Supplemental Methods

Asthma Histology Grading:

Hematoxylin- and -eosin stained lung slides were digitally scanned then randomly examined by a pathologist blinded to experimental arms or the mouse genotype. The scoring system used was adapted from the model proposed by Furness (1). Briefly, the following nine parameters were assessed: smooth muscle hyperplasia/hyperthrophy, inflammation, globet cell hyperplasia, alveolar septa thickening, vascular thickening, peribronchiolar fibrosis, degree of airway accumulation, degree of airway contraction, perivascular lymphoid aggregates. Each parameter was given a score of 0 (for no change), 1 (mild change), 2 (moderate change), and 3 (intense change). The reported score is a sum of the aforementioned parameters.

Immunostaining Lungs that had not been subjected to a bronchoalveolar lavage were inflated with 10% formalin/phosphate buffer (Fisher, Pittsburg PA) at constant pressure (25cmH_2O) and fixed overnight at room temperature. Paraffin-embedded sections (5μm thick) were stained at 4°C overnight with a rabbit anti-5-lipoxygenase antibody (1:125; catalog number sc-20785, Santa Cruz Biotechnology Inc., Dallas, TX), a rabbit anti-CLCA3 antibody (1:12500; catalog number Ab46512, Abcam, Cambridge, MA) or a mouse anti-Mucin 5AC (MUC5AC) antibody (1:1000; catalog number Ab3649, Abcam); followed by incubation with a HRP-conjugated goat-anti rabbit secondary antibody (1:500; catalog number PI-1000, Vector Laboratories, Burlingame, CA) or a goat anti-mouse where indicated (1:200; catalog number BA-9200 Vector Laboratories). Lungs sections were also stained with a goat anti-PIGF antibody (1:500; catalog number sc-1882, Santa Cruz Biotechnology Inc.) at 4°C, followed by incubation with a HRP-conjugated horse-anti goat (1:200; catalog number AP-9500-1, Vector Laboratories). All sections were counterstained with hematoxylin. Digital images of stained sections were captured using a Leica microscope (Buffalo Groove, IL).

Bronchoalveolar lavage To collect the bronchoalveolar lavage fluid (BALF), lungs were lavaged following tracheostomy with 1ml of 1x Hank's balanced salt solution (HBSS). BALF was depleted of cells by centrifugation at 2000 rpm at 4℃; supernatant was stored at -80℃. Cell pellets were resuspended in 1xPBS/ 2% BSA solution and counted using an automated cell counter (Hemavet, Drew Scientific, Waterbury CT). For the differential count, the remaining BAL cells were cytopspun on slides (Shandon Lipshaw, Pittsburgh, PA) and stained with the Diff-Quick solution (Fisher Scientific, Pittsburg PA); an average of two hundred cells were enumerated per slide by an examiner blinded to the experimental details.

Western blot and MUC5AC ELISA Lungs were harvested 48 hours after the last HDM challenge and stored at -80C. Proteins from lung homogenates were analyzed by SDS page electrophoresis and blotted with the anti Clca3 (catalog number Ab46512, Abcam) and GAPDH antibodies, respectively. The software My Image Analysis (Thermo Scientific) was used to perform quantification of bands.

To quantify Muc5AC in BALF collected 48 hours post HDM challenge, immunoassay plates were coated with 1ug/ml of capture antibody (anti-Muc5AC (K20) goat polyclonal (catalog number sc-16903, Santa Cruz Biotechnology Inc.) in 1X saline and incubate overnight at 4°C. Plates were blocked with 1X Carbo-free blocking solution (Vector Laboratories, Burlingame, CA) at room temperature for 1 hour, and incubated with serial dilutions of Carbo-free diluted BALF samples overnight at 4°C. Following a 1 hour incubation with biotinylated wheat germ agglutinin (diluted in 1X Carbo-free blocking solution) at room temperature, Muc5AC was detected with streptavidin–HRP conjugated antibody (kit catalog number 555248, BD Biosciences, San Diego, CA) added to the wells for an hour-long incubation at RT. Unbound reagent was washed out at each step with a 0.05% Tween/1X saline solution. Subsequently, TMB substrate solution (BD Biosciences, San Diego, CA) was added to the wells and incubated for approximately 5 minutes until color

developed. The enzymatic reaction was stopped with a 2N sulfuric acid solution and the plate was read using a spectrophotometer set at 450nm.

HDM-specific IgE Levels of HDM-specific IgG and IgE were measured in the plasma collected after the last HDM challenge using ELISA. ELISA was performed as follows: wells were coated with 0.01% HDM overnight, and then blocked with 1%BSA /1XPBS solution for an hour at room temperature (RT). Diluted samples were loaded onto plates and incubated for an hour, followed by an hour incubation with either a biotinylated rat anti-mouse IgE (clone R35-118) or IgG (clone A85-1) antibody (catalog numbers 553419 and 550331, BD Biosciences). Subsequently, streptavidin–HRP conjugated antibody (kit catalog number 555248, BD Biosciences) was added to the wells for an hour incubation at RT. Excess unbound reagent at each step was removed with washes with a 0.1% Tween/1X saline solution. HRP substrate solution (BD Biosciences) was added to wells and incubated for approximately 5 minutes until color developed. The enzymatic reaction was stopped with a 2N sulfuric acid solution and the plate was read using a spectrophotometer set at 405nm.

Cytokine analysis

Cytokines in the BALF and plasma were quantified using a multiplex biomarker panel (Chemokine Panel I) and Luminex xMAP technology (Millipore, Billerica, MA), following the manufacturer's instructions. Total BALF IL13 levels (free and IL13Ra2–bound) were measured as followed. Briefly, wells were coated with 50µl of anti-IL13 (4µg/ml; AF-413-NA, R&D Systems, Minneapolis, MN) overnight at 4^0C. After all free IL13 present in BALF samples is complexed to recombinant IL13Ra2/Fc chimera (10ng/ml; R&D 539-IR) following 1h incubation at 37^0C, samples are added to wells and incubated for 2h at room temperature, followed by a 1h incubation with biotinylated IL13Ra2 (100ng/ml; R&D BAF539) and a 30min incubation with Steptavidin-HRP (1/200; R&D DY998). Finally, TMB substrate (BD OptEIA, BD Biosciences) is added to each well and incubated

in the dark for 15-25min. Reaction is stopped with 2N sulphuric acid and changes in OD measured at 450nm.

Leukotriene analyses Urinary cysteinyl leukotrienes (CysLTC$_4$, D$_4$, E$_4$) levels were extracted using an affinity column (Cayman Chemicals, Ann Arbor, MI). Tritium-labeled LTD$_4$ was spiked in to assess the percentage of sample recovery after extraction. CysLT extracts were measured using ELISA kits (Cayman Chemicals), according to the manufacturer's instructions. Urinary creatinine levels were assayed using a chemical assay (R&D) and served as internal reference for the normalization of CysLT amounts in each sample.

Flow cytometry Lungs from chimeric C57/BL6 and HbS, and BALB/c mice were harvested 48 hours after HDM challenge. Cells were incubated at 37℃ for 45 min in DMEM/DNAse media containing 1mg/ml of dispase/collagenase (Roche). Lung cells were mechanically disrupted by passage through a 70-μm cell strainer and mashing with a syringe rubber. Cells were centrifuged and resuspended in DMEM, and cell viability was assessed using trypan blue exclusion dye. Lung single cell suspensions from chimeric mice were stained with CD45.1 (clone A20, catalog number BD553775, BD Biosciences) and CD45.2 antibodies (clone 104, catalog number BD558702, BD Biosciences) and a live dead dye (Aqua; ref L34957; Invitrogen, Grand Island, NY) to exclude dead cells.

To characterize the repertoire of lung immune cells in BALB/c and chimeric mice, T cells were stained with a panel of fluorescently labelled antibodies, CD3 (clone 17A2; catalog number 100216; BioLegend, San Diego, CA), CD8b (clone YTS156.7.7; catalog number 126610; BioLegend), CD4 (clone RM4-5; catalog number 100510 or 100528; BioLegend), and CD44 (clone IM7; catalog number 103020; BioLegend). Intracellular staining with an IL-13 antibody (clone eBio13A; ref 12-7133-82; eBioscience) identified Th2 cells gated out of

$CD44^+/CD4^+$ cell population. Dendritic cells (DC) were identified as MHCII+ (I-A/I-E; clone M5/114.15.2; catalog number 107622 BioLegend) and CD11c+(clone N418; catalog number 117312; BioLegend), they were further divided into two subgroups as CD11bhigh (clone M1/70; catalog number 101216; BioLegend) and $CD11b^{low}$, $CD103^+$ (clone 2E7; catalog number 121416; BioLegend). Inflammatory cells in the lungs, BAL cell pellets and blood were stained with antibody against the following cell subsets: eosinophils were identified as CD45+ (clone 30-F11, catalog number BD553080, BD Biosciences), Siglec F^{high} (clone E50-2440, catalog number BD552126, BD Biosciences), $CD11b^{high}$ (clone M1/70, catalog number BD552850, BD Biosciences), and $CD11c^-$ (clone HL3, catalog number BD561119, BD Biosciences); alveolar macrophages were identified by $F4/80^+$ (clone BM8, catalog number 123108, BioLegend), $CD11b^-$ and $CD11c^+$, and $MHC\text{-}II^+$ (clone M5/114.15.2, catalog number BD562363, BD Biosciences). Sorted lung eosinophils and macrophages were collected in RNase free- solution for further Q-PCR analysis. Fluorescently labelled antibodies were purchased from BD Biosciences and e BioLegend. The labeled cells were acquired on a 6-color FACSCanto flow cytometer and their analysis conducted on BD FACSDiva software (Becton-Dickinson Corporation, Mountainview, CA).

In vitro HDM 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 150,000 cells/well in duplicate or triplicate. The cells were then cultured in the presence of HDM (30µg/ml) for 5 days. Supernatants were collected and replicates were pooled before being stored at -20C.

