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Eosinophils Regulate Dendritic Cells and Th2 Pulmonary Immune Responses following Allergen Provocation

Elizabeth A. Jacobsen,* Katie R. Zellner,[†] Dana Colbert,[†] Nancy A. Lee,[†] and James J. Lee*

Reports have recently suggested that eosinophils have the potential to modulate allergen-dependent pulmonary immune responses. The studies presented expand these reports demonstrating in the mouse that eosinophils are required for the allergen-dependent Th2 pulmonary immune responses mediated by dendritic cells (DCs) and T lymphocytes. Specifically, the recruitment of peripheral eosinophils to the pulmonary lymphatic compartment(s) was required for the accumulation of myeloid DCs in draining lymph nodes and, in turn, Ag-specific T effector cell production. These effects on DCs and Ag-specific T cells did not require MHC class II expression on eosinophils, suggesting that these granulocytes have an accessory role as opposed to direct T cell stimulation. The data also showed that eosinophils uniquely suppress the DC-mediated production of Th17 and, to smaller degree, Th1 responses. The cumulative effect of these eosinophil-dependent immune mechanisms is to promote the Th2 polarization characteristic of the pulmonary microenvironment after allergen challenge. *The Journal of Immunology*, 2011, 187: 000–000.

Allergic asthma is a chronic inflammatory disease thought to be initiated by innate pulmonary immune responses that subsequently activate the adaptive immune system against specific environmental assaults. This adaptive immunity and the characteristically persistent Th2 inflammation are often dominated by the presence of activated T cells (1) and eosinophils (2) in the lung even in mild cases of asthma. In this paradigm, eosinophils and T cells are generally considered disparate mediators of the inflammation, with T cells acting as immune regulators and eosinophils as end-stage destructive effector cells. However, recent studies suggest that this perspective of eosinophil effector functions is too narrow. Specifically, in mouse models of allergic respiratory inflammation, eosinophils appear to play a role during the secondary immune responses leading to the activation and proliferation of Ag-specific memory T cells (3, 4) and the subsequent recruitment of newly formed T effector cell populations to the lung (5, 6).

Dendritic cells (DCs) have long been classified as “professional” APCs fundamental for both T cell activation and immune tolerance. Specifically, several elegant studies have demonstrated that lung DCs have a unique capacity to engulf exogenous Ag and migrate to lung draining lymph nodes (LDLNs) to present Ags to

T cells (7). These DCs are uniquely necessary for the induction of allergic pulmonary pathologies in mouse models of allergic respiratory inflammation (8) and are increased in the lungs of asthmatic patients (9). Mouse models that conditionally deplete DCs by injection of targeted toxins during allergen provocation completely abolished activation of T cells and pulmonary inflammation, indicating DCs are essential for induced allergen-specific pathologies (10, 11). This dependency is highlighted by adoptive transfer studies of myeloid DCs into the lungs of sensitized mice and their restoration of allergic pulmonary inflammation, including eosinophil recruitment to the lung (8, 10).

Leukocytes other than DCs have also been shown to have the ability to promote and/or modulate the activation and polarization of T cells (12), and thus represent particularly problematic observations for paradigms suggesting the singular importance of DC-mediated events. In particular, two independent studies using genetically engineered mouse models deficient in eosinophils demonstrated that it was possible to correlate the loss of eosinophils with significant reductions in Th2 pulmonary pathologies [e.g., *PHIL* (5) and *Δdbl-GATA1* (6)]. Mice with a partial eosinophil deficiency, such as IL-5 knockout (13) and IL-5/eotaxin double-knockout (4) mice, have also been described to have impaired Th2 responses to allergen. In addition, eosinophils have been shown to have DC-like functions, such as the expression of MHC class II (MHC II) and costimulatory receptors (14–16), a capacity to traffic to LDLNs (14, 17), and the ability to stimulate Th2 cytokine production from Ag-specific CD4⁺ T cells (3, 18). Eosinophils are also capable of releasing immune modulators such as Th2 cytokines (e.g., IL-4 and IL-25), Th1 cytokines (e.g., IL-12), or suppressive mediators (e.g., indoleamine 2,3-dioxygenase and TGF-β) (19, 20). Collectively, these data suggest that the presence of these granulocytes may elicit underappreciated immune regulatory functions in the lung.

In this report, we suggest a solution to the paradox of the dual importance of both eosinophils and DCs in allergen-specific T cell responses. The studies presented capitalize on unique adoptive cell transfer approaches possible in the mouse as part of reductionist strategies defining mechanisms underlying immune responses. Specifically, we present data demonstrating that rather than mutually exclusive activities, eosinophils and DCs act in concert,

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Abbreviations used in this article: BAL, bronchoalveolar lavage; DC, dendritic cell; ICS, inhaled corticosteroids; i.t., intratracheal; LDLN, lung draining lymph node; MHC II, MHC class II.

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particularly in the LDLNs where T cell activation and polarization primarily occur during allergen challenge in mice (21). Specifically, our studies demonstrate that eosinophils induce the accumulation of mature myeloid DCs to LDLNs during allergen challenge. The activation of T cells in response to this DC accumulation was independent of MHC II expression on eosinophils, suggesting eosinophils are not required APCs in the secondary immune responses. Instead, we demonstrate through adoptive transfer of activated myeloid DCs that the ability of these transferred cells to polarize pulmonary immune responses was a function of the presence of eosinophils. That is, in the presence of eosinophils (i.e., wild type recipient mice), transfer of myeloid DCs leads to characteristically Th2-polarized immune responses in the lung. In contrast, in the absence of eosinophils (i.e., *PHIL* recipient mice), the transferred myeloid DCs promote a mixed Th2/Th17/Th1 phenotype and the accumulation of pulmonary neutrophils. Significantly, the Th17 component and the induced neutrophilia of this response in *PHIL* recipient mice were suppressed by cotransfer of eosinophils during allergen challenge. These results imply an important function for eosinophils in modulating the balance between Th2 and Th17 pathways generated by DCs during allergen challenge and, in turn, the development of allergic respiratory inflammation.

Materials and Methods

Mice

All studies were performed with mice on the C57BL/6J background. Eosinophil-deficient *PHIL* mice (22) and IL-5^{-/-} mice (13) were generated from established institutional colonies. MHC II knockout mice (B6.129S2-H2^{dIA1-Ea/J}) were purchased from The Jackson Laboratory (Bar Harbor, ME). MHC II^{-/-} mice were crossed with IL-5-expressing transgenic mice (NJ.1638) (23) to generate MHC II^{-/-} IL-5 transgenic mice to isolate MHC II^{-/-} donor eosinophils. Mice were maintained in ventilated microisolator cages housed in the specific pathogen-free animal facility at the Mayo Clinic Arizona. Protocols and studies involving animals were performed in accordance with National Institutes of Health and Mayo Foundation institutional guidelines.

OVA sensitization/challenge protocols and adoptive cell transfer strategies

Unless indicated otherwise, mice were sensitized with 100- μ l injections (i.p.) of 400 μ g/ml OVA grade VI (Sigma-Aldrich) and 2.25 mg Imject Alum (Thermo Scientific) on days 0 and 14 of the protocols. In studies involving no adoptive transfers or only eosinophil adoptive transfers, mice were challenged with aerosolized OVA (grade VI) at a concentration of 1% (w/v) for 25 min on days 24–26 and assessed on day 28. Time-course studies are indicated as hours after first OVA challenge. I.p. transfer of eosinophils was completed by adoptively transferring 4×10^7 eosinophils on day 24 before OVA challenge. Experiments including DC adoptive transfer were completed as described in previously published studies (8, 24). In brief, intratracheal (i.t.) transfer of $2-3 \times 10^6$ million bone marrow-derived, OVA-pulsed myeloid DCs (30 μ l saline) into OVA-sensitized mice (or nonsensitized where indicated) were performed immediately before 1% OVA challenge on day 24. Continued OVA challenges of these mice occurred on days 25–27 (20 min each day), and end-point assessments were performed on day 29. Where indicated, some mice received both 4×10^7 eosinophils by i.p. transfer and $2-3 \times 10^6$ DCs by i.t. transfer on day 24, with 1% OVA challenges on days 24–27; end-point assessments were performed on day 29. Controls include saline-sensitized mice, saline-challenged mice, and mice receiving saline transfers rather than cell transfers. Each of these OVA protocols and adoptive cell transfer strategies are outlined in Supplementary Fig. 1.

In vivo lymph node proliferation assay

Four hours before harvesting LDLNs of OVA-treated (i.e., OVA-sensitized/challenged) mice on day 28, the animals were injected (i.p.) with BrdU (3 mg/mouse; Sigma-Aldrich). BrdU incorporation into T cells was assessed by colabeling cells with Abs to TCR- β and following manufacturer's recommendations for the FITC BrdU Flow Kit (BD Biosciences).

Collection and cell differentials of bronchoalveolar lavage fluid-derived leukocytes

Bronchoalveolar lavage fluid (BAL) assessments were completed as described previously (5). Cell differentials of each sample were performed on ≥ 300 cells.

