

Allergic pulmonary inflammation in mice is dependent on eosinophil-induced recruitment of effector T cells

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The current paradigm surrounding allergen-mediated T helper type 2 (Th2) immune responses in the lung suggests an almost hegemonic role for T cells. Our studies propose an alternative hypothesis implicating eosinophils in the regulation of pulmonary T cell responses. In particular, ovalbumin (OVA)-sensitized/challenged mice devoid of eosinophils (the transgenic line *PHIL*) have reduced airway levels of Th2 cytokines relative to the OVA-treated wild type that correlated with a reduced ability to recruit effector T cells to the lung. Adoptive transfer of Th2-polarized OVA-specific transgenic T cells (OT-II) alone into OVA-challenged *PHIL* recipient mice failed to restore Th2 cytokines, airway histopathologies, and, most importantly, the recruitment of pulmonary effector T cells. In contrast, the combined transfer of OT-II cells and eosinophils into *PHIL* mice resulted in the accumulation of effector T cells and a concomitant increase in both airway Th2 immune responses and histopathologies. Moreover, we show that eosinophils elicit the expression of the Th2 chemokines thymus- and activation-regulated chemokine/CCL17 and macrophage-derived chemokine/CCL22 in the lung after allergen challenge, and blockade of these chemokines inhibited the recruitment of effector T cells. In summary, the data suggest that pulmonary eosinophils are required for the localized recruitment of effector T cells.

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Abbreviations used: BAL, bronchoalveolar lavage; i.n., intranasal(ly); i.t., intratracheal(ly); MBP, major basic protein; MDC, macrophage-derived chemokine; TARC, thymus- and activation-regulated chemokine.

The activation, proliferation, and recruitment of T cells to the lungs of allergic asthmatic patients have been shown to promote the immune responses generally associated with this disease, such as airway Th2 cytokine production, the recruitment/accumulation of eosinophils, and induction of pulmonary pathologies (1). The importance of T lymphocytes in mouse models of allergic respiratory inflammation is also highlighted in studies showing that depletion of T cells in allergen-provoked mice inhibits asthma pathologies, including eosinophil recruitment to the lungs (2). Interestingly, although eosinophil recruitment to the lung has been a defining characteristic of allergic respiratory inflammation, occurring, for example, even in mild forms of asthma (3), causative links between pathologies and specific eosinophil-mediated activities have remained unresolved (e.g., reference 4 vs. reference 5), with some

clinical studies even discounting significant roles for eosinophils (6). Instead, most papers have implied a nearly unidirectional immune regulatory mechanism by which T cells promote the inflammation of asthma with the role of eosinophils remaining ambiguous and the subject of debate. This ambiguity results, in part, because the definition and significance of unique eosinophil effector functions have also remained debatable, with the primary role of eosinophils restricted to destructive effector cells mediating tissue damage (7). Specifically, the dominant effector functions ascribed to eosinophils have been hypothesized to result from the production of reactive oxygen species (i.e., respiratory burst) and the release of cationic granule proteins (i.e., eosinophil degranulation) (8). This perspective is so pervasive that it often excludes discussion of other effector functions (for review see reference 9).

Recent studies of eosinophils and their associated activities have nonetheless revealed a

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wealth of complexity that extends beyond the notion that these leukocytes are primarily a delivery system for their cationic and toxic granule proteins. Two areas of research are primarily responsible for this changing paradigm. First, studies in the mouse have overwhelmingly shown both the absence of extensive eosinophil degranulation in most Th2 inflammatory models and the lack of any significant contribution by eosinophil granule proteins to the disease pathologies associated with pulmonary allergen provocation (10, 11). Yet, in studies using mice congenitally deficient of eosinophils, the unique absence of these granulocytes leads to a diminution of Th2 immune responses in both acute and chronic models of allergen provocation (5, 12). Second, studies assessing immune responses associated with allergen provocation or parasite infection have implicated eosinophils as potential immune regulatory cells capable of modulating T cell responses and, in turn, local tissue inflammation. For example, eosinophils have been shown to express key mediators of T cell inhibition, activation, and polarization, such as IL-2, IL-4, indoleamine 2,3-dioxygenase, TGF- β , and IL-10 (9). In addition, recent studies have highlighted the potential role of eosinophils as antigen-presenting cells as part of immune responses leading to T cell proliferation/activation in response to allergen provocation in the lung (13), as well as in response to parasite infection (14). Collectively, these studies suggest that eosinophils may be critical cells regulating Th2 immune responses in the lung after allergen provocation, and their role may even supercede the functions of T cells (for review see reference 15).

In this study, we provide support for the novel hypothesis that a primary role of eosinophils in allergic pulmonary responses is to elicit the localized recruitment/accumulation of effector T cells in the lung. In particular, our data using the eosinophil-deficient transgenic strain of mice known as *PHIL* (5) and two independent models of allergic respiratory inflammation showed that the attenuation of Th2 immune responses in the absence of eosinophils (i.e., relative to eosinophil-sufficient wild-type animals) was accompanied by a concomitant and specific loss of T cells from the lung and regional draining lymph nodes. Moreover, using adoptive cell transfer of both eosinophils and allergen-specific activated effector T cells, we were able to show that eosinophil-mediated activities were required for the recruitment of T cells to the lung, and that one of the mechanisms of this recruitment is likely through the induced production of the Th2-chemoattracting chemokines thymus- and activation-regulated chemokine (TARC)/CCL17 and macrophage-derived chemokine (MDC)/CCL22 in the lung.

