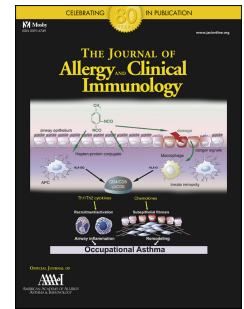


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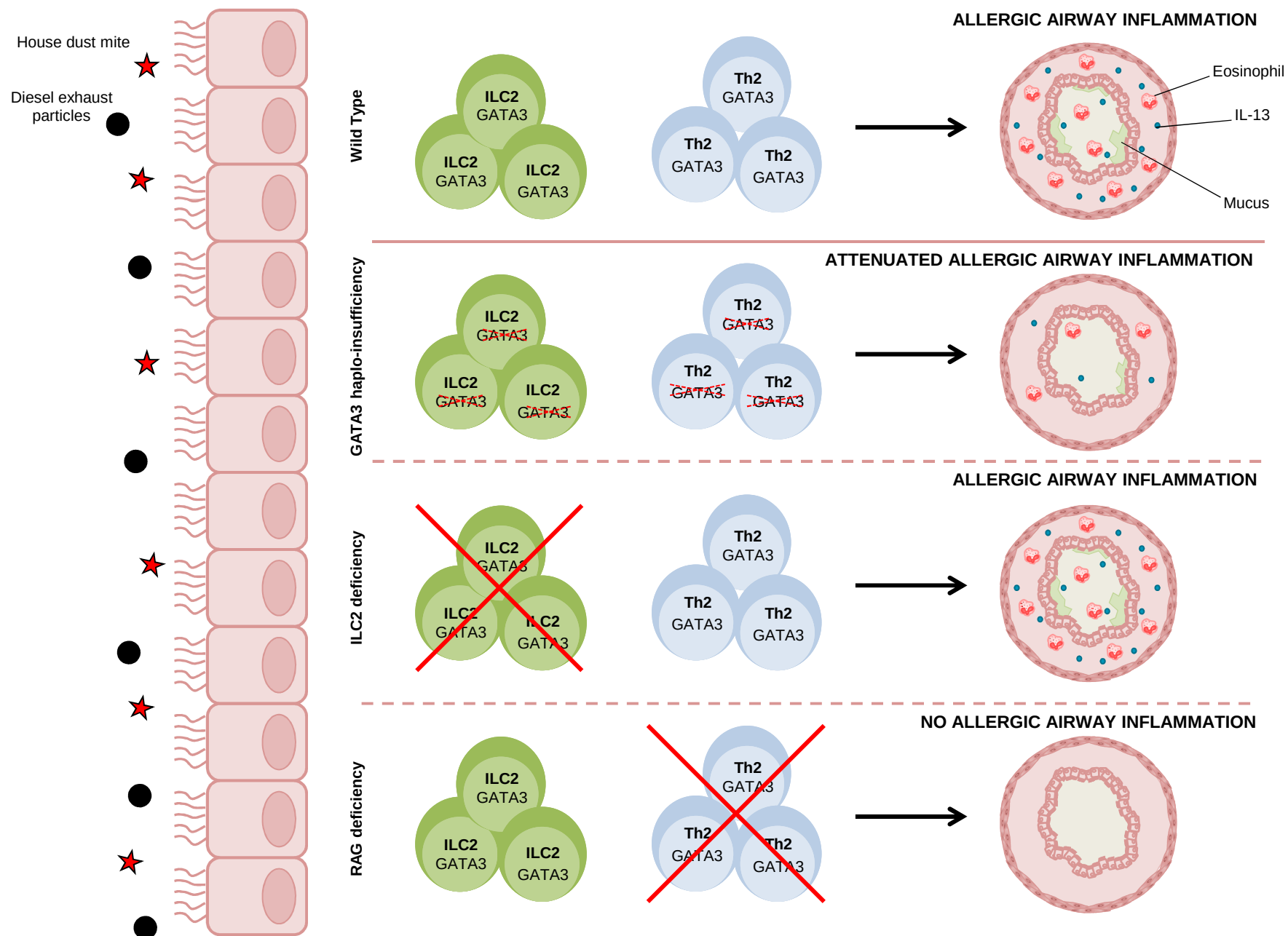
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Role of ILC2 and Th2 cells in pollutant-enhanced allergic airway inflammation



Dysregulation of type 2 innate lymphoid cells and Th2 cells
impairs pollutant-induced allergic airway responses

Katrien C. De Grove^{1*}, Sharen Provoost^{1*}, Rudi W. Hendriks², Andrew N. J. McKenzie³,
Leen J. M. Seys¹, Smitha Kumar¹, Tania Maes¹, Guy G. Brusselle^{1#}, Guy F. Joos^{1#}.

¹ Department of Respiratory Medicine, Laboratory for Translational Research in Obstructive
Pulmonary Diseases, Ghent University Hospital, Ghent, Belgium.

² Department of Pulmonary Medicine, Erasmus MC, Rotterdam, The Netherlands

³ MRC Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge, CB2 0QH, UK

* The authors equally contributed to this work. # The authors equally supervised this work.

Corresponding author:

Sharen Provoost

Department of Respiratory Medicine

Ghent University Hospital

Blok B, 4th floor

De Pintelaan 185

9000 Ghent

Belgium

sharen.provoost@UGent.be

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ABSTRACT

Background: Whereas the prominent role of T helper 2 (Th2) cells in type 2 immune responses is well established, the newly identified type 2 innate lymphoid cells (ILC2) can also contribute to the orchestration of allergic responses. Several experimental and epidemiological studies have provided evidence that allergen-induced airway responses can be further enhanced upon exposure to environmental pollutants, such as diesel exhaust particles (DEP). However, the components and pathways responsible remain incompletely known.

Objective: To investigate the relative contribution of ILC2 and adaptive Th2 cell responses in a murine model of DEP-enhanced allergic airway inflammation.

Methods: Wild-type (WT), $Gata3^{+/nls lacZ}$ (Gata-3 haploinsufficient), $ROR\alpha^{fl/fl}IL7R^{Cre}$ (ILC2-deficient) and $Rag2^{-/-}$ mice were challenged with saline, DEP or house dust mite (HDM), or combined DEP+HDM. Airway hyperresponsiveness as well as inflammation and intracellular cytokine expression in ILC2 and Th2 cells in the bronchoalveolar lavage fluid and lung tissue were assessed.

Results: Concomitant DEP+HDM exposure significantly enhanced allergic airway inflammation, characterized by increased airway eosinophilia, goblet cell metaplasia, accumulation of ILC2 and Th2 cells, type 2 cytokine production and airway hyperresponsiveness, compared to sole DEP or HDM. Reduced Gata-3 expression decreased the number of functional ILC2 and Th2 cells in DEP+HDM exposed mice, resulting in an impaired DEP-enhanced allergic airway inflammation. Interestingly, whereas the DEP-enhanced allergic inflammation was marginally reduced in ILC2-deficient mice that received combined DEP+HDM, it was abolished in DEP+HDM-exposed $Rag2^{-/-}$ mice.

Conclusion: These data indicate that dysregulation of ILC2 and Th2 cells attenuates DEP-enhanced allergic airway inflammation. In addition, a crucial role for the adaptive immune system was shown upon concomitant DEP+HDM exposure.

KEY MESSAGES

- Dysregulation of type 2 innate lymphoid cells and T helper 2 cells by targeting Gata-3 is associated with an attenuation of diesel-induced allergic airway inflammation.
- The adaptive immune system has a crucial role in diesel-enhanced allergic airway inflammation.

KEY WORDS

Diesel exhaust particles – house dust mite – type 2 innate lymphoid cell (ILC2) – Th2 response - asthma

ABBREVIATIONS*Abbreviations used*

AHR: airway hyperresponsiveness

BALF: bronchoalveolar lavage fluid

DC: Dendritic cells

DEP: Diesel exhaust particles

Gata3: Trans-acting T-cell-specific transcription factor

HDM: House dust mite

IN: Intranasal

ILC2: Type 2 innate lymphoid cell

MLN: Mediastinal lymph nodes

ROR α : RAR-related orphan receptor alpha

TSLP: Thymic stromal lymphopoietin

WT: Wild type

INTRODUCTION

Asthma is a chronic disorder of the conducting airways that is associated with a reversible airway obstruction, chronic airway inflammation, airway remodeling, and airway hyperresponsiveness¹. It is a heterogeneous disease where multiple phenotypes can be distinguished based on clinical characteristics and inflammatory profile. Asthma that originates during childhood (early-onset asthma) mostly has an atopic component^{2, 3} and is typically considered as mainly a T helper 2 (Th2) driven disease⁴.

In addition to the adaptive immune system, the airway epithelium has gained great importance during the initiation and maintenance of the allergic and asthmatic cascade. In particular, it has been shown that upon allergen exposure, several epithelial cytokines – such as interleukin (IL)-25, IL-33 and thymic stromal lymphopoietin (TSLP) – are involved in the pathogenesis of asthma^{5, 6}. Moreover, several genes discovered in genome wide association studies (i.e. IL-33, IL-1RL1, TSLP) support a key role for these cytokines^{7, 8}. On the one hand, these epithelial-derived cytokines have the capability to activate the adaptive immune system by stimulating Th2 polarizing dendritic cells (DC)⁵. On the other hand, the recently identified type 2 innate lymphoid cells (ILC2) also become activated by these cytokines⁹⁻¹¹. Analogous with the Th2 cells, ILC2 require the transcription factor Gata-3 and are a potent source of the type 2 cytokines IL-5 and IL-13, which are able to induce lung eosinophilia and mucus hypersecretion¹¹⁻¹⁵. Studies in Rag^{-/-} mice have shown that these ILC2 are crucial players in allergic airway responses¹⁶. Even in the absence of the adaptive immune system, ILC2 were able to mediate eosinophilia, goblet cell metaplasia, type 2 cytokine production and airway hyperresponsiveness (AHR)¹⁷⁻¹⁹. In addition, mice that were ILC2-deficient due to the targeting of the transcription factor ROR α had decreased type 2 immune responses^{14, 20, 21}. Interestingly, it was reported that ILC2 and T cells interact with each other and that this crosstalk could contribute to the maintenance, proliferation and activation of both ILC2 and Th2 cells²¹⁻²³.