Quantitative RT-PCR RNA was extracted from mouse whole lungs using qiazol (Qiagen) solution. For the quantification of PlGF expression in sorted lung inflammatory cells, RNA was isolated with RNA-STAT from alveolar macrophages and eosinophils. RNA extracts from total bone marrow cells were used as positive control for *PlGF* expression. *PlGF*, *5-LO*, *FLAP*, *Cysltr1*

and β-actin mRNA expression was assessed by real time quantitative PCR using custom TaqMan assays (Applied Biosystems). cDNA extracts from human nasal epithelial cells (2), were used to assess the expression of human PIGF using a Taqman custom assay (Applied Biosystems).

1. Furness MC, Bienzle D, Caswell JL, DeLay J, and Viel L. Immunohistochemical Identification of Collagen in the Equine Lung. *Veterinary Pathology Online.* 2010;47(5):982-90.
2. Guajardo JR, Schleifer KW, Daines MO, Ruddy RM, Aronow BJ, Wills-Karp M, and Hershey GKK. Altered gene expression profiles in nasal respiratory epithelium reflect stable versus acute childhood asthma. *Journal of Allergy and Clinical Immunology.* 2005;115(2):243-51.

Supplementary Figures

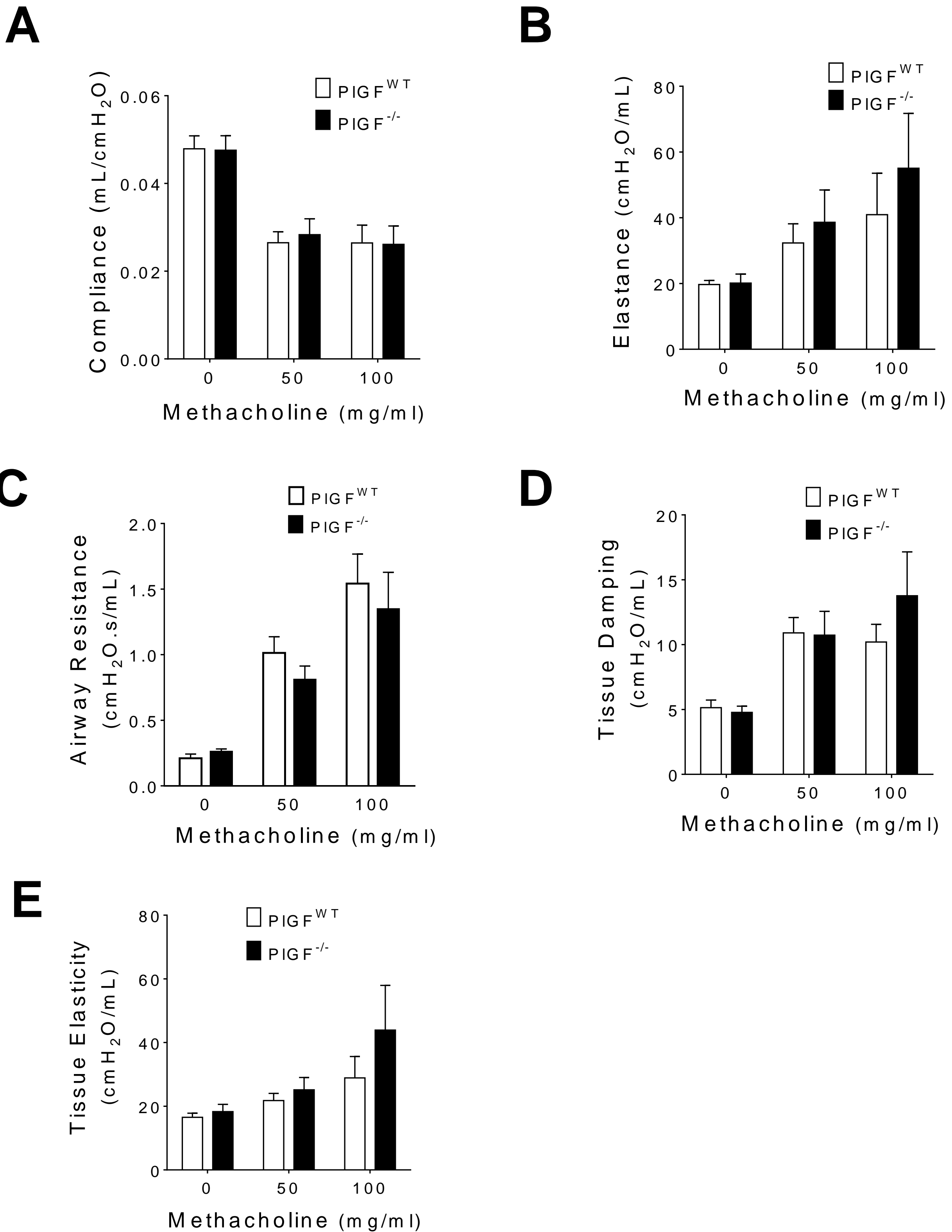
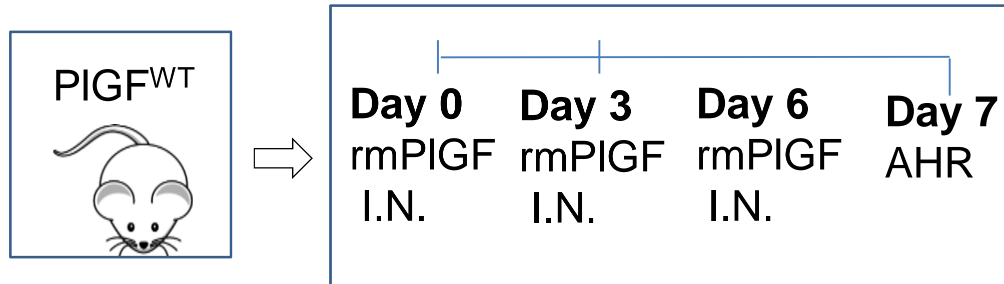


Figure S1. Baseline lung mechanics parameters of BALB/c mice. Lung mechanical parameters were measured using the single-compartment model. **(A)** compliance, **(B)** elastance, **(C)** airway resistance, **(D)** tissue damping an **(E)** tissue elasticity were assessed in unchallenged PlGF^{WT} and PlGF $^{-/-}$ mice in response to increasing concentrations of methacholine. Statistical analysis using two-way-ANOVA showed no significant difference in any groups: 7-10 animals per group. All data are presented as mean ± s.e.m. Results are from two independent experiments.

Figure S2

A



B

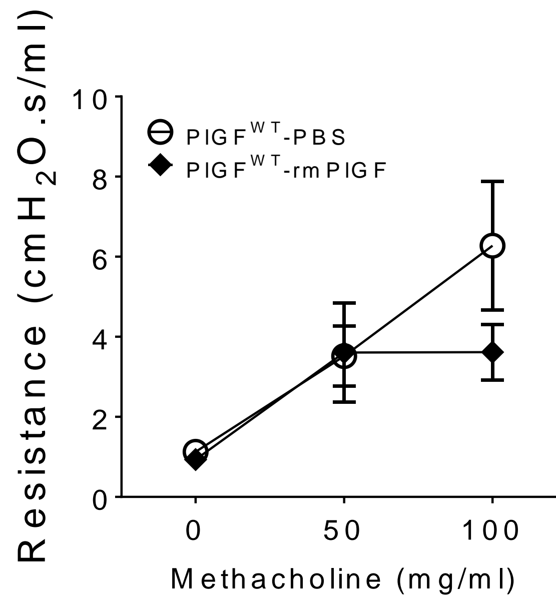
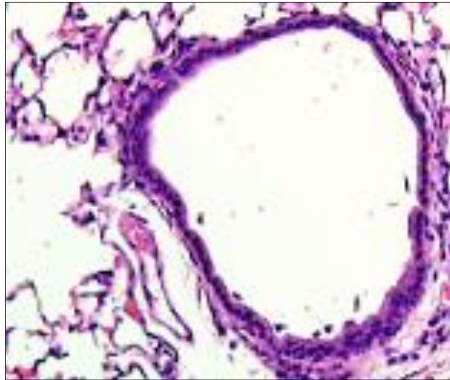
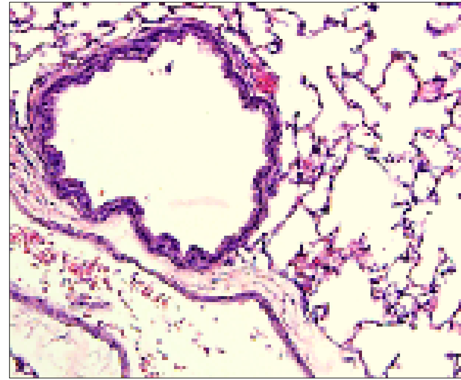


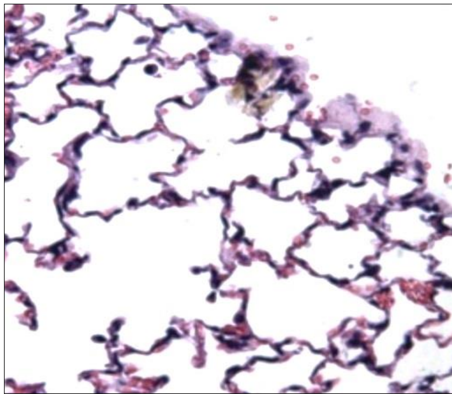
Figure S2. Direct exposure of airways to excessive PIGF is not sufficient to induce AHR in mice BALB/c. AHR was assessed in PIGF^{WT} mice treated with intranasal doses of recombinant mouse PIGF (rmPIGF) or vehicle control (PBS). No significant difference in any groups, two-way ANOVA; 7-10 animals per group. All data are presented as mean ± s.e.m. Results are representative of one experiment.