RESULTS

The reduction of allergen-induced pulmonary pathologies in OVA-treated *PHIL* mice is accompanied by a corresponding decrease in pulmonary Th2 immune responses

We used a novel eosinophil-deficient transgenic line (*PHIL*), which is congenitally devoid of eosinophils without disturbing the production of other hematopoietic lineages (5), to

unequivocally show the relationship between eosinophils and the development of pulmonary Th2 immune responses in a mouse model of acute respiratory inflammation. Specifically, *PHIL* mice and C57BL/6J wild-type controls were sensitized (days 0 and 14) with OVA/Alum and challenged with an OVA aerosol on days 24, 25, and 26 (control animals received saline alone), and endpoints of pathology were analyzed on day 28 (Fig. S1 A, available at <http://www.jem.org/cgi/content/full/jem.20071840/DC1>). In contrast to wild-type controls, *PHIL* mice were unable to induce a pulmonary eosinophilia (Fig. 1 A) or elevate bronchoalveolar lavage (BAL) Th2 cytokines (IL-4, IL-5, and IL-13) in response to allergen (Fig. 1 B). Previous hypotheses characterizing the immune responses in the lung after allergen provocation have implicated T cells in the production of Th2 cytokines (1). Furthermore, mouse models of chronic allergic asthma have correlated reduced airway eosinophilia in CCR3 knockout mice and eosinophil-deficient mice with reduced lymphocyte infiltration and hindered Th2 pathologies (12). This led us to determine the numbers of lymphocytes accumulating in the airways of allergen-provoked *PHIL* mice relative to wild-type animals as a possible explanation of the reduced Th2 responses in the absence of eosinophils. These assessments showed that lymphocyte numbers in the airways of OVA-treated *PHIL* mice were significantly reduced as compared with similarly treated wild-type controls (Fig. 1 C). Our studies also demonstrated that the decreased lymphocyte infiltration into the airways of *PHIL* mice was not transient and that the loss of eosinophils had consequences for the extended kinetics of lymphocyte accumulation. Specifically, *PHIL* and wild-type controls were allergen challenged and analyzed at different time points after allergen challenge (Fig. S1 B). BAL cell accumulation data demonstrated that the eosinophil infiltration of the airways in wild-type mice occurred maximally 4 d after allergen challenge and were resolved (i.e., returned to baseline levels) within 2 wk (Fig. 1 D). In contrast, although lymphocyte infiltration of the lungs in wild-type mice followed a similar kinetic pattern early (i.e., achieving a maximum at ~4 d after challenge), these cells remained elevated during the subsequent 15-d period after allergen challenge (Fig. 1 E). However, these data also showed that in the absence of eosinophils, the kinetics of lymphocyte accumulation in the airways are disrupted with neither the maximal increase at 4 d after allergen challenge nor the sustained elevation of lymphocytes occurring in OVA-treated *PHIL* mice (Fig. 1 E). Thus, allergen-mediated eosinophil recruitment to the lungs of mice appears to be necessary for the pulmonary recruitment and accumulation of lymphocytes, and the corresponding production of BAL Th2 cytokines.

OVA-treated *PHIL* mice fail to accumulate both CD4⁺ and CD8⁺ T cells in the airways, lungs, and regional draining lymph nodes

The lack of significant accumulation of total lymphocytes and the reduced Th2 cytokine expression suggested the potential that both CD4⁺ and CD8⁺ T cell accumulation in the lungs