Besides allergen exposure, it has become well accepted that traffic-related particulate matter, such as diesel exhaust particles (DEP), also contributes to the development and exacerbation of asthma²⁴⁻²⁶. For instance, epidemiological studies reported a correlation between high DEP levels and the frequency of symptomatic episodes in allergic children²⁷. In addition, combined

allergen + DEP administration during controlled human exposure studies resulted in increased allergen-specific immunoglobulins and type 2 cytokine responses²⁸. Furthermore, concomitant DEP + house dust mite (HDM) exposure in murine models enhanced eosinophilia, immunoglobulin production, AHR and remodeling²⁹. However, the mechanisms underlying the enhanced effects of DEP on allergen-induced airway inflammation remain largely unknown. Several studies suggested that the airway epithelium could be an important player since particulate matter was also able to stimulate the release of several epithelial-derived cytokines, such as TSLP and IL-33, which can lead to enhanced DC maturation and Th2 responses³⁰⁻³³. However, whether this also activates ILC2 is unknown.

In this paper, we investigate the relative contribution of ILC2 and the adaptive immune system in the enhancing effects of DEP on allergen-induced airway inflammation. We show in a murine model that concomitant exposure to a clinically relevant allergen (i.e. HDM) and DEP enhances several allergic airway responses, including airway eosinophilia, goblet cell metaplasia, increased ILC2 and Th2 cells, type 2 cytokine production and AHR. Since Gata-3 is an important transcription factor during the development and function of both ILC2 and Th2 cells³⁴, we used haploinsufficient $Gata3^{+/-nlslacZ}$ mice – which have a reduced expression of Gata-3³⁵ – to demonstrate that the enhancing effects of DEP on allergic airway inflammation depend on Gata-3 mediated regulation of ILC2 and Th2 cells. Moreover, to examine the specific contribution of ILC2 in the model of DEP-enhanced allergic airway inflammation, we used a conditionally targeted $ROR\alpha^{fl/fl}$ mouse that, when intercrossed with IL-7 receptor (IL7R)-Cre mice, yields an ILC2-deficient mouse strain in which other lineages are unaffected²¹. Finally, to investigate the functional role of the adaptive immune system in this model, we used $RAG2^{-/-}$ mice which lack mature T and B cells³⁶. We demonstrated that ILC2 marginally contributed to DEP-enhanced allergic airway responses, whereas the adaptive immune system appeared critical to orchestrate the enhanced effect of DEP on allergic airway inflammation and AHR.

MATERIAL & METHODS

Mice

Female C57BL/6 mice (6-8 weeks old) were obtained from the Jackson Laboratory. C57BL/6 $Gata3^{+/nlslacZ}$ and their WT littermates were a kind gift of Dr. R. Hendriks (Department of pulmonary medicine, Erasmus MC, Rotterdam, The Netherlands)³⁵ and bred in our animal facility of Ghent University. $ROR\alpha^{fl/fl}IL7R^{Cre}$ mice and $ROR\alpha^{fl/fl}IL7R^{+/+}$ controls were on a C57BL/6 background²¹. C57BL/6 $Rag2^{-/-}$ mice³⁶ and their wild-type (WT) controls were purchased from Taconic. All *in vivo* manipulations were approved by the Animal Ethical Committee of the Faculty of Medicine and Health Sciences of Ghent University.

Intranasal instillation of reagents

DEP (SRM 2975) was purchased from the National Institute for Standards and Technology (NIST). HDM (Dermatophagoides pteronyssinus) was obtained from Greer laboratories. Saline, 1µg HDM extract dissolved in saline, 25µg DEP suspended in saline or a combination of DEP+HDM were delivered intranasally to isoflurane anesthetized mice using a continuous flow vaporizer on day 1, 8 and 15. Two days after the last challenge, mice were sacrificed with a lethal dose intraperitoneal pentobarbital.

Bronchoalveolar lavage fluid (BALF)

A tracheal cannula was inserted and BALF was recuperated by instillation of 3 x 300µl 1% Hank's balanced salt solution (HBSS) supplemented with 1% bovine serum albumin (BSA) and 6 x 500µl HBSS supplemented with ethylenediaminetetraacetic acid (EDTA). The lavage fractions were pooled and total cell counts were measured using a Bürker chamber. Differential cell counts were performed on cytopsin after a May-Grünwald-Giemsa staining. The remaining cells were used for flow cytometry.

Lung and mediastinal lymph node single cell suspensions

The pulmonary circulation was rinsed with saline supplemented with EDTA to remove the intravascular pool of cells. Lungs and mediastinal lymph nodes (MLN) were minced and incubated for 45 minutes in digestion medium (roswell park memorial institute medium (RPMI)-1640 supplemented with 5% foetal calf serum (FCS), 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.

Mediastinal lymph node cell culture

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

Flow cytometry

BALF cells and single lung suspensions were stained with a combination of anti-mouse fluorochrome-conjugated monoclonal antibodies against: CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), CD69 (H1.2F3), Ly6C (AL-21), Ly6G (1A8), MHCII (2G9), Siglec-F (E50-2440) (all BD Biosciences); CD3 (145-2C11), CD90.2 (30.H12) (all Biolegend); CD5 (53-7.3), CD11c (N418), CD25 (PC61.5), CD127 (A7R34), CD45R (RA3-6B2), NK1.1 (PK136), TCRβ (H57-597) (all eBioscience). For cytoplasmatic cytokine staining, cells were stimulated for 4 hours with ionomycin and phorbol 12-myristate 13-acetate (PMA), supplemented with brefeldin A and monensin at 37°C for 4h. The intracellular fixation and permeabilization buffer set (eBioscience) was used for fixation and cell permeabilization. The following antibodies were used: PE-conjugated anti-IL-5 (TRFK5), anti-IL-13 (eBio13A) and isotype-matched controls (eBioscience). Data acquisition was performed on a FACSCalibur flow cytometer running CellQuest software or a LSR II cytometer running DiVa software. 250.000 events were collected. Cell subsets were analyzed using FlowJo software. Representative flow cytometric density plots and gating strategy of all analyzed cell populations in BALF and lung tissue are shown in Fig. S1 and S2 respectively.

Histology

The left lung was fixated with 4% paraformaldehyde (PFA) and embedded in paraffin. 3µm transversal sections were stained with a Congo Red staining to identify eosinophils or with a Periodic Acid-Schiff (PAS) staining to analyze goblet cells. Quantitative measurements were performed using a Zeiss KS400 Image analyzer platform.

Protein measurements

IL-4, IL-5 and IL-13 levels in BALF or MLN supernatants were measured by commercially available ELISA kits (R&D systems). In lung homogenates, IL-25 and IL-33 were measured with ELISA (R&D systems) following the manufacturer's instructions. HDM-specific IgG1 was determined on serum collected by retro-orbital bleeding. For detection, plates were coated with HDM extract. Serum was added, followed by an HRP-conjugated polyclonal goat anti-mouse IgG1 antibody (Bethyl Laboratories). The plate was read at 490nm. HDM-IgG1 levels were reported in optical densities (OD). All samples were on the same plate and experimental data from different plates are not co-presented.

Airway hyperresponsiveness (AHR)

Airway hyperresponsiveness in response to increasing doses carbachol (0, 20, 40, 80, 160, 320, 640 µg/kg) was measured 48 hours after the last intranasal instillation using the forced oscillation technique (Flexivent System, SCIREQ, Montreal, QC, Canada). Neuromuscular blockade was induced by injecting 1mg/kg pancuronium bromide intravenously. A “snapshot perturbation” maneuver was imposed to measure the resistance (R) of the whole respiratory system (airways, lung and chest wall).

Statistical analysis

Statistical analysis was performed with SPSS, version 22.0. Non parametric tests (Kruskal-Wallis and Mann-Whitney-U) were used to compare different groups, according to the

218 standard statistical criteria. Values were reported as mean $\pm$ SEM. P-values < 0.05 were
219 considered as significant.

