence of CD4⁺ T-cells isolated from WT and ST2^{-/-} mice exposed to either saline, DEP, HDM or HDM + DEP (Figure 6A). The absence of ST2 on BMDC logically prevented IL33-stimulated BMDC from promoting IL13 secretion by WT CD4⁺ T-cells (Figure S6A). Similarly, HDM ± DEP stimulated BMDC were less efficient at promoting Th2 responses (Figure S6B) confirming the importance of IL33 primed DC in Th2 responses.

Interestingly, IL33 stimulated BMDC not only induces IL13 secretion by CD4⁺ T cells but, also IL17A (Figure 6B). IL13 and IL17A release was significantly decreased when these Th2/Th17 cells lacked ST2 (Figure 6B). When WT BMDC were stimulated with HDM instead of IL33, the increase in Th2/Th17 cytokine secretion was not significant (Figure 6C). However, co-exposure of BMDC to HDM and DEP induced enhanced IL13 and IL17A secretion from WT but not ST2^{-/-} CD4⁺ T-cells (Figure 6C). Taken together these findings point to a role for IL33 signaling in both T-cells and dendritic cells in driving allergen-induced Th2/Th17 responses.

4 | DISCUSSION

In this study, we have confirmed that exposure to DEP alone generates an immune response dominated by neutrophils, inflammatory cytokines (ie, IL6), and T-cells expressing IL17A (Th17 and γδT-cells), whereas co-exposure with a common aeroallergen (HDM) potentiates HDM-induced Th2 responses and airway hyper-responsiveness. We have identified an alarmin, IL33, that is induced by DEP and, using mice deficient for ST2, we demonstrated a crucial role for IL33 in promoting diesel pollution-mediated increased allergic asthma severity.

While this manuscript was being finalized, a study by De Grove et al came to a similar conclusion.²² The authors exposed C57Bl/6 mice intranasally on days 1, 8 and 15 to either saline, DEP (25 μg

of SRM 2975)+0.05% Tween 80, HDM (Greer; 1 µg of total protein) or a combination of DEP and HDM and assessed lung inflammation two days later. Only mice co-exposed to HDM + DEP demonstrated increased IL-33 lung levels, Th2 inflammation, and mild AHR.²² Neutralizing IL-33, by administering soluble ST2 after each intranasal challenge, significantly decreased Th2 inflammation but not AHR.²² Importantly, in our model, ST2-deficient BALB/c mice exposed to HDM + DEP demonstrate a 50% decrease in AHR, the most clinically relevant outcome in a mouse model of allergic airway disease. Further, the fact that both studies reach an overall similar conclusion, despite using different mouse strains, sources of DEP, challenge protocols and approaches to blocking IL33 signaling, strengthens both studies and strongly support a role for IL33 in driving the observed synergic increase in Th2 inflammation observed in both studies. Additionally, we have demonstrated that IL33 is less susceptible to steroid treatment than Th2 cytokines and our data uniquely highlight the contribution of IL33 to the pulmonary accumulation of innate and adaptive IL5, IL13 and IL17A producing lung cells in this diesel pollution mediated model of severe experimental asthma.

Innate cells such as ILC2, basophils, mast cells, and eosinophils constitutively express ST2 and have been shown to release Th2 cytokines upon IL33 stimulation.²³ Most mouse models of allergic airway disease are not dependent on IgE and/or basophils,^{24,25} and the mast cell contribution to AHR depends on the mouse model utilized.^{23,25} Eosinophils contribute to AHR in C57Bl/6 mice but are dispensable in BALB/c mice.²⁶ ILC2 represent a major source of IL5 and IL13 in the lungs,²⁷ whereas ILC3 are rare (Figure 4F). ILC2 levels were only mildly increased following 9 exposures to HDM + DEP and differences between wild type and ST2-deficient mice did not reach significance, even when mice were segregated by sex. De Grove et al observed a significant accumulation of ILC2 in the BALF of HDM + DEP challenged mice that was alleviated in ST2^{-/-} mice.²² However, the contribution of ILC2 to DEP-induced exacerbation of allergic airway responses appears limited, as the same group previously demonstrated that mice lacking all ILCs (RORα^{fl/fl} IL7R^{Cre} C57Bl/6 mice) only showed partial decreases in Th2 inflammation following co-exposure to HDM and DEP.²⁸ Hence, ILC2s are unlikely to play a major role in HDM + DEP induced AHR.