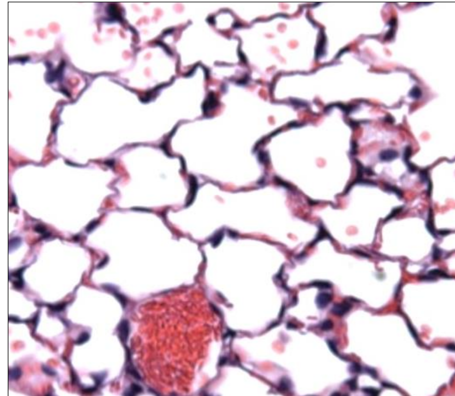
Figure S3

PlGF ^{WT} PlGF $^{-/-}$









Saline

Figure S3. Saline exposed-BALB/c mice do not develop lung inflammation. Representative H&E lung sections of PlGF^{WT} and PlGF$^{-/-}$ mice after IP sensitization and IT challenge with Saline, 20X magnfication.

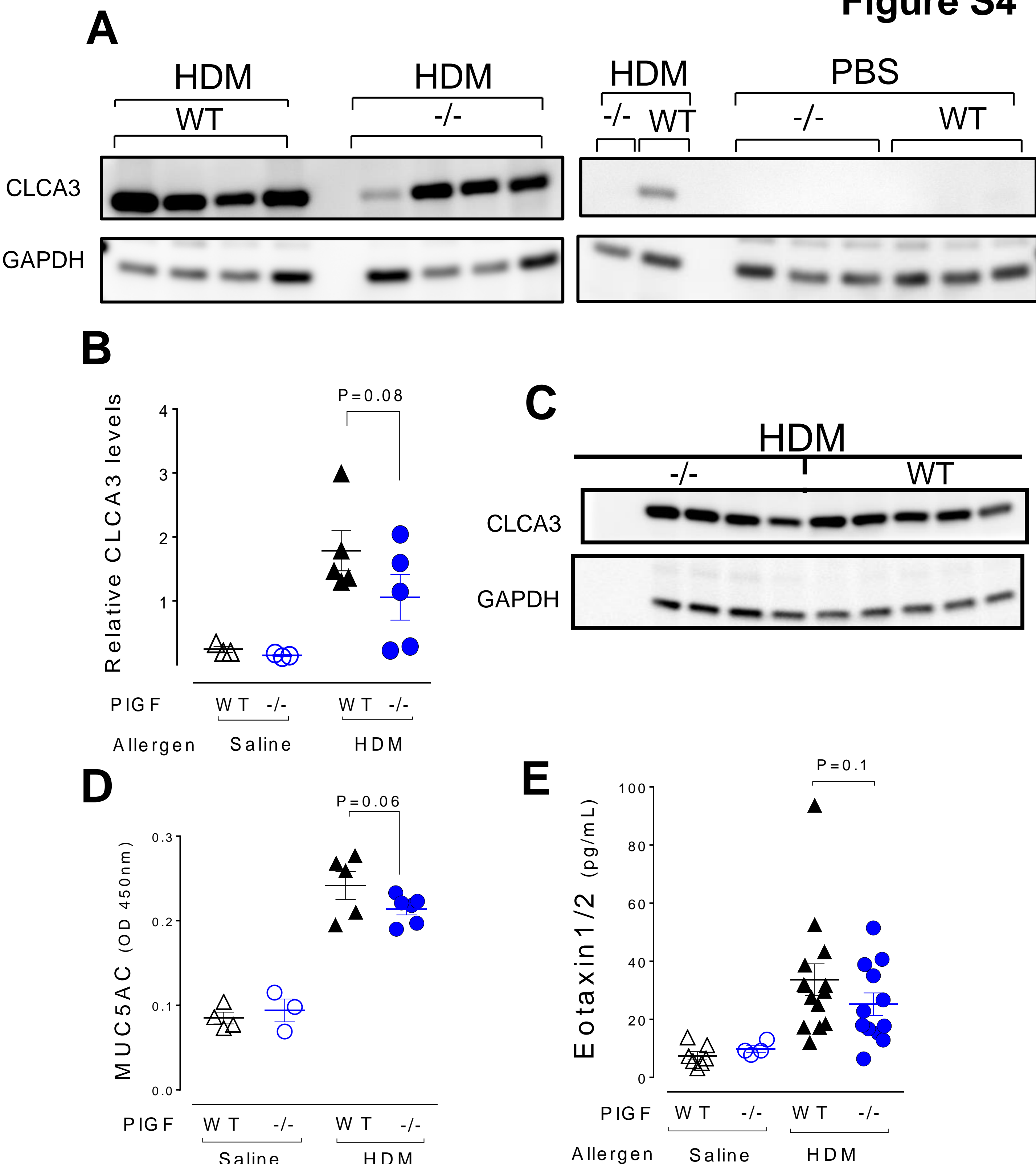
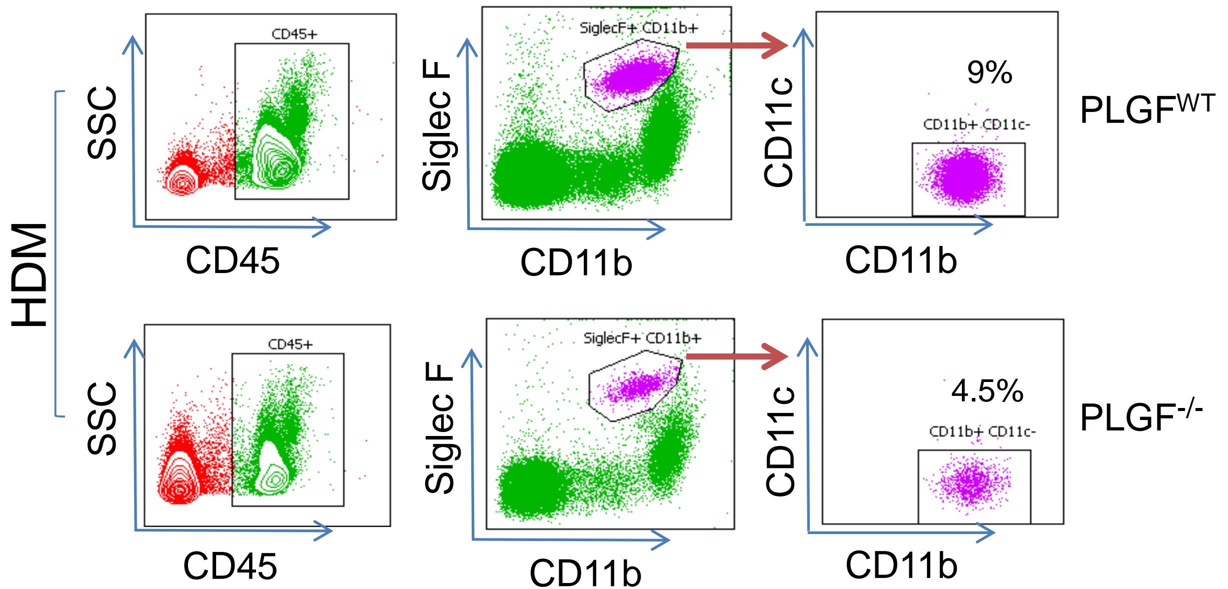


Figure S4. Allergen-specific goblet cell response and eotaxin levels in BALB/c mice. (A) CLCA3 expression in lung homogenates of HDM exposed BALB/c mice. **(B)** Quantification of CLCA3 western blot in A. **(C)** Additional blots showing CLCA3 expression in lungs of HDM exposed BALB/c mice. **(D)** BALF Muc5AC levels in HDM exposed mice measured by ELISA. **(E)** Eotaxin levels measured in the BALF of HDM-exposed BALB/c mice. Each dot represents in an animal. Statistics: Student's t test. All data are presented as mean ± s.e.m. Results are from one experiment (A-B, D), or representative of a distinct single experiment (C). Results in E are representative of two independent experiments.

Figure S5



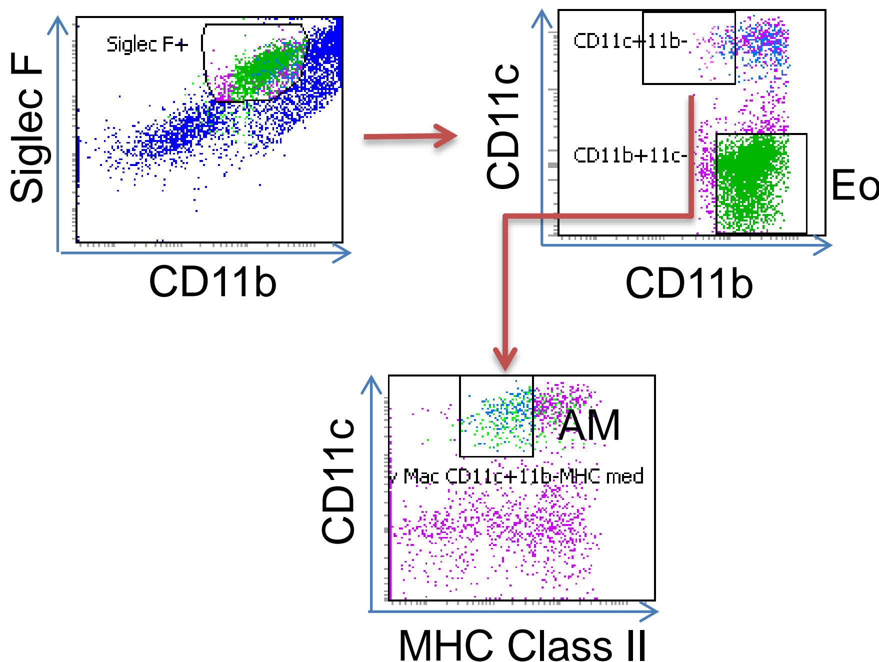
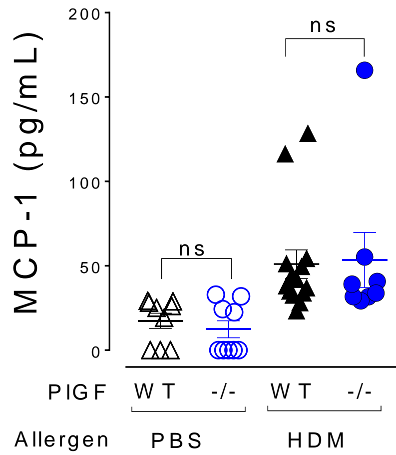
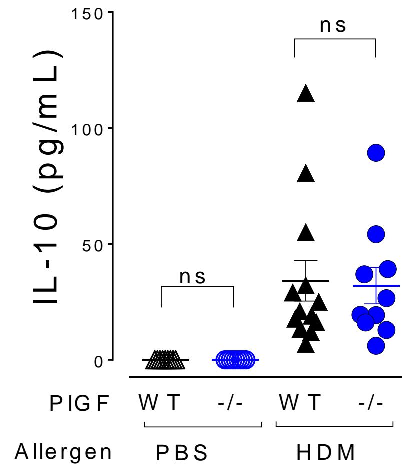
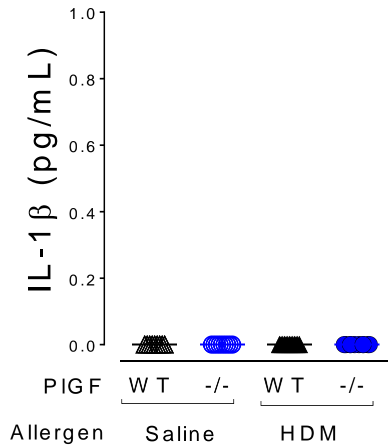