220

RESULTS

Exposure to diesel exhaust particles enhances house dust mite-induced airway inflammation

C57BL/6 mice were exposed to saline, DEP or HDM alone, or combined DEP+HDM (Fig. 1A). In order to have a model wherein we could optimally examine the potential adjuvant capacities of DEP on HDM-induced airway inflammation, mice were exposed to doses of DEP and HDM that elicited almost no inflammatory response on their own (dose titrations for HDM are shown in Fig. S3, BALF inflammation to low versus high doses of DEP was previously shown³²). Exposure to DEP alone slightly increased the amount of DC, neutrophils and CD4⁺ T cells in the BALF, in comparison with saline-exposed mice (Fig. 1D, E and G). Administration of HDM induced a modest increase of DC, CD4⁺ T cells, CD8⁺ T cells and eosinophils in the BALF when compared with the saline group (Fig. 1D, G, H and I). In contrast, combined exposure to DEP+HDM greatly enhanced the allergic airway immune responses. Concomitant exposure to DEP+HDM elicited a marked increase in the epithelial-derived cytokines IL-25 and IL-33 (Fig. 1B, C). Moreover, the numbers of DC, neutrophils, ILC2, CD4⁺ T cells, CD8⁺ T cells and eosinophils were significantly increased in the BALF of WT mice that received combined DEP+HDM compared with the three control groups (Fig. 1D-I). Of note, all ILC2 expressed ST2 (data not shown), resembling natural ILC2³⁷. Co-exposure of DEP+HDM was also associated with a significant enhancement of inflammatory cells in the lung tissue (Fig. S4). Furthermore, histological examination revealed increased peribronchovascular eosinophilic inflammation and goblet cell metaplasia upon simultaneous DEP+HDM exposure (Fig. 1J, K).

Combined exposure to DEP+HDM increases type 2 cytokine production, HDM-specific IgG1 and induces airway hyperresponsiveness

Typical type 2 cytokines were evaluated in the BALF and supernatant of HDM-restimulated MLN that were obtained from WT mice exposed to saline, DEP or HDM alone, or combined DEP+HDM. In the BALF of mice exposed to DEP+HDM, elevated IL-5 and IL-13 levels were found compared to the three control groups, whereas HDM or DEP alone elicited no response (Fig. 2A, B). In the supernatant of HDM-restimulated MLN, concomitant DEP+HDM exposed mice had marked higher levels of IL-4, IL-5 and IL-13, compared to the control groups (Fig. 2C-E). In contrast, exposure to DEP alone was associated with a modest increase in IL-4 and IL-13 in comparison with the saline group (Fig. 2C, E). Sole HDM

administration slightly increased IL-5 and IL-13 levels in the MLN compared with the saline-exposed group (Fig. 2D, E). Furthermore, combined DEP+HDM exposed mice had significantly elevated HDM-specific IgG1 titers in serum, when compared to saline-, DEP- and HDM-exposed control groups (Fig. 2F). Additionally, mice that were concomitantly exposed to DEP+HDM showed AHR in comparison with the three control groups (Fig. 2G).

Reduced Gata-3 expression impairs airway eosinophilia and mucus metaplasia upon combined DEP+HDM exposure

Gata-3 is an important transcription factor for the development of ILC2 and Th2 cells³⁴. Since the Gata-3 gene copy number is positively correlated with both ILC2 and Th2 function³⁸⁻⁴⁰, we evaluated the effect of reduced Gata3 expression in our model of DEP-enhanced allergic airway inflammation. Gata3^{+/-nls lacZ} mice, in which one allele is disrupted by insertion of a beta-galactosidase reporter³⁵, and WT littermates were exposed to saline, DEP or HDM alone, or the combination of DEP+HDM. Concomitant DEP+HDM exposure in Gata3^{+/-nls lacZ} mice resulted in DC, neutrophil, CD4⁺ T cell and CD8⁺ T cell numbers that were comparable to those in WT littermates, whereas ILC2 and eosinophil numbers in the BALF were significantly reduced (Fig. 3A-F). Moreover, histological analyses in DEP+HDM exposed Gata3^{+/-nls lacZ} mice revealed a diminished peribronchial eosinophilic inflammation and goblet cell metaplasia compared to their littermate controls (Fig. 3G, H). Also upon sole HDM exposure, a reduced eosinophilia (Fig. 3G) and goblet cell metaplasia (Fig. 3H) was observed in the Gata3^{+/-nls lacZ} mice in comparison with littermates. Comparable HDM-specific IgG1 levels in the serum were found in WT and Gata3^{+/-nls lacZ} mice that were exposed to DEP+HDM (Fig. 3I). Moreover, concomitant exposure to DEP+HDM led to a similar increase in airway responsiveness in Gata3^{+/-nls lacZ} mice and WT littermates, when compared with their HDM control groups (Fig. 3J).

Reduced Gata-3 expression decreases type 2 cytokine production by ILC2 and CD4⁺ T cells upon combined DEP+HDM exposure

We further assessed the type 2 cytokine production in Gata3^{+/-nls lacZ} mice and WT littermates in response to combined DEP+HDM. Exposure to DEP+HDM led to reduced IL-13 levels in BALF of Gata3^{+/-nls lacZ} mice, compared to littermates (Fig. 4A). In addition, intracellular type 2 cytokine production in both ILC2 and CD4⁺ T cells was investigated in BALF and lung tissue. The increased numbers of IL-13 positive ILC2 and CD4⁺ T cells that were observed in BALF of DEP+HDM WT mice were significantly decreased in DEP+HDM exposed

Gata3^{+/-nlslacZ} mice (Fig. 4B, C). Moreover, IL-5 and IL-13 expressing ILC2 were significantly diminished in lung single cell suspensions of Gata3^{+/-nlslacZ} mice independent of the exposure, compared to littermates (Fig. 4D, E). The increased percentages of IL-5 and IL-13 positive CD4⁺ T cells that were observed in the co-exposed DEP+HDM Gata3^{+/-nlslacZ} mice did not significantly differ from their WT controls. In contrast, Gata3^{+/-nlslacZ} mice exposed to sole HDM had significant lower IL-5 and IL-13 positive CD4⁺ T cells in comparison with the littermates (Fig. 4F, G).

ILC2 marginally contribute to DEP-enhanced allergic airway inflammation

Since Gata-3 haploinsufficiency affects both the innate and adaptive component, we next examined the specific contribution of ILC2 in the enhancing effects of DEP on allergic airway inflammation. For that, we exposed RORα^{fl/fl}IL7R^{Cre} (ILC2-deficient) mice²¹ and their corresponding RORα^{fl/fl}IL7R^{+/+} controls to HDM and DEP+HDM. As expected, ILC2 numbers in the BALF were abolished in RORα^{fl/fl}IL7R^{Cre} mice, independent of the exposure (Fig. 5A). Concomitant DEP+HDM exposed RORα^{fl/fl}IL7R^{Cre} mice had significant reduced DC in the BALF (Fig. 5B), whereas the BALF neutrophils, CD4⁺ T cells, CD8⁺ T cells and eosinophils tended to decrease compared to RORα^{fl/fl}IL7R^{+/+} controls (Fig. 5C-F). Histological analyses further revealed a similar peribronchial eosinophilia and goblet cell metaplasia between DEP+HDM exposed RORα^{fl/fl}IL7R^{Cre} mice and controls (Fig. 5G-H). Moreover, the increased BALF IL-5 (data not shown) and IL-13 levels found upon DEP+HDM exposure in the BALF and supernatants of restimulated MLN did not differ between RORα^{fl/fl}IL7R^{Cre} mice and RORα^{fl/fl}IL7R^{+/+} controls (Fig. 5I-J). Levels of HDM-specific IgG1 were also comparable between DEP+HDM exposed RORα^{fl/fl}IL7R^{Cre} mice and their controls (Fig. 5K).

The adaptive immune system has a crucial role in DEP-enhanced allergic airway inflammation

To investigate how the adaptive immune system contributes to the DEP-enhanced allergic airway inflammation, we exposed WT and Rag2^{-/-} mice – which lack an adaptive immune system³⁶ – to saline, DEP or HDM alone, or combined DEP+HDM. As expected, Rag2^{-/-} mice had no mature CD4⁺ T cells in the lung, whereas the proportion of lung ILC2 was increased, independent of the exposure (Fig. 6A, B). Rag2^{-/-} mice and WT controls that were co-exposed to DEP+HDM had similar ratios of DC and neutrophils in the BALF (Fig. 6C, D). However, combined DEP+HDM exposure resulted in a complete abolishment of BALF eosinophils

323 compared to the WT controls (Fig. 6E). Histological analyses further revealed a severely
324 reduced eosinophilia and abolished goblet cell metaplasia in the Rag2^{-/-} mice that received
325 combined DEP+HDM in comparison with WT controls (Fig. 6F, G). Additionally, combined
326 DEP+HDM exposure failed to increase BALF IL-5 (data not shown) and IL-13 levels in
327 BALF and supernatants of restimulated MLN in Rag2^{-/-} mice compared with their WT
328 controls (Fig. 6H, I). The modest inflammation that was seen in the WT mice upon sole HDM
329 exposure was also completely abolished in the Rag2^{-/-} mice. Independent of the exposure,
330 there was no HDM-specific IgG1 production in Rag2^{-/-} mice, compared to WT controls (data
331 not shown). Furthermore, RAG2^{-/-} mice had no increase in airway responsiveness in response
332 to DEP + HDM (Fig. 6J).

DISCUSSION

In this paper, we demonstrated that dysregulation in ILC2 and Th2 cell numbers and function by targeting Gata-3 was associated with an attenuated airway inflammation upon concomitant DEP+HDM exposure. Moreover, we showed a critical role of the adaptive immune system to DEP-enhanced allergic responses and AHR, whereas ILC2 only marginally contribute to DEP-enhanced allergic airway inflammation.

