

ORIGINAL ARTICLE

Asthma and Lower Airway Disease

PAG1 limits allergen-induced type 2 inflammation in the murine lung

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Abstract

Background: Phosphoprotein associated with glycosphingolipid-enriched microdomains 1 (PAG1) is a transmembrane adaptor protein that affects immune receptor signaling in T and B cells. Evidence from genome-wide association studies of asthma suggests that genetic variants that regulate the expression of PAG1 are associated with asthma risk. However, it is not known whether PAG1 expression is causally related to asthma pathophysiology. Here, we investigated the role of PAG1 in a preclinical mouse model of house dust mite (HDM)-induced allergic sensitization and allergic airway inflammation.

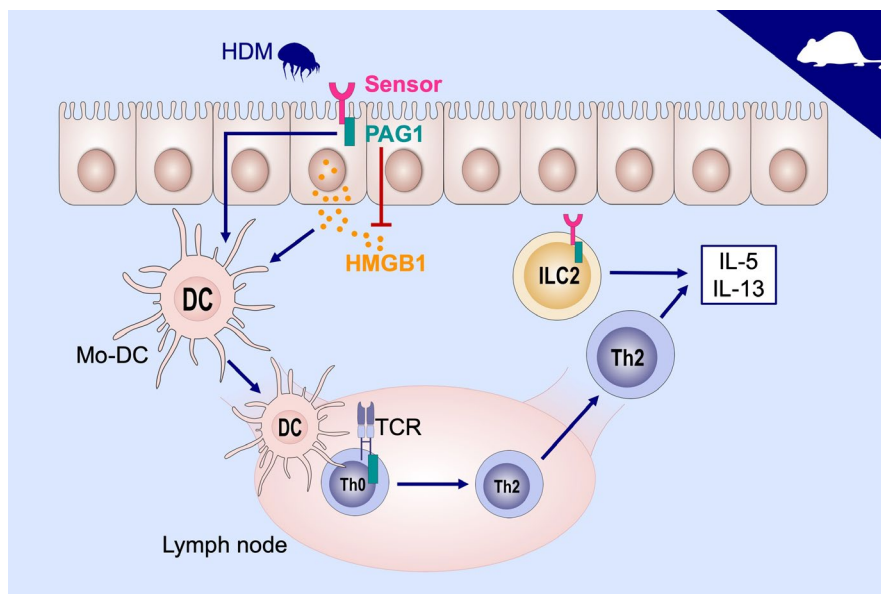
Methods: *Pag1*-deficient (*Pag1*^{-/-}) and wild-type (WT) mice were sensitized or sensitized/challenged to HDM, and hallmark features of allergic inflammation were assessed. The contribution of T cells was assessed through depletion (anti-CD4 antibody) and adoptive transfer studies.

Results: Type 2 inflammation (eosinophilia, eotaxin-2 expression, IL-4/IL-5/IL-13 production, mucus production) in the airways and lungs was significantly increased in HDM sensitized/challenged *Pag1*^{-/-} mice compared to WT mice. The predisposition to allergic sensitization was associated with increased airway epithelial high-mobility group box 1 (HMGB1) translocation and release, increased type 2 innate lymphoid cells (ILC2s) and monocyte-derived dendritic cell numbers in the mediastinal lymph nodes, and increased T-helper type 2 (T_H2)-cell differentiation. CD4⁺ T-cell depletion studies or the adoptive transfer of WT OVA-specific CD4⁺ T cells to WT or *Pag1*^{-/-} recipients demonstrated that the heightened propensity for T_H2-cell differentiation was both T cell intrinsic and extrinsic.

Conclusion: PAG1 deficiency increased airway epithelial activation, ILC2 expansion, and T_H2 differentiation. As a consequence, PAG1 deficiency predisposed toward allergic sensitization and increased the severity of experimental asthma.

KEYWORDS

HMGB1, house dust mite, PAG1, T_H2 cells, type 2 inflammation



GRAPHICAL ABSTRACT

PAG1 deficiency negatively regulates the release of epithelial HMGB1 following allergic sensitization. PAG1 deficiency promotes allergic sensitization by increasing the recruitment of monocyte-derived DCs and type-2 innate lymphoid cells and enhanced differentiation of CD4⁺ T cells into TH2 cells. Consequently, allergen-induced type 2 inflammation is aggravated in the lungs of PAG1-deficient mice. HDM, house dust mite; HMGB1: high-mobility group box 1; Mo-DC, monocyte-derived DCs; PAG1, phosphoprotein associated with glycosphingolipid-enriched microdomains 1

1 | INTRODUCTION

Allergic asthma often begins in early childhood when children are exposed to airborne allergens, such as house dust mite (HDM), cockroach, pollen, animal dander, and fungal spores.¹ Airway exposure to allergens can lead to the activation of airway epithelial cells (AECs) and the release of type 2 instructive cytokines, such as IL-33, IL-25, thymic stromal lymphopoietin (TSLP), and HMGB1, which promote a pro-type 2 microenvironment. These epithelia-derived cytokines activate dendritic cells (DCs) and ILC2s, which contribute to the priming of CD4⁺ T_H2 cells.²⁻⁴ Both ILC2s and T_H2 cells produce an array of type 2 cytokines (IL-4, IL-5, IL-9, and IL-13) which orchestrate the pathological features of asthma including granulocyte infiltration into the airways, excessive mucus production, airway wall remodeling, and airway hyperreactivity.⁵ However, key knowledge gaps remain in relation to the signaling cascades that regulate the pro-type 2 microenvironment and lead to stable type 2 airway inflammation.

Characterizing the contribution to disease pathophysiology of genes identified through genome-wide association studies (GWAS) of asthma can potentially help address such knowledge gaps. One such gene is PAG1, whose expression is determined by genetic variants associated with asthma risk.^{6,7} PAG1 encodes a transmembrane phosphotyrosylated adaptor protein, which resides in lipid rafts and is ubiquitously expressed. Although PAG1 has been found to be dispensable for T-cell development and function,^{8,9} some studies report that PAG1 is a negative regulator of T-cell immune responses. Upon T-cell receptor (TCR) stimulation, PAG1

inhibits T-cell activation via protein tyrosine kinase CSK (C-terminal SRC kinase).¹⁰⁻¹² In the setting of experimental autoimmune encephalomyelitis, loss of PAG1 leads to T-cell hyperactivation and the induction of a detrimental T-helper type 1 (T_H1) response. However, whether PAG1 expression affects the development of T_H2 responses is not known. PAG1 also inhibits the SRC kinase Lyn following B-cell receptor signaling and B-cell activation.¹³ In contrast, PAG1 in mast cells acts as a feedback-loop inhibitor. Following FcεRI activation on mast cells, SRC kinases hyperphosphorylate PAG1. This results in increased recruitment of CSK to lipid rafts which in turn inactivates SRC kinases.¹⁴⁻¹⁶ Thus, PAG1 down-regulates mast cell activation and degranulation.

Given the importance of T, B, and mast cells in allergic asthma,¹⁷ the ability of PAG1 to influence the activation of these cells, and its association with asthma risk, we hypothesized that PAG1 regulates allergic sensitization and development of allergic airway inflammation. To test this hypothesis, we used a preclinical mouse model of HDM-induced allergic airway inflammation.

2 | MATERIALS AND METHODS

2.1 | Mouse strains

Pag1-deficient mice (*Pag1*^{-/-}) on C57Bl/6J background, kindly provided by Dr Jonathan Lindquist,¹⁸ were imported to Australia, re-derived at The University of Queensland, and then housed and bred in a specific-pathogen-free (SPF) facility at QIMR Berghofer Medical Research Institute. C57Bl/6J OT-II mice (express T-cell

receptor specific for chicken ovalbumin 323-339 peptide in the context of I-A^b) were obtained from the animal facility of QIMR Berghofer Medical Research Institute. All animal experiments were approved by the Animal Ethics Committee of QIMR Berghofer Medical Research Institute.

2.2 | Allergen model

To evaluate innate immune responses during sensitization to allergen, 6- to 12-week-old mice were sensitized intranasally with 10 µg of HDM (Citeq) or PBS at day 0 under isoflurane-induced anesthesia. Mice were euthanized 3 days later and airway inflammation measured as described in the section below. To evaluate adaptive immune responses that develop after re-exposure to allergen, sensitized mice were subsequently challenged with 3 µg of HDM or PBS at days 14-17. Mice were euthanized 3 hours after the final challenge.

