SUPPLEMENTARY MATERIAL

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Flow cytometry of lung and lung draining lymph nodes

Single cell suspensions of lungs and lung draining lymph nodes (caudal mediastinal) were isolated as described previously[1]. Lungs were digested in collagenase I (Life Technologies) for 1 hour at 37C, homogenized with frosted glasses slides by mechanical disruption, lysed of red blood cells, filtered through a 40um nylon filter, and washed with buffer (PBS, 0.5% (w/v) BSA, 2mM EDTA). Single cell suspensions of lung draining lymph nodes were obtained by mechanical disruption with glass slides followed by washing and filtering through 40uM mesh filters. Cell counts were completed after the last wash before staining for flow cytometry. Single-cell suspensions were stained for 25 min on ice with cell type-specific antibodies after blockade of Fc receptors using 1µg/µl of Fc blocker (CD16/32; BD Biosciences). Live-dead staining was completed using LIVE/DEAD® Fixable Dead Cell Stains (LifeTechnologies). Antibodies included: TCR-β (H57-597; BD Biosciences), CD4 (RM4-5; eBiosciences), CD44 (IM27; eBiosciences), CCR3 (83101; R&D Systems,) Siglec-F (E50-2440; BD Biosciences), Gr1 (RB6-8C5; eBiosciences), F4/80 (BM8; eBiosciences,), CD11c (HL3; BD Biosciences), CD11b (M1/70; eBiosciences), CD206 (C068C2; Biolegend), and IL-13 (eBio13A; eBiosciences). Flow cytometry was performed on a cytofluorimeter (Cyan; DAKO). Intracellular IL-13 was detected by culture of lung suspension with IL-33 (30ng/ml) for 2.5 hours in the presence of brefeldin and monesin then fixed and permeabilized following manufacturer's instructions (eBiosciences). Data acquisition and analysis were performed using Summit (version 4.3; Dako) software. Calculations of cell numbers are completed by counting total cellularity of whole lung or LDLN single cell suspensions and multiplying with the percent of the population as determined by flow cytometry.

Bronchoalveolar lavage

BAL assessments were completed as described previously [2]. Supernatant from BAL were assessed for mouse IL-17, IL-13, IFN-γ, TARC, and MDC levels using immunoassay kits (R&D Systems) according to the manufacturer's instructions. The limits of detection for each ELISA assay were 5-10pg/mL.

Histology and immunohistochemistry

Resected lungs were inflated with 10% formalin for 24 hours and paraffin embedded as described previously (REF). Serial sections (5μm) of mouse lungs were either stained with hematoxylin and eosin (general histology) or Periodic Acid Schiff (PAS, goblet cell metaplasia/airway epithelial cell mucin accumulation) or subjected to immunohistochemistry using an antibody to arginase I (LS-B4660). De-paraffinized sections underwent heat-antigen retrieval with DAKO citrate pH6.0 Target Retrieval Solution for 30 minutes in steamer. Slides were rinsed in TBS buffer and then incubated at RT for 10 minutes in Peroxidase and Alkaline Phosphatase Blocking Reagent (Dual Endogenous Enzyme-Blocking Reagent. Then were stained with rabbit polyclonal arginase I (1:250) in Background Reducing Antibody Diluent (DAKO) for 1 hr RT and then washed three times. HRP-conjugated EnVision™ anti-rabbit (DAKO) was added for 30 minutes at RT then slides were washed three times. Antibody was detected with DAB (diaminobenzidine) from DAKO with methyl green counterstain. Arginase I+ large mononuclear macrophage cells were identified by morphology and counted per lung section per individual animal with the entire lung section counted for n>6 samples per cohort in a total of three independent experiments. Total area of the lung was measured using DP2-BSW v2.2 Olympus Software (Olympus Corporation) to determine number of arginase I^+ cells per mm^2 of lung area.