The immunological mechanisms by which DEP can promote allergen-induced airway inflammation are largely unknown. To unravel these cellular and molecular mechanisms, we set up a mouse model with concomitant exposure to a clinical relevant allergen (i.e. HDM) and DEP. To have an optimal model wherein the potential adjuvant effects of DEP on HDM-induced allergic airway inflammation can be evaluated, we downtitrated the dose of DEP and HDM until they elicited minimal inflammatory responses on their own. We demonstrated that concomitant exposure to DEP+HDM markedly enhanced multiple features of allergic inflammation, characterized by an eosinophilic response, goblet cell metaplasia, ILC2 and Th2 cell accumulation, type 2 cytokine production, elevated HDM-specific IgG1 levels and AHR. In accordance with our findings, several experimental and epidemiological studies already provided evidence regarding the synergistic ability of DEP on allergic airway inflammation^{25, 27-29}. Although these studies already demonstrated the enhanced effects on airway remodeling, AHR, eosinophilic inflammation and immunoglobulin production, insights concerning the number and function of the recently identified ILC2s in response to concomitant exposure to DEP+HDM were lacking.

Epithelial cells are the first barrier to encounter several inhaled allergens and particles. In response to these particulates, the epithelium can release cytokines and chemokines to direct the recruitment and activation of several innate and adaptive immune cells^{5, 6}. Upon HDM for instance, it was shown that IL-25 and IL-33 levels were upregulated, contributing to the observed airway and lung inflammation^{41, 42}. Although we observed no increased IL-25 and IL-33 response upon sole DEP or HDM, combined DEP+HDM exposure elicited an increase of IL-25 and IL-33 in particular, suggesting that DEP works synergistically with HDM to induce release of epithelial cytokines in the environment.

These epithelial-derived cytokines share the capacity to stimulate Th2 development by polarizing DC on the one hand⁵ and to activate ILC2 on the other hand^{9, 10}. Whereas the role of Th2 cells in the pathogenesis of asthma is well established⁴³, it was recently shown that ILC2 can also substantially contribute to allergic airway inflammation¹⁶. Moreover, ILC2 can mediate AHR, independent of the adaptive immune system^{18, 19}. Importantly, we found that exposure to DEP+HDM increased the number of cytokine expressing ILC2 and Th2 cells in the alveolar space, suggesting that type 2 cytokine production of both cell types contributes to the adjuvant effects of DEP on allergic airway inflammation. Although the numbers of type 2 expressing ILC2 in the lung did not increase upon combined DEP+HDM exposure, ILC2 could still be critical in initiating and maintaining the DEP-enhanced type 2 immune responses. At least in response to the pollutant ozone, it was suggested that the increased activation of ILC2 was associated with an enhanced eosinophilic inflammation towards *Aspergillus fumigates*⁴⁴.

Since Gata-3 is an important transcription factor during the development and function of ILC2 as well as Th2 cells³⁴, we investigated the effects of Gata-3 modulation in our model of DEP-enhanced allergic airway inflammation. At baseline, Gata3^{+/-nlslacZ} mice had lower functional ILC2 in the lung, while effects on Th2 cells were limited. In response to DEP+HDM, both IL-13 expressing ILC2 and Th2 cells were attenuated in BALF of Gata3^{+/-nlslacZ} mice, suggesting that the observed reduction in type 2 airway inflammation could be the result of diminished functional ILC2 and Th2 cells in the bronchoalveolar space. In addition, considering that co-exposure of DEP+HDM only tended to decrease the type 2 cytokine expressing Th2 cells in the lung whereas the ILC2 were abolished, the imbalance of functional ILC2 and Th2 cells in the lung could contribute to the reduced eosinophilic inflammation. Importantly, the reduction of type 2 expressing ILC2 and Th2 cells in Gata3^{+/-nlslacZ} mice had no effect on the development of AHR in response to DEP+HDM. Interestingly, the modest inflammation that was observed in response to only HDM was also greatly reduced in the Gata3^{+/-nlslacZ} mice. In line, exposure of Gata-3 mutant mice to ovalbumin inhibited the allergic airway inflammation⁴⁵. Moreover, therapeutic targeting of Gata-3 in a clinical trial involving allergic asthmatics also attenuated both late and early allergen-induced asthmatic responses⁴⁶.

To further assess the relative contribution of the innate (i.e. ILC2) and adaptive (i.e. Th2 cells) arm in our model of DEP-enhanced allergic airway inflammation, we used ROR α ^{fl/fl}IL7R^{Cre}

(ILC2-deficient) mice and Rag2^{-/-} mice respectively. Intriguingly, we found that the DEP-enhanced allergic airway inflammation only tended to decrease in RORα^{fl/fl}IL7R^{Cre} mice, whereas typical type 2 immune responses such as eosinophilia, mucus metaplasia and type 2 cytokine production were completely abolished in Rag2^{-/-} mice that received combined DEP+HDM. In addition, Rag2^{-/-} mice exposed to DEP+HDM failed to develop AHR. This suggests that the presence of an adaptive immune system or at least an adequate interaction of adaptive immune cells with ILC2 is required in mediating the adjuvant capacity of DEP on HDM-enhanced allergic airway inflammation and AHR. Of note, depletion of CD4⁺ or CD8⁺ T cells in a murine model with intraperitoneal exposure to DEP+ovalbumin was associated with abrogated cytokine production and ovalbumin-specific immunoglobulin responses in the peritoneal exudate fluid⁴⁷. Interestingly, whereas it was previously shown that ILC2 were critical in T-cell independent allergic lung inflammation¹⁷, the modest inflammation in response to HDM in our model was completely abolished in the Rag2^{-/-} mice. The amount of administered HDM could therefore be important, suggesting that doses of HDM that elicit limited biological inflammation on their own are unable to activate ILC2 and to drive the allergic airway inflammation in absence of an adaptive immune system. Furthermore, it could be that ILC2 are of less importance in subchronic responses than during the acute phases of an inflammatory response. This was supported by research performed in a papain model, where the secondary responses were more likely Th2 dependent¹⁴. Moreover, it was suggested that the communication between ILC2 and Th2 cells by a MHCII-mediated dialog or specific cytokine secretion could be crucial to substantiate their effects on allergic airway inflammation²¹⁻²³. It seemed however that upon concomitant DEP+HDM exposure activation of Th2 cells appeared relatively independent of ILC2, opposed to previous reports where ILC2 were crucial for the initiation of Th2 responses towards (relatively high) doses of HDM and papain^{14, 20}.

Taken together, although a significant role for ILC2s has been demonstrated in several models of allergen-induced inflammation, our findings suggest that co-exposure to multiple environmental factors, such as particulate pollutants and allergens (i.e. HDM), modulate the contribution of ILC2 and Th2 cells to allergic airway inflammation and AHR.

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FIGURE LEGENDS

Figure 1: Exposure to diesel exhaust particles enhances house dust mite-induced airway inflammation. WT mice were exposed to saline (white bar), 25µg DEP (striped bar), 1µg HDM (checked bar) or DEP+HDM (black bar) on day 1, 8 and 15. **A**, Schematic overview of our model of DEP-enhanced HDM-induced airway inflammation. **B-C**, IL-25 (**B**) and IL-33 (**C**) protein levels in lung were determined by ELISA. **D-I**, DC (CD11c^{high}, low autofluorescent and MHCII⁺) (**D**), neutrophils (**E**), ILC2 (Lin⁻ (CD3⁻, CD5⁻, NK1.1⁻, TCRβ⁻, CD11c⁻, CD11b⁻ and CD45R⁻) CD25⁺ CD90⁺) (**F**), CD4⁺ T cells (CD3⁺ CD4⁺ CD8⁻) (**G**), CD8⁺ T cells (CD3⁺ CD8⁺ CD4⁻) (**H**) and eosinophils (**I**) in BALF were determined by flow cytometry except neutrophils and eosinophils that were determined on cytopsin. **J-K**, Representative photomicrographs and quantification of peribronchovascular eosinophils (**J**) and PAS-stained lung specimen (**K**). Results are expressed as mean ± SEM. n = 7-8 mice per group. * p < 0.05. Data are representative of three independent experiments. Representative flow cytometric density plots and gating strategy are shown in Fig. S1.

Figure 2: Combined exposure to DEP+HDM increases type 2 cytokine production, HDM-specific IgG1 and induces airway hyperresponsiveness. WT mice were exposed to saline (white bar), 25µg DEP (striped bar), 1µg HDM (checked bar) or DEP+HDM (black bar) on day 1, 8 and 15. **A-B**, IL-5 (**A**) and IL-13 (**B**) protein levels in BALF were determined by ELISA. **C-E**, IL-4 (**C**), IL-5 (**D**), IL-13 (**E**) protein levels in the supernatants of HDM-restimulated MLN were determined by ELISA. **F**, HDM-specific IgG1 titers in serum were determined by ELISA. **G**, Airway resistance (R) of saline (black line), DEP (blue line), HDM (green line) and DEP+HDM (red line) exposed mice was measured in response to increasing doses of carbachol. Results are expressed as mean ± SEM. n = 7-8 mice per group. * p < 0.05. Data are representative of two independent experiments.