To investigate the role of CD4⁺T cells during allergic sensitization, 250 µg of anti-CD4⁺ depleting antibody (GK1.5; made in-house) or isotype IgG (Sigma) was administered intraperitoneally. Twenty-four hours later, mice were sensitized with 100 µg of HDM per day for three consecutive days and euthanized 72 hours after the last exposure. To evaluate the role of PAG1 in CD4⁺ T-cell priming and differentiation, 0.25 × 10⁶ FACS-sorted OT-II T cells (CD3ε⁺CD4⁺TCR-Vβ5.1,5.2⁺TCR-Vα2⁺) labeled with carboxyfluorescein succinimidyl ester (CFSE) were injected intravenously 2 hours prior to intranasal ovalbumin (OVA; 10 µg; Sigma) and HDM (10 µg) administration. Five days later, mediastinal lymph nodes (mLNs) were analyzed for OT-II T-cell expansion and differentiation.

2.3 | Assessment of airway inflammation

Bronchoalveolar lavage (BAL) was obtained by flushing the lungs with 600 µL of ice-cold PBS. Following centrifugation, BAL fluids (BALF) were harvested and stored at -80°C for cytokine measurement. Red blood cells (RBCs) were lysed using Gey's lysis buffer, and cell suspensions were used to enumerate immune cells by flow cytometry. Lung lobes were harvested for flow cytometry, histology, and protein assay. For cytokine measurement in the lung tissue, lung lobes were homogenized in radioimmunoprecipitation assay buffer (Sigma) using Tissue-Tearor (BioSpec). Cell-debris-free supernatants were harvested after centrifugation and stored at -80°C until used for cytokine assay.

2.4 | Cell culture

To assess effector cytokine production, mLNs from allergen sensitized and/or challenged mice were harvested, single cell suspensions were prepared, and 1 × 10⁶ cells were seeded in 96-well flat-bottom plates with 200 µL of culture medium (RPMI-1640 medium [Gibco] supplemented with 10% fetal calf serum [GE Healthcare], 1 mmol/L sodium pyruvate [Gibco], 2 mmol/L L-glutamine [Gibco], 20 mmol/L

HEPES [Gibco], 100 U/mL penicillin-streptomycin [Gibco], and 10 mmol/L 2-mercaptoethanol [Sigma]). Cells were stimulated *ex vivo* with HDM (20 µg/mL) or OVA (10 µg/mL). Culture supernatants were harvested 4 days later and stored at -80°C prior to cytokine detection.

Bone marrow (BM) cells were flushed from the femurs and tibias of naïve WT and *Pag1*^{-/-} mice with cold culture medium using a 25-gauge needle and syringe under sterile conditions. Cells were passed through a 70 µmol/L cell strainer (BD Biosciences) to prepare single cell suspension. RBCs were lysed as mentioned above. The frequency of ILC2s was evaluated in BM by flow cytometry. BM cells were seeded in 200 µL medium (1 × 10⁶ cells) and stimulated with recombinant IL-33 (10 ng/mL) and IL-2 (10 ng/mL). Cell-free supernatants were harvested 4 days later and stored at -80°C prior to cytokine analysis.

2.5 | Cell isolation and flow cytometry

Single cell suspensions were prepared from lung tissues and mLNs by mechanical disruption against a 70 µmol/L cell strainer. RBCs were lysed as mentioned above, and cells were washed with staining buffer (2% FCS in PBS). To inhibit nonspecific binding, lung, mLN, and BM cells were incubated with an Fc block antibody (2.4G2) for 30 minutes at 4°C. Cells were then stained for 30 minutes at 4°C with various fluorochrome-conjugated monoclonal antibodies as appropriate (Table S1). For intracellular staining, cells were processed using the FoxP3/transcription factor fixation/permeabilization kit (eBioscience) as per the manufacturer's instruction before staining with FoxP3 (MF23, BD Biosciences) or GATA3 antibody (TWAJ, PE, eBioscience) for 30 minutes at 4°C. Finally, cells were washed with staining buffer and were run on a BD Fortessa IV flow cytometer (BD Biosciences). Data were acquired using the FACSDiva software (ver 8.0; BD Biosciences) and analyzed with the FlowJo software (ver 10; TreeStar).

2.6 | Lung tissue immunohistochemistry

Paraffin-embedded tissue sections were prepared and processed for staining as previously described.¹⁹ Briefly, lung biopsy specimens were rehydrated and incubated in citrate buffer followed by heating in a pressure cooker microwave for antigen retrieval. After washing the sections, 10% goat serum was added to block nonspecific binding. Sections were then incubated overnight at room temperature with rabbit anti-mouse HMGB1 antibody (EPR3507, Abcam). The following day, sections were washed and incubated with an anti-rabbit alkaline phosphatase secondary antibody (Sigma) for 60 minutes. After washing, color was developed with warp red chromogen (Biocare Medical). Sections were counter-stained with hematoxylin and mounted with DePeX (Sigma). Cytoplasmic translocation of HMGB1 in AECs was quantified as described previously.⁴ Lung biopsies were stained with periodic acid-Schiff (PAS) reagent, and mucus hypersecretion was evaluated using a semi-quantitative scoring system as described previously.²⁰