Histology

Histopathologic changes of the airways were assessed as described previously (5). Formalin-fixed and paraffin-embedded sections of mouse lungs were stained with H&E or periodic acid-Schiff.

Lymph node and lung cell isolation

Total pulmonary leukocytes were recovered from lungs perfused with PBS-EDTA before removal, placed in complete RPMI-1640 media with 175 U/ml collagenase type I (Invitrogen), 2 mM CaCl₂, and digested for 45 min at 37°C. Single-cell suspensions of lung and lymph nodes were obtained by homogenization with frosted glass slides followed by passing through a 40- μ m nylon filter to remove larger aggregates of cells per tissue. RBCs were lysed by brief exposure to PharmLyse (BD Biosciences). Cell counts were completed after the last wash before staining for flow cytometry or lymph node culture assays.

Flow cytometry analysis

Single-cell suspensions were stained for 25 min on ice with cell type-specific Abs after blockade of Fc receptors using 1 μ g/ μ l Fc blocker (CD16/32; BD Biosciences). Abs used for staining unique cell types were as follows: Abs used to characterize T cell populations were CD4 (RM4-5; eBiosciences), TCR- β (H57-597; BD Biosciences), and CD44 (IM27; eBiosciences). Eosinophils were identified by Abs to CCR3 (83101; R&D Systems) and Siglec-F (E50-2440; BD Biosciences). Macrophages were stained with F4/80 (BM8; BD Biosciences). DC subtypes and activation status were stained with CD11c (HL3; BD Biosciences), Gr1 (RB6-8C5; eBiosciences), B220 (RA3-6B2; eBiosciences), MHC II (AF6-120; BD Biosciences), and CD11b (M1/70; eBiosciences). Flow cytometry was performed on a cytofluorimeter (Cyan; DAKO). 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 percentage of the population as determined by flow cytometry.

Cytokine assays

Mouse IL-17, IL-13, and IFN- γ levels were assessed using immunoassay kits (R&D Systems) according to the manufacturer's instructions. The limits of detection for each ELISA assay were 5–10 pg/ml.

Lymph node culture

Single-cell suspensions of LDLNs were plated for 72 h on a 96-well plate at 5×10^5 /well with either complete RPMI-1640 media alone or OVA (grade VI; Sigma-Aldrich) that had been previously treated with 200 μ g/ml Detoxi-Gel endotoxin removing gel (Thermo Scientific). Supernatants were analyzed by ELISA.

Culture and selection of bone marrow-derived myeloid OVA-pulsed DCs

Bone marrow cells were isolated from naive 6- to 12-wk-old C57BL/6J wild-type mice, and RBCs were lysed by PharmLyse treatment followed by washing with cold MACS buffer (PBS, 0.5% [w/v] BSA, 2 mM EDTA). Lymphocytes were removed from the bone marrow by negative selection with magnetic beads conjugated with Abs to CD45R/B220 and CD90/Thy1.2 as per the manufacturer's recommendations (Miltenyi Biotech). The negative fraction was plated (2×10^5 cells/ml) in complete RPMI media supplemented with GM-CSF (30 ng/ml; R&D Systems) and IL-4 (10 ng/ml; R&D Systems) for 2–3 d. Sequentially lower doses of GM-CSF were used with 20 ng/ml on day 3 and 10 ng/ml on day 6, as described previously (25). On day 8 or 9, cells were resuspended in fresh complete RPMI-1640 media containing 10 ng/ml GM-CSF and 200 μ g/ml Detoxi-Gel-treated OVA grade VI (Sigma-Aldrich). Sixteen to 18 h later, the cells were collected and stained with Abs to select myeloid DCs (F4/80⁺CD11c⁺B220⁺) for adoptive transfers. Cells were sorted by FACSARIA (BD Biosciences), washed in cold PBS twice, and resuspended for i.t. transfer into mice. No significant differences in results were seen between sorted and unsorted bone marrow-derived OVA-pulsed DCs (data not shown).

Isolation of mouse peripheral blood eosinophils

Eosinophils were isolated from IL-5-expressing transgenic mice (NJ.1638) or NJ.1638/MHC II^{-/-} as previously described (5). In brief, peripheral blood pooled from mice was layered onto single-step Histopaque 1119 gradient (Sigma-Aldrich). The eosinophil-containing interface was then treated briefly (<10 s) with ice-cold distilled water to lyse any remaining RBCs before adding 1/10 volume of 10× PBS (i.e., cells are returned to a final concentration of 1× PBS). No differences in T cell proliferation were found when cells were lysed with the ammonium chloride-based PharmLyse solution. Eosinophils of >99% purity were isolated according to the manufacturer's recommendations (Miltenyi Biotec) by negative selection with magnetic beads conjugated with Abs to CD45R/B220 and CD90/Thy1.2.

Statistics

All data are derived from at least three independent experiments each with cohort sizes of one to six mice (error bars, SEM; **p* < 0.05, ***p* < 0.01, ****p* < 0.001, unpaired two-tailed Student *t* test).

Results

Peripheral eosinophils are required for T cell activation

We used mice that are eosinophil-sufficient (wild type), eosinophil-low (IL-5^{-/-} mice) (13), and eosinophil-null (*PHIL* mice) (5, 22) to test the hypothesis that eosinophil levels and their specific tissue localization effect T cell accumulation/activation during allergen provocation. Wild type, IL-5^{-/-}, and *PHIL* mice were sensitized with i.p. injections of OVA/Alum (days 0 and 14) and challenged via the airways with a 1% OVA aerosol (OVA-treated) on days 24, 25, and 26 (control animals received saline alone; Supplemental Fig. 1A). The partial (IL-5^{-/-}) and complete (*PHIL*) absence of eosinophils in these mice were each accompanied by a loss of the OVA-induced CD4⁺ T cell accumulation in the lung as com-

pared with OVA-treated wild type animals (Fig. 1A). However, injection of these OVA-treated mice with BrdU (4 h before end-point assessments on day 28) to measure T cell proliferation in LDLNs showed that only the complete loss of eosinophils (i.e., *PHIL* mice), and not the partial (85%) loss (i.e., IL-5^{-/-} mice; data not shown), blocked T cell proliferation in the lymphatic compartment (Fig. 1B). Moreover, the failure to induce proliferation in the LDLNs of *PHIL* mice was not unique to the OVA allergen because sensitization and challenge with a more environmentally relevant allergen, ragweed, produced similar results (Supplemental Fig. 2). To bypass the complete absence of peripheral eosinophils in *PHIL* mice and promote their recruitment to LDLNs after OVA provocation, we adoptively transferred eosinophils by i.p. injection; this strategy has been shown to promote leukocyte recruitment promptly to LDLN within hours of injection (3, 11). *PHIL* mice received 4 × 10⁷ eosinophils by i.p. transfer 1 h before the first OVA challenge (Supplemental Fig. 1B). This was sufficient to return eosinophil percentages in the LDLNs to levels similar to those observed in OVA-treated wild type mice (Fig. 1C). It is noteworthy that as previously reported (3), transfer of eosinophils via the peritoneal cavity restored eosinophil accumulation only to the lymphatic compartments of *PHIL* mice and not in the lung itself, where eosinophil levels remained >98% lower relative to wild type animals. In addition, unlike the trafficking of mononuclear cells such as dendritic cells (26) or T cells (27), eosinophil movement through lymphatic circulation occurred independently of CCR7 expression (Supplemental Fig. 3). Concomitant with eosinophil accumulation to the LDLNs of OVA-treated *PHIL* mice, CD4⁺ T cell numbers in the LDLNs increased to levels equivalent to that observed in OVA-treated wild type