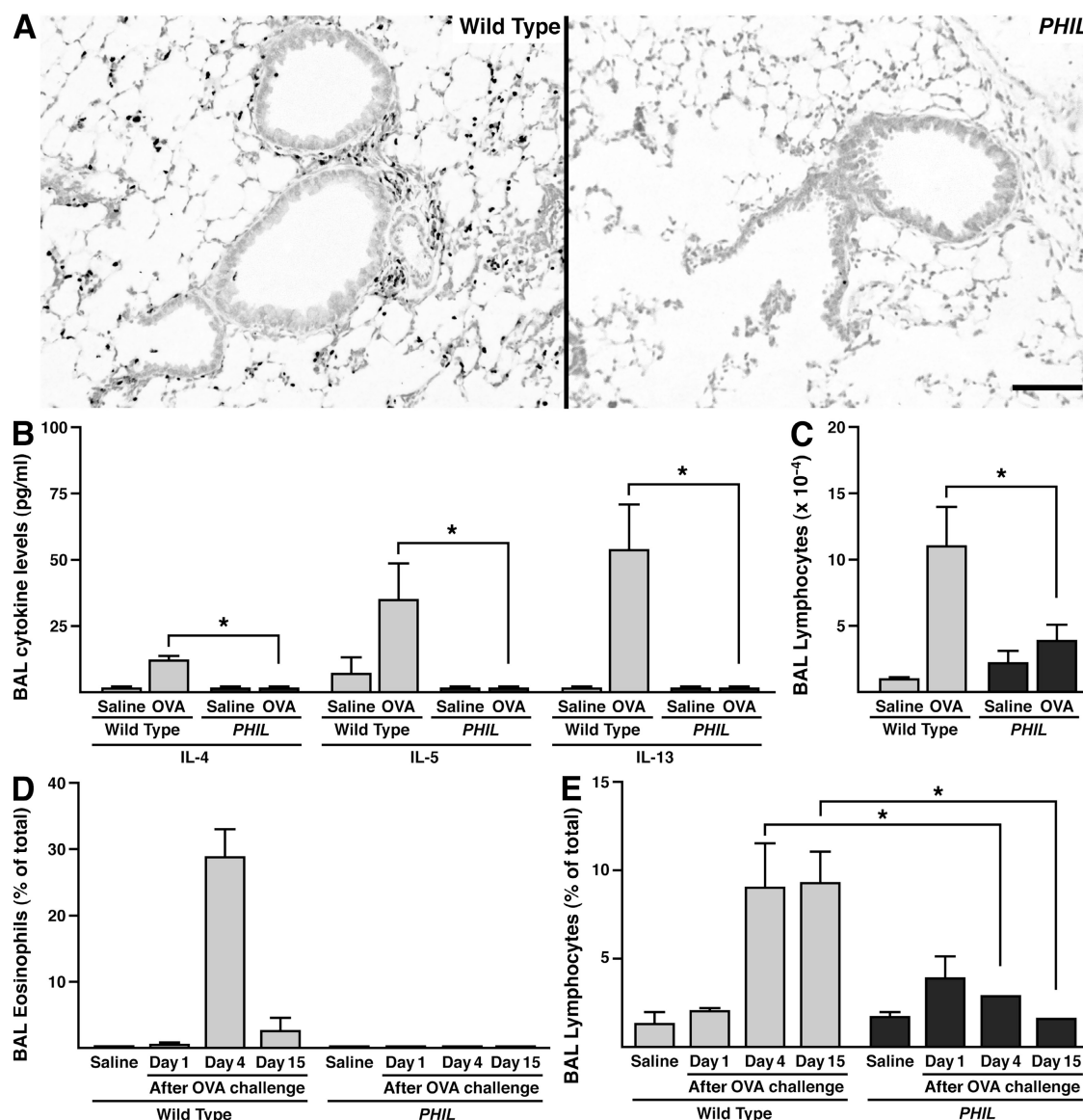


Figure 1. Allergen-mediated pulmonary Th2 inflammatory responses and lymphocyte infiltration of the lung are attenuated in the absence of eosinophils. Wild-type and *PHIL* mice were subjected to the acute OVA sensitization/challenge (control animals received saline alone) protocol described in Materials and methods and Fig. S1 A. (A) Immunohistochemistry using an anti-mouse MBP rat mAb (black) demonstrated the absence of infiltrating eosinophils in OVA-treated *PHIL* mice relative to wild-type controls. Bar, 100 μ m. (B) Allergen-induced increases in BAL Th2 cytokine levels (as assessed by ELISA) failed to occur in *PHIL* mice as compared with wild-type controls. (C) Assessments of BAL cellularity demonstrated that lymphocyte numbers are significantly reduced in OVA-treated *PHIL* mice relative to wild-type controls. Kinetic assessments (Fig. S1 B) of OVA-induced eosinophil (D) and lymphocyte (E) accumulation in the BAL of wild-type versus *PHIL* mice 1, 4, and 15 d after OVA challenge showed that in the absence of eosinophils, the early and sustained influx of lymphocytes into the lung after allergen challenge is abolished. Data presented are the means \pm SE. *, $P < 0.05$.

may be altered in OVA-treated *PHIL* mice (15). Leukocytes from the airways (i.e., BAL), whole-lung digests, caudal mediastinal lymph nodes, and splenocytes from OVA-sensitized/aerosol-challenged *PHIL* and wild-type animals were analyzed by flow cytometry for populations of CD4⁺ and CD8⁺ T cells (Fig. 2). In contrast to OVA-treated wild-type controls, which displayed significant increases in both T cell subtypes relative to saline control groups, OVA-treated *PHIL*

mice failed to display allergen-mediated increases in either CD4⁺ or CD8⁺ T cells in the airways, lungs, and regional draining lymph nodes. This lack of T cell accumulation was restricted to the lungs and lymph nodes, as spleens from these mice did not statistically differ in their total numbers of CD4⁺ and CD8⁺ T cells, suggesting an inability to accumulate these cells specifically to pulmonary compartments after OVA provocation.

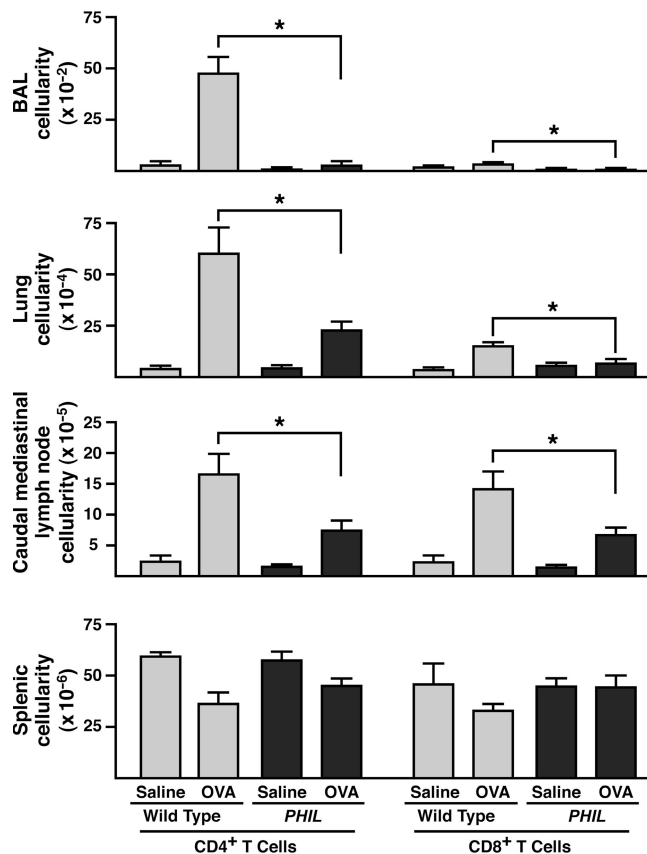


Figure 2. The OVA-induced accumulation of both CD4⁺ and CD8⁺ T cells is significantly reduced in the airways, lungs, and regional draining lymph nodes in the absence of eosinophils. Flow cytometric assessments of leukocytes derived from BAL, whole-lung digests, and lung draining lymph nodes showed that in the absence of eosinophils (i.e., *PHIL*), significant reductions of both CD4⁺ and CD8⁺ T cell subtypes are observed in all three pulmonary compartments after OVA provocation. This observation was shown to be lung specific, as assessments of the splenic cellularity of these mice showed no difference in either CD4⁺ or CD8⁺ T cell numbers between OVA-treated wild-type and *PHIL* mice. In each case, single-cell suspensions were counted, and the fractional composition of each suspension was assessed by flow cytometry based initially on scatter to establish a lymphocyte gate, and then stained with antibodies for the T cell markers CD3, CD4, and CD8. Data presented are the means \pm SE. *, $P < 0.05$.