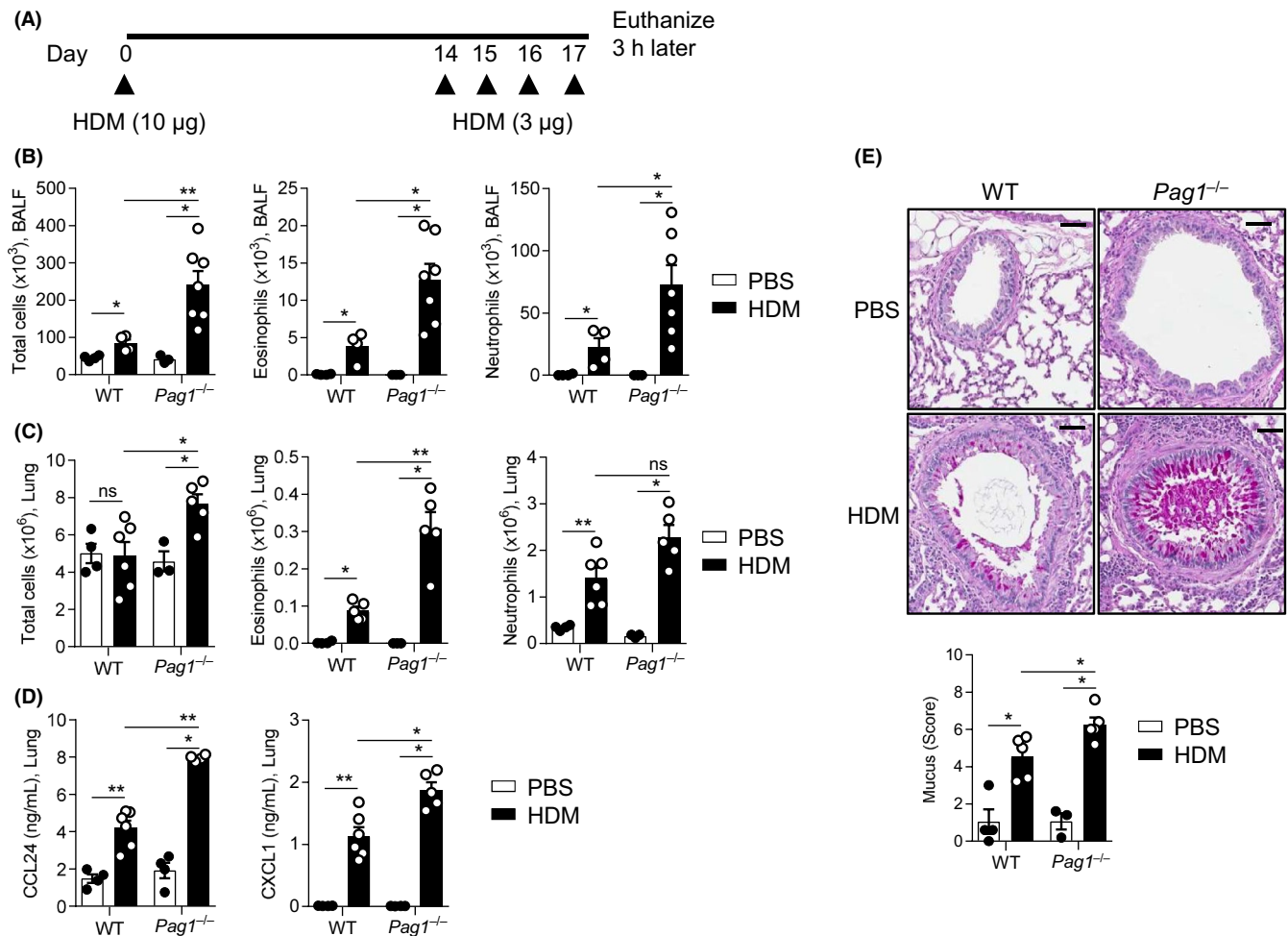


FIGURE 1 PAG1 deficiency exaggerates airway inflammation after re-exposure to HDM. A, Study design. WT and *Pag1*^{-/-} mice were sensitized intranasally with 10 µg of HDM at day 0 and challenged at days 14, 15, 16, and 17 with 3 µg of HDM. BALF and lung tissues were harvested 3 h after the final challenge. B, Total cell count and the number of eosinophils (SigF⁺CD11b⁺Ly6G⁻CD11c⁻) and neutrophils (SigF⁺CD11b⁺Ly6G⁺CD11c⁻) in BALF. C, Total cell count and the number of eosinophils and neutrophils in lung tissues. D, Expression of CCL24 and CXCL1 in lung homogenates. E, Representative images of periodic acid-Schiff (PAS) staining of paraffin-embedded lung sections (x200 magnification; Scale bar = 50 µm). Semi-quantitative histological scores of PAS staining in lung airways. Results are shown as mean ± SEM of n = 3-7 mice per group of a single experiment and are representative of two experiments. *P < .05, **P < .01, and ns = not significant

2.7 | Cytokines and immunoglobulins assay

Cytokine concentrations in BALF, lung homogenate, and lymph node cell supernatants were measured by ELISA using commercially available kits as per the manufacturer's instructions: IL-10, IL-17A, and IFN-γ (BioLegend); CCL24, KC, IL-13, and IL-33 (R & D systems); and IL-4 and IL-5 (BD Biosciences). IL-13 concentrations in lung homogenates were measured using an IL-13 enhanced sensitivity cytometric bead assay kit (BD Biosciences). Serum HDM-specific IgG1 was measured as described previously.⁴

2.8 | Statistical analysis

Data were presented as mean ± standard error of mean (SEM), and an unpaired 2-tailed Mann-Whitney U test was used for comparisons. A P value of <0.05 was considered significant. Statistical

analyses were performed using Prism version 7.02 software (GraphPad) (NS, not significant; *P < 0.05; **P < 0.01; ***P < 0.001).

3 | RESULTS

3.1 | PAG1 deficiency exaggerates airway inflammation after re-exposure to HDM

To investigate the contribution of PAG1 to allergen-induced type 2 inflammation, WT (*Pag1*^{+/+}) and *Pag1*^{-/-} littermate controls were intranasally sensitized with 10 µg of HDM or PBS, and then challenged 2 weeks later for four consecutive days with 3 µg HDM or PBS (Figure 1A). As expected, this acute model of HDM-induced experimental asthma resulted in increased numbers of immune cells in the BALF and lungs of WT mice (Figure 1B and C). Importantly, total cell numbers, eosinophils, and neutrophils in

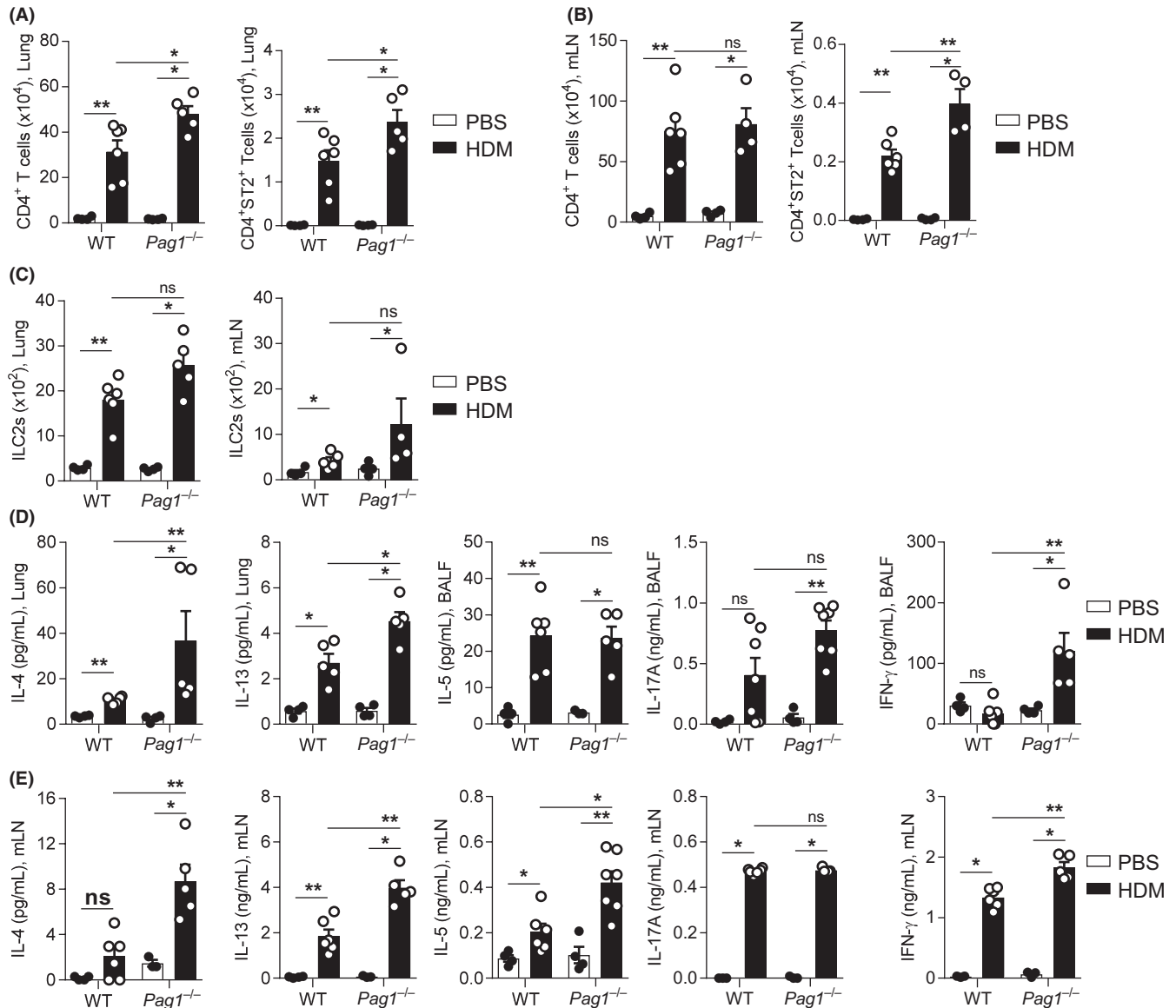


FIGURE 2 PAG1 deficiency exaggerates type 2 inflammation after re-exposure to HDM. WT and *Pag1*^{-/-} mice were sensitized and challenged intranasally with HDM. BALF and lung tissues were harvested 3 h after the final challenge. A and B, Number of CD4⁺ T cells (CD3ε⁺CD4⁺CD8⁻) and T_H2 cells (ST2⁺CD4⁺ T) in the lungs and mLN. C, Number of ILC2s (CD3ε⁻CD19⁺CD45R⁺CD11c⁻Gr-1⁻CD11b⁺NK1.1⁻CD90.2⁺CD25⁺ST2⁺) in the lungs and mLN. D, Concentration of IL-4 and IL-13 in lung homogenates and IL-5, IL-17A, and IFN-γ in BALF. E, mLN were harvested from sensitized-challenged mice and cells were stimulated ex vivo with HDM for 4 d. Concentration of IL-4, IL-13, IL-5, IL-17A, and IFN-γ was measured in cell-free supernatants. Results are shown as mean ± SEM of n = 4–7 mice per group of a single experiment and are representative of two experiments. *P < .05, **P < .01, and ns = not significant

BALF were significantly higher in *Pag1*^{-/-} mice compared to WT mice (Figure 1B). Lung eosinophils, but not neutrophils, were also significantly higher in *Pag1*^{-/-} mice compared to WT mice (Figure 1C). This response was associated with increased expression of eosinophil and neutrophil recruiting chemokines CCL24 (eotaxin-2) and CXCL1 in lung homogenates of *Pag1*^{-/-} mice (Figure 1D). Additionally, the proportion of mucus-producing cells in the airways was greater in *Pag1*^{-/-} mice compared to WT mice (Figure 1E), although serum levels of HDM-specific IgG1 were similar between WT and *Pag1*^{-/-} mice (Figure S1A).