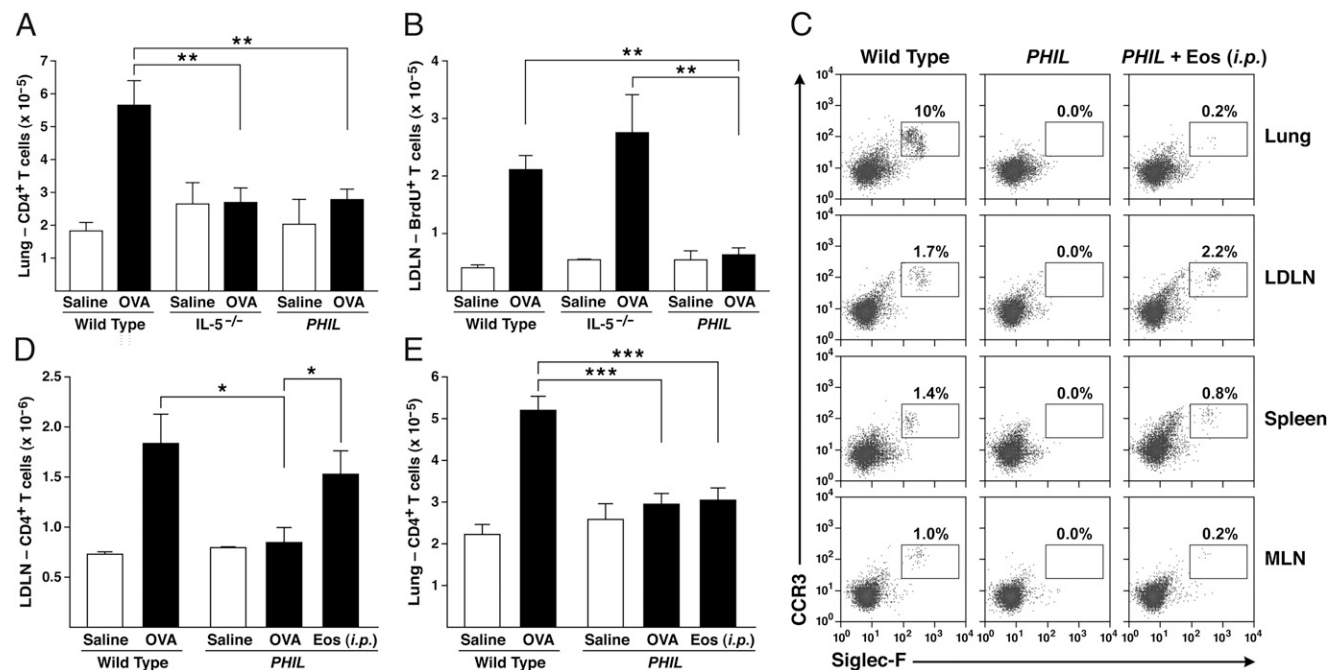


FIGURE 1. Peripheral eosinophils are required for T cell activation in LDLNs of OVA-treated mice. **A**, Wild type, IL-5 knockout (IL-5^{-/-}), and *PHIL* mice were subjected to OVA sensitization on days 0 and 14, acute OVA challenge on days 24–26 (OVA treated), and assessed on day 28. Control animals were treated with saline alone. CD4⁺/TCR-β⁺ T cell numbers were determined by flow cytometry of total cells in the lungs of OVA-treated mice. **B**, Mice treated as in **A** were injected with BrdU (4 h before sacrifice) and assessed for total TCR-β⁺/BrdU⁺ T cells by flow cytometry (i.e., cell proliferation). **C**, Eosinophil and CD4⁺ T cell numbers in mice were determined (day 28) in OVA-treated wild type, *PHIL*, and *PHIL* mice that were also transferred (i.p.) with 4 × 10⁷ eosinophils (Eos [i.p.]) 1 h before the first OVA challenge. Representative FACS plots are shown of eosinophils (Siglec-F⁺/CCR3⁺) in the lung, LDLNs, spleen, and a nonpulmonary lymphoid compartment (mesenteric lymph node [MLN]). Numbers above boxes indicate percentage of eosinophils out of the total live population. CD4⁺/TCR-β⁺ T cell numbers were assessed by flow cytometry of total cells in the LDLNs (**D**) and lungs (**E**) of OVA-treated mice after eosinophil adoptive transfer. All data are derived from at least three independent experiments, each with cohort sizes of two to six mice (mean ± SEM). **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (unpaired two-tailed Student *t* test).

animals (Fig. 1D). Similar to OVA-treated $IL-5^{-/-}$ mice, the accumulation was limited to the LDLNs; that is, i.p. transfer of eosinophils into OVA-treated *PHIL* mice did not induce the accumulation of $CD4^{+}$ T cells into the lungs (Fig. 1E). These data demonstrate that the localization of eosinophils to the LDLNs was necessary for, and limited to, T cell accumulation in LDLNs in response to airway allergen challenge of OVA-treated *PHIL* mice.

Eosinophils do not require MHC II to activate T cells

Eosinophils have been demonstrated to express MHC II molecules and potentially mediate Ag presentation in vivo as part of immune responses leading to the T cell activation (14, 15, 18). We adoptively transferred (i.p.) MHC II-deficient eosinophils into OVA-treated *PHIL* mice to determine whether the T cell activation/proliferation occurring in the LDLNs of recipient animals was a consequence of MHC II-dependent Ag presentation by eosinophils. These studies showed that migration of MHC II^{+/+} or MHC II^{-/-} eosinophils to LDLNs was equivalent (Fig. 2A). More importantly, transfer of either MHC II^{+/+} or MHC II^{-/-} eosinophils into OVA-treated *PHIL* recipients promoted comparable levels of $CD4^{+}$ effector T cell accumulation in LDLNs (Fig. 2B), showing that eosinophils do not require expression of MHC II to promote the activation of T cells in the LDLNs after allergen challenge.

The accumulation of myeloid dendritic cells is dependent on eosinophils

Commonly accepted paradigms on DC functions suggest an independence between eosinophils and LDLN T cell activation. However, our results demonstrating that eosinophils are required for T cell proliferation, as well as studies describing eosinophil-

mediated effects on DCs (28, 29), suggested a possible eosinophil dependence of DC activities in secondary immune responses after allergen challenge. To test this hypothesis, LDLNs in OVA-treated wild type versus OVA-treated *PHIL* mice were examined with and without cotransfer of eosinophils to determine the relative effects of eosinophils on activated MHC II^{hi} DC accumulation in the LDLNs. Significantly, OVA-treated *PHIL* mice have reduced numbers of DCs in the LDLNs as compared with OVA-treated wild type mice (Fig. 3A). This reduction occurred without changes in basophil levels in the LDLN, thus suggesting that eosinophils alone and not basophils are correlated with DC levels (Supplemental Fig. 4). Furthermore, the induced DC accumulation in the LDLN of OVA-treated *PHIL* mice was restored by i.p. adoptive transfer of MHC II^{+/+} or MHC II^{-/-} eosinophils (Fig. 3A). Significantly, the presence, although reduced, of eosinophils in the LDLNs of OVA-treated $IL-5^{-/-}$ mice was also sufficient to induce accumulation of activated MHC II^{hi} DCs.

Myeloid dendritic cells, in particular, have been suggested to be key agonists of pathways linked with pulmonary allergic immune responses (7). Assessments of the DC subtypes present in the lungs and LDLNs of OVA-treated wild type and *PHIL* mice 5 d after the first OVA challenge revealed that OVA-treated *PHIL* mice have equivalent (i.e., relative to wild type) percentages of lymphocytic DCs ($F4/80^{-}/CD11b^{+}/Gr1^{+}/CD11c^{+}$), plasmacytoid DCs ($F4/80^{-}/CD11b^{-}/Gr1^{+}/CD11c^{+}$), and monocyte inflammatory DCs ($F4/80^{-}/CD11b^{+}/Gr1^{+}/CD11c^{-}$) in both the lungs and LDLNs (Fig. 3B). However, myeloid DC ($F4/80^{-}/CD11b^{+}/Gr1^{-}/CD11c^{+}$) levels were lower in both the lung and LDLNs of OVA-treated *PHIL* mice relative to control OVA-treated wild type animals (Fig. 3B). Moreover, the total number of myeloid DCs were significantly reduced in the lungs of OVA-treated *PHIL* mice as compared with wild type mice ($0.47 \times 10^6 \pm 0.7 \times 10^5$ versus $1.02 \times 10^6 \pm 1.5 \times 10^5$, respectively; $p = 0.007$). In addition, the number of myeloid DCs in the LDLNs ($2.56 \times 10^5 \pm 0.3 \times 10^5$ versus $5.90 \times 10^4 \pm 0.8 \times 10^5$, OVA-treated *PHIL* versus wild type, respectively; $p = 0.0007$), mirrored that of LDLN MHC II^{hi} DCs. Thus, myeloid DC accumulation in both the lungs and LDLNs of mice after allergen provocation appears to be a function of one or more eosinophil activities.

Several studies have demonstrated that myeloid DCs migrate from the lung to the LDLNs within ~10 h after aerosolized allergen challenge, peaking at ~40 h (30). Thus, we assessed DC levels at various time points after allergen challenge to determine whether OVA-treated *PHIL* mice had recruited DCs at a rate similar to OVA-wild type mice and simply failed to accumulate these cells in draining pulmonary lymphoid compartments because of a change(s) in their survival (i.e., LDLN half-life). The LDLNs of OVA-treated wild type mice accumulated eosinophils and MHC II^{hi} activated DCs within 20 h of OVA challenge (Fig. 3C); myeloid DC ($F4/80^{-}/CD11b^{+}/Gr1^{-}/CD11c^{+}$) populations mirrored MHC II^{hi} DCs ($MHC II^{hi}/CD11c^{+}$). In contrast, OVA-treated *PHIL* mice failed to accumulate DCs within 20 h of allergen challenge, suggesting a deficiency in recruitment of these lung DCs to the LDLN. These data demonstrated that the presence of eosinophils in the LDLN is necessary to promote myeloid DC recruitment to LDLNs and, in turn, T cell activation during OVA provocation.