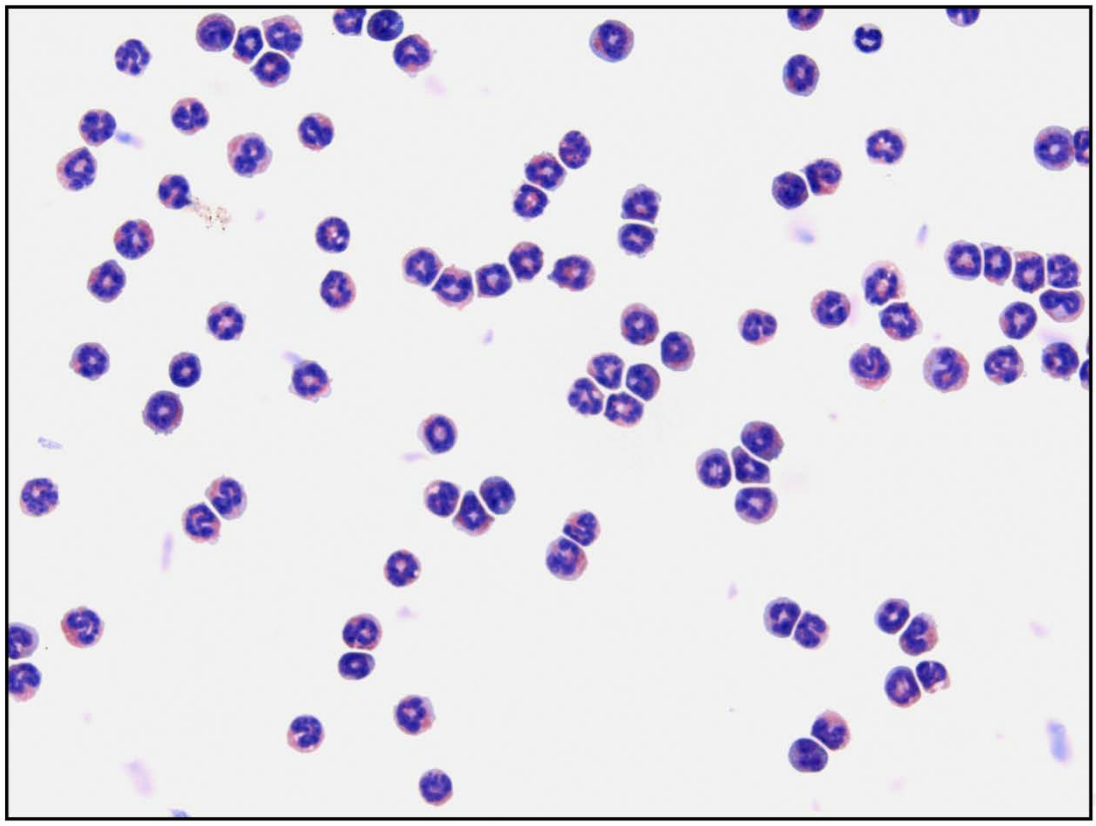
REFERENCES

1. Jacobsen EA, Zellner KR, Colbert D, Lee NA, Lee JJ. Eosinophils regulate dendritic cells and Th2 pulmonary immune responses following allergen provocation. *J Immunol* 2011; 187:6059-68.
2. Jacobsen EA, Ochkur SI, Pero RS, Taranova AG, Protheroe CA, Colbert DC, et al. Allergic Pulmonary Inflammation in Mice is Dependent on Eosinophil-induced Recruitment of Effector T Cells. *Journal of Experimental Medicine* 2008; 205:699-710.

SUPPLEMENTARY FIGURES

Supplementary Figure 1

A



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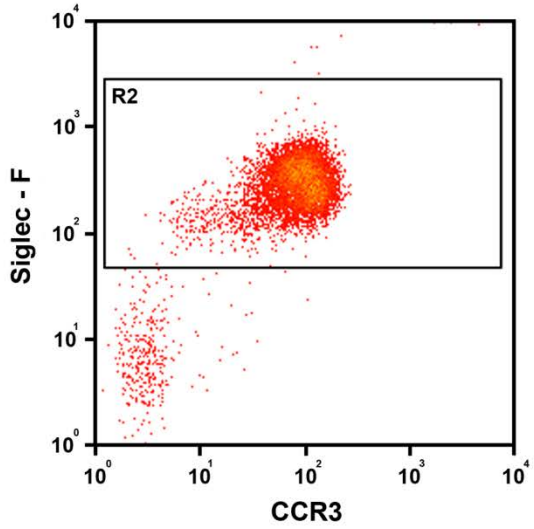
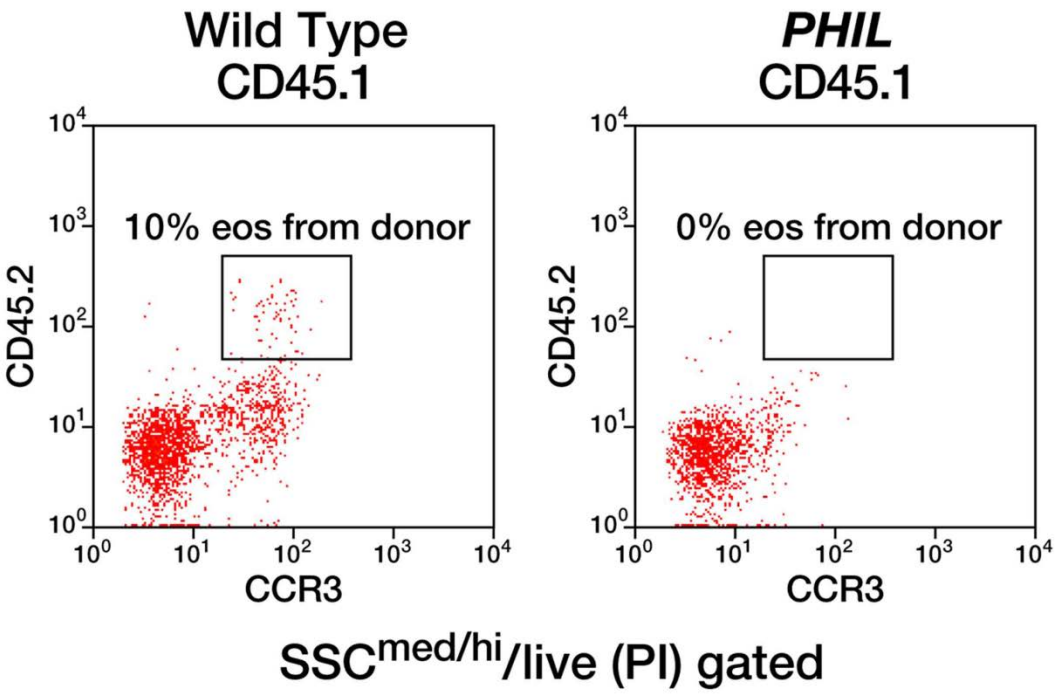