3.2 | PAG1 deficiency exaggerates type 2 inflammation after re-exposure to HDM

The increase in eosinophils, CCL24, and mucus production observed in the acute model of HDM-induced asthma suggested that the absence of PAG1 increases the magnitude of allergen-induced type 2 inflammation. The number of CD8⁺ T cells was not affected by the absence of PAG1 (Figure S1B). However, the absolute number of CD4⁺ T cells in the lung, but not in the mLN, was higher in the *Pag1*^{-/-} mice (Figure 2A and B). The number of ST2-expressing CD4⁺ T cells (T_H2

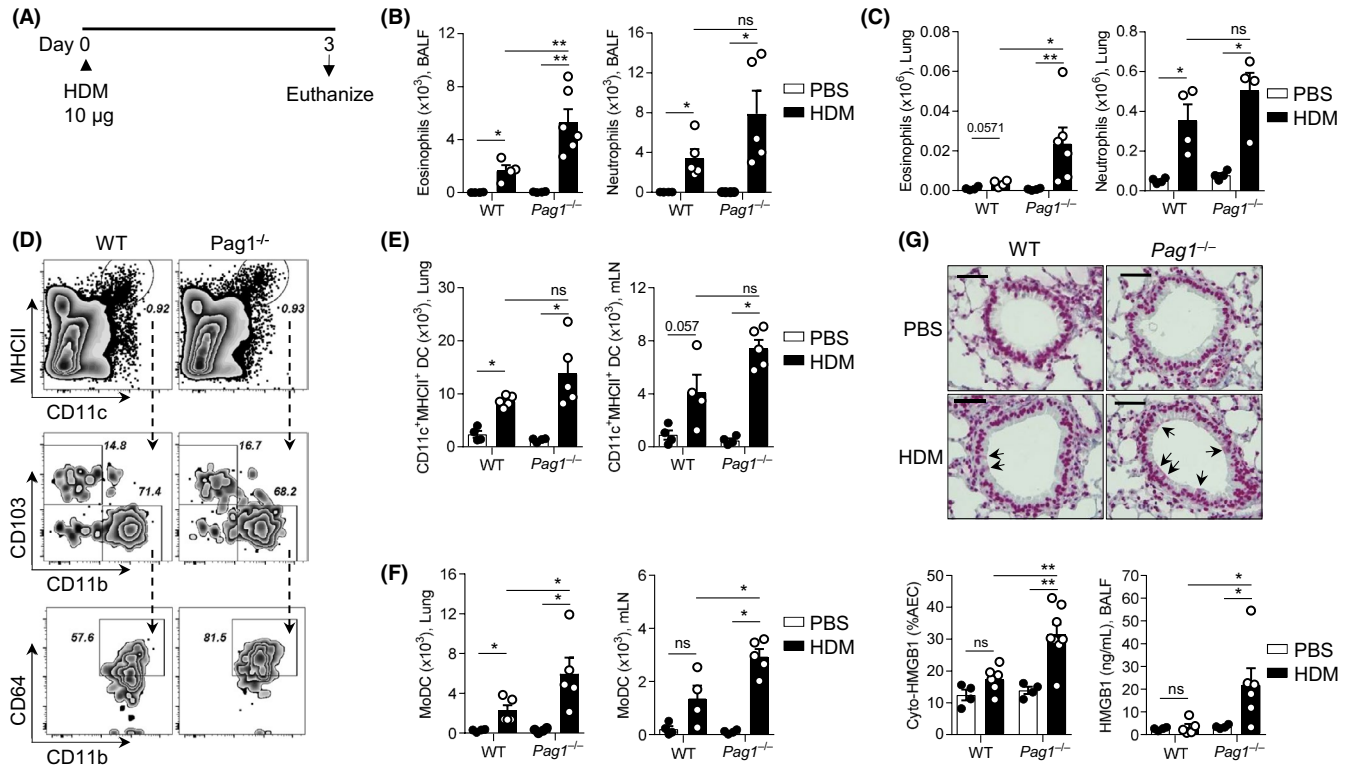


FIGURE 3 PAG1 deficiency increases innate immune responses to HDM exposure. A, Study design. WT and *Pag1*^{-/-} mice were intranasally sensitized with 10 µg of HDM at day 0, and endpoints were analyzed 3 d later. B and C, Number of eosinophils and neutrophils in BALF and lung tissues. D, Gating strategy of lung DC subsets in mice sensitized with HDM. E, Number of total cDCs (CD11c^{Hi}MHCII⁺) in lung and mLN. F, Number of MoDCs (CD11c^{Hi}MHCII⁺ + CD103⁺CD11b⁺CD64⁺) in lung and mLN. G, Representative images of HMGB1 immunostaining on paraffin-embedded lung sections (×400 magnification; Scale bar = 50 µm). Arrows indicate cyto-HMGB1. Quantification of cytoplasmic HMGB1 in lung AECs and HMGB1 release in BALF. Results are shown as mean ± SEM of n = 4–8 mice per group of a single experiment and are representative of two experiments. **P* < .05, ***P* < .01, and ns = not significant

cells) was also significantly higher in the lung and mLN of *Pag1*^{-/-} mice (Figure 2A and B). In contrast, the number of type 2 innate lymphoid cells (ILC2s; gated as CD3⁺CD19⁺CD45⁺CD11c⁺Gr-1⁺CD11b⁺NK1.1⁺CD90.2⁺CD25⁺ST2⁺) after HDM sensitization and challenge was not different between WT and *Pag1*^{-/-} mice (Figure 2C). Consistent with the increased number of T_H2 cells, the concentration of IL-4 and IL-13 was significantly higher in the lungs of *Pag1*^{-/-} mice compared to WT mice (Figure 2D); however, IL-4 and IL-13 were undetectable in BALF. The level of IL-5, IL-17A, and IL-10 in the BALF was comparable between the two strains, whereas IFN-γ was significantly higher in *Pag1*^{-/-} mice compared to WT mice (Figure 2D; S1C). To assess the HDM-specific cytokine response, mLN were excised and the cells were re-stimulated ex vivo with HDM. We found that the concentration of IL-4, IL-13, IL-5, and IFN-γ was significantly higher in mLN from *Pag1*^{-/-} mice (Figure 2E), whereas there was no difference in IL-17A (Figure 2E) and IL-10 (S1C) levels between WT and *Pag1*^{-/-} mice. The elevated type 2 immunity may occur from a lack of immunoregulation by regulatory T cells¹⁹; however, similar to the levels of IL-10, the number of FoxP3⁺ regulatory T cells in the lung and mLN was not different between WT and *Pag1*^{-/-} mice (S1C-D). Taken together, these findings demonstrate that PAG1 deficiency exacerbates type 2 inflammation in our acute model of HDM-induced asthma.