Adoptive transfer of OVA-pulsed myeloid DCs is sufficient for DC accumulation and T cell activation in the LDLNs of eosinophil-deficient mice

We performed adoptive transfer experiments of activated (i.e., OVA-pulsed) myeloid DCs similar in design of other studies (8, 10, 31) to circumvent the limitation of eosinophil-dependent DC activation/recruitment. This allowed for the assessment of activated myeloid

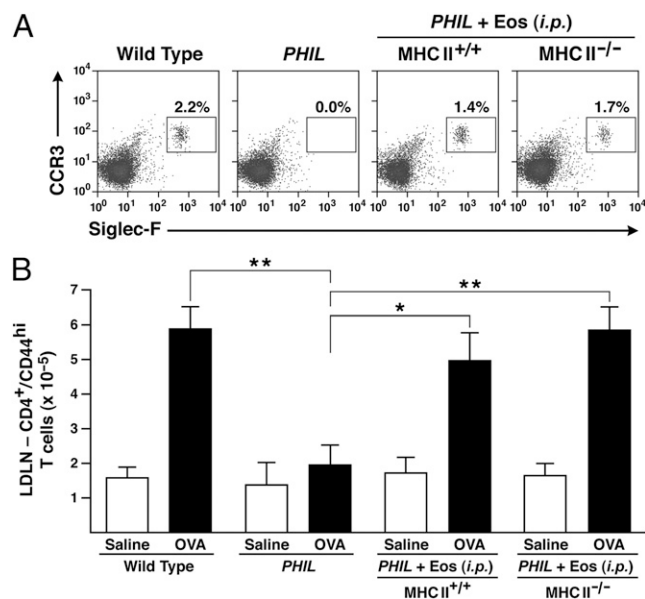
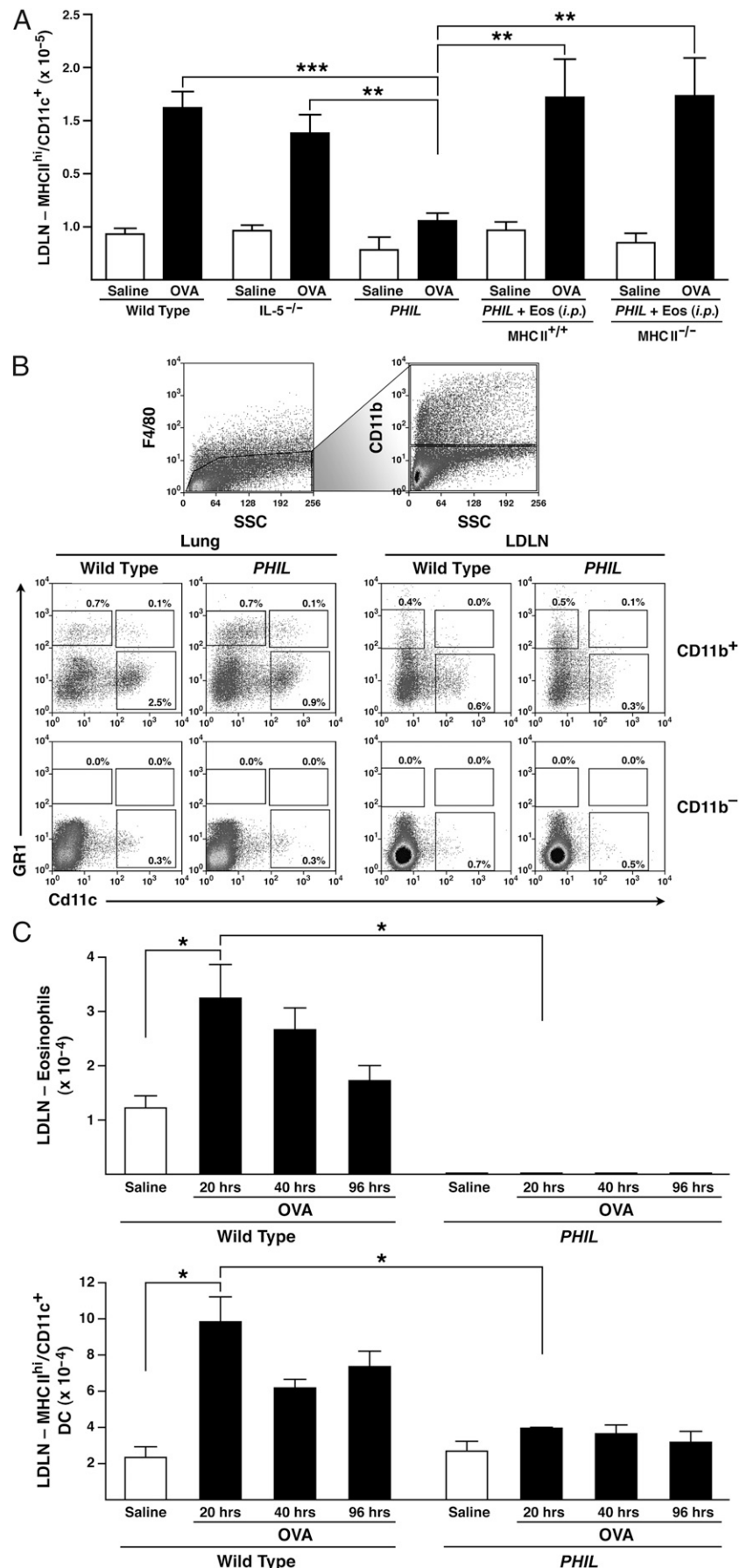


FIGURE 2. MHC II expression on eosinophils is not necessary for T cell activation. OVA-treated wild type and *PHIL* mice, as well as OVA-treated *PHIL* mice after adoptive transfer (i.p.) of either 4×10^7 MHC II^{+/+} or MHC II^{-/-} eosinophils (Eos [i.p.]) 1 h before the first OVA challenge (day 24), were assessed on day 28 of the protocol; control animals were challenged with saline alone. **A**, Representative FACS plots of LDLN cells demonstrate the presence of eosinophils (Siglec-F⁺/CCR3⁺). Numbers above boxes indicate percentage of eosinophils out of the total live population. **B**, Effector T cell ($CD4^{+}/TCR-\beta^{+}/CD44^{hi}$) numbers were determined in LDLNs from OVA-treated mice after eosinophil adoptive transfer. All data are derived from at least three independent experiments, each with cohort sizes of two to four mice (mean $\pm$ SEM). * $p < 0.05$, ** $p < 0.01$ (unpaired two-tailed Student *t* test).

FIGURE 3. Eosinophils are required for activation/accumulation of myeloid DCs. **A**, OVA-treated wild type and *PHIL* mice, as well as OVA-treated *PHIL* mice after adoptive transfer (i.p.) of either 4×10^7 MHC II^{+/+} or MHC II^{-/-} (Eos [i.p.]) 1 h before the first OVA challenge (day 24), were assessed on day 28 of the protocol; control animals were challenged with saline alone. OVA-sensitized wild type, IL5^{-/-}, and *PHIL* mice challenged with either OVA or saline are shown for comparison. LDLNs of OVA-treated recipient *PHIL* mice were assessed for the presence of MHC II^{hi}CD11c⁺ DCs by flow cytometry as calculated from total single-cell suspensions of LDLNs. **B**, Lungs and LDLN from OVA-treated wild type and *PHIL* mice that did not undergo eosinophil adoptive transfer were assessed for various DC populations. Total cell suspensions were gated on the F4/80⁻ (nonmacrophage) population and then gated into CD11b⁺ and CD11b⁻ groups. Percentages of total populations of these were determined for lymphocytic DC (F4/80⁻/CD11b⁺/Gr1⁺/CD11c⁺), plasmacytoid DC (F4/80⁻/CD11b⁻/Gr1⁺/CD11c⁺) and monocytic inflammatory DC (F4/80⁻/CD11b⁺/Gr1⁺/CD11c⁻), and myeloid DC (F4/80⁻/CD11b⁻/Gr1⁻/CD11c⁺) populations. **C**, Kinetic assessment of eosinophil and activated DC (MHC II^{hi}CD11c⁺) accumulation in the LDLNs of OVA-treated wild type versus *PHIL* mice. DC and eosinophil numbers were determined by collecting LDLN for flow cytometry analysis at 20, 40, and 96 h after the first challenge of the three OVA challenges. All data are derived from at least three independent experiments, each with cohort sizes of two to five mice (mean $\pm$ SEM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (unpaired two-tailed Student *t* test).



DCs to induce immune responses in the absence of eosinophils. Specifically, bone marrow-derived, OVA-pulsed myeloid DCs were adoptively transferred i.t. on the first day of allergen challenge into either OVA-sensitized *PHIL* or nonsensitized *PHIL* mice (Supplemental Fig. 1C); OVA-sensitized wild type animals served as controls. These adoptively transferred OVA-pulsed DCs accumulated in the LDLNs in all mice receiving DCs, including nonsensitized *PHIL* mice, demonstrating that recruitment of activated DCs to LDLNs is eosinophil independent in this system (Fig. 4A). Despite the presence of DCs in the LDLNs of nonsensitized *PHIL* mice after adoptive transfer, production of effector T cells is deficient (Fig. 4B), demonstrating that OVA-specific memory T cells are required for their activation into effector T cells. Furthermore, the increase in effector T cells that occurred in OVA-treated *PHIL* mice receiving activated DCs was equivalent to the levels observed in OVA-treated wild type mice receiving DCs.