Supplementary Figure 1

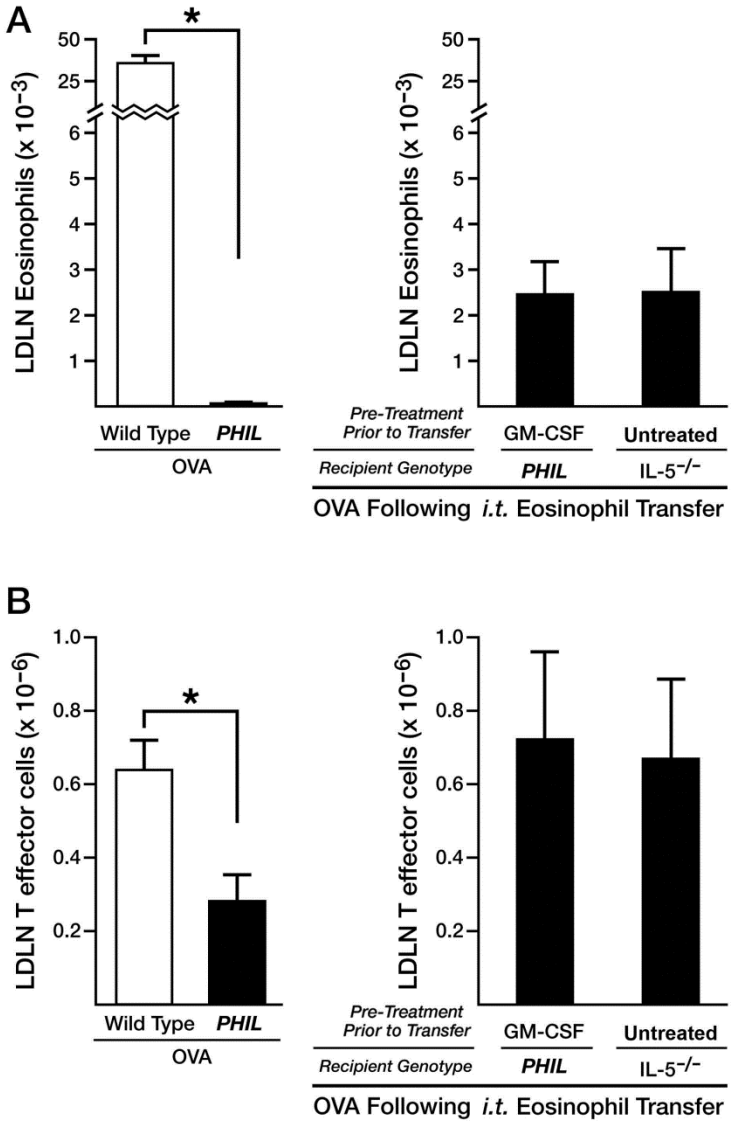
Purified eosinophils from the blood of IL-5 transgenic NJ.1638 mice represent a pure population of mature peripheral eosinophils with typical morphology and cell surface marker profile. (**A**) Cytospin preparations and Diff-Quick staining of eosinophils isolated via density gradient centrifugation followed by cell depletion with magnetic beads revealed that this purification strategy yielded cell populations that were mature, morphologically unremarkable, and representative of typical peripheral eosinophilic granulocytes. (**B**) Flow cytometric assessments of eosinophils purified from the blood of **NJ.1638** mice displayed a cell surface marker profile characteristic of mature peripheral eosinophil populations (i.e, $CCR3^{+}$/$Siglec\text{-}F^{+}$).