3.3 | PAG1 deficiency increases innate immune responses to HDM exposure

Next, to address the role of PAG1 in the development of sensitization against HDM, we exposed WT and *Pag1*^{-/-} mice to 10 µg of HDM and measured the immune response at day 3 (Figure 3A). HDM exposure induced a significant eosinophilic and neutrophilic infiltrate into the airways and lungs of WT mice (Figure 3B and C). Similar to our findings in the acute challenge model, the number of eosinophils, but not neutrophils, in the lung and BALF was significantly greater in *Pag1*^{-/-} mice (Figure 3B and C). Next, we investigated whether PAG1 deficiency altered the recruitment of dendritic cells (DCs) during the sensitization phase. The total numbers of conventional DCs (cDCs; gated as CD11c^{Hi}MHCII⁺), cDC2 (CD11c^{Hi}MHCII⁺CD11b⁺CD103⁺CD64⁺), and cDC1 (CD11c^{Hi}MHCII⁺CD11b⁺CD103⁺CD64⁺) in the lungs or mLN were not affected by PAG1 deficiency (Figure 3D and E, Figure S2A and B). However, the number of monocyte-derived DCs (MoDCs; gated as CD11c^{Hi}MHCII⁺CD103⁺CD11b⁺CD64⁺) was significantly higher in *Pag1*^{-/-} mice compared to WT mice (Figure 3D,F).

We have previously shown that HDM induces allergic sensitization by promoting the nuclear-to-cytoplasmic translocation

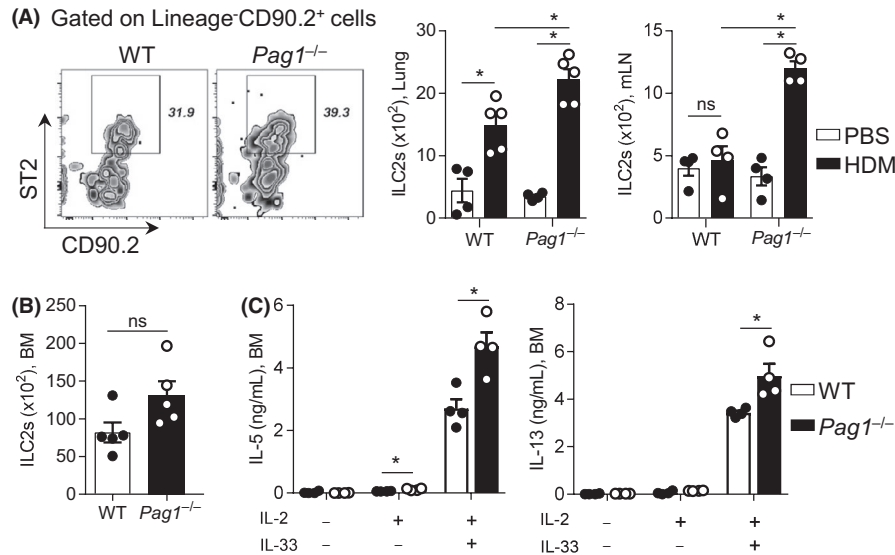


FIGURE 4 PAG1 suppresses ILC2 cytokine production. A, Representative flow cytometry staining of lung ILC2s in mice sensitized with HDM and number of ILC2s (CD3^ε⁻CD19⁻CD45R⁻CD11c⁻Gr-1⁻CD11b⁻NK1.1⁻CD90.2⁺CD25⁺ST2⁺) in the lungs and mLNs 3 d after HDM sensitization. B, Enumeration of ILC2s (CD3^ε⁻CD19⁻CD45R⁻CD11c⁻Gr-1⁻CD11b⁻NK1.1⁻CD90.2⁺CD25⁺ST2⁺) in BM of naïve mice. C, BM cells were simulated with the indicated cytokines for 4 d, and IL-5/IL-13 were measured in cell-free supernatants. Results are shown as mean ± SEM of n = 4–5 mice per group of a single experiment and are representative of two experiments. *P < .05, **P < .01, and ns = not significant

and downstream release of HMGB1 from the airway epithelium.⁴ Here, we found that the percentage of AECs with cytoplasmic HMGB1 was significantly higher in *Pag1*^{-/-} mice compared to WT mice (Figure 3G). Furthermore, the concentration of HMGB1 in the BALF was significantly higher in *Pag1*^{-/-} compared to WT mice following HDM exposure (Figure 3G). The expression of IL-33, another T_H2 instructive cytokine,²¹ was also elevated in the BALF in response to HDM exposure; however, its concentration did not differ between WT and *Pag1*^{-/-}-deficient mice (Figure S2C).

3.4 | PAG1 suppresses ILC2 cytokine production

ILC2s belong to the family of innate immune cells, and in response to epithelial alarmins, ILC2s rapidly produce effector cytokines such as IL-5 and IL-13. Thus, ILC2s can induce T/B-cell independent type 2 inflammation in the airways and are also suggested to contribute to T_H2-cell differentiation.^{5,22} In the sensitization phase, ILC2 numbers in the lungs and mLNs were significantly higher in *Pag1*^{-/-} compared to WT mice (Figure 4A). Next, we investigated whether PAG1 regulates cytokine production by ILC2s. Stimulation of total bone marrow (BM) cells with IL-33 induces the production of type 2 cytokines IL-5 and IL-13 primarily by BM ILC2s.²³ Hence, we stimulated total bone marrow (BM) cells from naïve WT and *Pag1*^{-/-} mice with IL-2 and IL-33. Although there was an equivalent number of ILC2s in the bone marrow of WT and *Pag1*^{-/-} mice, *Pag1*^{-/-} BM cells produced significantly more type 2 cytokines IL-5 and IL-13 following IL-2/IL-33 stimulation compared to WT BM cells (Figure 4B and C).

3.5 | CD4⁺ T cells contribute to an exaggerated HDM-induced type 2 inflammation in *Pag1*^{-/-} deficient mice

Three days after HDM sensitization, the number of CD4⁺ T cells was similar between WT and *Pag1*^{-/-} mice; however, we found an increased number of T_H2 cells in the lungs of *Pag1*^{-/-} mice (Figure 5A and B). As expected at this early time point, IL-5 and IL-13 expression was undetectable in the lungs. Next, to investigate the contribution of T cells to the exaggerated type 2 inflammation in *Pag1*^{-/-} mice, we treated WT and *Pag1*^{-/-} mice with an anti-CD4 depleting antibody and then exposed the mice to HDM on 3 consecutive days (Figure 5C). We used a higher dose of HDM in order to elicit a measurable type 2 cytokine response at day 5. As expected, the anti-CD4 treatment depleted CD4⁺ T cells in the lungs and mLNs of WT and *Pag1*^{-/-} mice without affecting the CD8⁺ T-cell (Figure S3) or ILC2 numbers (Figure 5D). In both WT and *Pag1*^{-/-} mice, CD4⁺ T-cell depletion significantly decreased the number of airway eosinophils and lung IL-13 levels but did not affect neutrophil numbers or IL-5 levels in the BALF (Figure 5E and F). Importantly, despite CD4⁺ T-cell depletion, airway eosinophil numbers and the concentration of IL-5 and IL-13 were still higher in *Pag1*^{-/-} mice compared to WT mice (Figure 5E and F). Collectively, these results demonstrate that CD4⁺ T cells contribute to the exaggerated HDM-induced type 2 inflammation observed in *Pag1*^{-/-} mice; however, as the type 2 response was still greater in *Pag1*^{-/-} compared to WT mice after CD4⁺ T-cell depletion, this suggests that other cell types (eg, AECs, ILC2s, DCs) are also contribute to this process.