Eosinophil-dependent T cell activation and recruitment each represent mechanisms leading to the reduced pulmonary T cell accumulation in OVA-treated *PHIL* mice

Mouse models of allergen-mediated Th2 inflammation have been attributed to the activities (e.g., Th2 cytokine expression) of particular subsets of CD4⁺ T cells in the lung (e.g., CD4⁺/CD25⁺ cells [i.e., proliferating T cells and/or regulatory T cells; reference 16], NKT cells [CD4⁺/NK1.1⁺; reference 17]), and effector T cells (CD4⁺/CD62L^{-/lo}/CD44^{hi} [18]), as well as CD8⁺ T cells (19). To define the extent of the effects on pulmonary T cell recruitment in the absence of eosinophils, the numbers of CD4⁺ T cells within each subset were determined in OVA-treated *PHIL* mice relative to their accumulation

in wild-type control animals. The lungs of OVA-treated *PHIL* mice displayed a significant reduction in accumulating CD4⁺/CD25⁺ T cells and CD4⁺/NK1.1⁺ NKT cells (Fig. 3 A) relative to OVA-treated wild-type controls. Furthermore, examination of the T cell subtypes in OVA-treated *PHIL* versus wild-type mice showed that significant accumulation of effector T cells (CD4⁺/CD62L^{-/lo}/CD44^{hi}) in the lungs and regional draining lymph nodes occurred only in wild-type animals and was absent in OVA-treated *PHIL* mice (Fig. 3 B). Interestingly, the regional draining lymph nodes of OVA-treated *PHIL* mice also displayed a significant reduction in memory (CD4⁺/CD62L^{med/hi}/CD44^{hi}) but not naive (CD4⁺/CD62L^{hi}/CD44^{-/lo}) T cells (Fig. 3 B). Furthermore, assessments of CCR7 expression (i.e., a memory/effector T cell marker that appears to participate in the recruitment of T cells to lymph nodes in allergen challenge models [20]) also demonstrated that CD4⁺/CCR7⁺ T cells were reduced in OVA-treated *PHIL* draining lymph nodes as compared with wild-type controls (0.65 ± 0.12 vs. $2.18 \pm 0.35 \times 10^5$, respectively; $P < 0.05$). These phenomena appear to be specific to the pulmonary compartments, as splenocyte numbers of effector, memory, and naive T cells are unchanged between OVA-treated *PHIL* and wild-type control animals. Thus, the loss of these T cell populations in the lungs and regional draining lymph nodes of OVA-treated *PHIL* mice suggested that the absence of eosinophils led to the loss of T cell proliferation/activation, a loss of recruitment pathways leading to T cell accumulation in the lung, or both.

Several previous studies have demonstrated that eosinophils participate in the proliferation/activation of naive and memory T cells into effector T cells (e.g., through antigen presentation and co-stimulation [14, 21–23]), which may be evident by the reduced numbers of memory/effector T cells in the regional draining lymph nodes of OVA-treated *PHIL* mice. Conversely, the impaired recruitment of all T cell subsets (CD8⁺, CD4⁺/CD25⁺, CD4⁺/NK1.1⁺, and CD4⁺/CD62L^{-/lo}/CD44^{hi}) into the lung may implicate a larger and more profound failure in T cell recruitment, extending beyond potential eosinophil activities mediating allergen-specific T cell proliferation/activation. We assessed the potential of each of these eosinophil-dependent mechanisms as contributors to pulmonary T cell accumulation in two independent experiments.

First, in eosinophil-mediated T cell proliferation/activation, our inability to recruit OVA-specific Th2 cells after the adoptive transfer of eosinophils into the airways of OVA-sensitized and -challenged *PHIL* mice (unpublished data) suggested that eosinophils may be required for Th2 cell generation. This potential pathway was further defined in *ex vivo* studies demonstrating that eosinophils were required for the elaboration of Th2 immune responses after allergen challenge. Specifically, splenocytes from OVA-sensitized or OVA-sensitized and -challenged *PHIL* mice (splenocytes from wild-type animals were used as controls) were isolated, and their abilities to elaborate IL-13 were assessed after coculture with media alone, OVA, or a nonspecific stimulus (i.e., cross-linking of surface receptors with anti-CD3 and -CD28