Supplementary Figure 2



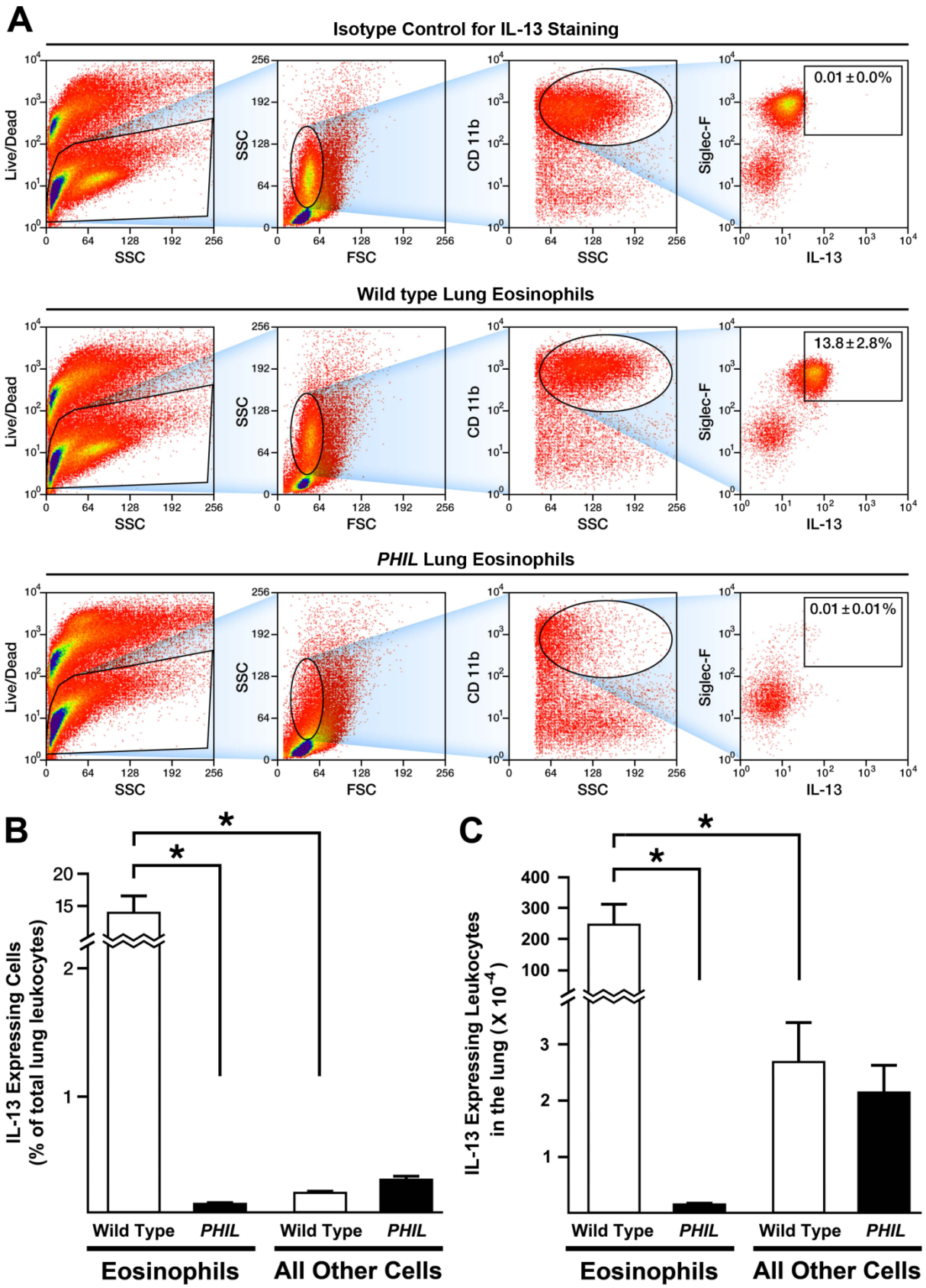
Untreated eosinophils fail to migrate to LDLN of OVA challenged *PHIL* mice following eosinophil adoptive transfer. Wild type and ***PHIL*** mice on a CD45.1 background were subjected to OVA sensitization on day 0 and 14, acute OVA challenge on days 24-26 (OVA-treated), and assessed on day 28. Untreated blood eosinophils from NJ.1638 IL-5 transgenic mice (CD45.2 background) were adoptively transferred (*i.t.*) into the lungs of animals on days 24-27 of the protocol and assessed on day 28. Lung draining lymph nodes were isolated and single cell suspensions were gated by FSC/SSC and Siglec F^+ staining. Gated cells were further assessed for presence of donor eosinophils ($CD45.2^+/CCR3^+$) compared to recipient eosinophils ($CD45.2^-/CCR3^+$). As shown in the histogram, ~10% of LDLN eosinophils of wild type mice are donor-derived, demonstrating their ability to migrate from the to the LDLN of wild type mice. Conversely, the LDLN of ***PHIL*** mice did not contain any detectable donor eosinophils.