Adoptive transfer of OVA-pulsed myeloid DCs into PHIL leads to neutrophilic inflammation that is reduced by restoration of peripheral eosinophils

OVA-treated *PHIL* mice receiving OVA-pulsed DCs (i.t.) displayed histopathologic changes, including goblet cell metaplasia/

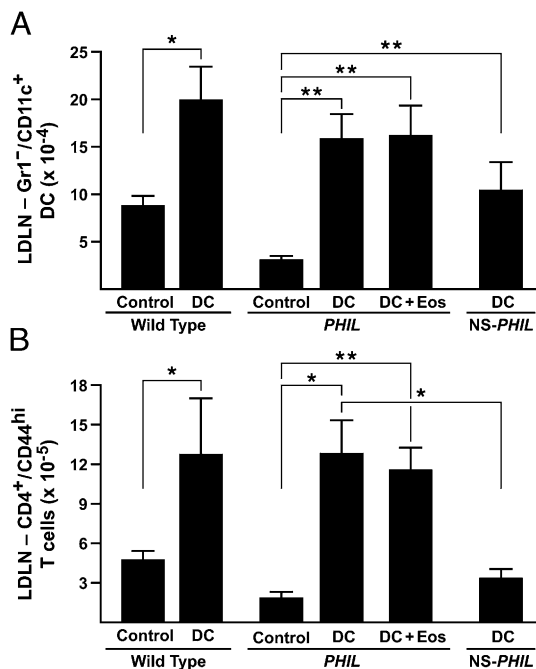


FIGURE 4. OVA-pulsed myeloid DCs are sufficient for DC accumulation and T cell activation in the LDLNs of eosinophil-deficient mice. Bone marrow-derived and OVA-pulsed DCs (2–3 million cells) were transferred into the lungs of OVA-treated (OVA-sensitized/challenged) wild type or *PHIL* mice (i.t.) on day 24 of the acute OVA protocol and assessed on day 29. DC represents mice receiving DCs via i.t. transfer; Control represents vehicle i.t. control. An additional cohort of OVA-treated *PHIL* mice also were adoptively transferred with 4×10^7 eosinophils i.p. on the same day (i.e., day 24) as the DC transfer (DC + Eos). Nonsensitized but OVA-challenged *PHIL* recipients adoptively transferred (i.t.) with DCs (NS-*PHIL*) were included as controls to determine the necessity of Ag-dependent memory T cell activation. OVA sensitized/challenged wild type and *PHIL* mice subjected to the acute OVA protocol without cell transfer were performed as additional controls. A, DCs (Gr1⁺CD11c⁺) and (B) effector (CD4⁺/TCRβ⁺/CD44^{hi}) T cell populations in LDLNs were assessed in each group of mice by flow cytometry. All data are derived from at least four independent experiments, each with cohort sizes of two to four mice (mean ± SEM). * $p < 0.05$, ** $p < 0.01$ (unpaired two-tailed Student *t* test).

airway epithelial cell mucin accumulation that was indistinguishable from OVA-treated wild type control animals (Fig. 5A). OVA-treated *PHIL* mice transferred with DCs (i.t.) and eosinophils (i.p.) were comparable in their histopathology to OVA-treated *PHIL* mice transferred only with DCs. Marked elevations in BAL total cellularity also occurred in OVA-treated *PHIL* mice receiving OVA-pulsed DCs relative to nonsensitized *PHIL* animals (Fig. 5B). However, a significant change occurred in the composition of the BAL cellularity. In OVA-treated *PHIL* DC-recipient mice, the OVA-induced BAL cellularity was predominantly a neutrophilic and lymphocytic infiltrate. The significance of this airway neutrophilia is that it was Ag dependent (i.e., memory T cell dependent) because nonsensitized *PHIL* DC-recipient mice (i.e., mice with no OVA-specific memory T cells) failed to develop airway inflammation (Fig. 5A). In comparison, OVA-treated wild type mice receiving OVA-pulsed DCs developed both airway inflammation and a significant eosinophilic infiltrate in the airways with no increase in neutrophil levels (Fig. 5B). Surprisingly, despite the induced neutrophilia in the OVA-treated DC-recipient *PHIL* mice, the BAL cytokine milieu was Th2 polarized with elevated expression levels of cytokines such as IL-13 and undetectable BAL levels of either IFN-γ or IL-17 (Fig. 5C). Thus, in the absence of eosinophils, OVA-mediated immune responses were mixed with a decidedly Th2 polarized cytokine response in the lung that nonetheless was accompanied by an induced airway neutrophilia. Significantly, OVA-treated *PHIL* mice receiving cotransfer of eosinophils with the OVA-pulsed DCs resulted in a significant reduction in the percentage of neutrophils in the BAL as compared with *PHIL* mice transferred with OVA-pulsed DCs alone (Fig. 5B), suggesting that eosinophils are key mediators of modulating the pulmonary cellular infiltrate after OVA challenge.

Eosinophils establish the character of induced pulmonary immune responses to allergen by suppressing Th17 immunity in the LDLNs after OVA challenge

The increased presence of neutrophils and lymphocytes as part of the BAL cellular infiltrate of OVA-treated *PHIL* mice after the transfer of OVA-pulsed DCs suggested that in the absence of eosinophils, a change in the character of the OVA-induced memory T cell responses occurred. The role of eosinophils in modulating the unique phenotype of LDLN effector T cell populations was determined by coculturing OVA with LDLN cells from each of these groups of mice and assessing their expression of the cytokines IL-17 (Th17), IFN-γ (Th1), and IL-13 (Th2). LDLN cells from OVA-treated wild type mice after adoptive transfer (i.t.) of OVA-pulsed DCs displayed a distinct Th2 polarized phenotype, expressing only IL-13 (Fig. 6). In contrast, OVA-treated *PHIL* animals receiving OVA-pulsed DCs had a mixed effector T cell phenotype with increased levels of IFN-γ (Th1), IL-13 (Th2), and IL-17 (Th17). Strikingly, adoptive transfer of eosinophils into OVA-treated *PHIL* recipients that also received OVA-pulsed DCs selectively resulted in the suppression of the induced Th17 responses (coinciding with a reduction of the airways neutrophilia; Fig. 5B) in these mice and, to a lower, yet not significant degree, the Th1 responses; this leads to the emergence of a Th2 polarized immune response (Fig. 6). These data demonstrate a unique role for eosinophils in suppressing Th17 and possibly Th1 events after transfer of OVA-pulsed DCs that changes the character of the induced pulmonary immune response(s).

Discussion

Allergen exposure to the airways of sensitized mice results in a cascade of events that have been characterized as Th2-dominated immune responses leading to histopathologies and lung dysfunction.