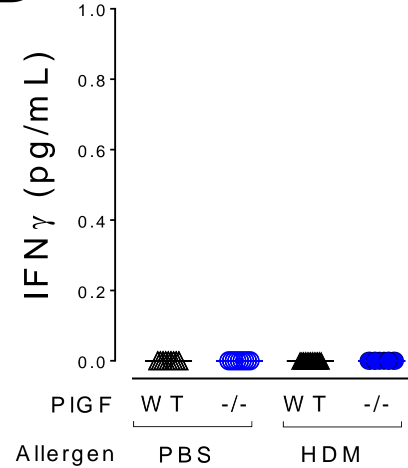
Figure S5. Flow cytometry analysis of blood and lung eosinophils, and alveolar macrophages in HDM-exposed BALB/c mice. (A) Blood eosinophils were gated out of a $CD45^+$ population as $Siglec^+$, $CD11b^+$ and $CD11c^-$ cells. **(B)** Lung eosinophils (Eo) and alveolar macrophages (AM) were isolated from whole lung. Cells were first gated on $F4/80^+$, then Eo were identified as $F4/80^+$, $Siglec^+$, $CD11b^+$ cells; whereas AM were $F4/80^+$, $Siglec^+$, $CD11b^-$, $CD11c^+$, $MHCII^+$.

Figure S6









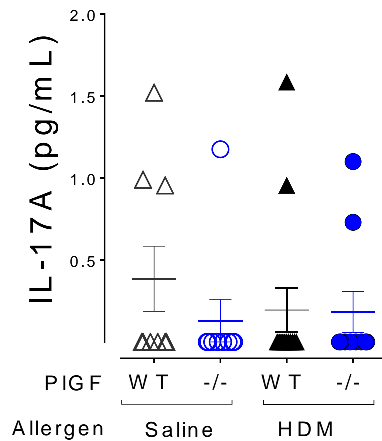


Figure S6. T-cell derived cytokines in BALB/c mice. (A-E) MCP-1, IL-10, IL1-β, IFN-γ and IL-17A cytokines measured in BALF of HDM-exposed BALB/c mice using ELISA. Each dot represents in an animal. No difference was observed in any of the groups (statistics: Mann Whitney test). All data are presented as mean ± s.e.m. Results are from three independent experiments (A-E).

Figure S7

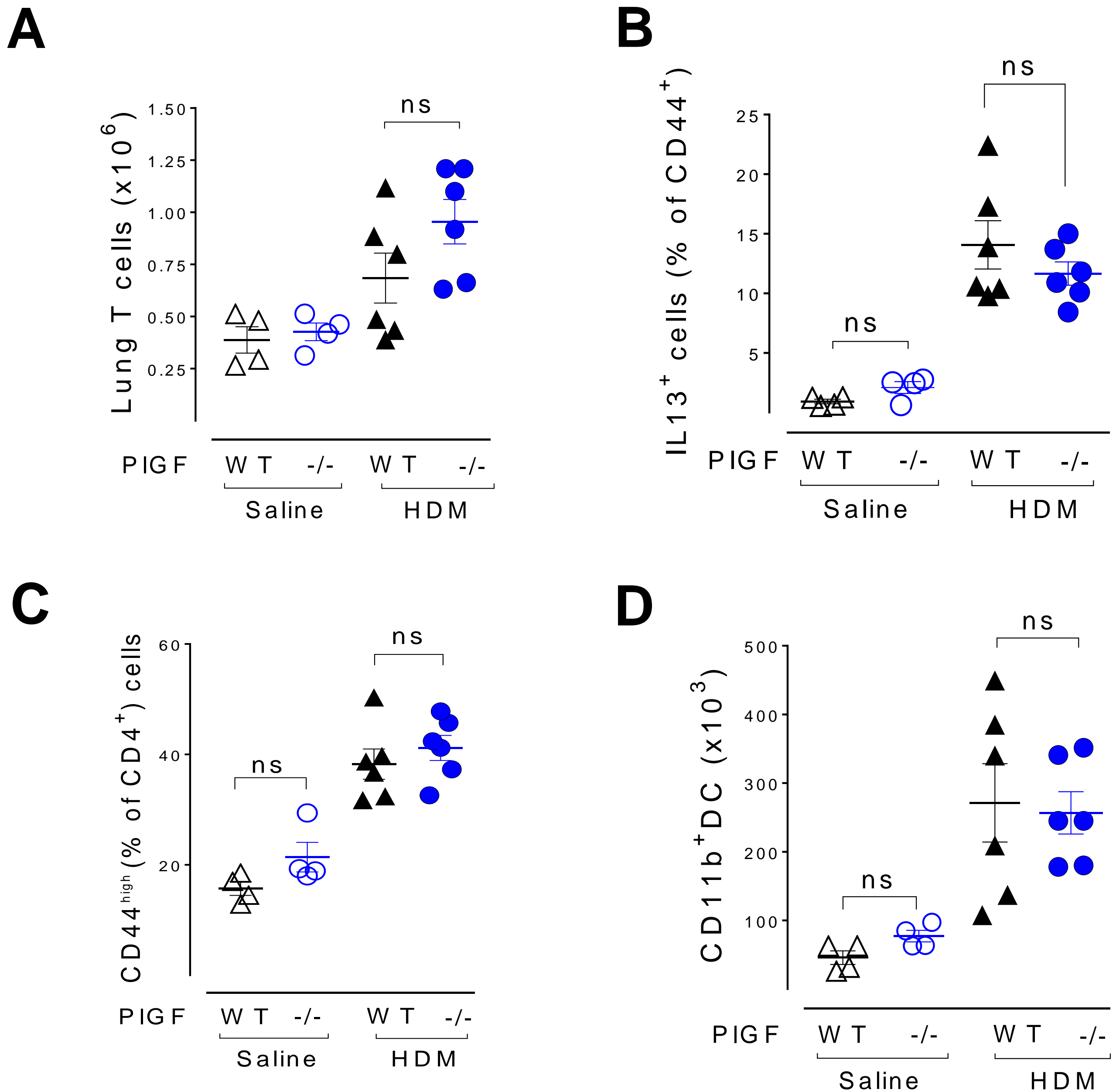


Figure S7. Th2 cells subsets and dendritic cells in BALB/c mice in presence and absence of the allergen. Frequency of lung **(A)** T cells, **(B-C)** effector T cells, and **(D)** dendritic cells (DC). Using total lung cell-count, frequencies were assessed 48 hours after the last HDM or saline exposure by flow cytometry. No significant difference (ns) in any groups, Mann Whitney test; each mouse is represented as a single symbol on the graphs. All data are presented as mean ± s.e.m. Results are representative of one experiment (A-D).