Supplementary Figure 3



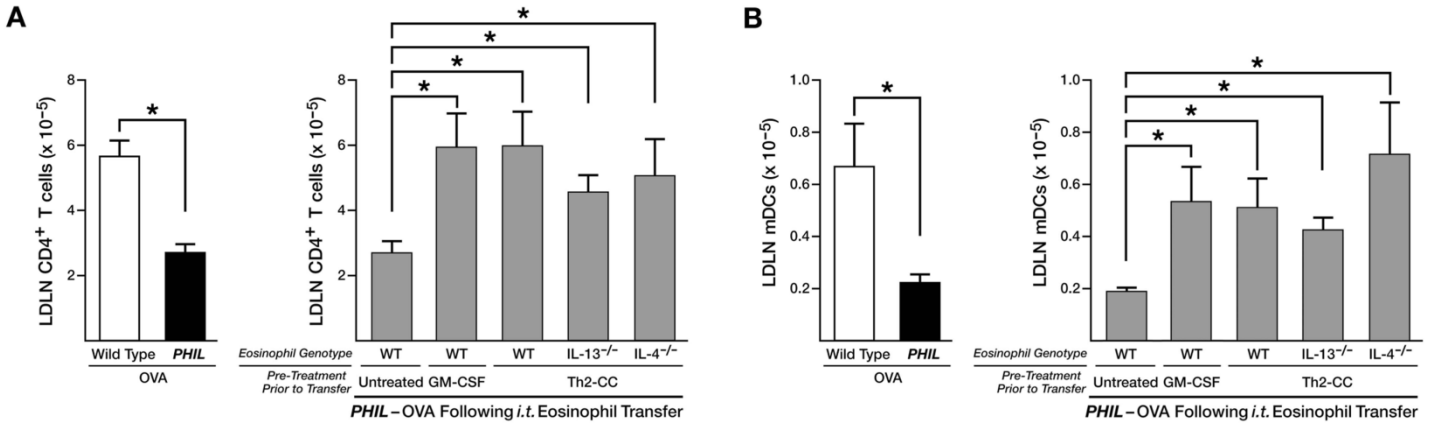
GM-CSF pre-treatment of eosinophils is sufficient upon adoptive transfer (*i.t.*) into OVA-treated *PHIL* mice to elicit the recruitment of eosinophils and, in turn, activate T cells to LDLN at the same levels that occur in OVA-treated IL-5$^{-/-}$ mice. Wild type, IL-5$^{-/-}$, and ***PHIL*** mice were subjected to OVA sensitization on day 0 and 14, acute OVA challenge on days 24-26 (OVA), and assessed on day 28. ***PHIL*** mice were adoptively transferred (*i.t.*) with wild type eosinophils pre-treated with GM-CSF for 24-48 hours prior to adoptive transfer (*i.t.*) on days 24-27 of the protocol whereas IL-5$^{-/-}$ mice received wild type eosinophils that were not treated with any exogenous cytokines (Untreated). Control animals were treated with saline alone. The accumulation of (**A**) eosinophils (Gr1^{+}/CCR3^{+}/Siglec-F^{+}) and (**B**) CD4^{+} effector T cells (CD4^{+}/TCR-β^{+}/CD44^{hi}) in the LDLNs were determined by flow cytometry. All data are derived from at least three independent experiments each with cohort sizes of 2-6 mice (Mean ± SEM). *p<0.05