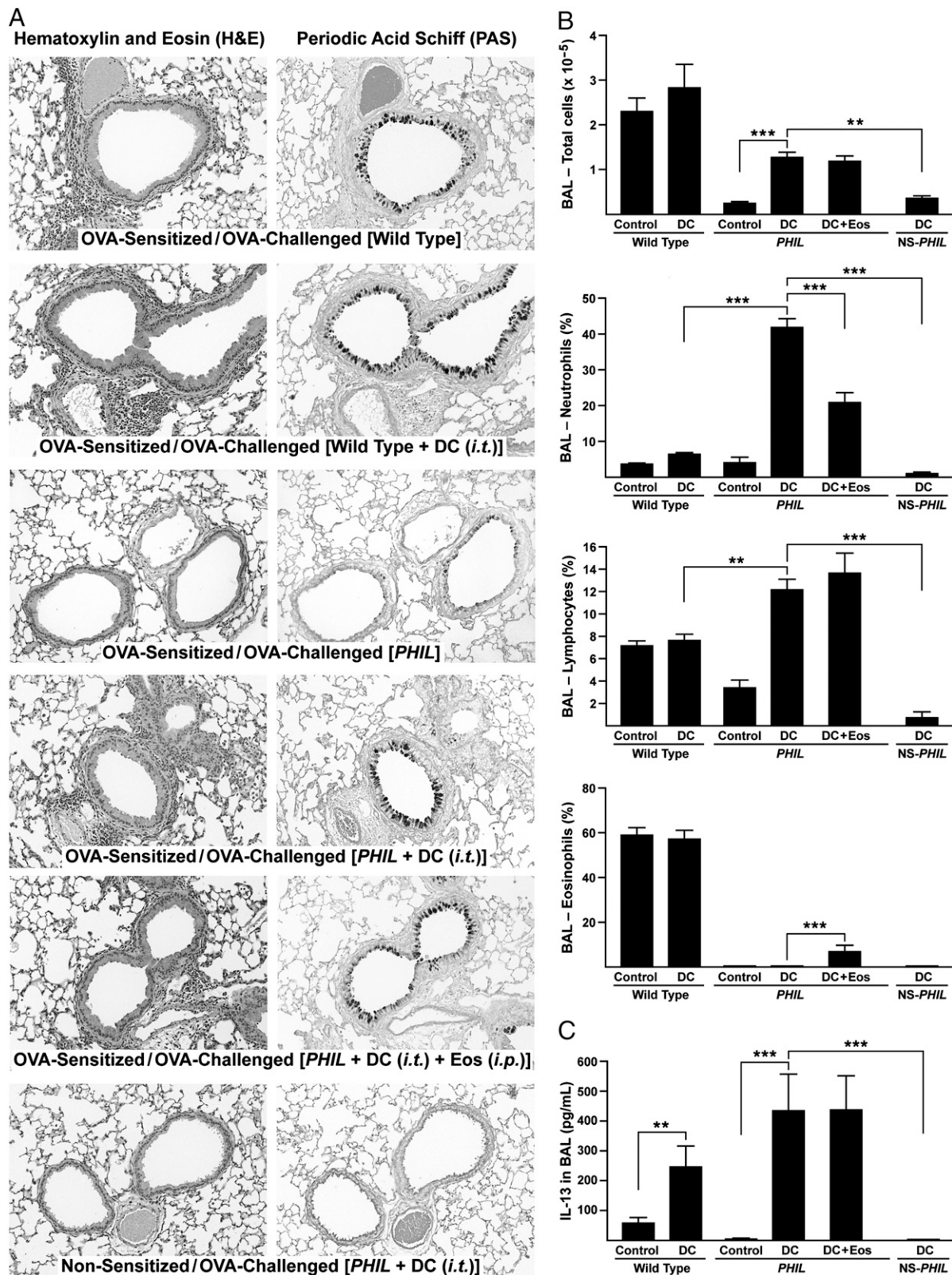


FIGURE 5. Transfer of OVA-pulsed myeloid DCs into *PHIL* leads to neutrophilic inflammation. Bone marrow-derived and OVA-pulsed DCs ($2\text{--}3$ million cells) were transferred into the lungs of OVA-sensitized/challenged wild type and *PHIL* mice by i.t. instillation +DC (i.t.) on the first day of OVA challenge (day 24) and assessed on day 29. An additional cohort of OVA-treated *PHIL* mice also were adoptively transferred with 4×10^7 eosinophils i.p. on the same day (i.e., day 24) as the DC transfer (+ DC [i.t.] + Eos [i.p.]). In addition, bone marrow-derived, OVA-pulsed, myeloid DCs were transferred (i.t.) into a cohort of OVA-naïve recipient *PHIL* mice that were subsequently OVA-challenged (nonsensitized/OVA-challenged [NS-*PHIL*]) to determine the necessity of Ag-dependent memory T cell activation. OVA sensitized/challenged wild type and *PHIL* mice subjected to the acute OVA protocol without cell transfer were performed as additional controls. A, Representative images of lung sections assessing cellular inflammation (H&E staining) and goblet cell metaplasia/airway epithelial cell mucin accumulation (periodic acid–Schiff staining, dark stain). B, Airway cellular infiltration was measured by calculating total BAL cellularity and differentials of neutrophils, lymphocytes, and eosinophils. DC represents mice receiving (Figure legend continues)

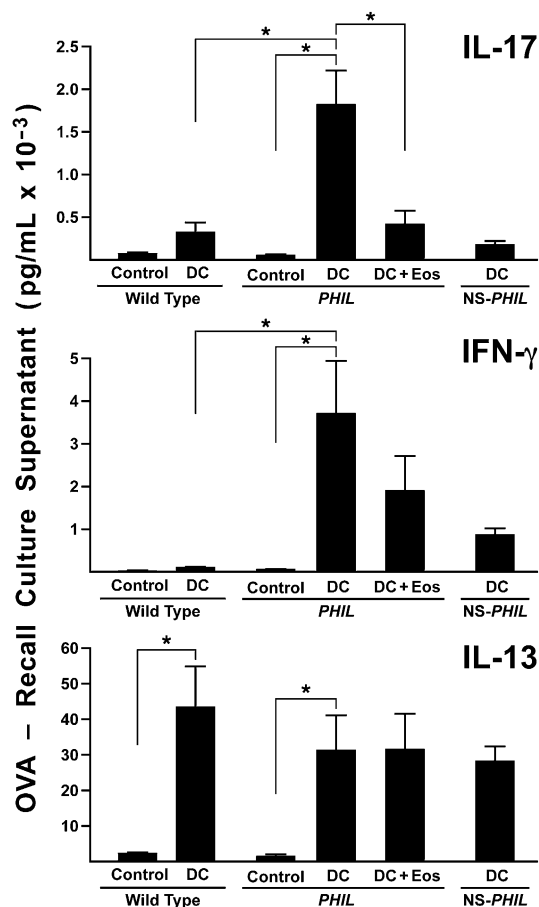


FIGURE 6. Eosinophil-dependent suppression of Th17 immune responses after transfer of OVA-pulsed myeloid DCs. Bone marrow-derived and OVA-pulsed DCs (2–3 million cells) were transferred into the lungs of OVA-treated (OVA-sensitized/challenged) wild type or *PHIL* mice on day 24 of the acute OVA protocol described and assessed on day 29. DC represents mice receiving DCs via i.t. transfer; Control represents transfer of vehicle alone. An additional cohort of OVA-treated *PHIL* mice also were adoptively transferred with 4×10^7 eosinophils i.p. on the same day (i.e., day 24) as the DC transfer (DC + Eos). Nonsensitized but OVA-challenged *PHIL* recipients adoptively transferred (i.t.) with DCs are indicated by NS-*PHIL*. LDLN-derived cells from each cohort of mice were incubated for 72 h in the presence of OVA, and culture supernatant levels of IL-17, IFN- γ , and IL-13 were assessed by ELISA. All data are derived from at least four independent experiments, each with cohort sizes of two to four mice (mean $\pm$ SEM). * $p < 0.05$ (unpaired two-tailed Student *t* test).

tion pathognomonic of asthma. Although eosinophil accumulation in the lung has been considered a hallmark part of these Th2-dominated pulmonary immune responses [occurring in both patients (32) and animal models (33)], the definition of specific eosinophil-mediated mechanisms within the immune cascade associated with allergen provocation still has remained unclear. The seemingly incongruent observation that both dendritic cells (8, 10, 11) and eosinophils (5, 6) are necessary for Th2 pulmonary immune responses suggest that previously unrecognized (and/or poorly understood) nodes exist whereby these two cells interact

as part the development of pulmonary immune responses. The data presented in this study now provide evidence for these nodes.

Our studies demonstrated that mice deficient in eosinophils were unable to promote the Th2 polarization of the lung microenvironment after allergen provocation because of a T cell activation/proliferation deficiency in LDLNs. This observation was linked, in part, to a failure of DCs to accumulate in the LDLNs of OVA-treated *PHIL* mice, suggesting a previously underappreciated mechanism by which the steady-state presence of circulating/lymphatic eosinophils are necessary for pulmonary DC emigration to the LDLNs after allergen challenge. The mechanism of eosinophil-induced recruitment/accumulation of DCs is unknown but may rely on release of eosinophil mediators to influence maturation/activation (29) and/or chemoattraction (29, 34). Significantly, adoptive transfer of eosinophils into *PHIL* mice only modulated the accumulation of the DC subtype known to induce pulmonary inflammation (i.e., MHC II^{hi} myeloid DCs) and did not elicit the accumulation of Ag tolerance-inducing plasmacytoid DCs (35). In addition, adoptive cell transfer studies of MHC II-deficient eosinophils into *PHIL* recipient mice demonstrated that MHC II expression on eosinophils was not required for LDLN T cell proliferation in this allergen challenge model system. Thus, although eosinophils have the potential to act as APCs (14–16), it appears that the multitude of overlapping pathways offered by the various professional APCs available in the pulmonary compartment (in particular, dendritic cells) are both necessary and sufficient providers of the APC activities needed for immune responses to allergen provocation.

The adoptive transfers of activated OVA-pulsed DCs into wild type versus *PHIL* mice suggest a larger than previously suspected role for eosinophils in the Th2-associated pulmonary immune responses that occur after allergen challenge. In particular, these data suggested a reciprocal correlation exists between eosinophils and T cell immune responses such that in the absence of eosinophils, allergen-dependent T cell immunity is skewed and becomes a mixed Th2/Th17/Th1 collection of responses that now induces a pulmonary neutrophilia. This role for eosinophils in DC-mediated T cell polarization provides a parsimonious explanation for observations from several earlier studies investigating the activation and polarization of T cells in allergen-mediated pulmonary immune responses. For example, two recent studies (31, 36) demonstrated that in the absence of DCs (or the targeted knockout of MHC II expression on DCs), LDLN T cell proliferation occurs in mice, but the Th2 polarization of these T cells after allergen challenge is dependent on another (i.e., non-DC) MHC II-expressing cell(s). As a consequence, mice deficient in non-DC MHC II-expressing cells experience development of a neutrophilic and IFN- γ -dependent inflammation, although the status of Th17 was unknown in these models. An additional example of the proposed importance of one or more non-DC MHC II-expressing cells on the skewing of Ag-induced immune responses is provided in studies depleting basophils with MAR-1 (Fc ϵ RI α) Ab in a lung inflammation model (37). Significantly, this Ab also reduced eosinophil levels in these mice (31). In doing so, this leaves open the possibility that eosinophils and not basophils were the contributors skewing the immune responses in that study.