Interestingly, ST2 deficiency impaired HDM + DEP induced accumulation of IL17A-producing lung cells (IL5⁺IL17A⁺CD4⁺ T-cells, γδT-cells and to a lesser degree Th17 cells) suggesting that blocking IL33 signaling affects not only Th2 responses. Indeed, we show that in vitro stimulation of BMDC with IL33 also promotes IL17A release by T-cells expressing ST2. This is consistent with functional ST2 reported on Th1 and Th17 cells under inflammatory conditions.^{29,30} Furthermore, IL33 has been shown to induce BMDC to release IL6 in a dose dependent fashion and co-culture of IL33 stimulated BMDC with CD4⁺ T-cells promotes IL33-induced IL17A release.^{9,20} Accordingly, IL33 stimulated BMDC promoted Th17 responses via IL1β and IL6.²⁰ Taken together with our findings that DEP and HDM exposures promote IL6 release and HDM + DEP induced IL6

secretion is impaired in ST2^{-/-} lung cells, the results suggest that DEP + HDM co-exposure induces IL6 secretion, in part via IL33 stimulated DC, promoting a mixed Th2/Th17 response.

ST2 deficiency prevented the increased accumulation of IL5⁺IL17A⁺ pathogenic T-cells in the lungs of HDM + DEP co-exposed mice. Transfer experiments in mice with in vitro derived Th2 cells and Th2/Th17 cells have demonstrated that T-cells expressing both Th2 and Th17 cytokines are more pathogenic than classic Th2 cells.⁷ Similar dual positive Th2/Th17 cells were found in a population of patients with severe asthma and demonstrated resistance to glucocorticoid treatment in vitro.⁶ These cells are distinct from pathogenic Th2 cells (CRTH2⁺CD161⁺), which express high levels of IL5, IL9, IL13, and little to no IL17A and which respond to innate cytokine (TSLP, IL25, IL33) to a greater extent than classic Th2 cells.^{31,32} The respective importance of pathogenic Th2 cells expressing large amounts of IL5³³ and these pathogenic IL5⁺IL17A⁺ producing T-cells remains to be determined using IL5 and IL17A reporter mice.

Th2 and Th17 responses have been presented as antagonistic,³⁴ notably when IL17A is produced by γδT-cells.³⁵ However, it has also been suggested that Th17 responses are needed for optimal Th2 responses.^{36,37} Indeed, co-exposure to both IL13 and IL17A has been demonstrated to exacerbate IL13-induced AHR by enhancing IL13/STAT6 signaling.³⁸ Taken together with our finding that IL17A contributes to DEP-induced exacerbation of allergic airway disease,³ we propose a model whereby IL17A not only exacerbates asthma by directly promoting smooth muscle contraction independent of IL13,³⁹ but also exacerbates Th2 signaling via promoting STAT6 activation within cells critically involved in asthma like epithelial cells and smooth muscle cells. The IL5/IL13/IL17A producing CD4⁺ T-cells that we have identified in HDM + DEP exposed mice, would be ideally suited to deliver these concomitant IL5/IL13 and IL17A signals that promote asthma exacerbation.