Supplementary Figure 4



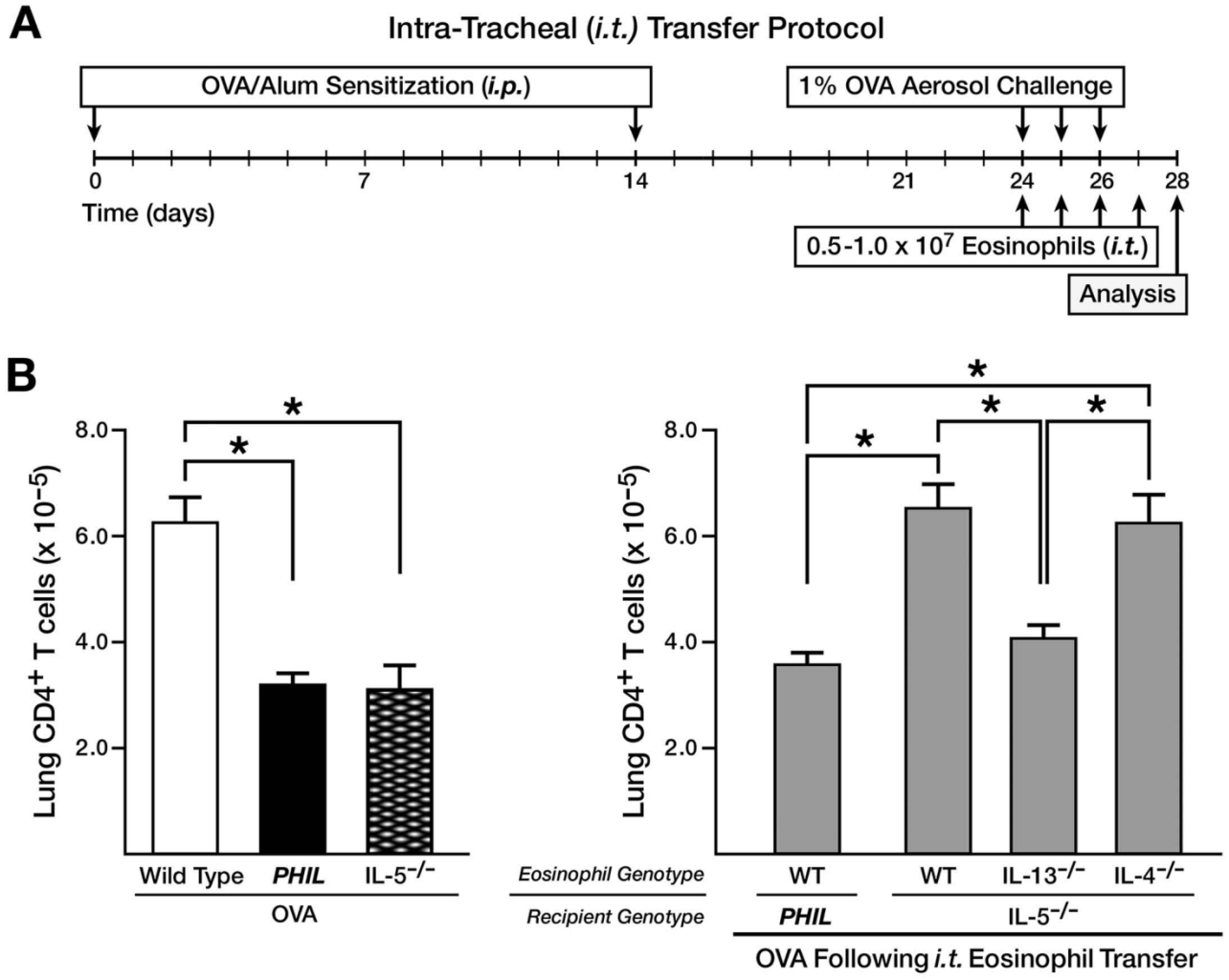
The majority of eosinophils produce IL-13 in the lungs OVA-treated wild type mice. Wild type and ***PHIL*** mice were subjected to OVA sensitization on day 0 and 14, acute OVA challenge on days 24-26 (OVA-treated), and assessed on day 28. Single cell suspensions of lung cells from OVA-treated wild type or OVA-treated ***PHIL*** mice were cultured with IL-33, brefeldin and monesin, and then stained for intracellular IL-13. (**A**) Flow cytometry plots show gating for eosinophils by side scatter and forward scatter after exclusion of dead cells. Eosinophils were further selected by CD11b expression and Siglec-F expression ($CD11b^{+}Siglec\text{-}F^{+}$). The specificity of IL-13 within eosinophils by this method is shown by negative staining in the isotype control and by the absence of staining in OVA-treated ***PHIL*** mice. (**B**) Greater than 12% of total cells in the lung expressing IL-13 are eosinophils. (**C**) The distribution of IL-13 expressing eosinophils vs. all other IL-13 expressing cells demonstrated the predominance of $IL\text{-}13^{+}$ eosinophils in the lung.