DCs via (i.t.) transfer; DC + Eos represents cotransfer of DCs (i.t.) and eosinophils (i.p.); Control represents vehicle transfer; and NS-*PHIL* represents nonsensitized/OVA-challenged *PHIL* recipient mice receiving DCs via i.t. transfer. C, BAL levels of IL-13 were measured from each group of mice. IL-17 and IFN- γ levels were also performed using these assessments and demonstrated that neither cytokine was detectable in the BALs of these mice. All data are derived from at least four independent experiments, each with cohort sizes of one to four mice (mean $\pm$ SEM). ** $p < 0.01$, *** $p < 0.001$ (unpaired two-tailed Student *t* test).

The mechanisms by which LDLN eosinophils suppress the production of Th17/Th1 cells are unknown; however, they appear to modulate DC-induced memory T cell polarization as they proliferate during their transition into effector T cells. That is, the presence of eosinophils in the immune environment where DC-mediated T cell activation/polarization is occurring provides (or in the case of *PHIL* mice, fails to provide) additional signals to suppress the differentiation of Th17, and to some extent Th1, cells (38). Potential mechanisms by which eosinophils may modulate DC–T cell interactions abound. For example, Yang and colleagues (29) demonstrated that human eosinophil granule proteins can stimulate DCs through a TLR2-dependent pathway, which has been shown to be an important pathway to suppress Th17 responses (39) and induce Th2 pulmonary inflammation (40). Moreover, other studies have demonstrated that eosinophils are also a potentially significant source of the DC-modulating cytokine IL-25 that augments Th2 responses (41). Still other studies have shown that eosinophils express additional cytokines/enzymes with the potential ability(ies) to alter the relative balance of immune responses such as Th2 (IL-4) versus Th1 (IL-12) versus immunosuppressive responses characterized by expression of TGF- β and indoleamine 2,3-dioxygenase (19, 20). Finally, eosinophils may selectively activate or suppress T cells derived from a mixed pool of Th17/Th1/Th2 memory T cells that are plastic in phenotype (42).

The potential significance of eosinophil–DC–T cell interactions as part of cellular/molecular mechanism occurring in subsets of asthma patients offers unique and/or alternative explanations for confounding observations often associated with the care of these patients. Three examples are of particular interest. First, viral infections or postviral complications represent significant events linked with asthma exacerbation leading to hospital visits (43). The assumption has been that viral-mediated lung pathology simply synergized with the difficulties already experienced by asthmatics leading to exacerbation events in this population (44). In addition, studies have demonstrated increased susceptibility to viral infections and extended/greater viral load, suggesting deficiencies in production of Th1 cytokine responses because of augmented Th2 cytokine production (45, 46). The hypothesis articulated in this article suggests that in asthmatic patients experiencing a viral infection, some DCs arriving in LDLNs encounter activated eosinophils that may differentially suppress Th17/Th1 events. This, in turn, would reduce targeted antiviral immune responses, increasing the likelihood of infection and/or its severity (47). Moreover, the suppression of Th17/Th1 immune responses by activated eosinophils may also lead to the disproportional production of viral Ag-specific Th2 effector cells that upon recruitment to the lung enhance allergic respiratory inflammation in these patients (48). Second, the paradigm of eosinophil–DC–T cell interactions suggests that inhaled corticosteroids (ICS) may attenuate local immune responses in the lung via a novel eosinophil-dependent pathway. Specifically, in addition to the ability of ICS to eliminate eosinophil activities through cytotoxic effects (49), ICS treatment of patients would disrupt eosinophil-mediated positive regulatory loops promoting the production of Th2 effector cells in LDLNs and their subsequent recruitment to the lung. It is noteworthy that this same eosinophil-mediated disruption of immune-regulatory loops may also provide an explanation for the observation that the most significant effect of anti-IL-5–based treatment of asthma patients (i.e., mepolizumab) (50, 51) is not acute improvement of lung function with the resolution of symptoms, but the reduction of exacerbation events over time. Third, the proposed Th17-suppressive character of eosinophils may also provide a mechanism to explain the symptoms/pathologies experienced by patients with neutrophil-dominated severe asthma. In

particular, corticosteroid administration that reduces pulmonary and LDLN-associated eosinophils may also have the additional consequence of inducing/exacerbating severe and neutrophilic asthma (52) through increased Th17 production in these patients (53).

We suggest that the respective activities and importance of pulmonary and LDLN eosinophils may be greater than previously suspected. In particular, the roles of eosinophils as unique mediators of local immune responses provide new insights as to the larger contributory role of this leukocyte in the complex inflammatory cascades leading to disease pathology. We would also suggest that these roles are likely to extend beyond asthma and allergic diseases, and may provide explanations for the involvement of eosinophils in other inflammatory diseases that have been linked with these cells, including helminth infection, gastrointestinal diseases, cancer, and organ transplant rejection (54).

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Disclosures

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1.

Timelines describing the acute OVA sensitization/aerosol challenge protocol and the adoptive transfer addendums to this base protocol. Details of this protocol and the adoptive transfer addendums as described in Materials and Methods.

Supplementary Figure 2.

***PHIL* mice fail to induce proliferation of T cells in the lung draining lymph nodes (LDLNs) in a model of acute ragweed allergen sensitization/challenge protocol.** Ragweed-treated mice were sensitized with a 100µl injection (*i.p.*) of a 1:1 ratio of 400µg ragweed (Greer)/ml and 2.25 mg of Imject Alum on days 0 and 7. Sensitized mice received an intranasal (*i.n.*) challenge with 20µl of 5mg/ml ragweed on days 14, 15 and 16 and assessed on day 18. Mice were given an *i.p.* injection of BrdU (3mg/mouse) four hours prior to LDLN harvest on day 18. BrdU labeled T cells (BrdU⁺/TCRαβ⁺) in LDLNs were assessed by flow cytometry of single cell suspensions. All data are derived from at least two independent experiments each with cohort sizes of 2-4 mice (error bars, s.e.m.). **p*<0.05 (unpaired two-tailed student's *t*-test).

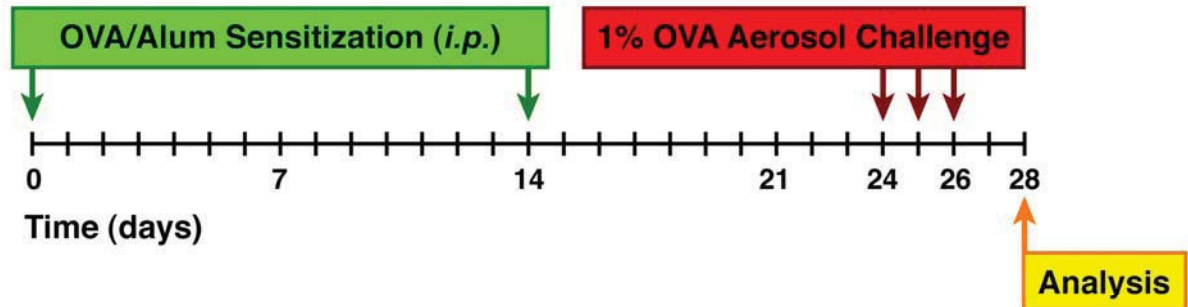
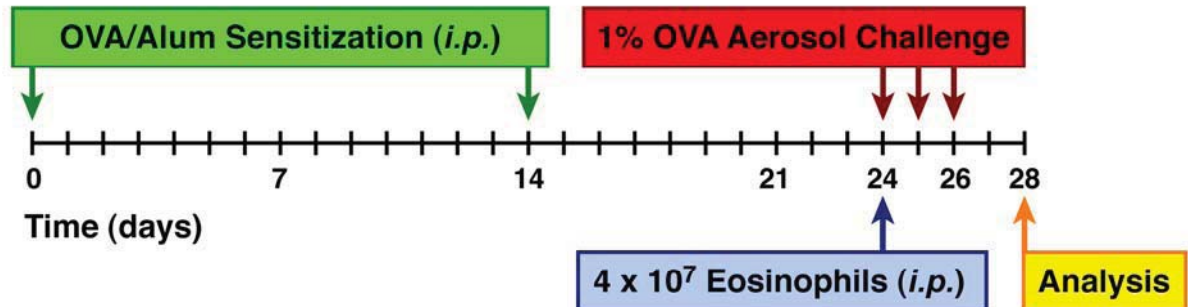
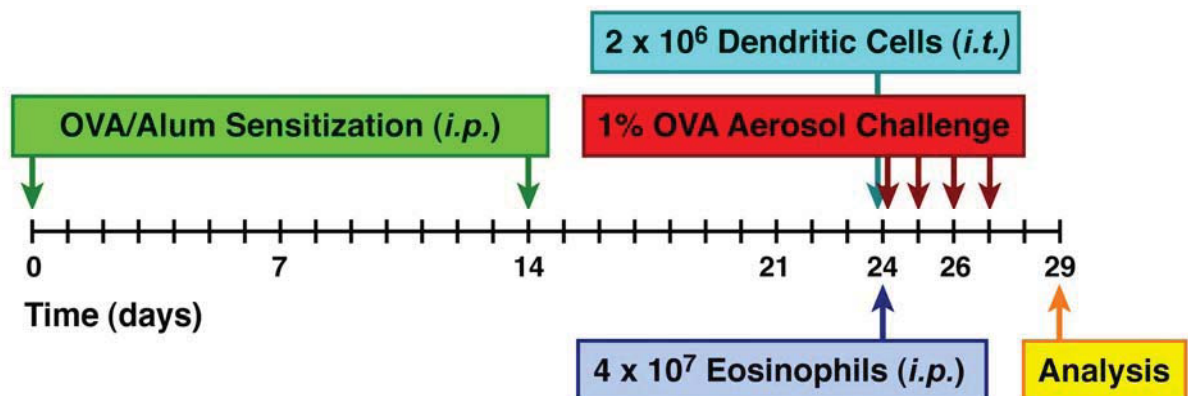
Supplementary Figure 3.