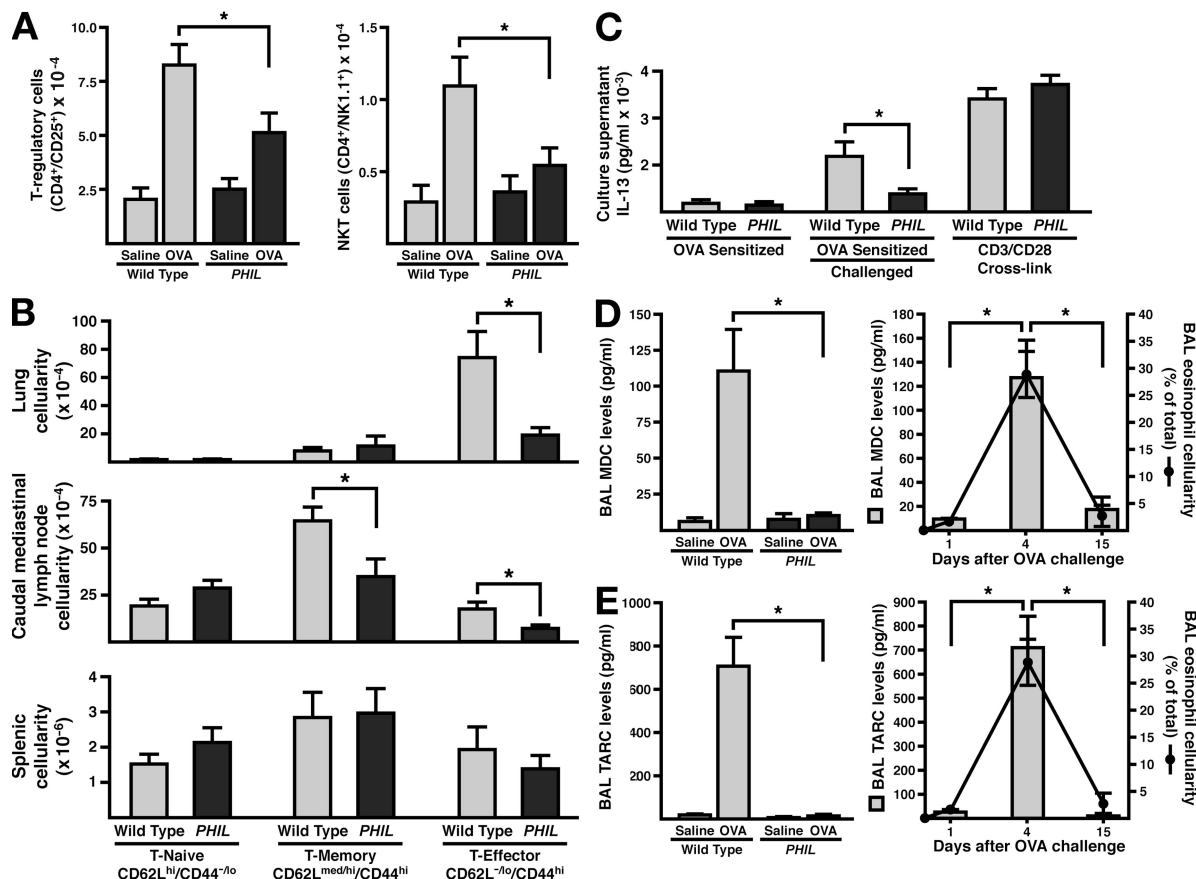


Figure 3. OVA-treated *PHIL* mice display reduced accumulation of CD4⁺ T cell subtypes, intrinsic defects in allergen-specific T cell activities, and the loss of allergen-induced TARC and MDC expression. (A) Determinations of T cell subsets for whole-lung digests, regional draining lymph nodes, and spleen were completed by examining total cell numbers between OVA-treated wild-type versus *PHIL* mice. OVA-treated wild-type versus *PHIL* mice show that CD4⁺/CD25⁺ (T regulatory/proliferation) and CD4⁺/NK1.1⁺ (NKT) cells were elevated in the lungs of OVA-treated wild-type mice but not in OVA-treated *PHIL* mice. (B) Effector T cells (CD4⁺/CD62L^{hi}/CD44^{lo}) were reduced in lungs and caudal mediastinal (i.e., regional draining) lymph nodes. Furthermore, caudal mediastinal lymph nodes also had reduced numbers of memory T cells (CD4⁺/CD62L^{med}/CD44^{hi}). Naive T cell (CD4⁺/CD62L^{hi}/CD44^{lo}) numbers remained unchanged between wild-type and *PHIL* mice in both the spleen and pulmonary compartments. (C) Splenocytes from sensitized and sensitized/challenged *PHIL* and wild-type mice were cultured for 96 h in the presence of media alone, 200 µg/ml OVA, or cross-linked anti-CD3/CD28. Measurement of IL-13 in the supernatant revealed an inability for sensitized/challenged *PHIL* splenocytes to generate wild-type levels of this cytokine. (D) BAL chemokine levels were determined by ELISA from wild-type and *PHIL* mice subjected to the acute OVA sensitization/challenge protocol described in Fig. S1 A. Significantly, although substantive increases in MDC and TARC were observed in the BAL of OVA-treated eosinophil-sufficient wild-type animals (i.e., relative to saline control mice), increased levels of these Th2 cell chemokines did not occur in the lungs of OVA-treated *PHIL* mice (i.e., in the absence of eosinophils). Kinetic assessments (Fig. S1 B) of MDC and TARC in the BAL of post-OVA-challenged wild-type mice showed that the appearance of these Th2 cell chemokines correlated with the levels of OVA-induced airway eosinophilia. Data presented are the means ± SE. *, P < 0.05.

antibodies; Fig. 3 C). These studies demonstrated that the absence of eosinophils (i.e., *PHIL* mice) leads to a defect in OVA-specific Th2 cytokine production. That is, this observed defect was restricted to OVA exposure *ex vivo*, as T cell responses to a nonspecific stimulus were equivalent between splenocytes isolated from *PHIL* versus wild-type mice. Significantly, this OVA-specific defect in IL-13 production was limited to animals that were both OVA-sensitized and aerosol-challenged, suggesting an impairment in the activation/proliferation of OVA-specific memory T cells into polarized Th2 effector T cells in OVA-treated *PHIL* mice. This immunoregulatory role for eosinophils represents a likely mechanism contributing to the attenuated immune responses

in OVA-treated *PHIL* mice and is part of our ongoing studies further characterizing the role of eosinophils in activation/co-stimulation of T cells (unpublished data).

Second, in eosinophil-dependent recruitment/accumulation of T cells to the lung, the impaired accumulation of multiple T cell subsets in OVA-treated *PHIL* mice (i.e., CD8⁺, CD4⁺/CD25⁺, CD4⁺/NK1.1⁺, and CD4⁺/CD62L^{hi}/CD44^{lo} T cells) suggested that eosinophils may be required for generalized pathways that promote localized T cell recruitment. The ability of all of these T cell subtypes to express CCR4 (24–26) also provides a potential mechanism by which eosinophils would be able to mediate the recruitment of T cells to sites of Th2 inflammation. Several lines of evidence further suggested that

the CCR4 chemokine ligands TARC and MDC were likely candidates by which lung-infiltrating eosinophils may elicit the recruitment of T cells: (a) TARC and MDC have been described as part of the mechanisms mediating T cell recruitment in several mouse models of Th2 inflammation (26); (b) induced airway epithelial and macrophage expression of TARC and MDC correlates with the pulmonary eosinophilia associated with allergic respiratory inflammation (27), and the levels of each of these chemokines are elevated in the sputum of asthmatic patients as compared with otherwise normal individuals (28); and (c) microarray analyses of gene expression comparing mRNA levels from BAL-derived eosinophils of OVA-treated mice versus blood eosinophils of OVA-naïve animals showed that TARC and MDC were each significantly elevated (greater than fourfold) in BAL-derived eosinophils (unpublished data) (29).

Assessments of MDC and TARC levels in the BAL of OVA-treated *PHIL* and wild-type mice confirmed that the expression of these chemokines was linked to the presence of pulmonary eosinophils. That is, OVA-sensitized/aerosol-challenged wild-type animals displayed a significant elevation (relative to saline controls) in BAL levels of MDC and TARC that did not occur in OVA-treated *PHIL* mice (Fig. 3 D). In contrast, the BAL levels of other chemokines such as eotaxin-1 and -2 were not statistically different between *PHIL* and wild-type mice (not depicted). Kinetic assessments of MDC and TARC expression in OVA-treated wild-type animals showed that this expression correlated with the induced pulmonary eosinophil infiltrate, with BAL chemokine levels closely paralleling BAL eosinophilia after OVA challenge (Figs. 3 E). Significantly, the levels of each of these Th2 cell chemokines in OVA-treated *PHIL* mice were indistinguishable from saline controls throughout the time period examined, suggesting that eosinophil-induced expression of MDC and TARC may be a contributing (possibly responsible) mechanism for allergen-mediated T cell recruitment to the lung.