Supplementary Figure 5



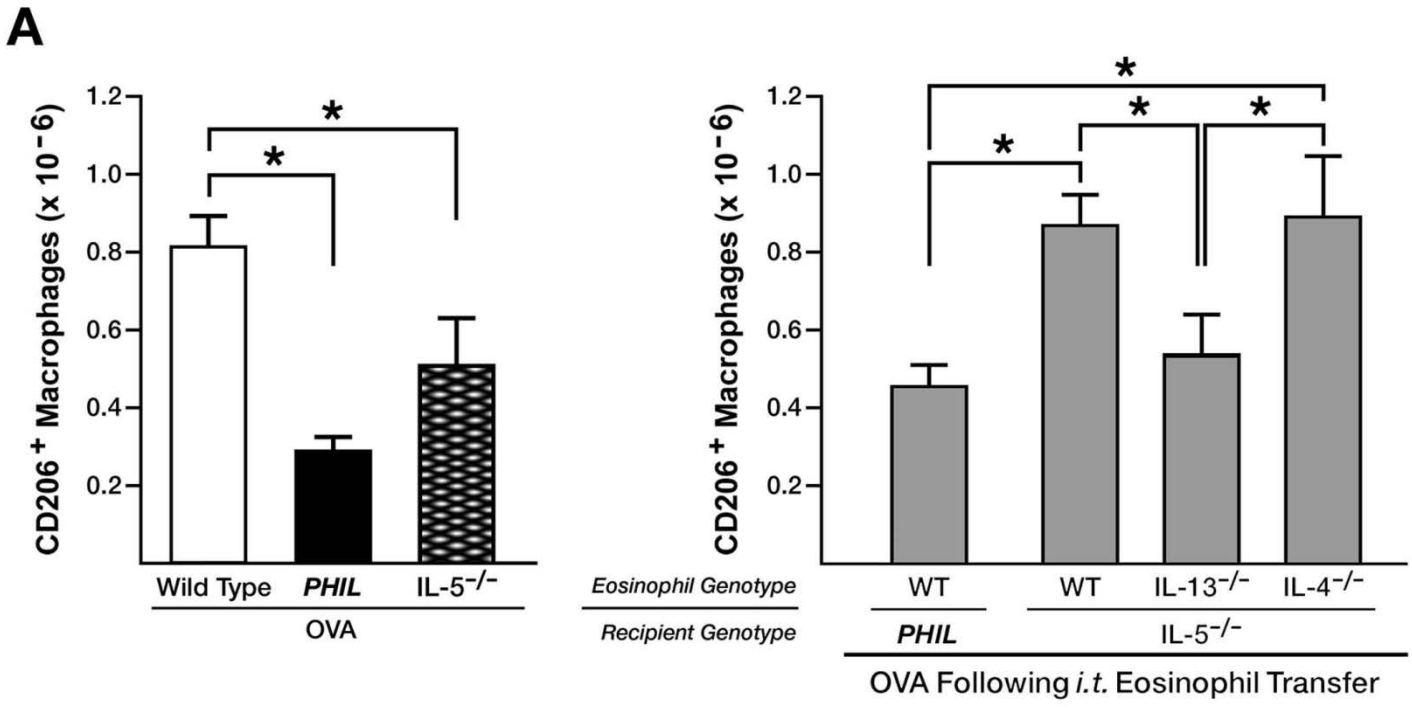
Eosinophil-derived IL-13, and not IL-4, promotes the T cell and DC accumulation that occurs in the LDLNs of OVA challenged PHIL mice following eosinophil adoptive transfer. The accumulation of CD4+ T cells (CD4+/TCR-β+) and myeloid DCs (F4/80-/Gr1-/CD11c+) in the LDLNs (panels (A) and (B), respectively) of OVA-treated wild type and ***PHIL*** mice were determined by flow cytometry relative to the accumulations occurring OVA-treated ***PHIL*** mice following adoptive transfer (*i.t.*) of wild type eosinophils that were untreated (no cytokines), pre-treated with GM-CSF, or pre-treated with a Th2-cc cytokine cocktail (i.e., GM-CSF, IL-4, IL-33) for 24-48 hours prior to adoptive transfer (*i.t.*) into the lungs of mice on days 24-27 of the protocol. Additional groups of OVA-treated ***PHIL*** mice were adoptively transferred (*i.t.*) with IL-$13^{-/-}$ or IL-$4^{-/-}$ eosinophils pre-treated with a Th2-cc cytokine cocktail (i.e., GM-CSF, IL-4, and IL-33). All data are derived from at least three independent experiments each with cohort sizes of 2-6 mice (Mean ± SEM.). *$p<0.05$