In conclusion, DEP-induced IL33 contributes to the observed adjuvant effect of DEP on allergen-induced experimental asthma severity, as assessed by AHR, by promoting a mixed type 2/ type 17 response. The relative resistance to steroid treatment of HDM + DEP induced IL33 likely contributes to the inability of dexamethasone treatment to abrogate allergic AHR in this model of DEP mediated severe asthma. While therapeutic treatment of HDM + DEP exposed C57Bl/6 mice with soluble ST2 failed to decrease inflammation,²² treatment of HDM + DEP exposed BALB/c mice with an IL17A neutralization antibody in combination with dexamethasone significantly decreased AHR compared to steroid treatment alone.⁴ Hence, targeting the IL33 pathway, which impacts both Th2 and Th17 responses, may still benefit poor steroid responders with severe asthma related to high levels of exposure to traffic related diesel pollution.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

EB, B.R and PB performed the experiments and contributed to the acquisition and analysis of the data. E.B and G.K-H. contributed to the conception, design and interpretation of the data and wrote the manuscript. All authors revised and approved the final version.

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SUPPORTING INFORMATION

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

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Figure S1

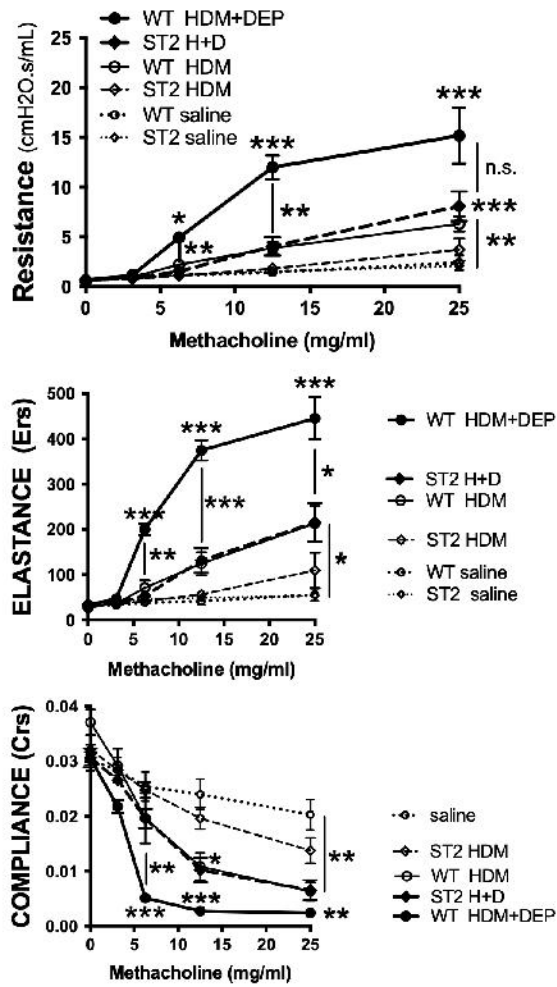


Figure S3

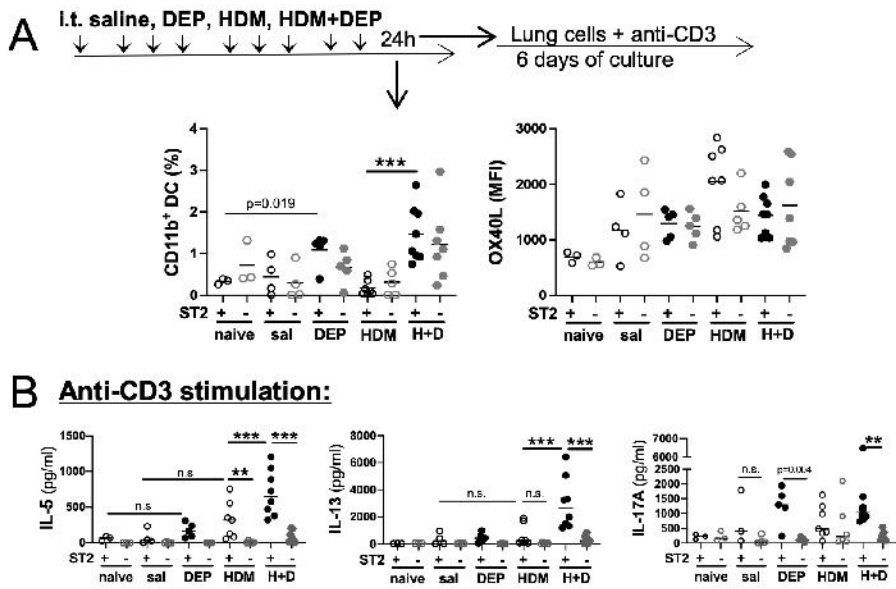


Figure S4

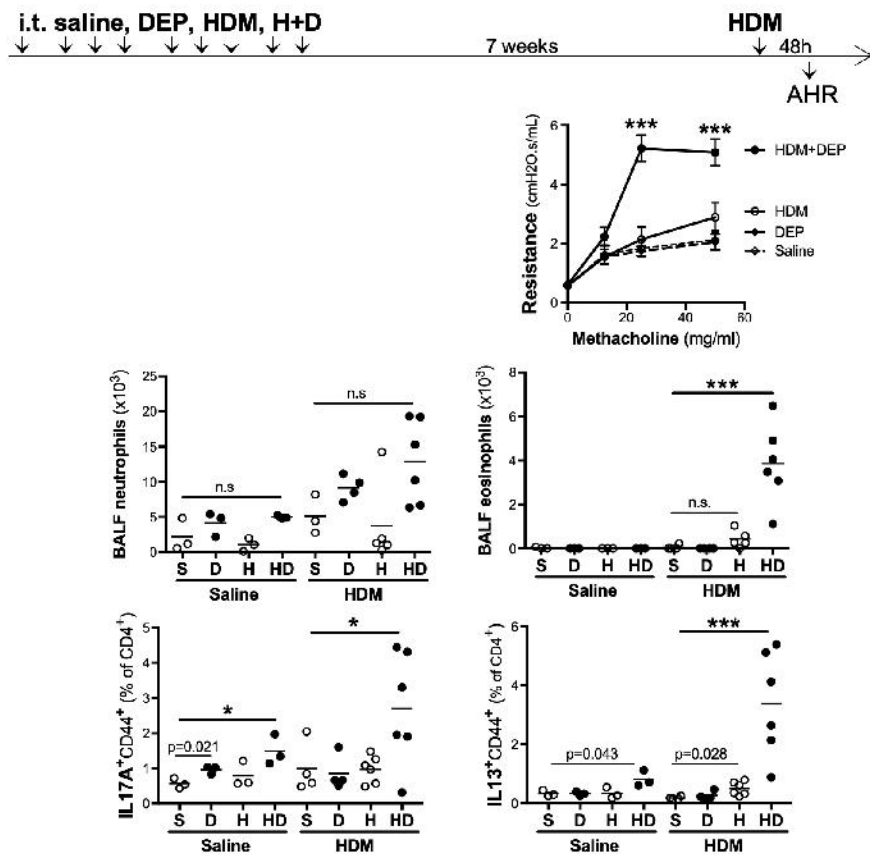


Figure S2

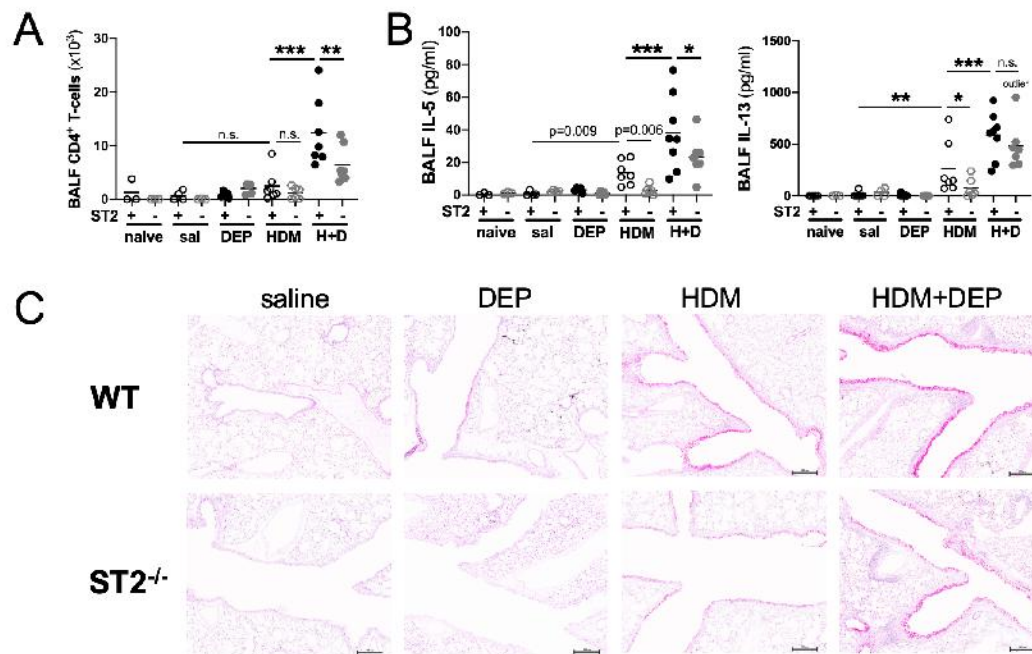
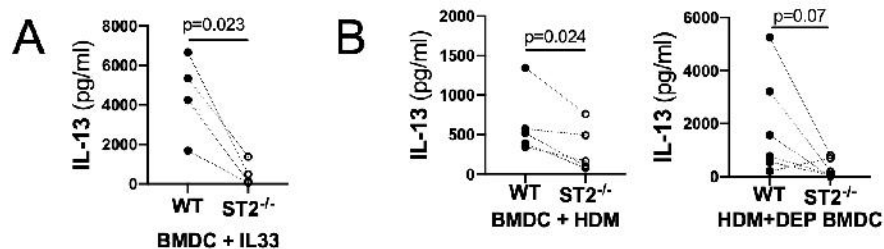


Figure S6

i.t. saline, DEP, HDM, HDM+DEP
 ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ 24h → CD4⁺ Tcells + stimulated WT or ST2^{-/-} DC
 7 days of culture



Supplementary Table 1: Antibody table used for flow cytometry

	clone	Vendor	REF#
Lineage: FITC anti-mouse CD3	17A2	BioLegend	100204