Whether this phenotype was T cell intrinsic or instead, mediated by an upstream cellular event, remained unclear. To address this, we injected PAG1-sufficient CFSE-labeled OVA-specific transgenic OT-II

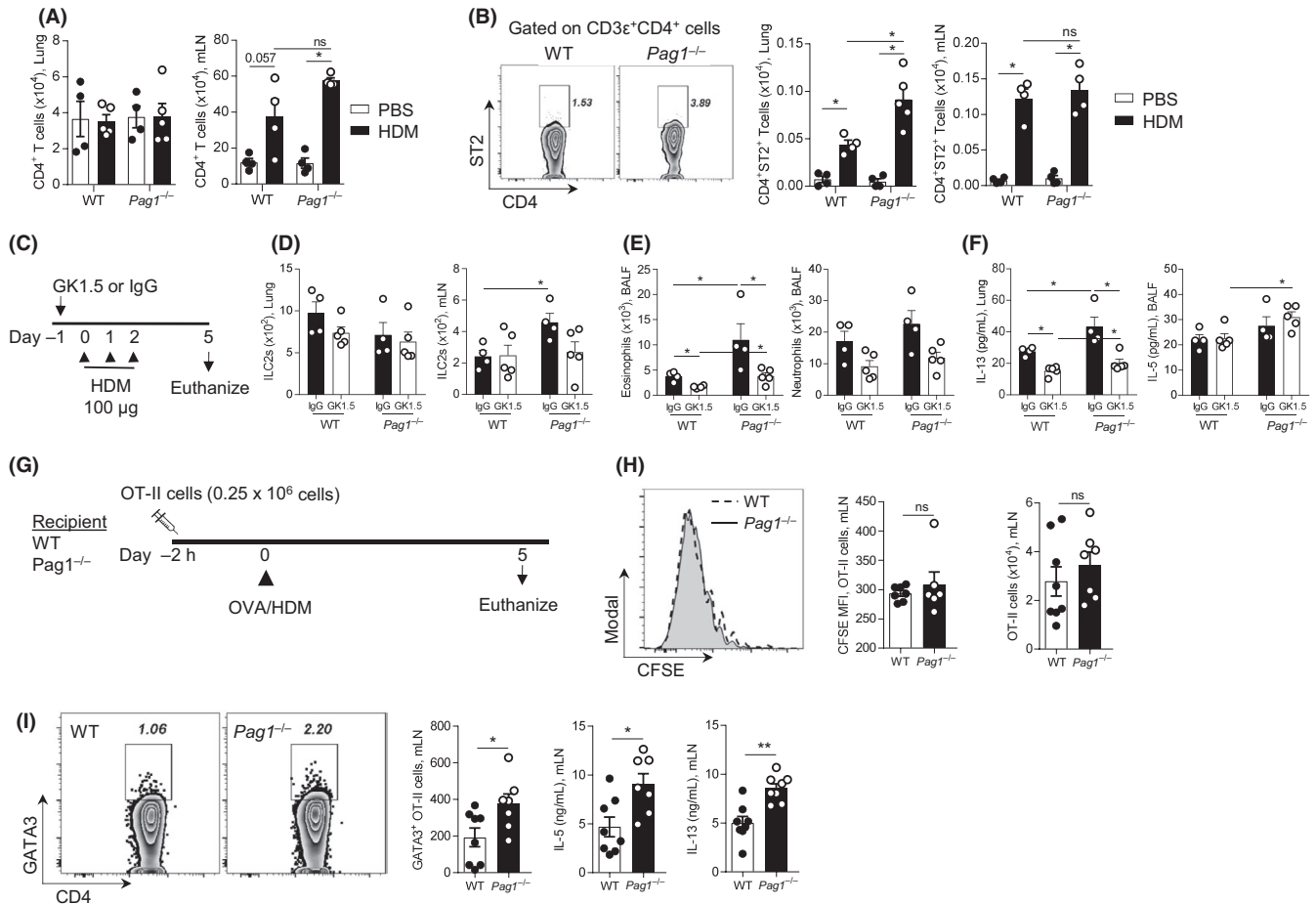


FIGURE 5 CD4⁺ T cells contribute to an exaggerated HDM-induced type 2 inflammation in *Pag1*-deficient mice. A, Number of CD4⁺ T cells in the lungs and mLNs. B, Flow cytometry analysis of ST2⁺CD4⁺ T cells in the lungs of HDM sensitized mice and enumeration of CD3ε⁺CD4⁺ T cells and T_H2 cells (ST2⁺CD4⁺ T) in the lungs and mLNs 3 d after HDM sensitization. C, Study design. WT and *Pag1*^{-/-} mice were administered a CD4⁺ T-cell depletion antibody GK1.5 or IgG control antibody. Twenty-four hours later, mice were sensitized with 100 μg of HDM at day 0, 1, and 2, and endpoints were analyzed at day 5. D, Number of ILC2s in the lungs and mLNs. E, Number of eosinophils and neutrophils in BALF. F, Concentration of IL-13 in lung homogenates and IL-5 in BALF. G, WT and *Pag1*^{-/-} mice were injected with 0.25 × 10⁶ CFSE-labeled OVA-specific OT-II cells. Two hours later, mice were given OVA and HDM and endpoints were assessed 5 d later. H, Representative flow cytometry plot and quantification of OT-II cell (CD3ε⁺CD4⁺TCR-Vβ5.1,5.2⁺TCR-Vα2⁺) proliferation and number of OT-II cells in mLNs. I, Quantification of GATA3 expressing OT-II cells. IL-5 and IL-13 expression in the ex vivo recall assay. Results are shown as mean ± SEM of n = 4–8 mice per group of a single experiment and are representative of two experiments. *P < .05, **P < .01, and ns = not significant

cells into WT and *Pag1*^{-/-} mice. We then exposed the mice to OVA (the cognate ligand of the TCR on the OT-II cells) and HDM, the latter effectively employed as an adjuvant to prevent tolerance. After 5 days, the proliferative response and cytokine production of the OT-II cells in the mLNs were analyzed (Figure 5G). In both WT and *Pag1*^{-/-} recipient mice, OT-II cells expanded equivalently in response to OVA/HDM (Figure 5H). Importantly, the number of GATA3⁺ OT-II cells as well as the production of IL-5 and IL-13 was significantly higher in *Pag1*^{-/-} recipient mice (Figure 5I). These data demonstrate that the propensity for T_H2-cell differentiation in *Pag1*^{-/-} mice is not intrinsic to CD4⁺ T cells.

4 | DISCUSSION

PAG1 was recently implicated in asthma pathophysiology based on results from a GWAS and functional follow-up studies.⁶ Here, we

demonstrated that PAG1 deficiency in mice enhances type 2 inflammation in the lung following exposure to HDM. PAG1 deficiency caused increased cytoplasmic translocation of epithelial HMGB1 and its release into the airway lumen following HDM sensitization. This phase was also characterized by increased numbers of MoDCs and ILC2s, and increased CD4⁺ T_H2-cell differentiation in *Pag1*^{-/-} mice. As a consequence, the production of type 2 cytokines and airway inflammation were heightened in *Pag1*^{-/-} mice in the challenge phase. Taken together, these results indicate that PAG1 expression suppresses innate immune signals that underpin the development of allergen-induced type 2 inflammation in the lung.

The airway epithelium acts as the first line of defense against inhaled allergens including HDM. We have previously shown that HDM sensitization triggers the nuclear-to-cytoplasmic translocation of epithelial alarmin HMGB1 and that this event is regulated by the sequential activation of toll-like receptor 4 (TLR4) and receptor for advanced

glycation end products.⁴ Once extracellular, HMGB1 can activate immature DCs, increase their antigen-presenting capability, and promote CD4⁺ T_H2 and T_H17-cell differentiation.²⁴ Here, we found increased HMGB1 translocation in AECs and increased HMGB1 in the airways of *Pag1*^{-/-} mice in the sensitization phase. How PAG1 regulates HMGB1 translocation and release remains unknown. PAG1 localization in AECs could not be confirmed immunohistochemically because the commercially available antibody was reactive in lung specimens of *Pag1*^{-/-} mice (data not shown). PAG1 is a raft-associated transmembrane adaptor protein (TRAP) composed of a short extracellular peptide domain, a transmembrane segment followed by a palmitoylation motif and a long cytoplasmic domain containing multiple phosphorylation sites.²⁵ TRAPs provide intracellular docking sites for other proteins and organize membrane-proximal signaling assemblies making them key mediators of immune receptor signaling. A number of TRAPs have been functionally implicated in TLR signaling.²⁶ For example, SLP adaptor and C-terminal Src kinase (CSK)-interacting membrane protein (SCIMP), a member of the TRAP family, directly interact with TLR4 facilitating its ligand-induced phosphorylation and drive the innate immune response.²⁷ Similar to SCIMP, PAG1 may also interact with TLR4 to regulate TLR4-mediated HMGB1 translocation and release in response to HDM sensitization. In addition, phosphorylated PAG1 recruits and binds to the tyrosine kinase CSK in lipid rafts and this PAG1/CSK complex inhibits SRC kinases. Upon PAG1 dephosphorylation, CSK dissociates from PAG1 allowing SRC kinase activation.²⁵ SRC kinases are key intermediates of TLR4 and RAGE signaling,²⁸⁻³⁰ and hence, the suppression of SRC kinases by the PAG1-CSK complex represents another mechanism through which PAG1 might limit the translocation and release of epithelial-derived HMGB1. Further studies are required to confirm these hypotheses.