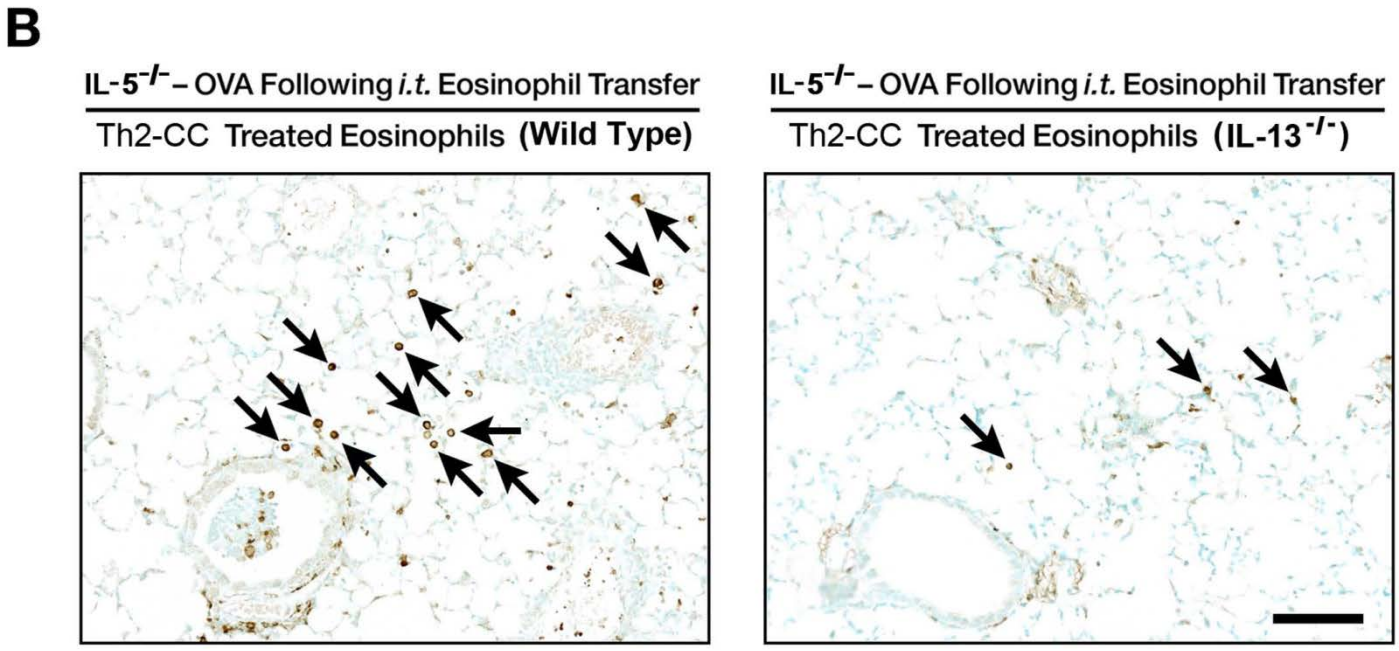
Supplementary Figure 6



Eosinophil-derived IL-13 contributes to T cell accumulation in the lungs of OVA-treated IL-5$^{-/-}$ mice. (**A**) Wild type, IL-5$^{-/-}$, and ***PHIL*** mice were subjected to OVA sensitization on day 0 and 14, acute OVA challenge on days 24-26 (OVA), and assessed on day 28. OVA-treated ***IL-5$^{-/-}$*** mice were adoptively transferred (*i.t.*) with wild type, IL-13$^{-/-}$, or IL-4$^{-/-}$ eosinophils that were pre-treated with a Th2-cc cytokine cocktail (i.e., GM-CSF, IL-4, IL-33) for 24-48 hours prior to adoptive transfer (*i.t.*) into the lungs of mice on days 24-27 of the protocol. Control groups included OVA-treated ***PHIL*** mice adoptively transferred (*i.t.*) with untreated wild type eosinophils (i.e., no cytokines) as well as cohorts of each genotype treated with saline alone. (**B**) The accumulation of CD4$^+$ T cells (CD4$^+$/TCR-β$^+$) in the lungs were determined by flow cytometry. All data are derived from at least three independent experiments each with cohort sizes of 2-6 mice (Mean ± SEM). *p<0.05

Supplementary Figure 7





Eosinophil-derived IL-13, but not IL-4, contributes to accumulation of alternatively activated (M2) macrophages in the lungs of OVA challenged IL-5$^{-/-}$ mice following eosinophil adoptive transfer. (**A**) M2 macrophage (CD206^{+}/F4/80^{+}/Gr1^{-}/CD11b^{+}) accumulation in the lung of OVA-treated wild type, ***PHIL***, and IL-5$^{-/-}$ mice was determined by flow cytometry relative to OVA-treated ***IL-5$^{-/-}$*** mice were adoptively transferred (*i.t.*) with wild type, IL-13$^{-/-}$, or IL-4$^{-/-}$ eosinophils that were pre-treated with a Th2-cc cytokine cocktail (i.e., GM-CSF, IL-4, IL-33) for 24-48 hours prior to adoptive transfer (*i.t.*) into the lungs of mice on days 24-27 of the protocol. Control groups included OVA-treated ***PHIL*** mice adoptively transferred (*i.t.*) with untreated wild type eosinophils (i.e., no cytokines) as well as cohorts of each genotype treated with saline alone. These data are derived from at least three independent experiments each with cohort sizes of 2-6 mice (Mean ± SEM). *p<0.05 (**B**) Representative images of lungs sections from OVA challenged IL-5$^{-/-}$ mice following adoptive transfer or either wild type or IL-13$^{-/-}$ eosinophil pre-treated with the Th2-cc cytokine cocktail (GM-CSF, IL-4, IL-33) stained for Arginase I by immunohistochemistry. Arginase I^{+} macrophages (arrows), identified as positive staining (brown color) cells with a unique morphology (large mononuclear cells with a low nuclear to cytoplasm ratio). Scale bar = 100μm.