Lymphatic trafficking of eosinophils from the intraperitoneal cavity to the LDLNs in response to OVA provocation does not require CCR7 expression. Peripheral blood eosinophils were isolated and adoptively transferred (via the peritoneal cavity (*i.p.*)) into OVA-treated wild type vs. ***PHIL*** (4×10^7 eosinophils one hour prior to the first OVA challenge). An additional cohort of OVA-treated ***PHIL*** mice were adoptively transferred (*i.p.*) with CCR7 deficient (CCR7^{-/-}) eosinophils derived from compound transgenic/gene knockout mice (i.e., mice resulting from a cross of IL-5 transgenic (NJ.1638 (19)) and CCR7 knockouts (B6.129P2(C)-Ccr7^{tm1Rfor/J}) (Jackson Laboratories). The equivalent ability of eosinophils (CCR3⁺/Siglec-F⁺) to migrate to LDLN irrespective of their CCR7 expression status was demonstrated by flow cytometry assessment. All data are derived from at least three independent experiments each with cohort sizes of 2-4 mice.

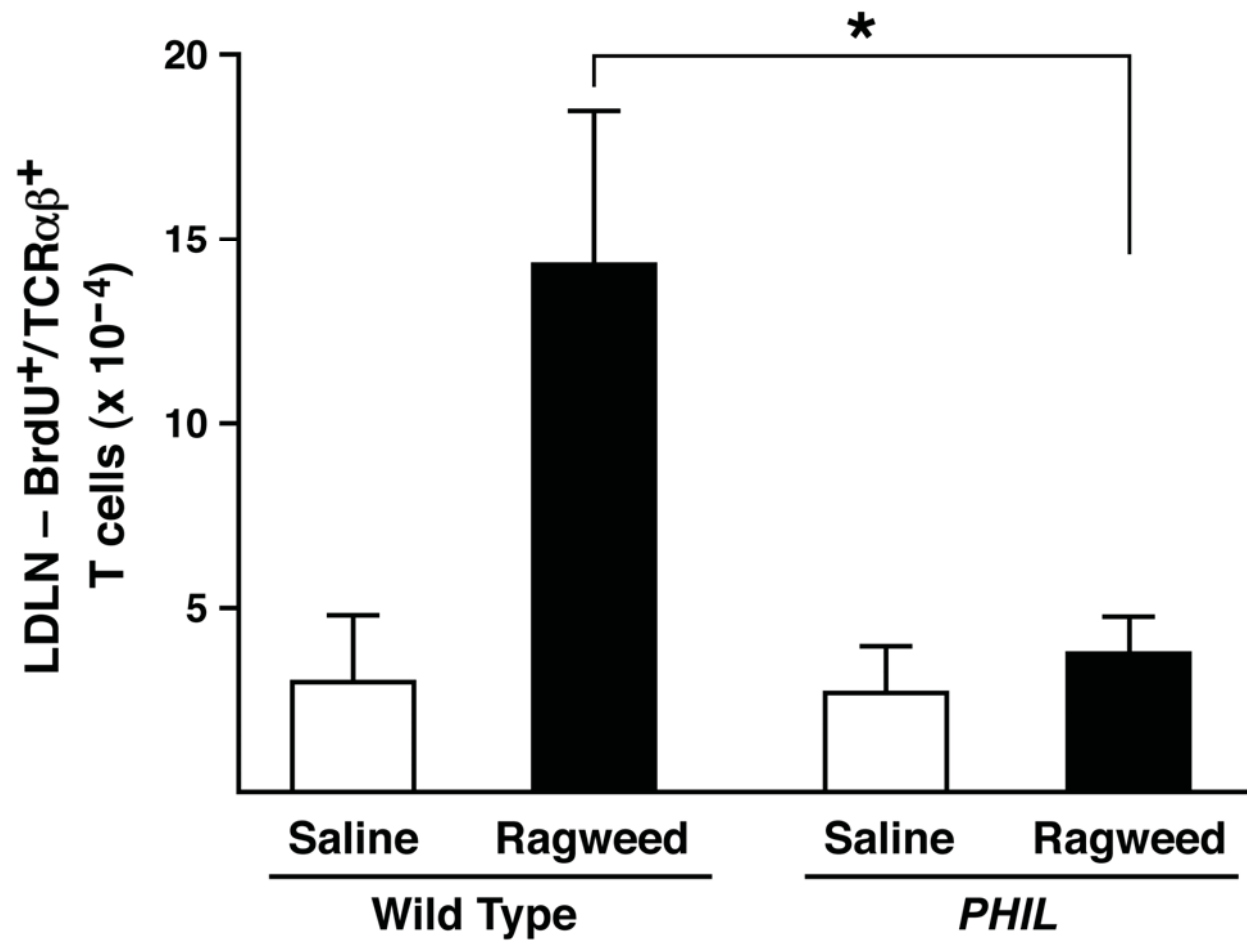
Supplementary Figure 4.

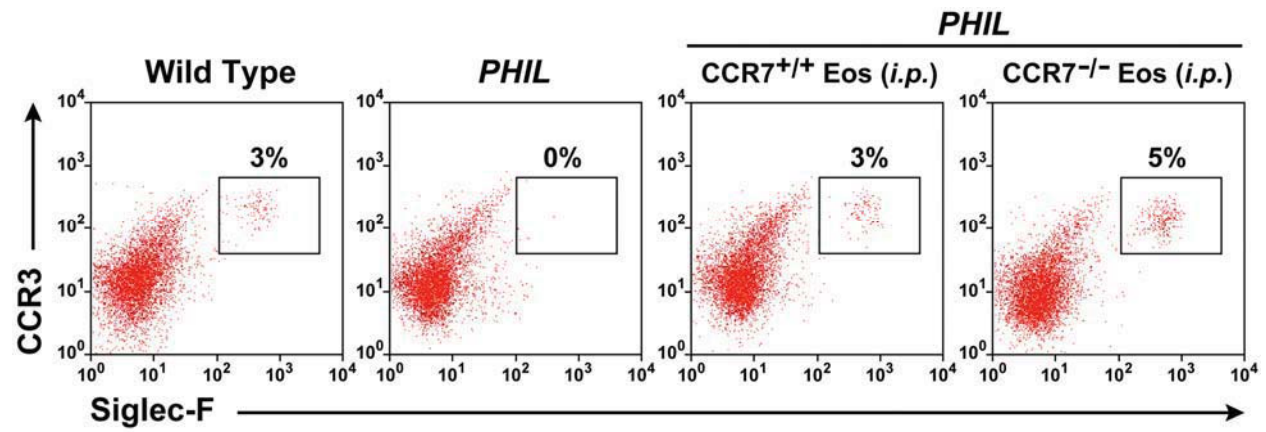
The presence of basophils in the LDLN of OVA-treated wild type and *PHIL* mice was equivalent and thus not dependent on the presence/absence of eosinophils. Basophils (B220⁻/CD4⁻/CD49b(DX5)⁺/IgERα1(MAR-1)⁺) cells were assessed in OVA-treated wild type and ***PHIL*** mice on day 28 of the acute OVA sensitization/challenge protocols. All data are derived from at least two independent experiments each with cohort sizes of 2-4 mice (MEAN ± SEM.).

Supplemental Figure 1.

A Acute Ovalbumin (OVA) Protocol**B Eos (*i.p.*) Protocol****C DC (*i.t.*) + Eos (*i.p.*) Protocol**

Supplemental Figure 2.



Supplemental Figure 3.

Supplemental Figure 4.