Figure 3: Reduced Gata-3 expression impairs airway eosinophilia and mucus metaplasia upon combined DEP+HDM exposure. WT and Gata3^{+/-nlsIacZ} mice were exposed to saline (white bar), 25µg DEP (striped bar), 1µg HDM (checked bar) or DEP+HDM (black bar). **A-F**, DC (CD11c^{high}, low autofluorescent and MHCII⁺) (**A**), neutrophils (**B**), ILC2 (Lin⁻ (CD3⁻, CD5⁻, NK1.1⁻, TCRβ⁻, CD11c⁻, CD11b⁻ and CD45R⁻) CD25⁺ CD90⁺) (**C**), CD4⁺ T cells (CD3⁺ CD4⁺ CD8⁻) (**D**), CD8⁺ T cells (CD3⁺ CD8⁺ CD4⁻) (**E**) and eosinophils (**F**) in BALF

were determined by flow cytometry except neutrophils and eosinophils that were determined on cytospin. **G-H**, Representative photomicrographs and quantification of congo red stained lungs (**G**) or PAS-stained mucus producing goblet cells (**H**) of DEP+HDM exposed WT and $Gata3^{+/nls lacZ}$ mice. **I**, HDM-specific IgG1 levels in serum were determined by ELISA. **J**, Airway resistance (R) in WT (full line) and $Gata3^{+/nls lacZ}$ (broken line) mice was measured in response to increasing doses of carbachol. Results are expressed as mean $\pm$ SEM. n = 7-9 mice per group. * p < 0.05.

Figure 4: Reduced Gata-3 expression decreases cytokine production by ILC2 and CD4⁺ T cells upon combined DEP+HDM exposure. WT and $Gata3^{+/nls lacZ}$ mice were exposed to saline (white bar), 25 μ g DEP (striped bar), 1 μ g HDM (checked bar) or DEP+HDM (black bar). **A**, IL-13 protein levels in BALF were determined by ELISA. **B-G**, BALF or lung cells were stimulated for 4 hours with PMA/ionomycin, intracellular labeled for cytokine production and analyzed using flow cytometry. Percentage IL-13 expressing ILC2 (Lin⁻ (CD3⁻, CD5⁻, NK1.1⁻, TCR β ⁻, CD11c⁻, CD11b⁻ and CD45R⁻) CD25⁺ CD90⁺) (**B**) or CD4⁺ T cells (CD3⁺ CD4⁺) (**C**) in BALF. Proportion of IL-5 (**D**) and IL-13 (**E**) producing ILC2 (Lin⁻ (CD3⁻, CD5⁻, NK1.1⁻, TCR β ⁻, CD11c⁻, CD11b⁻ and CD45R⁻) CD25⁺ CD90⁺) in the total lung tissue. Percentages IL-5 (**F**) and IL-13 (**G**) positive T cells (CD3⁺ CD4⁺) of total lung. Results are expressed as mean $\pm$ SEM. n = 7-8 mice per group. * p < 0.05. Representative flow cytometric histograms and density plots are shown in Fig. S1 (BALF) and Fig. S2 (lung).

Figure 5: ILC2 marginally contribute to DEP-enhanced allergic airway inflammation. $ROR\alpha^{fl/fl}IL7R^{Cre}$ mice and $ROR\alpha^{fl/fl}IL7R^{+/+}$ WT controls were exposed to 1 μ g HDM (checked bar) or DEP+HDM (black bar). **A-F**, ILC2 (Lin⁻ (CD3⁻, CD5⁻, NK1.1⁻, TCR β ⁻, CD11c⁻, CD11b⁻ and CD45R⁻) CD25⁺ CD90⁺) (**A**), DC (CD11c^{high}, low autofluorescent and MHCII⁺) (**B**), neutrophils (**C**), CD4⁺ T cells (CD3⁺ CD4⁺ CD8⁻) (**D**), CD8⁺ T cells (CD3⁺ CD8⁺ CD4⁻) (**E**) and eosinophils (**F**) in BALF were determined by flow cytometry except neutrophils and eosinophils that were determined on cytospin. **G-H**, Representative photomicrographs and quantification of congo red stained lungs (**G**) or PAS-stained mucus producing goblet cells (**H**). **I-K**, BALF IL-13 protein levels (**I**), IL-13 levels in the supernatants of HDM-restimulated MLN (**J**) and HDM-specific IgG1 levels in serum (**K**) were determined by ELISA. Results are expressed as mean $\pm$ SEM. n = 8 mice per group. * p < 0.05.

Figure 6: The adaptive immune system has a crucial role in DEP-enhanced allergic airway inflammation. WT and Rag2^{-/-} mice were exposed to saline (white bar), 25µg DEP (striped bar), 1µg HDM (checked bar) or DEP+HDM (black bar). **A-E**, Percentage CD4⁺ T cells (CD3⁺ CD4⁺ CD8⁻ CD69⁺) of total lung (**A**), percentage lung ILC2 (Lin⁻ (CD3⁻, CD5⁻, NK1.1⁻, TCRβ⁻, CD11c⁻, CD11b⁻ and CD45R⁻) CD25⁺ CD127⁺) (**B**), number of BALF DC (CD11c^{high}, low autofluorescent and MHCII⁺) (**C**), number of BALF neutrophils (**D**) and number of BALF eosinophils (**E**) were determined by flow cytometry except neutrophils and eosinophils that were determined on cytospin. **F-G**, Representative photomicrographs and quantification of congo red stained lungs (**F**) or PAS-stained mucus producing goblet cells (**G**) of DEP+HDM exposed WT and Rag2^{-/-} mice. **H-I**, BALF IL-13 protein levels (**H**) and IL-13 levels in the supernatants of HDM-restimulated MLN (**I**) were determined by ELISA. **J**, airway resistance (R) in WT (full line) and Rag2^{-/-} (broken line) mice was measured in response to increasing doses of carbachol. Results are expressed as mean ± SEM. n = 7-8 mice per group. * p < 0.05.