Lineage: FITC anti-mouse CD45R/B220	RA3-6B2	BioLegend	103206
Lineage: FITC anti-mouse/human CD11b	M1/70	BioLegend	101206
Lineage: FITC anti-mouse CD11c	N418	BioLegend	117306
Lineage: FITC anti-mouse Ly-6G/Ly-6C (Gr-1)	RB6-8C5	BioLegend	108406
Lineage: FITC anti-mouse FcεR1α	MAR1	BioLegend	134306
Lineage: FITC anti-mouse TER-119/Erythroid cells	TER-119	BioLegend	116206
FITC anti-mouse CD4	RM4-5	BioLegend	100510
PE anti-mouse/human IL-5	TRFK5	BioLegend	504304
PerCP-eFluor 710 anti-mouse IL13	eBio13A	eBioscience	46-7133-82
PerCP/Cy5.5 anti-mouse IFNγ	XMG1.2	BioLegend	505822
PE/Dazzle 594 anti-mouse CD4	RM4-5	BioLegend	100566
PE/Cy7 anti-mouse TCR γ/δ	GL3	BioLegend	118124
PE/Cy7 anti-mouse CD25	PC61	BioLegend	102016
Alexa Fluor 647 anti-mouse IL17A	TC11-18H10.1	BioLegend	506912
Alexa Fluor 700 anti-mouse CD3	17A2	BioLegend	100216
Alexa Fluor 700 anti-mouse CD45.2	104	BioLegend	109822
Pacific Blue anti-mouse CD90.2	30-H12	BioLegend	105324
Pacific Blue anti-mouse/human CD44	IM7	BioLegend	103020