Lung DCs form the critical bridge between innate and adaptive immunity. Both cDC2 and MoDC can present HDM antigens to naïve CD4⁺T cells in the mLN to initiate T_H2 differentiation.³¹ Here, we found that the exaggerated type 2 inflammation in *Pag1*^{-/-} mice was associated with increased accumulation of MoDCs but not cDC2s. HMGB1 can recruit and activate DCs and instruct DCs to trigger T_H2 responses following HDM sensitization.^{4,24,32} Thus, the elevated levels of extracellular HMGB1 after HDM exposure in *Pag1*^{-/-} mice may be responsible for the increased recruitment of MoDCs to the lung and mLN. As an innate source of type 2 cytokines, ILC2s also facilitate the differentiation of CD4⁺ T cells to T_H2 cells.⁵ Although we did not observe a difference in HDM-induced IL-33 expression between WT and *Pag1*^{-/-} mice, ILC2 numbers were significantly increased in the lungs of *Pag1*^{-/-} mice sensitized with HDM. In addition, *Pag1*^{-/-} BM cells were more responsive to IL-2/IL-33 stimulation, producing more IL-5/IL-13 (primarily by ILC2s) than WT BM cells. Although ILC2s are the dominant source of IL-5/IL-13 in IL-33 stimulated BM cells,²³ we cannot rule out the contribution of other cell types to this response. Further experiments with purified ILC2s are required to confirm the cell intrinsic function of PAG1 in ILC2s.

T-cell activation via the TCR is initiated by SRC kinases.³³ As mentioned above, TCR stimulation results in PAG dephosphorylation. As a result, CSK dissociates from PAG1 and allows SRC kinase activation

resulting in T-cell hyperactivation.^{10,11,34} In the HDM sensitized/challenged mice, PAG1 deficiency increased the numbers of CD4⁺ T_H2 cells (ST2⁺) and the production of IL-5 and IL-13 in the recall response. To resolve whether the elevated T_H2 response was a consequence of T cell-specific PAG1 deficiency, we adoptively transferred PAG1-sufficient naïve OT-II cells into WT and *Pag1*^{-/-} recipients and subsequently exposed the mice to HDM and OVA to activate innate immunity and promote T-cell differentiation. To avoid the expansion and contribution of endogenous recipient OVA-specific T cells, mice were euthanized 5 days later. We observed an increase in antigen-specific IL-5 and IL-13 production. Therefore, the elevated type 2 inflammatory response in *Pag1*^{-/-} mice is not dependent on PAG1 deficiency in T cells.

Classically, type 2 and type 1 immunity cross-regulate one another, that is, an increase in type 2 immunity is associated with a decrease in type 1 immunity and vice versa.³⁵ Interestingly, in this study, PAG1 deficiency not only led to increased type 2 cytokines expression but also increased the expression of type 1 cytokine IFN- γ expression. Although we did not explore the mechanism underpinning the elevated IFN- γ response, others have shown that PAG1 directly affects TCR signaling and suppresses IFN- γ expression by T cells.¹⁰

In conclusion, our data demonstrate that PAG1 is a negative regulator of HMGB1 translocation and release in response to HDM exposure. Lower expression of PAG1 promotes allergic sensitization by increasing the recruitment of MoDCs and ILC2s, which promote the differentiation of T_H2 cells. As a consequence, T_H2 responses and granulocytic airway inflammation are enhanced during the challenge phase. Our data imply a novel function of PAG1 in limiting HDM-induced type 2 inflammation in the lung and highlight PAG1 signaling as a potential novel therapeutic target for asthma.

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CONFLICTS OF INTEREST

The authors have no relevant conflicts of interest.

AUTHOR CONTRIBUTIONS

MAU designed and performed the work, analyzed and interpreted the data, and wrote the paper. CTV, NC, BC, MAAS, IS, and JS performed the work and analyzed the data. AV and JAL provided reagents and expert advice on experimental design. MARF and SP designed and supervised the work, contributed to study design and data interpretation, and edited the paper.

ETHICAL APPROVAL

All animal procedures were performed in accordance with the approval of the Animal Ethics Committee of QIMR Berghofer Medical Research Institute (ethics number P2315 A1706-607M).

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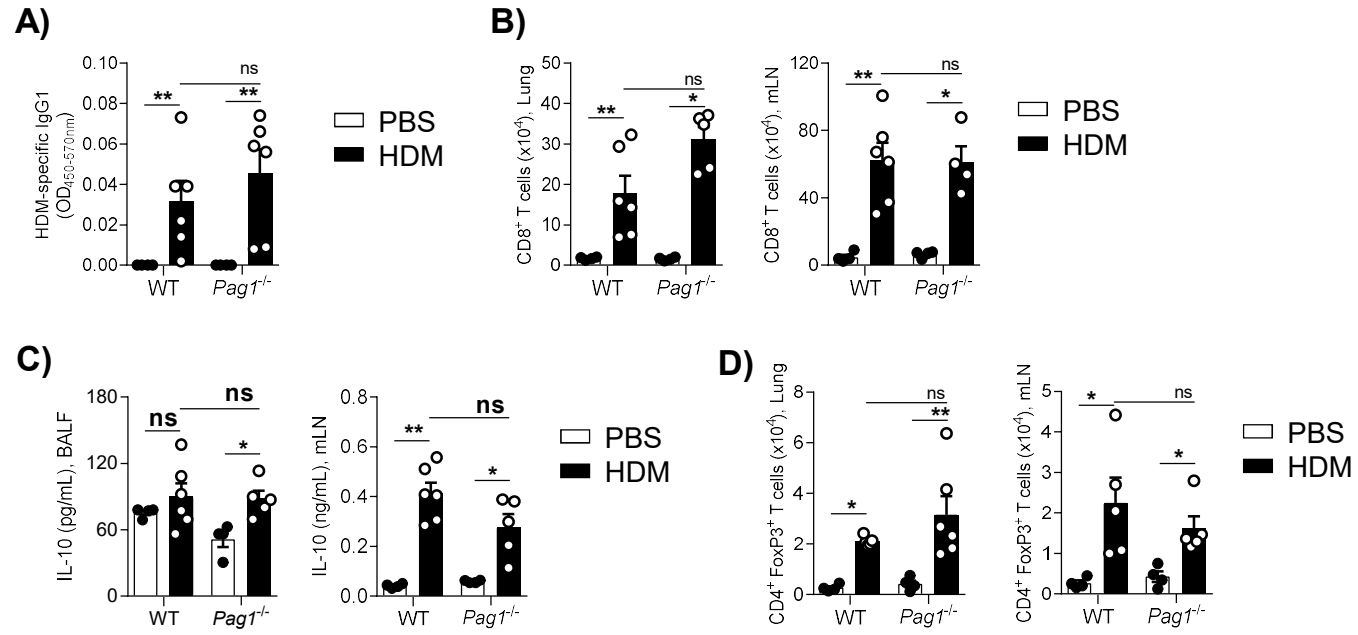
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SUPPORTING INFORMATION