Figure S8

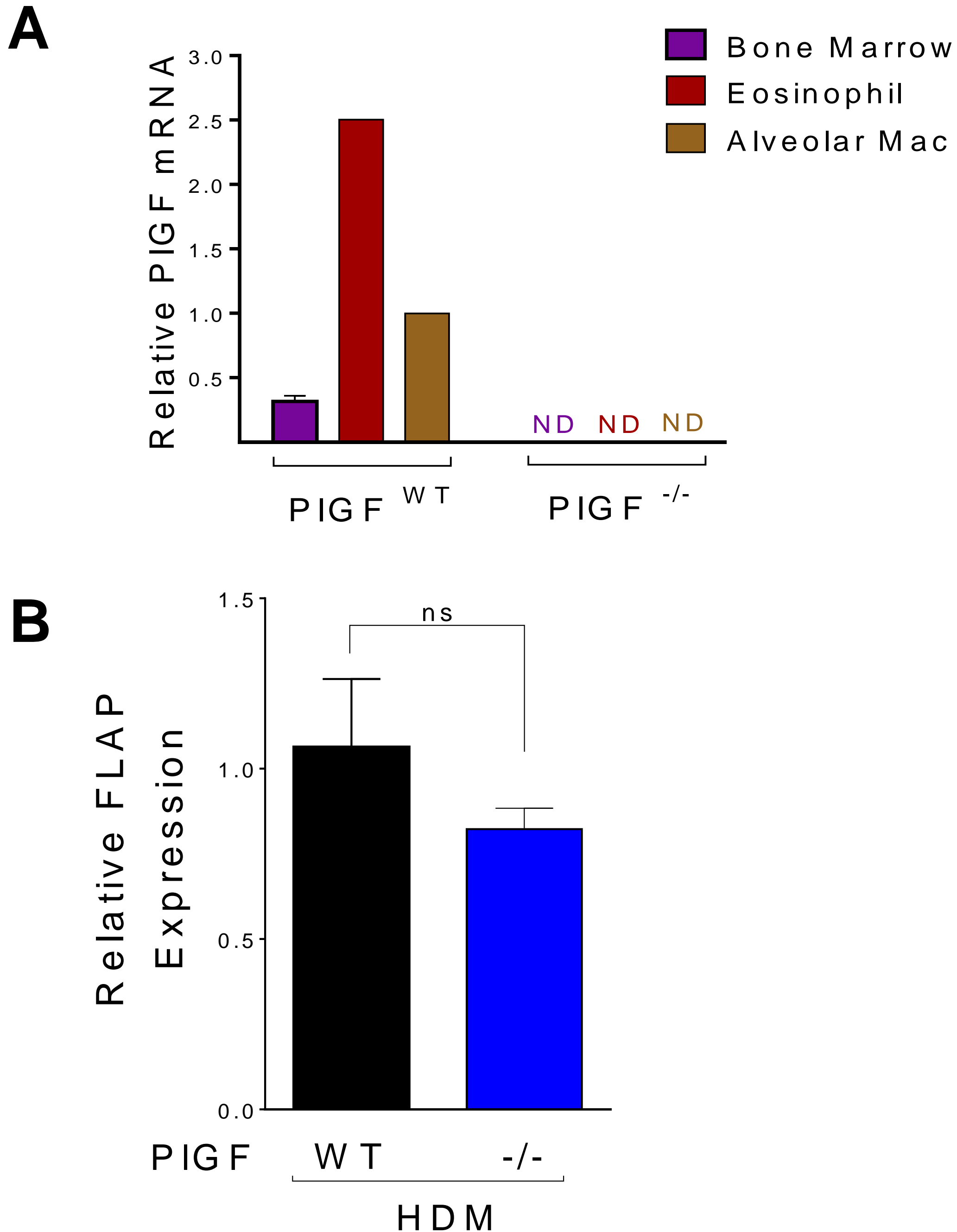
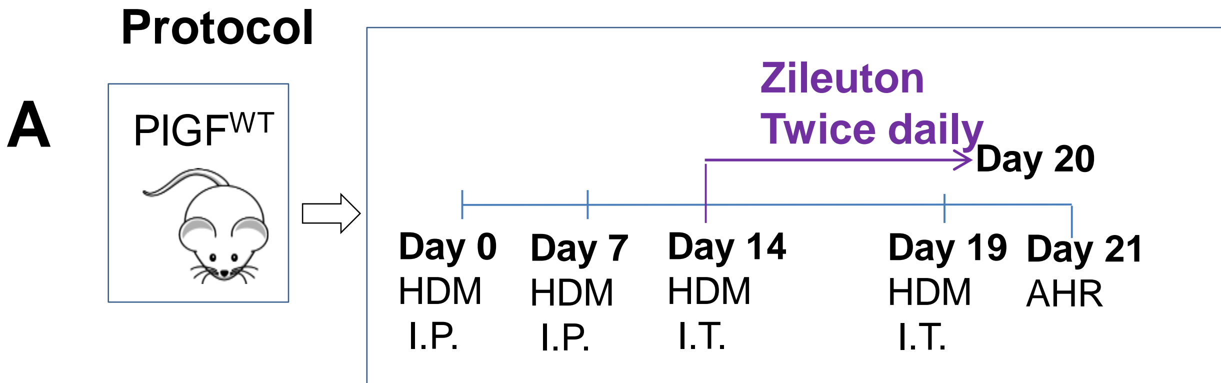


Figure S8. Expression of PlGF and FLAP in lungs of HDM exposed BALB/c mice. (A) Relative PlGF mRNA expression in the sorted lung inflammatory cells and bone marrow (BM) cells of PlGF^{WT} and PlGF$^{-/-}$ following HDM exposure. cDNA samples from each cell population from mice with the same genotype were pooled for the analysis. For the analysis of BM cells, three distinct PlGF ^{WT} mice were used for BM cells analysis. (**B**) Quantification of FLAP mRNA in lungs of HDM exposed BALB/c mice, n=4 mice per group; ns: no statistical difference (Mann Whitney's test). Results are representative of two distinct experiments (A-B).

Figure S9



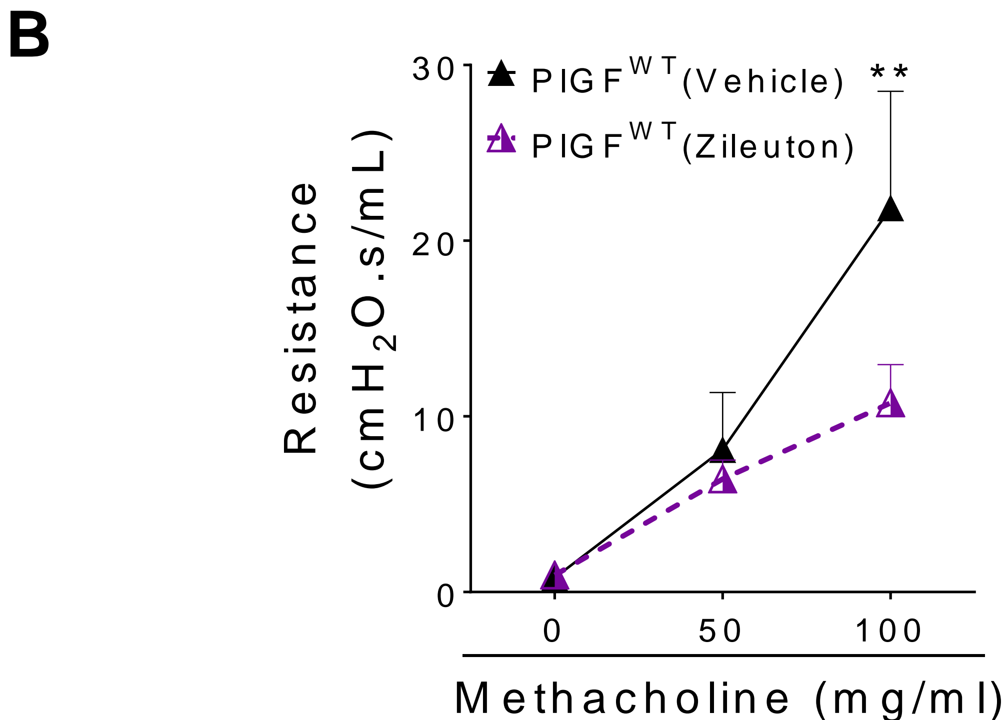


Figure S9. Zileuton reduces AHR in BALB/c mice. (A) Diagram of the induction of experimental AHR in BALB/c mice were sensitized to HDM or PBS intraperitoneally (IP) and then exposed to HDM via intratracheal instillation (IT) at the indicated time points; 48 hours after the last HDM exposure airway responsiveness was measured using the flexivent. Zileuton treatment was initiated at day 14 prior the first IT exposure, and administered twice daily until day 20. **(B)** Airway responsiveness of HDM- exposed PlGF^{WT} mice treated with zileuton; **$p<0.01$, two-way ANOVA; 5-8 animals per group. All data are presented as mean ± s.e.m. Results are representative of one experiment.

Figure S10

A

Cys-LT Rescue Protocol

PlGF^WT (BALB/C)

PlGF -/- (BALB/C)

1ug CystLT (I.T.)

D18 D19 D20

Day 0 HDM I.P.

Day 7 HDM I.P.

Day 14 HDM I.T.

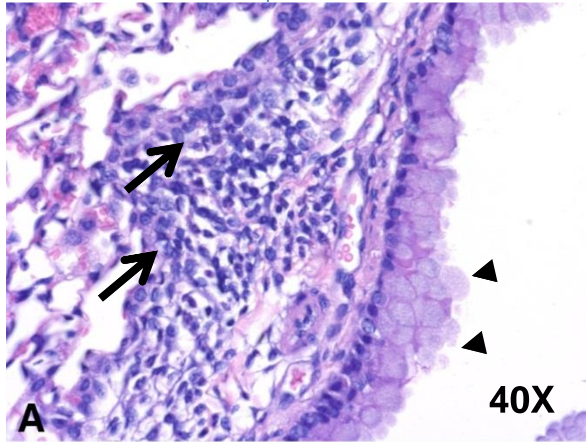
Day 19 HDM I.T.