Supplementary Table 2: Sequences of primers used for real time PCR

genes	Foward primer	bp	Reverse primer	bp
hprt	TGCCGAGGATTTGGA AAAAAG	20	CCCCCCTTGAGCACACAG	18
Hmox1	CCGAACATCGACAGCCCCACC	21	GGGGTTTCCCTCGGGGTGTCT	21
IL33	TCCCAACAGAAGACCAAAG	19	GATACTGCCAAGCAAGGAT	19
IL25	CCCAGCAAAGAGCAAGAACC	20	ACCCGATTCAAGTCCCTGTC	20
TSLP	TCAATCCTATCCCTGGCTG	19	GCATGAAGGAATACCACAATCTTA	24
IL13	GCTTGCCTTGGTGGTCTCGCC	21	GGGCTACACAGAACCCGCCA	20

All amplicons span at least one intron

Supplemental Figure legends:

Figure S1: AHR is ablated in ST2 deficient mice exposed to HDM and DEP. Airway resistance, elastance and compliance was measured a day after the last challenge (3-4 saline mice and 7 mice/group for HDM±DEP). Statistical significance was assessed by 2-way ANOVA followed by either Dunnett or Tuckey post hoc test for multiple comparisons (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ n.s.= not significant).

Figure S2: Th2 airway inflammation is decreased in ST2 deficient mice exposed to HDM and DEP. (A) BALF CD4⁺ T-cell counts were assessed by FAC and. (B) BALF IL5 and IL13 levels were assessed by ELISA (n=3-7 mice/group). Statistical significance was assessed by t-test (p=value) or 1-way ANOVA; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.= not significant). (C) Representative photomicrographs of PAS-stained large airway sections.

Figure S3: Lung dendritic cells are similar between WT and ST2 deficient mice. (A) Lung cells from Saline, DEP, HDM and HDM+DEP exposed mice were analyzed by FACS for CD11b⁺ MHCII⁺ CD11c⁺ dendritic cells. OX40 Ligand expression levels on CD11b⁺ DC was assessed by Mean Fluorescence Intensity (MFI). (B) IL5, IL13 and IL17A released by lung cells pre-exposed to either saline, HDM, or HDM+DEP was assessed by ELISA after 6 days of *in vitro* stimulation with 1µg/ml anti-CD3 (n=3-8 mice/group; significance was assessed by t-test (p=value) or 1-way ANOVA; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.= not significant).

Figure S4: Increased presence of HDM-specific memory cells 7 weeks after co-exposure to HDM+DEP promotes Th2/Th17 responses upon secondary HDM challenge. A single

i.t. challenge with either saline or 10µg of HDM was performed 7 weeks after the last exposure to either saline, DEP, HDM or HDM+DEP. AHR was assessed by FlexiVent 2 days later. Differential counts of BALF neutrophils and eosinophils levels reflect the frequency of lung CD4⁺ T-cells expressing IL17A and IL13 respectively (n=3-6 mice/group; significance was assessed by t-test (p=value) or 1-way ANOVA; *p<0.05, *** p<0.001, n.s.= not significant).

Figure S5: Minor impact of ST2 deficiency on HDM recall type 2 responses in mice pre-exposed to HDM+DEP. Following a single challenge with HDM (Figure 5B), IL5 producing lymphoid cells were identified by FACS following 4h of *ex vivo* PMA/ionomycin stimulation in the presence of Brefeldin A. (A) Representative FACS plots of IL5 producing none CD4⁺ T cells (mostly ILC2) and IL5⁺ CD4⁺ T-cells. (B) Frequencies of IL5 producing CD4⁺ T cells better reflect BALF IL5 levels (C) than IL5 producing innate cells (n=4-8 mice/group; 1-way ANOVA; *p<0.05,*** p<0.001, n.s.= not significant). (D) Representative photomicrographs of PAS-stained large airway sections.

Figure S6: IL33 signaling in bone marrow derived DC is contributing to HDM-induced IL13. BMDC from either wild type or ST2^{-/-} mice were exposed overnight to (A) IL33 or (B) HDM±DEP, washed and co-cultured for a week with CD4⁺ T-cell isolated from saline, DPE, HDM or HDM+DEP exposed mice. IL13 was measured in culture supernatants by ELISA and statistical significance determined by paired T-test.

Figure S5