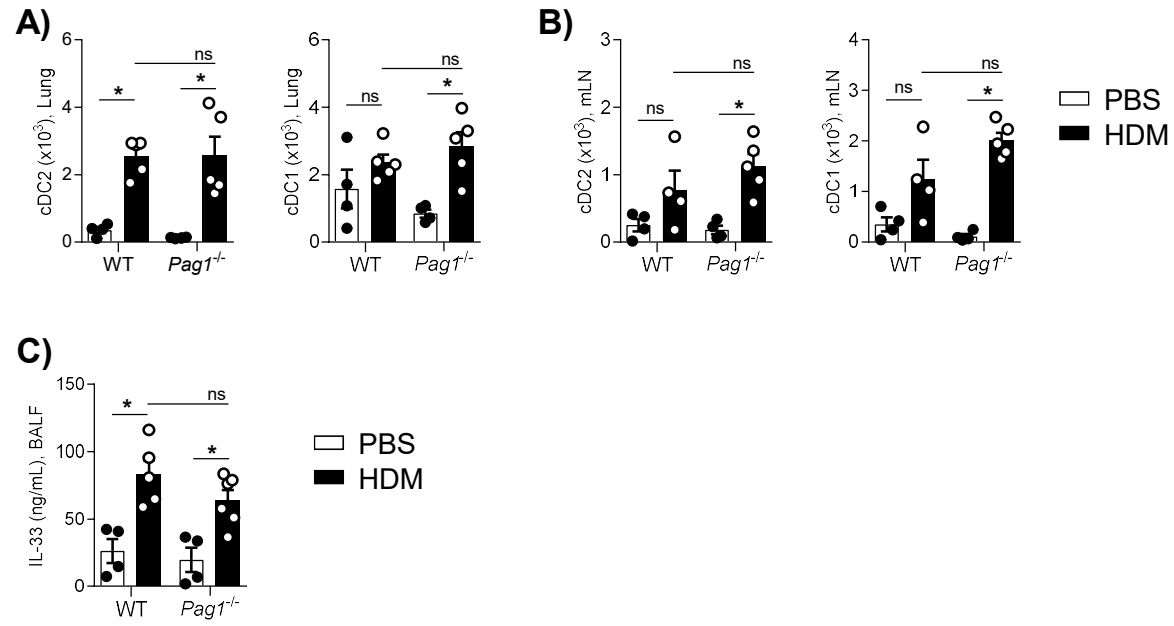
Additional supporting information may be found online in the Supporting Information section at the end of the article.

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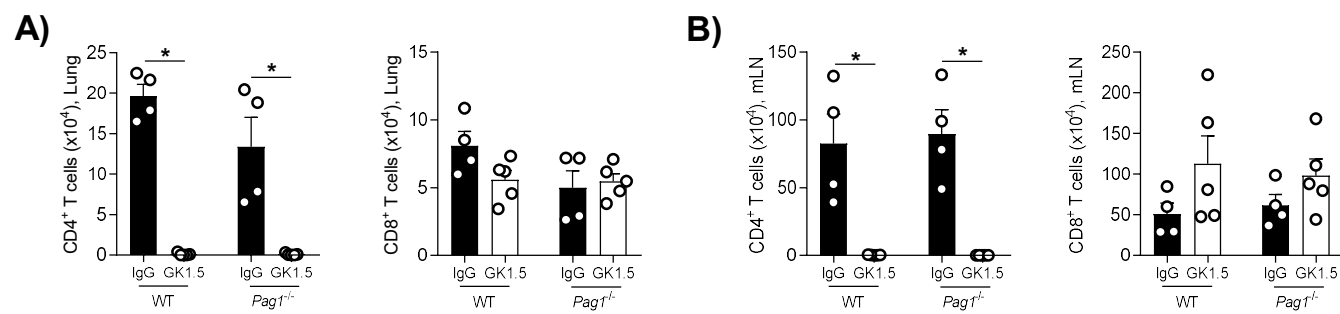
Supplementary Fig 1:



Supplementary Fig 2:



Supplementary Fig 3:



Supplementary Table

Table S1. Antibodies used for flow cytometry

Antibody	Conjugate	Clone	Supplier
Anti-mouse CD11b	PerCP-Cy5.5	M1/70	BD Biosciences, USA
Anti-mouse CD11b	FITC	M1/70	Miltenyi Biotech, Germany
Anti-mouse CD4	V500	RM4-5	BD Biosciences, USA
Anti-mouse CD90.2	APC-Cy7	53-2.1	BD Biosciences, USA
Anti-mouse Ly6G	FITC	1A8	BD Biosciences, USA
Anti-mouse Siglec F	AF647	E50-2440	BD Biosciences, USA
Anti-mouse B220	BV421	RA3-6B2	Biolegend, USA
Anti-mouse CD103	PE	2E7	Biolegend, USA
Anti-mouse CD11c	BV785	N418	Biolegend, USA
Anti-mouse CD11c	FITC	REA754	Miltenyi Biotech, Germany
Anti-mouse CD19	FITC and PE	1D3/CD19	Biolegend, USA
Anti-mouse CD25	BV650	PC61	Biolegend, USA
Anti-mouse CD3 ϵ	PE	145-2C11	Biolegend, USA
Anti-mouse CD3 ϵ	PE	145-2C11	eBioscience, USA
Anti-mouse CD3 ϵ	FITC	17A2	Miltenyi Biotech, Germany
Anti-mouse CD45	BV785	30-F11	Biolegend, USA
Anti-mouse CD8	PerCP-Cy5.5	53-6.7	Biolegend, USA
Anti-mouse CD64	BV421	X54-5/7.1	Biolegend, USA
Anti-mouse F4/80	FITC and PE	BM8	Biolegend, USA
Anti-mouse I-A/I-E	APC-Cy7	M5/114.15.2	Biolegend, USA
Anti-mouse NK1.1	FITC	PK136	Biolegend, USA
Anti-mouse ST2	APC	DIH9	Biolegend, USA
Anti-mouse TCR β	BV605	H57-597	Biolegend, USA
Anti-mouse TCR V β 5.1,5.2	APC	MR9-4	Biolegend, USA
Anti-mouse TCR V α 2	PerCP-Cy5.5	B20.1	Biolegend, USA
Anti-mouse CD45R	PE	RA3-6B2	eBioscience, USA
Anti-mouse CD45R	FITC	RA3-6B2	Miltenyi Biotech, Germany
Anti-mouse Gr-1	FITC	RBC-8C5	Miltenyi Biotech, Germany
Anti-mouse GATA3	PE	TWAJ	eBiosciences, USA
Anti-mouse FoxP3	APC, PE	MF23	BD Biosciences, USA

Supplementary figure legends

Supplementary figure 1. PAG1 deficiency does not affect serum IgG1 expression and the number of CD8⁺ T cells following HDM sensitization/challenge.

WT and *Pag1*^{-/-} mice were sensitized intranasally with 10 µg of HDM at day 0 and challenged intranasally at days 14, 15, 16 and 17 with 3 µg of HDM. BAL fluid and lung tissues were harvested 3 hours after the final challenge. (A) Serum level of HDM-specific IgG1. (B) Number of CD8⁺T cells in the lungs and mLNs. (C) Concentration of IL-10 in BALF and mLN cultures. (D) Number of regulatory T cells (FoxP3⁺CD4⁺ CD3⁺) in the lungs and mLNs. Results are shown as mean ± SEM of n= 4-6 mice per group of a single experiment and are representative of two experiments. **P*<0.05, ***P*<0.01 and ns = not significant.

Supplementary figure 2. PAG1 deficiency does not affect the number of cDC1 and cDC2 and IL-33 expression following HDM sensitization.

WT and *Pag1*^{-/-} mice were intranasally sensitized with 10 µg of HDM at day 0 and endpoints were analyzed 3 days later. (A-B) Number of cDC2 (CD11c^{Hi}MHCII⁺CD11b⁺CD103⁻CD64⁻) and cDC1 (CD11c^{Hi}MHCII⁺CD11b⁻CD103⁺CD64⁻) in the lungs and mLNs. (C) IL-33 concentration in BALF. Results are shown as mean ± SEM of n= 4-6 mice per group of a single experiment and are representative of two experiments. **P*<0.05 and ns = not significant.

Supplementary figure 3. Number of CD4⁺ and CD8⁺ T cells following CD4⁺T cell depletion during the sensitization phase.

WT and *Pag1*^{-/-} mice were administered a CD4⁺T cell depletion antibody GK1.5 or IgG control antibody. Twenty four hours later, mice were sensitized intranasally with 100 µg of HDM at day 0, 1 and 2, and endpoints were analysed at day 5. (A-B) Number of CD4⁺T and CD8⁺T cells in the lungs and mLNs. Results are shown as mean ± SEM of n= 4-5 mice per group of a single experiment and are representative of two experiments. **P*<0.05, ***P*<0.01 and ns = not significant.