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Figure 1

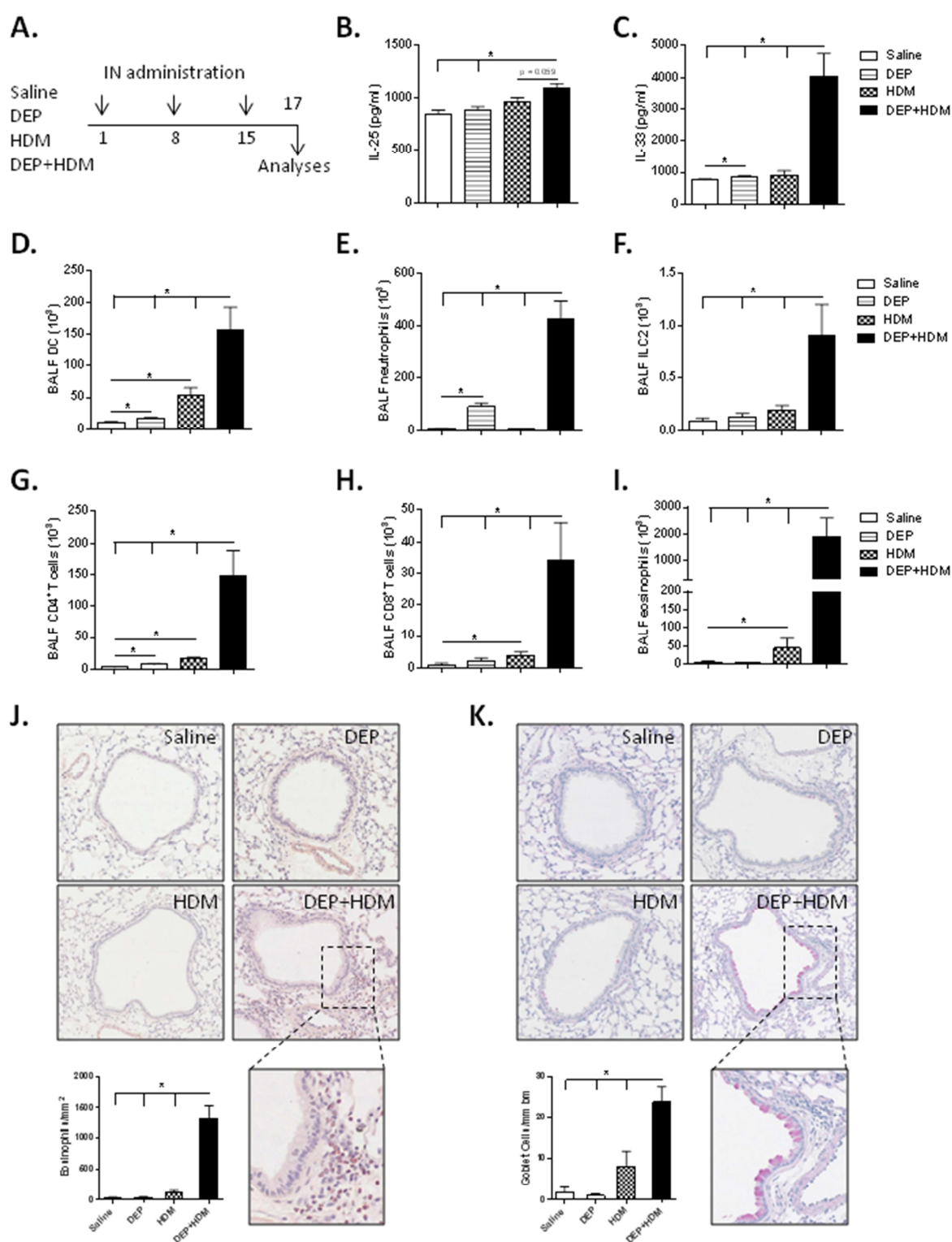


Figure 2

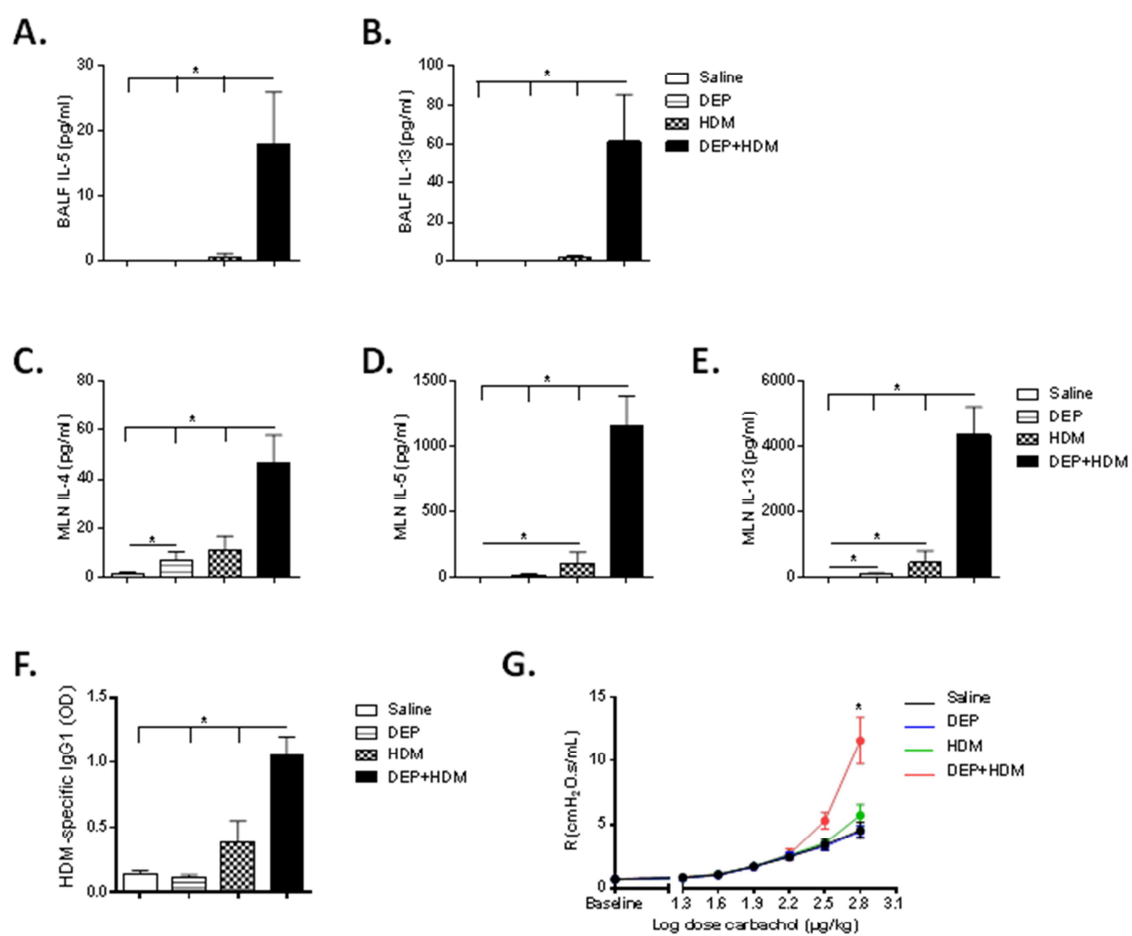


Figure 3

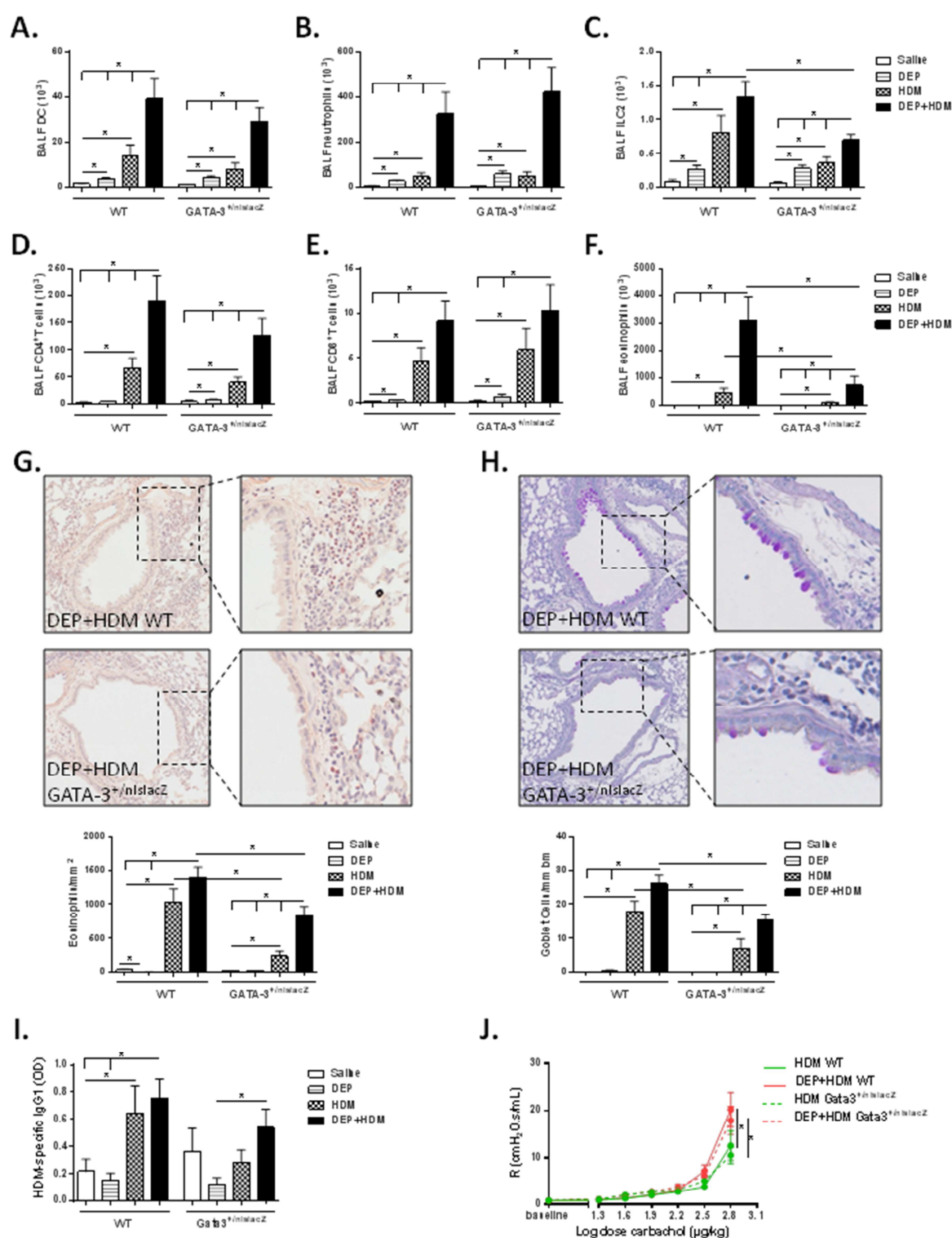


Figure 4

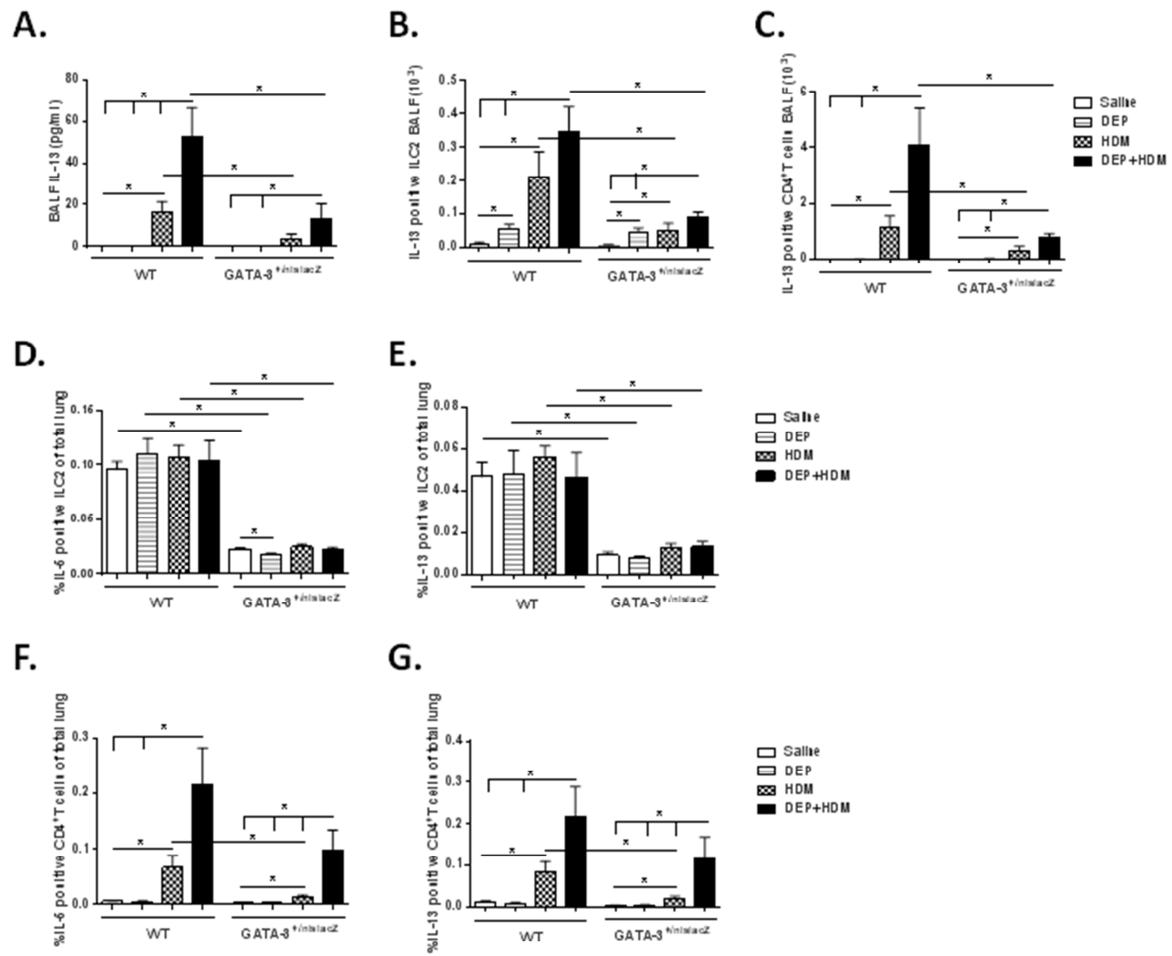


Figure 5

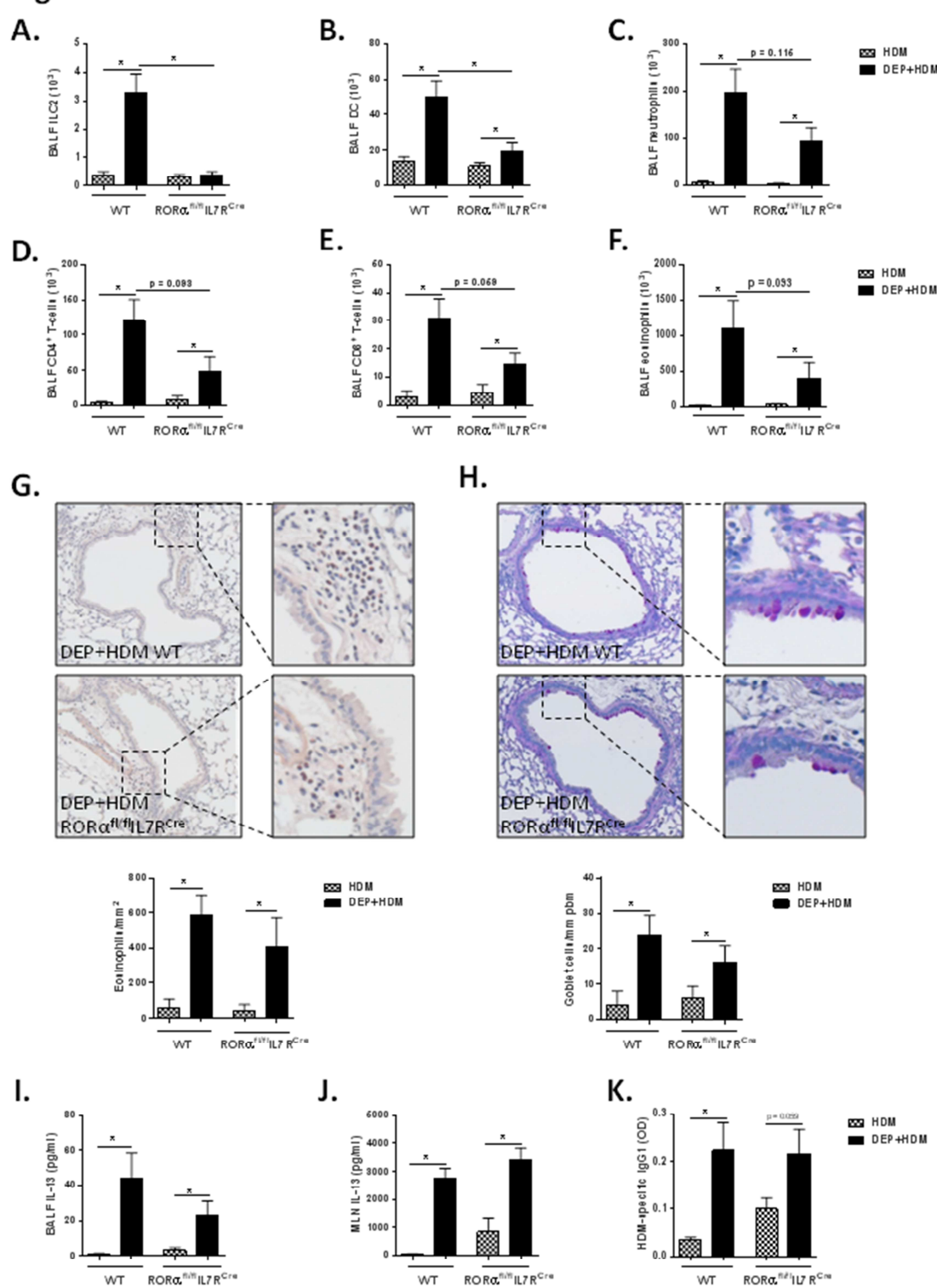
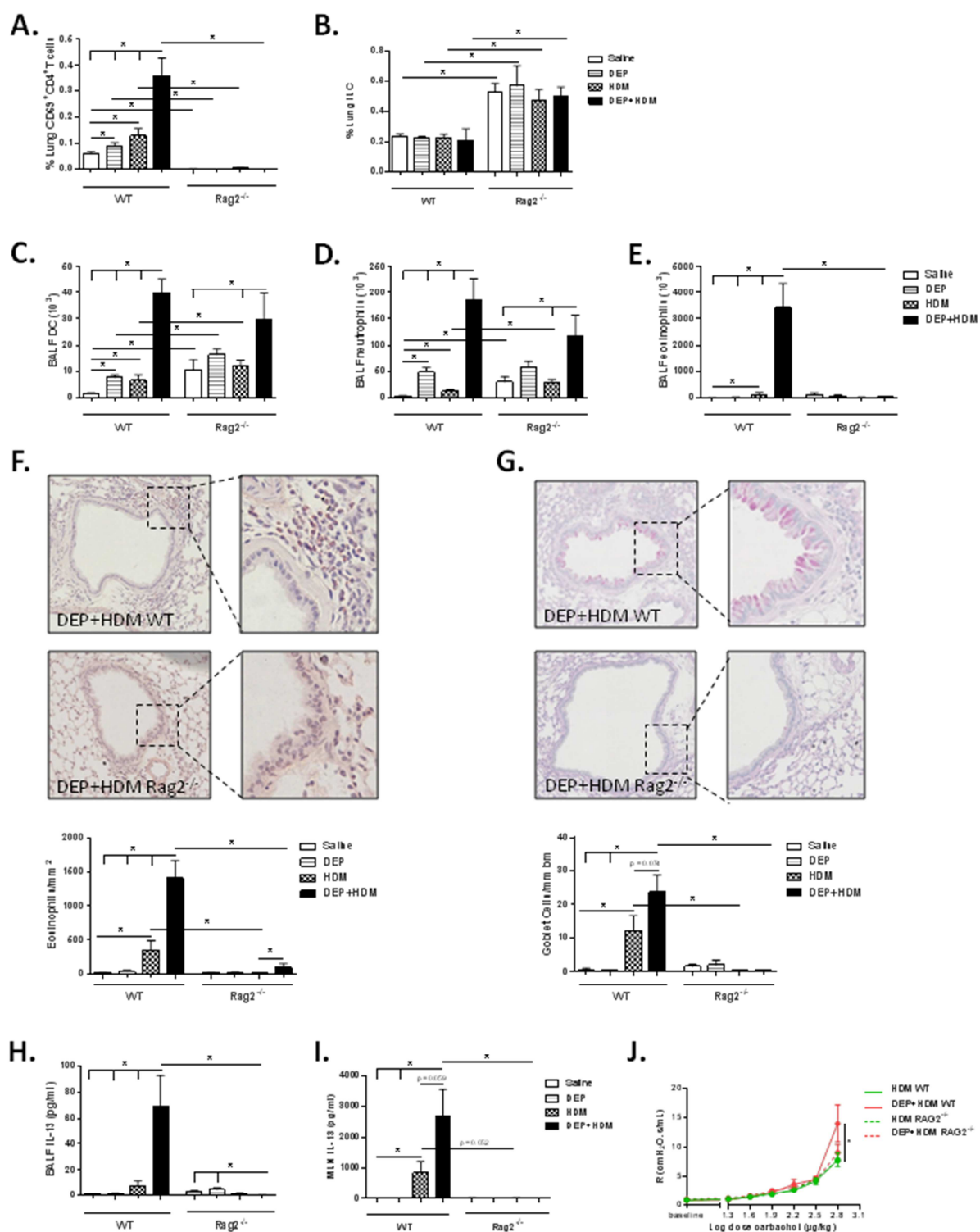


Figure 6



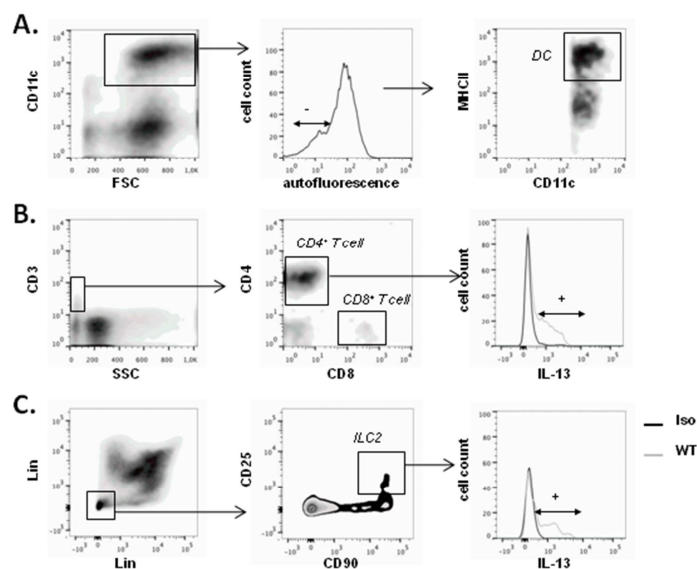


Figure S1: Representative density plots and gating strategy of all analyzed cell populations in BALF. WT mice were exposed to 25 μ g DEP + 1 μ g HDM. A, DC were gated as CD11c^{high}, low autofluorescent and MHCII⁺. B, CD4⁺ T cells were gated as CD3⁺, CD4⁺ and CD8⁺. CD8⁺ T cells were characterized as CD3⁺, CD4⁺ and CD8⁺. Intracellular IL-13 production of CD4⁺ T cells was investigated. C, ILC2 were identified as Lin⁺ (CD3⁺, CD5⁺, NK1.1⁺, TCR β ⁺, CD11c⁻, CD11b⁻ and CD45R⁻), CD25⁺ and CD90⁺ cells. Intracellular IL-13 production of ILC2 was investigated.