Restoration of the Th2 inflammatory lung responses of OVA-treated *PHIL* mice requires the adoptive transfer of both activated OVA-specific effector T cells and eosinophils

Adoptive cell transfers of OVA-specific Th2-polarized T cells into OVA-challenged *PHIL* recipient mice were performed to demonstrate the role of eosinophils in T cell recruitment and the development of Th2-driven immune responses in the lung. Specifically, adoptive transfer of OVA-specific Th2-polarized effector T cells allowed us to bypass requirements of eosinophil-dependent mechanisms of T cell proliferation/activation, testing the hypothesis that eosinophils contribute to the development of allergen-mediated Th2 immune responses through the recruitment of OVA-specific effector T cells. These studies capitalized both on our previous experience with eosinophil adoptive transfer (30) as well as the availability of OVA-specific TCR transgenic mice that allows the isolation/transfer of *in vitro* Th2-polarized OVA-specific effector T cells (OT-II). In this allergen challenge model system, naive eosinophil-sufficient wild-type

and eosinophil-deficient *PHIL* recipient mice were either injected (i.v.) once with PBS or 1.5×10^7 OT-II cells on day 0 of this protocol, followed by intranasal (i.n.) administration of 20 μ l of a 1 μ g/ μ l OVA solution prepared in saline on protocol days 0, 1, and 2 (Fig. 4 A). OVA-induced responses in these recipient mice were assessed on day 4 as the elaboration of Th2 cytokine expression and/or the development of allergen-induced pulmonary histopathology. Because of the eosinophil-deficient character of *PHIL* recipient mice and, therefore, the potential of artifactual events associated with the adoptive transfer of Th2-polarized OVA-specific effector T cells into these recipients, several control studies were performed to ensure that the proliferation and cell-surface activation profile of the adoptively transferred OT-II cells were not altered relative to wild-type control mice. For example, OT-II cells were labeled with the fluorescent marker CFSE, and the proliferation and cell-surface activation markers of these cells were assessed in OVA-challenged *PHIL* versus wild-type mice after adoptive transfer. As shown in Fig. S2 (available at <http://www.jem.org/cgi/content/full/jem.20071840/DC1>), OT-II cell proliferation (as judged by the dilution of CFSE staining intensity) was similar between *PHIL* and wild-type mice, indicating that eosinophils were not required for *in vivo* proliferation of these T cells. In addition, these CFSE-labeled cells maintained their memory ($CD4^+/CD62L^{med/hi}/CD44^{hi}$)/effector ($CD4^+/CD62L^{-/lo}/CD44^{hi}$) T cell surface phenotypes in OVA-challenged *PHIL* animals after adoptive transfer (Fig. S3).

A total of three independent experimental groups of OVA-challenged animals was examined using this adoptive transfer model to define the relative roles of eosinophils in the development of Th2 pulmonary immune/inflammatory responses (Fig. 4 A). Group I consisted of OVA-naïve wild-type and *PHIL* recipient mice receiving OT-II cells i.v. (controls received PBS vehicle alone) on protocol day 0, and saline intratracheally (i.t.) on protocol days 0, 1, 2, and 3. Group II consisted of OVA-naïve *PHIL* recipient mice after adoptive transfer (i.t.) of 10^7 eosinophils on protocol days 0, 1, 2, and 3. The success of eosinophil adoptive transfer was confirmed by the recovery of BAL eosinophils in hematoxylin and eosin-stained cytospin preparations (Fig. S4 A, available at <http://www.jem.org/cgi/content/full/jem.20071840/DC1>) and the identification of tissue-infiltrating eosinophils using major basic protein (MBP)-specific immunohistochemistry (Fig. S4 B) on protocol day 4. Finally, group III consisted of OVA-naïve *PHIL* recipient mice receiving both OT-II cells (i.v.) on protocol day 0 and adoptively transferred (i.t.) eosinophils on protocol days 0, 1, 2, and 3.

These studies showed that the adoptive transfer of OT-II cells (group I) or eosinophils (group II) alone into OVA-challenged *PHIL* recipient mice were each insufficient for the development of allergen-induced Th2 pulmonary responses (Fig. 4 B). Moreover, OVA-naïve wild-type and *PHIL* mice receiving OT-II cells by i.v. and saline by i.n. challenge did not display an increase in either Th2 cytokine levels or induced histopathologies (not depicted). In contrast, adoptive