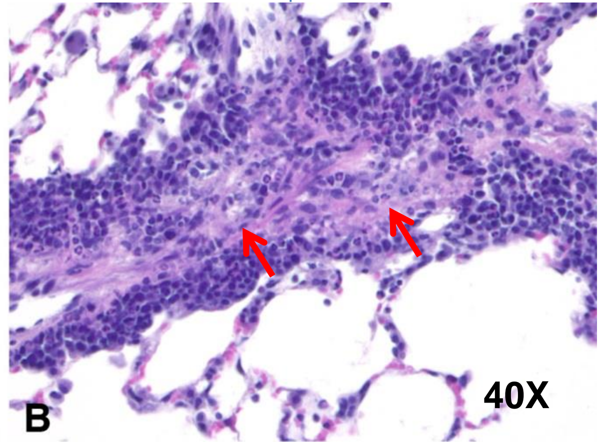
Day 21 AHR

B

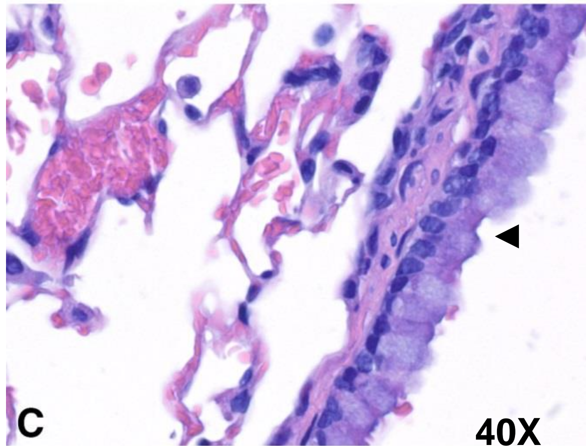
HDM

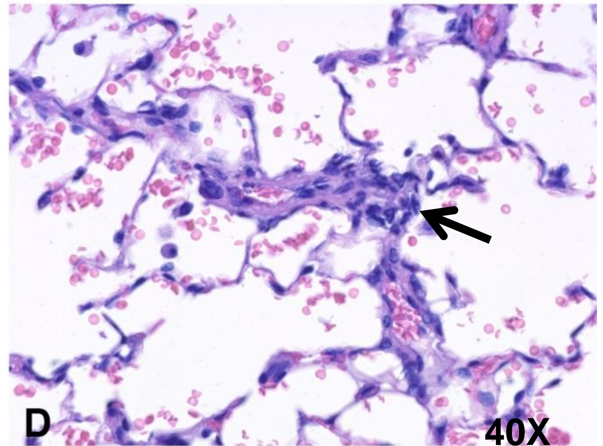
PlGF WT



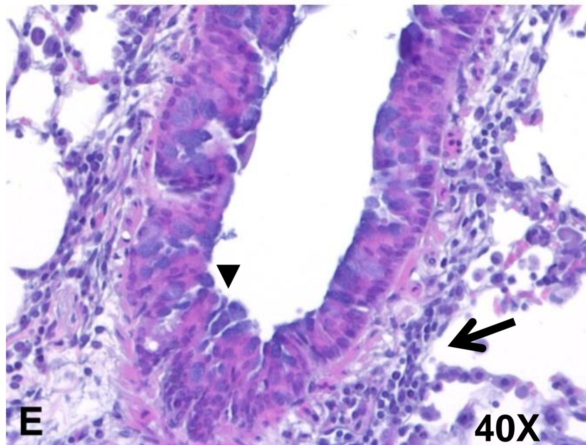


PlGF -/-





PlGF -/- +CysLT



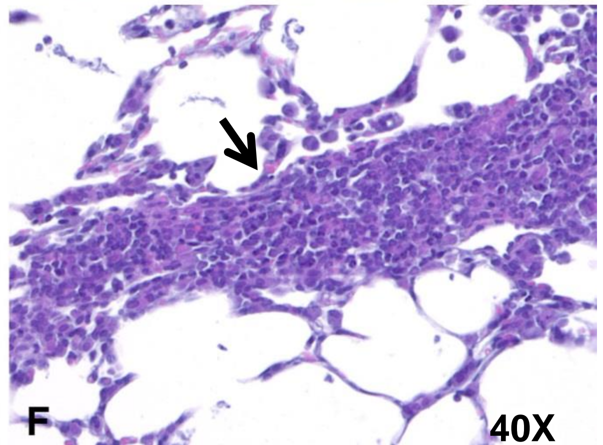


Figure S10

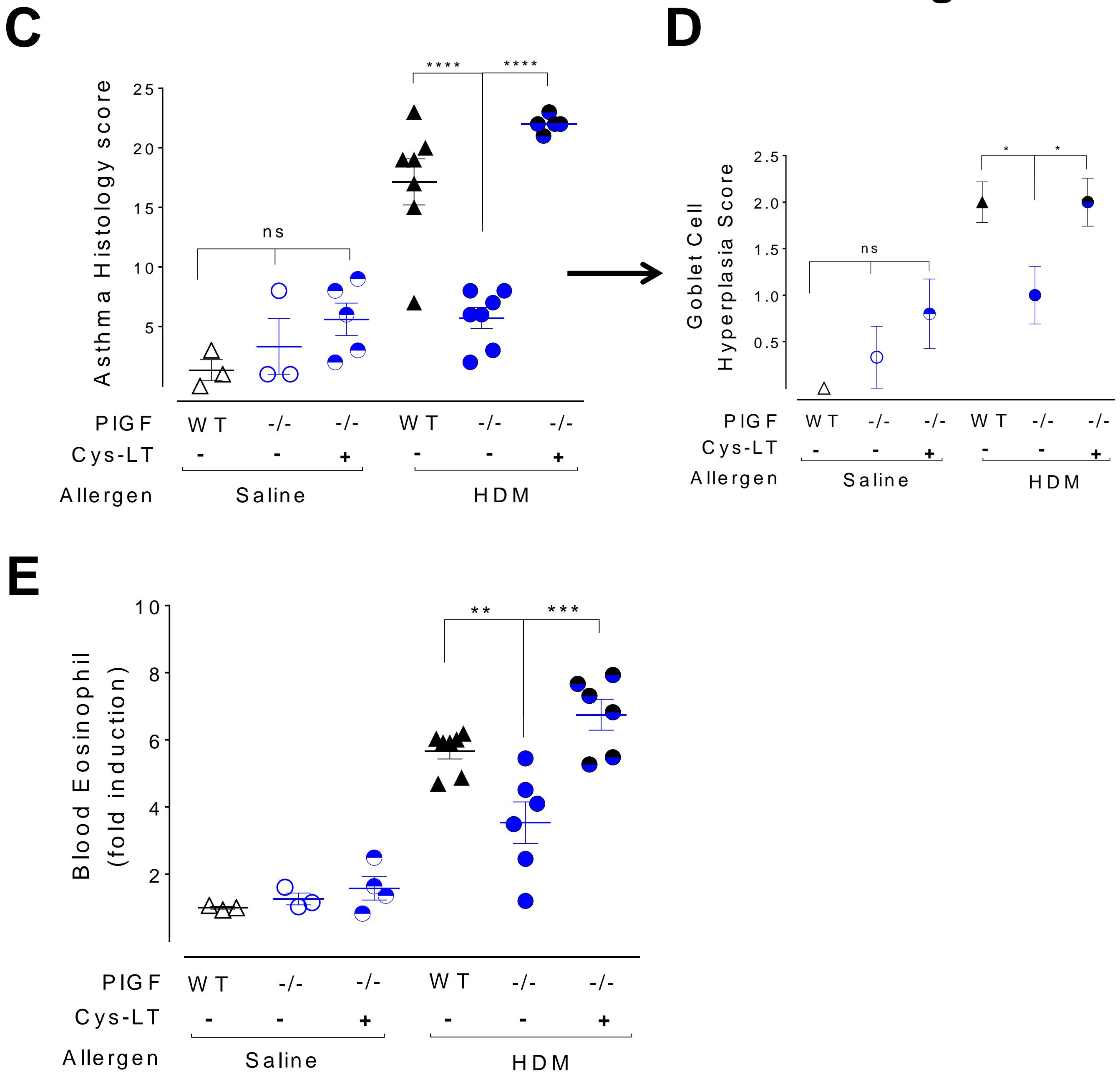
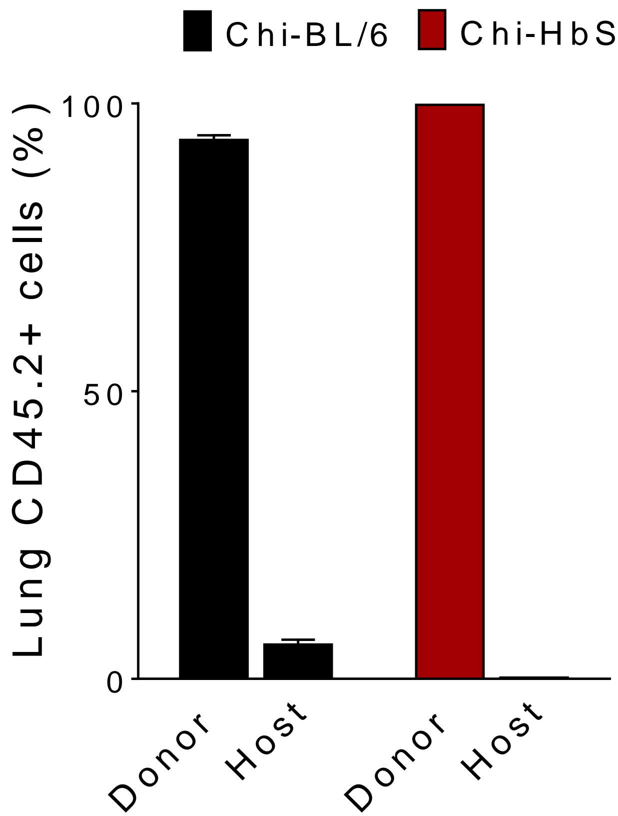
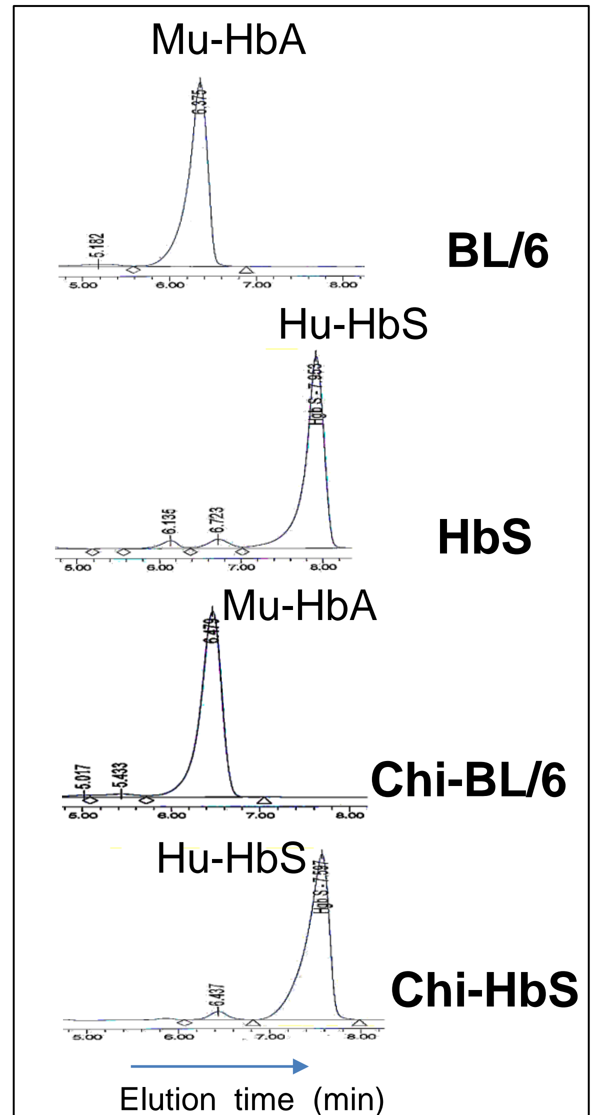


Figure S10. Leukotrienes treatment rescues allergen-induced inflammation in PlGF$^{-/-}$ mice. (A) Schematic diagram depicting the induction of AHR and CysLT treatment of BALB/c mice. **(B)** Representative H&E sections of CysLT- treated PlGF$^{-/-}$ and control treated- PlGF^{WT} and -/- mice showing smooth muscle proliferation, goblet cell hyperplasion and lung infiltrates. (**C**) Histological scoring of the lung inflammation features shown in A in HDM-exposed PlGF$^{-/-}$ and PlGF^{WT} mice treated with vehicle control or CysLT. (**D**) Score of goblet cell hyperlasia score of treated BALB/c mice. This parameter is a distinct parameter of the summed parameters scored in C. Each symbol represents 3 mice in the saline group and 5-6 mice in the HDM group. Data are presented as mean ±sem. **(E)** Fold increase in blood eosinophils counts measured by flow cytometry in vehicle and CysLT –treated PlGF^{WT} and PlGF$^{-/-}$ mice following allergen exposure. Each symbol represents an individual mouse on all graphs but D. *p<0.05,**p<0.01, ***p<0.0005 , ****p<0.0001 (one-way ANOVA). Results are from two experiments (C-D), and representative of a single experiments (E).