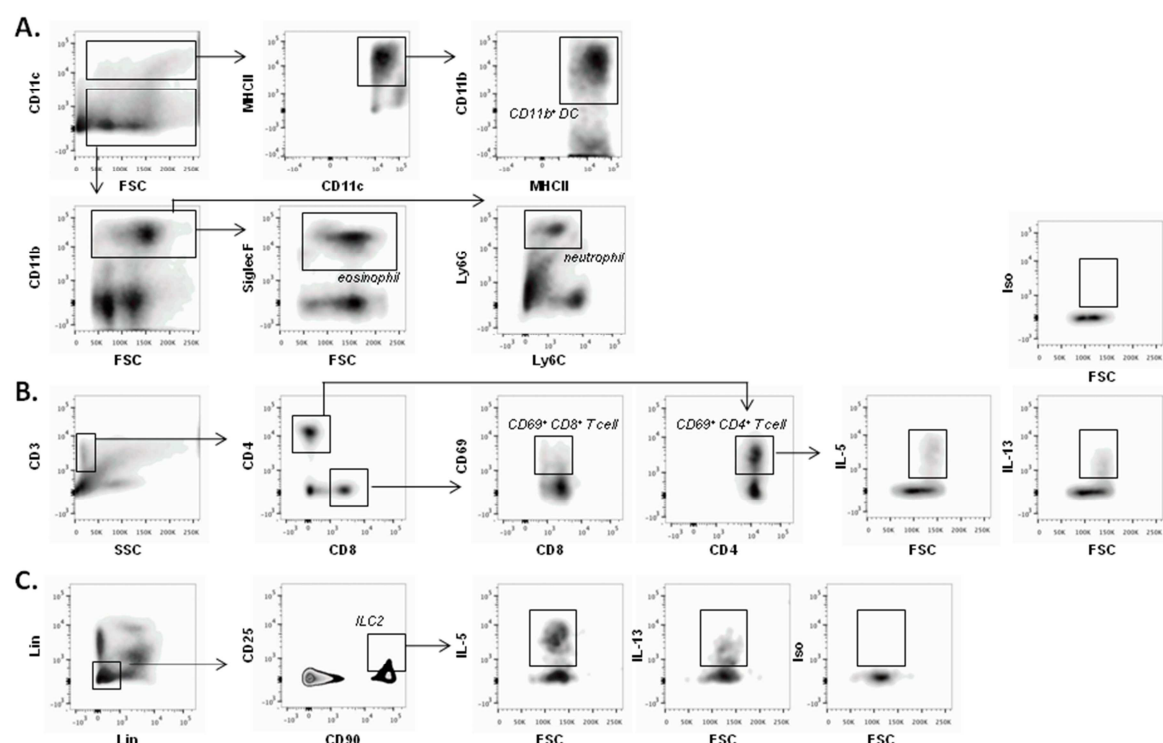


Figure S2: Representative density plots and gating strategy of all analyzed cell populations in lung tissue. WT mice were exposed to 25µg DEP + 1µg HDM. A, The pulmonary CD11b⁺ DC were identified as CD11c⁺, MHCII⁺ and CD11b⁺. Eosinophils were gated as CD11c⁺, CD11b⁺ and SiglecF⁺. Neutrophils were CD11c⁺, CD11b⁺, Ly6G⁺ and Ly6C⁺. B, CD4⁺ T cells were gated as CD3⁺, CD4⁺, CD8⁺ and CD69⁺. CD8⁺ T cells were characterized as CD3⁺, CD4⁺, CD8⁺ and CD69⁺. Intracellular IL-5 and IL-13 production of CD4⁺ T cells was investigated. C, ILC2 were identified as Lin⁺ (CD3⁺, CD5⁺, NK1.1⁺, TCRβ⁺, CD11c⁺, CD11b⁺ and CD45R⁺), CD25⁺ and CD90⁺ cells. Intracellular IL-5 and IL-13 production of ILC2 was investigated.

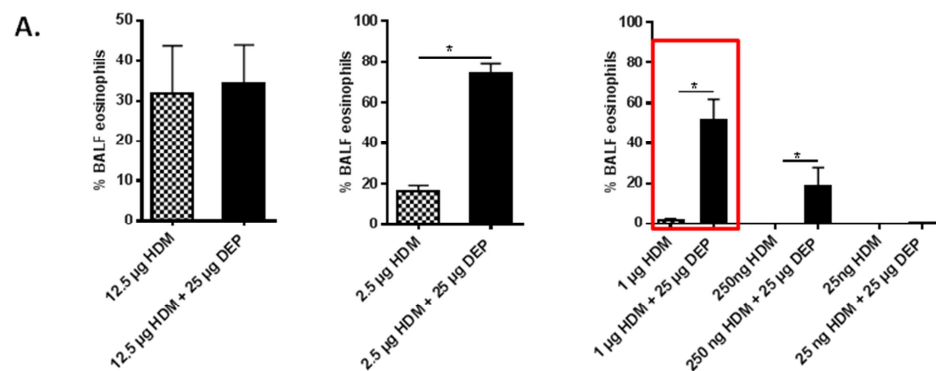


Figure S3: Titration of the HDM dose for the combined DEP+HDM model. A, C57BL/6 mice were exposed to decreasing doses of sole HDM (i.e. 12.5µg, 2.5µg, 1µg, 250ng, 25ng HDM) or combined 25µg DEP + decreasing doses of HDM on day 1, 8 and 15. On day 17, BALF eosinophils were determined on cytospins. Data presented on the different graph represent separate experiments. The red box represents the selected dose for the experiments in the manuscript. Results are expressed as mean $\pm$ SEM. n = 8 mice per group. *p < 0.05.

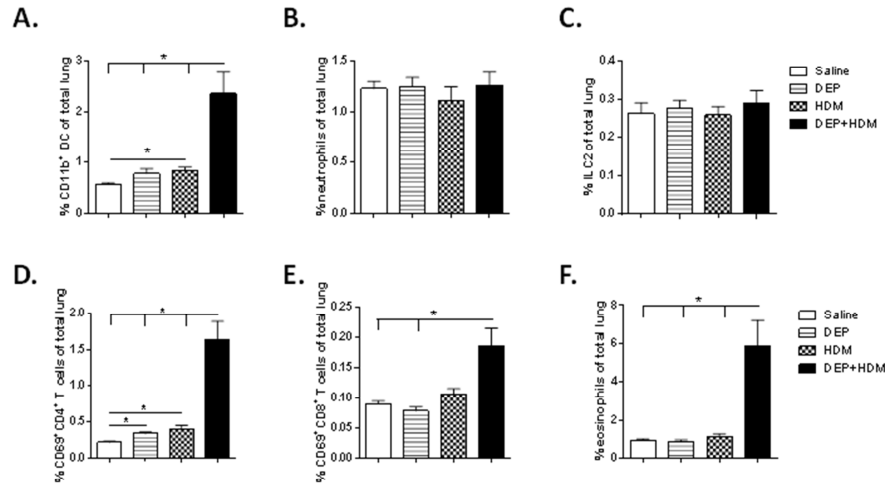


Figure S4: Adjuvant effect of DEP on HDM induced airway inflammation in the lung. WT mice were exposed to saline (white bar), 25μg DEP (striped bar), 1μg HDM (checked bar) or DEP+HDM (black bar) on day 1, 8 and 15. A-F, DC (CD11c⁺ MHCII⁺ CD11b⁺) (A), neutrophils (CD11c⁺ CD11b⁺ Ly6G⁺ Ly6C⁺) (B), eosinophils (CD11c⁺ CD11b⁺ Siglec-F⁺) (C), ILC2 (Lin⁻ (CD3⁻, CD5⁻, NK1.1⁻, TCRβ⁻, CD11c⁻, CD11b⁻ and CD45R⁻) CD25⁺ CD90⁺) (D), CD4⁺ T cells (CD3⁺ CD4⁺ CD8⁻ CD69⁺) (E) and CD8⁺ T cells (CD3⁺ CD8⁺ CD4⁻ CD69⁺) (F) in lung tissue were determined by flow cytometry. Results are expressed as the mean percentages of total lung ± SEM. n = 7-8 mice per group. *p < 0.05.