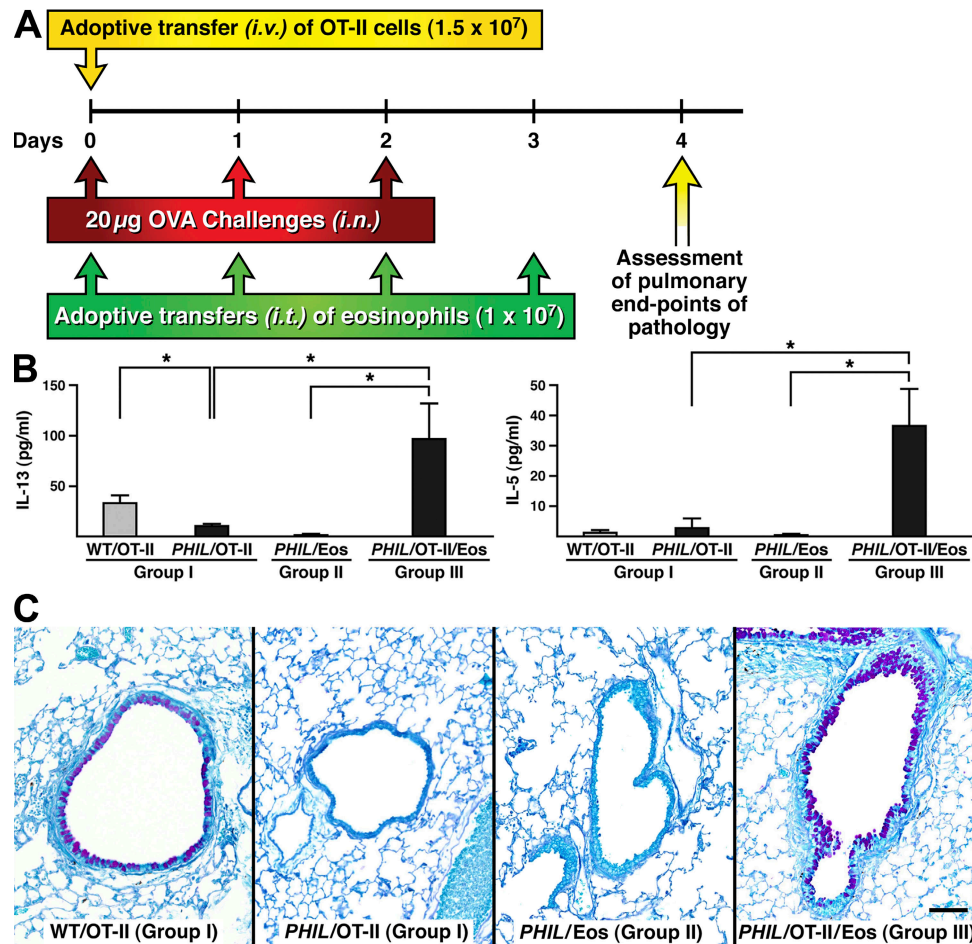


Figure 4. Adoptive transfer of both OVA-specific Th2-polarized effector T cells (OT-II) and eosinophils are required to generate Th2 pulmonary pathologies in *PHIL* mice. (A) Schematic timeline of the acute OVA challenge protocol associated with the adoptive transfer studies of Th2-polarized OVA-specific T cells (OT-II) and eosinophils. (B) Th2 BAL levels of the cytokines IL-13 and IL-5 (IL-4 was undetectable) were assessed by ELISA. *PHIL* mice receiving either OT-II cells (group I) or eosinophils (group II) alone showed no pathologies, whereas adoptive transfer of OT-II cells in *PHIL* mice together with the restoration of the absent pulmonary eosinophilia (group III) promoted significant increases in the BAL levels of the Th2 cytokines IL-13 and IL-5. Data presented are the means \pm SE. *, $P < 0.05$. (C) Assessments of goblet cell metaplasia and airway epithelial cell mucin accumulation by PAS staining of lung sections (purple) revealed that only the adoptive transfer of both OT-II cells and eosinophils to *PHIL* mice was capable of reconstituting this allergen-induced histopathology. *PHIL*/Eos, OVA-provoked *PHIL* mice after *i.t.* transfer of eosinophils; *PHIL*/OT-II, OVA-provoked *PHIL* mice after OT-II cell transfer; *PHIL*/OT-II/Eos, OVA-provoked *PHIL* mice after adoptive transfer of both OT-II cell (*i.v.*) and eosinophils (*i.t.*); WT/OT-II, OVA-provoked wild-type mice after OT-II cell transfer. Bar, 100 μ m.

transfer of both OT-II cells and eosinophils into *PHIL* mice (group III) led to significant increases in BAL levels of the Th2 cytokines (e.g., IL-5 and IL-13; Fig. 4 B); IL-4 was not significantly elevated above baseline. The development of Th2-driven histopathologies in each of these groups of mice paralleled the pattern of induced Th2 cytokine expression. That is, administration of either OT-II cells (group I) or eosinophils (group II) alone into OVA-challenged *PHIL* mice failed to result in the development of histopathologies such as the peribronchial aggregation of eosinophils (Fig. S4 B) and goblet cell metaplasia/airway epithelial cell mucin accumulation (Fig. 4 C). However, the adoptive transfer of both OT-II cells and eosinophils into OVA-challenged *PHIL*

mice (group III) resulted in goblet cell metaplasia/airway epithelial cell mucin accumulation that was comparable to that observed in OVA-challenged wild-type mice receiving OT-II cells (Fig. 4 C). These observations suggest that the eosinophil activities required for the development of Th2 responses extend beyond the potential effects on the proliferation/activation of OVA-specific effector T cells.

Eosinophils mediate T cell recruitment to the lung after adoptive transfer into OVA-challenged *PHIL* mice by an MDC/TARC-dependent mechanism

The allergen-induced pulmonary pathologies resulting from the combined adoptive transfer of OT-II cells and eosinophils

(group III) suggested that eosinophil-dependent recruitment of T cells may be a responsible mechanism for the immune/inflammatory responses that occur in the lungs of allergen-challenged mice. Indeed, unlike the transfer of individual leukocyte populations (group I and II), the concurrent adoptive transfer of both OT-II cells and eosinophils (group III) resulted in a significant increase in the recruitment of CD4⁺ T cells in the airways and lung tissue of OVA-challenged *PHIL* mice (Fig. 5 A); CD8⁺, CD4⁺/CD25⁺, and CD4⁺/NK1.1⁺ T cell profiles were similar to CD4⁺ T cell profiles (not depicted). Thus, adoptive transfer of eosinophils in the presence of OVA-specific Th2 effector T cells restored the recruitment of T cell subsets to the lung concurrent with the observed increases in Th2 immune-mediated pathologies.

To demonstrate that the recruitment of T cells to the lungs of OVA-challenged mice after adoptive cell transfer correlated with MDC and TARC expression, we measured the BAL levels of these chemokines in each of the adoptive transfer groups of mice. Significantly, the expression of MDC and TARC in the BAL of mice after adoptive cell transfer paralleled

the pattern of CD4⁺ T cell accumulation observed in these mice (Fig. 5 B). That is, *PHIL* mice receiving only OT-II cells (group I) or eosinophils (group II) were unable to elevate MDC and TARC to the levels observed in OVA-challenged wild-type mice receiving OT-II cells, whereas the administration of both OT-II cells and eosinophils to OVA-challenged *PHIL* mice was sufficient to return these chemokines to wild-type levels.