Figure S11





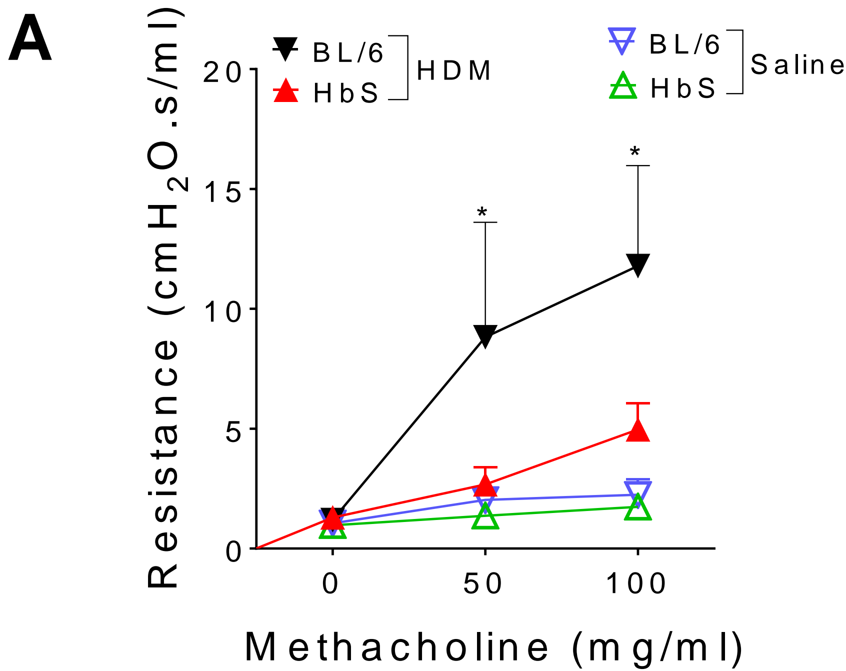
C

Blood Cell Counts

	Chi-BL/6	Chi-HbS
Hemoglobin (g/dl)	12.8 ± 0.19	4.6 ± 0.65
Hematocrit (%)	50.9 ± 1.17	18.4 ± 3.00
Reticulocytes (%)	0.8 ± 0.18	36.9 ± 1.9
WBC (10^3/ul)	4.9 ± 1.62	38.1 ± 7.53

Figure S11. Chimeric HbS mice develop characteristic features of sickle cell disease. (**A**) Donor derived-CD45 chimerism in lungs of chimeric host mice 4 months post transplantation, 4-5 animals per group. (**B**) Representative HPLC profiles showing globin chain composition of red blood cells from chimeric HbS and chimeric BL/6 mice 12 weeks post transplantation; muβ^{major} denotes adult hemoglobin tetramer in mice while Huβ^{S} indicates the human hemoglobin S tetramer. (**C**) Hematological parameters of chimeric mice 16 weeks post transplantation, 8-10 animals per group. All data are presented as mean ± s.e.m. Results are representative of one experiment.

Figure S12



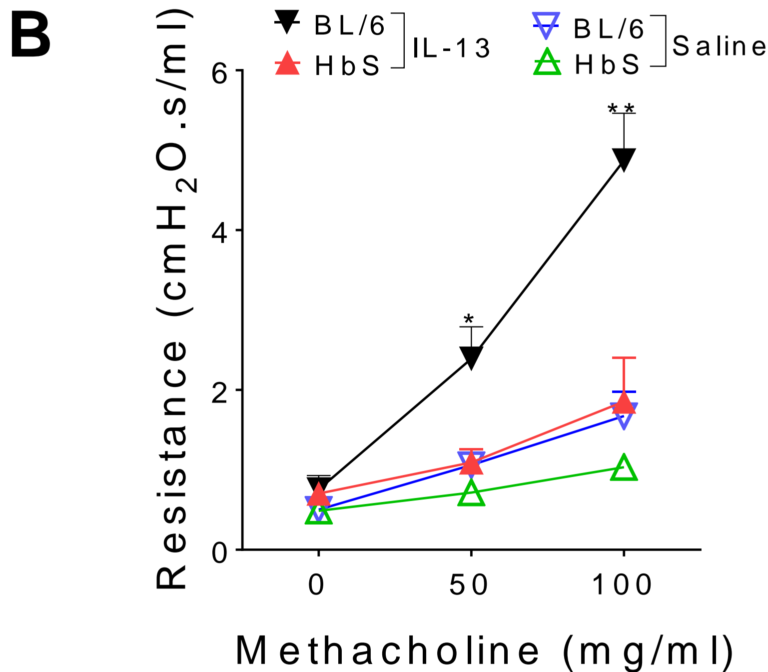


Figure S12. Blunted airway responsiveness in unmanipulated SCD mice.
(A) Airway responsiveness to methacholine of saline or HDM exposed wild- type BL/6 and HbS mice; *$p<0.05$ HDM-exposed BL/6 versus HDM-exposed HbS mice. **(B)** Airway responsiveness to methacholine of BL/6 and HbS mice following IL-13 or saline treatment; *$p<0.05$, ** $p<0.01$ IL-13-treated BL/6 versus IL-13-treated HbS, two-way ANOVA; 5-6 animals per group. Results are representative of two separate experiments (A-B).

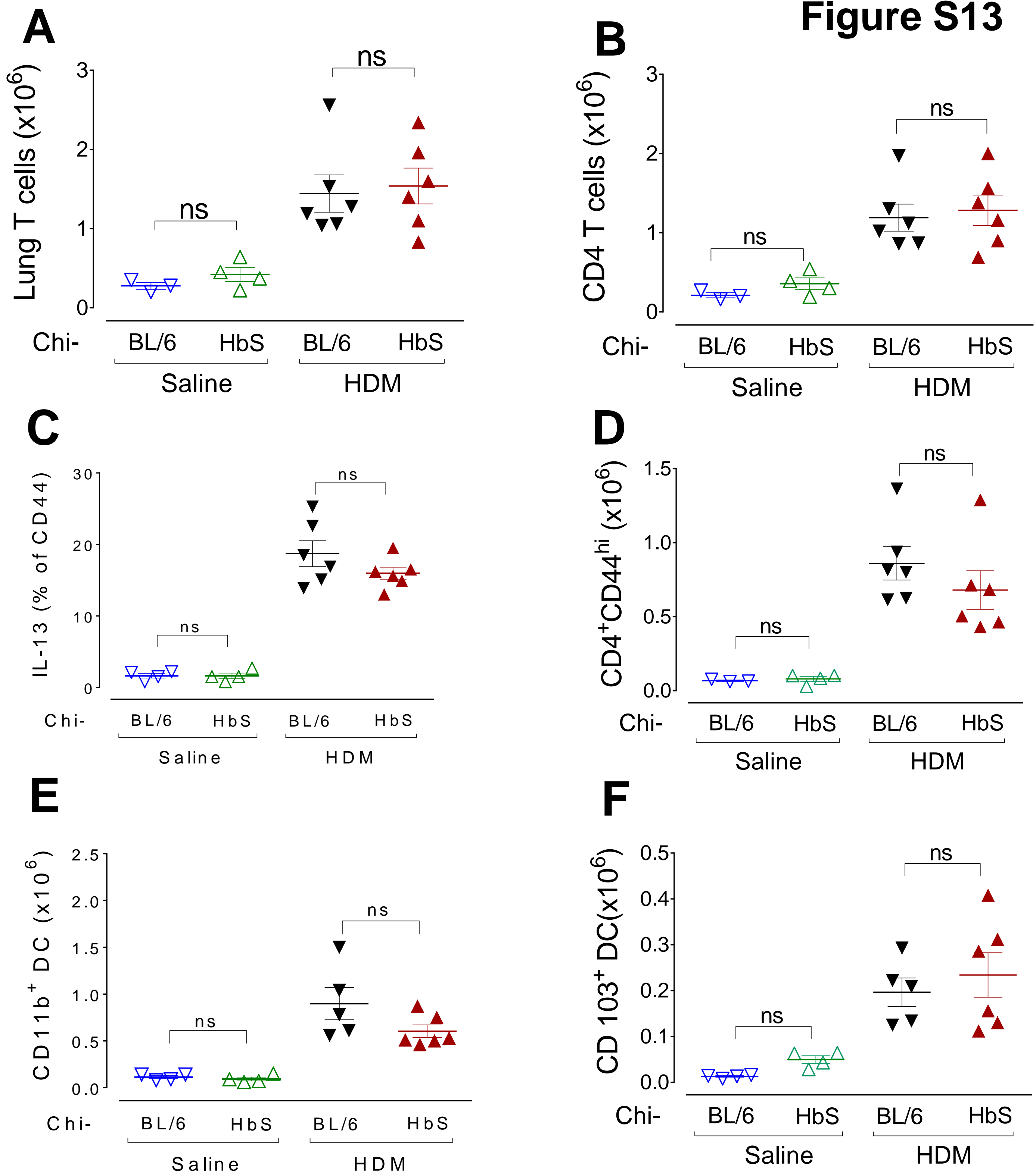


Figure S13. Th2 cells and dendritic cell subsets in chimeric mice in presence and absence of allergen. Frequency of lung **(A-B)** T cells**, (C-D)** effector T cells, and **(E-F**) dendritic cells **(DC).** Using total lung cell-count, frequencies were assessed 48 hours after the last HDM or saline exposure by flow cytometry. No significant difference (ns) in any groups, Mann Whitney test; each mouse is represented as a single symbol on the graphs. All data are presented as mean ± s.e.m. Results are representative of one experiment (A-F).

Figure S14

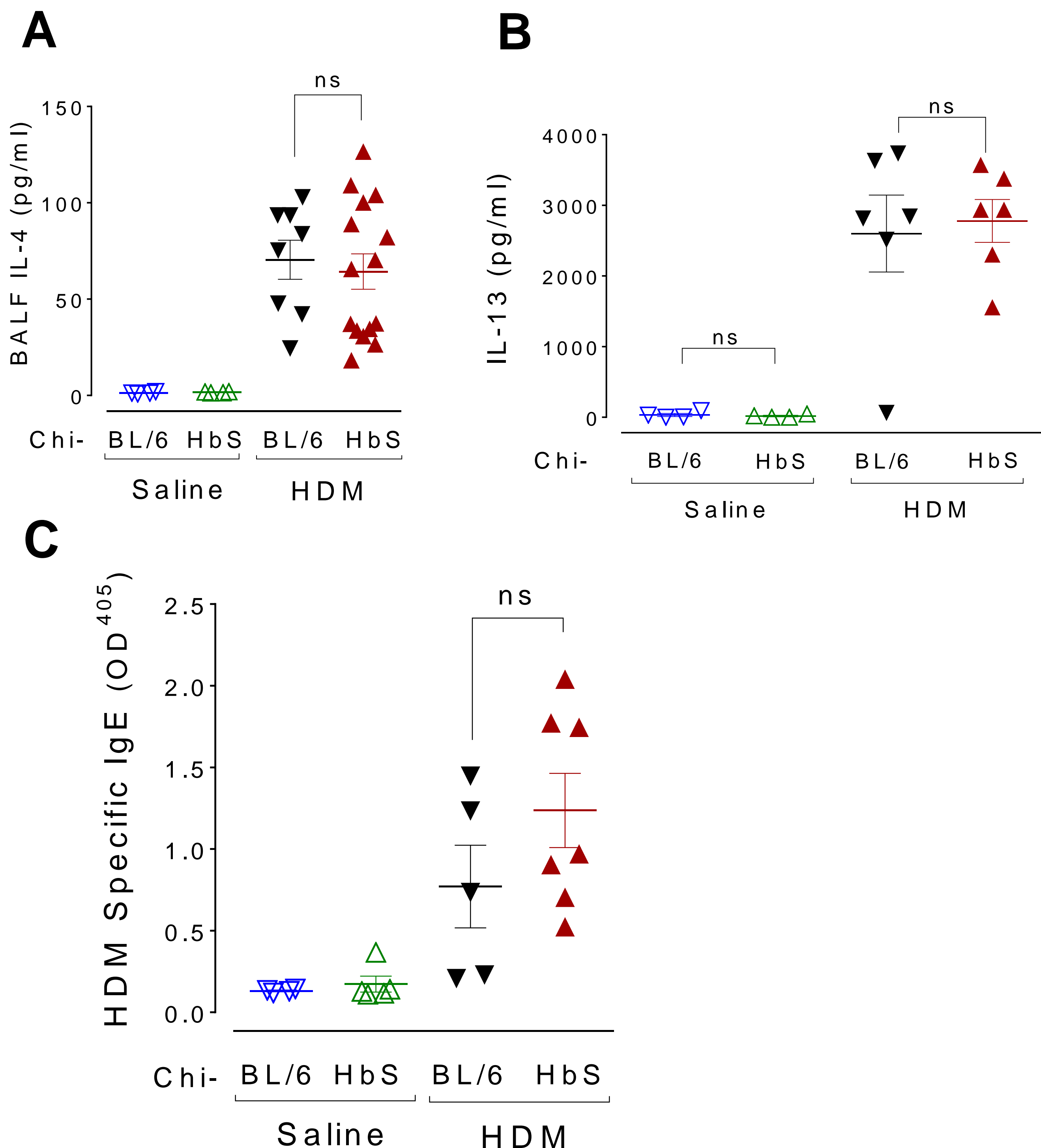


Figure S14. Th2 cytokine production and allergen-specific IgE levels in Chi-HbS mice. Levels of **(A)** IL-4 in the BALF and **(B)** IL-13 in cultured lung cells of HDM –exposed Chi-BL/6 and Chi-HbS mice. 48 hours following the last allergen challenge, harvested lung cells were cultured with HDM for 3-5 hours at 37̊C. Statistics: Mann Whitneys test, .ns non-significant. All data are presented as mean ± s.e.m. **(C)** HDM-specific IgE production in allergen challenged Chi-BL/6 and Chi-HbS mice measured in the plasma of chimeras 48 hours following the last intra-tracheal HDM challenge. Statistics: one-way ANOVA (Sidak); ns non-significant, ** p<0.01. All data are presented as mean ± s.e.m. Results are from two experiments (A), or representative of one experiment (B-C).

Figure S15

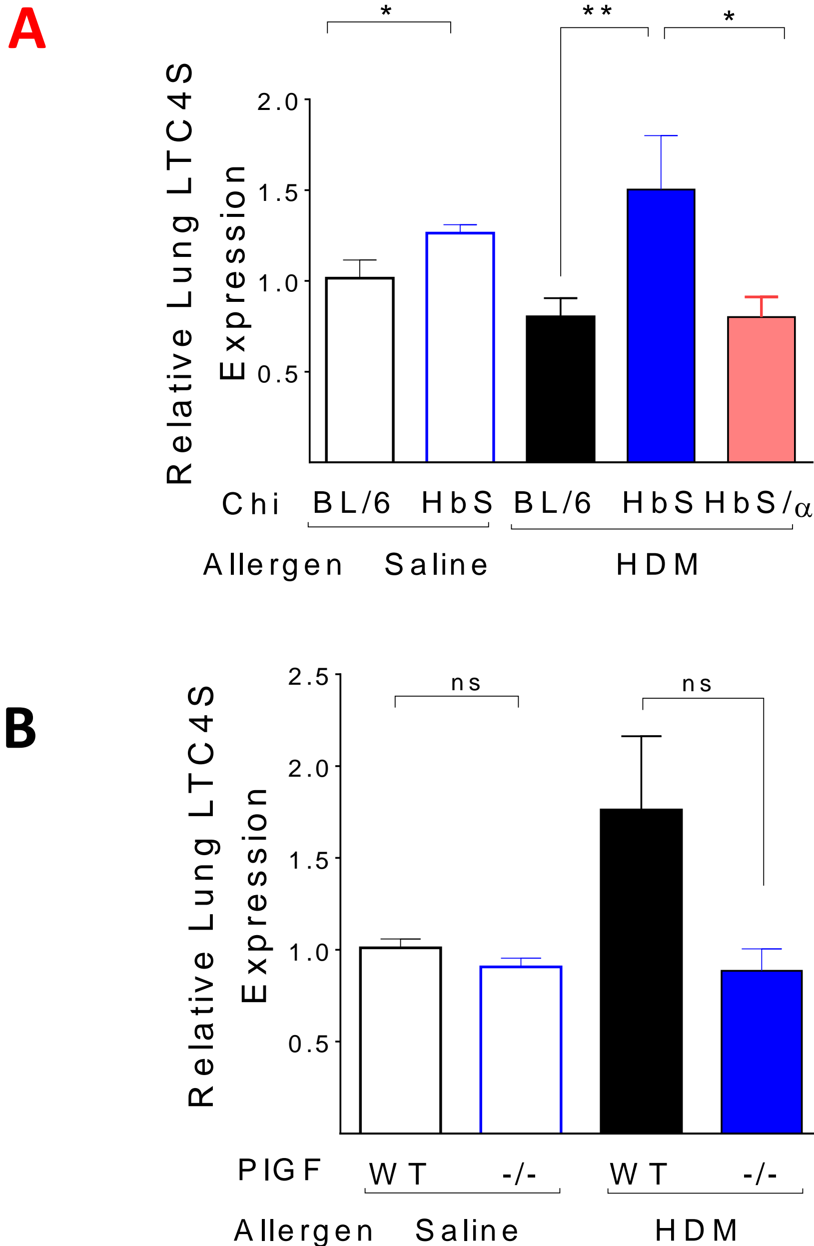


Figure S15. Lung LTC4S mRNA expression in chimeric sickle mice and PIGF deficient mice. (A) SCD mice: mRNA was extracted from saline or allergen sensitized lungs of Chi-BL/6 Chi-HbS, and PIGF antibody treated Chi-HbS/α mice and subjected to qRT PCR. Graph shows relative levels of mRNA. All data are presented as mean ± s.e.m. Statistics: Mann Whitney U test; * P<0.05. One-way ANOVA, Sidak"s test, * P<0.05, ** P<0.01; n=3 each for the saline groups of mice, n=8-12 each for the HDM exposed mice and n=10 for HbS mice treated with αPIGF. **(B) PIGF mice**: mRNA was extracted from saline or allergen sensitized lungs of PIGF deficient and WT Balb/C mice and subjected to qRT PCR. Graph shows relative levels of mRNA. All data are presented as mean ± s.e.m. Statistics:Mann Whitney's U test; ns non-significant (P value = 0.14 and 0.13 between the WT and -/- animals in the saline and HDM groups, respectively); n=7 each for the saline groups of mice, n=9 each for the HDM treated mice.