We performed antibody blocking studies to antagonize the activities of MDC and TARC after adoptive cell transfer to demonstrate that the recruitment of T cells in group III mice was dependent on the observed eosinophil-induced expression of these Th2 chemokines. Specifically, in addition to the assessments of CD4⁺ T cell recruitment to the lung after adoptive transfer of either OT-II cells (group I) or eosinophils (group II) alone, OVA-challenged *PHIL* mice adoptively transferred with both OT-II cells and eosinophils (group III) were treated with either isotype control antibody or neutralizing antibodies to TARC and MDC on days 0, 1, 2, and 3 of the protocol outlined in Fig. 4 A and Materials and

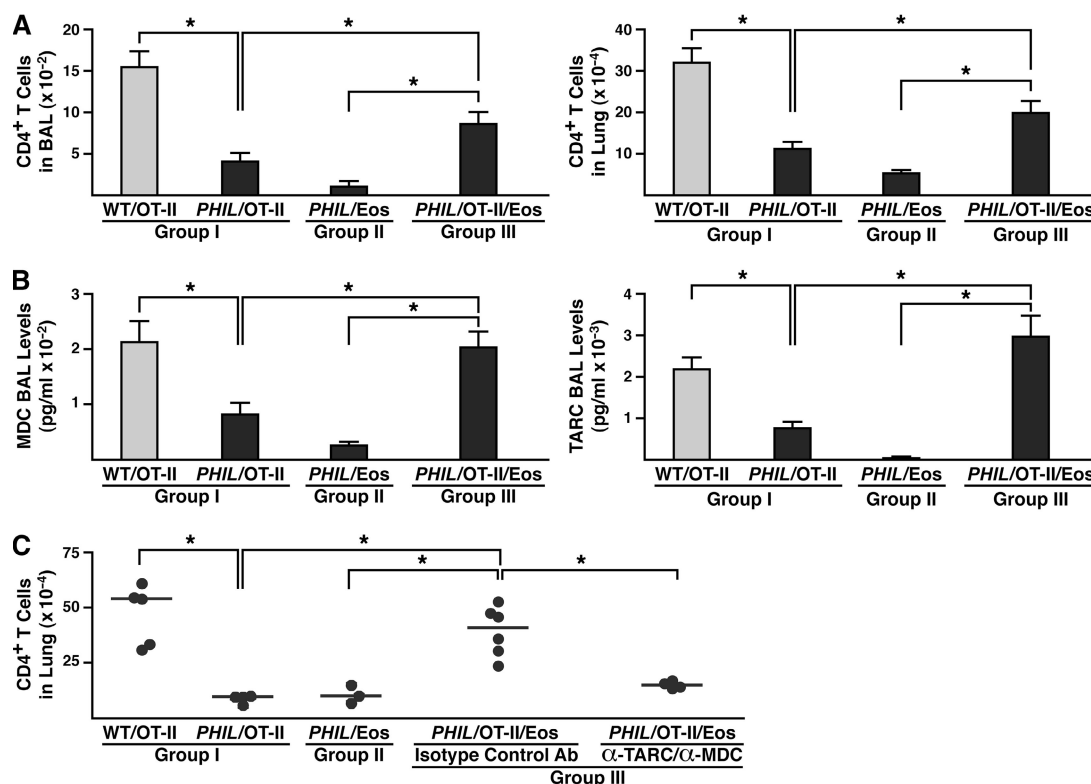


Figure 5. Eosinophil-dependent expression of TARC and MDC is required for CD4⁺ T cell recruitment to the lungs of allergen-challenged *PHIL* mice. (A) Wild-type and *PHIL* mice were adoptively transferred with either OT-II cells (group I) or eosinophils (group II) alone, or with both eosinophils and OT-II cells (group III). Assessments of the CD4⁺ T lymphocyte cellularity in BAL and whole-lung digests showed that in contrast to adoptive transfer of either OT-II cells (group I) or eosinophils (group II) alone, adoptive transfer of OT-II cells in *PHIL* mice together with the restoration of the absent pulmonary eosinophilia (group III) promoted OVA-induced CD4⁺ T cell accumulation in the lung. (B) ELISA measurements of these samples showed that BAL TARC and MDC levels are restored to wild-type levels in *PHIL* mice receiving both eosinophils and OT-II cells (group III). (C) Groups of mice were treated as in A, with the exception that group III mice were additionally administered either control polyclonal goat antibody (Isotype Control Ab) or neutralizing antibodies to TARC and MDC (α-TARC/α-MDC) on days 0, 1, 2, and 3. Whole-lung digests were analyzed for CD4⁺ T cell populations by flow cytometry. Horizontal bars represent means. Data presented are the means ± SE. *, *P* < 0.05.

methods. The antibody-mediated blockade of TARC and MDC in group III mice returned CD4⁺ T cell recruitment in these *PHIL* animals to the baseline levels observed in *PHIL* mice from groups I and II (Fig. 5 C). These observations extended to multiple T cell subsets (e.g., CD4⁺/CD62L^{-/lo}/CD44^{hi} effector T cells and CD8⁺ T cells) that were also dramatically reduced in the lungs of anti-TARC/-MDC-treated group III mice (not depicted). These studies showed that T cells are recruited to the lung by the eosinophil-dependent production of TARC and MDC and that this induced expression after allergen challenge is mechanistically linked to the T cell recruitment occurring in these mice.

DISCUSSION

The observation that eosinophils accumulating in the lungs after allergen provocation modulate local tissue immune responses through the specific recruitment of T cells suggests a novel mechanism of eosinophil effector function whose significance was previously underappreciated. This mechanism represents an expansion/extension of earlier studies investigating the potential importance of eosinophils and also provides an alternative interpretation of data initially noted by Lloyd et al. (26). Specifically, we suggest that the expression of eotaxins and perhaps other eosinophil agonist factors (e.g., leukotriene B₄) (31) immediately after allergen challenge elicits the recruitment of eosinophils and the expression of cytokines and growth factors that also promote eosinophil survival and activation. In turn, if and when these accumulating eosinophils achieve a critical level, their activities promote the local expression of Th2 chemokines (e.g., TARC and MDC) that leads to and/or amplifies the recruitment of T cells to the lungs and allergen-mediated Th2 inflammation. Thus, either by a direct (i.e., cell autonomous) or an indirect (i.e., induced from a third party cell type) mechanism, infiltrating lung eosinophils may mediate the induction of TARC and MDC that elicits the T cell recruitment necessary for the inflammatory responses associated with mouse models of asthma. Interestingly, studies assessing the initial leukocyte recruitment to the lungs in both human subjects and mouse models of allergic respiratory inflammation demonstrated similar kinetic patterns of eosinophil and lymphocyte accumulation into the airways (32, 33), implying that eosinophil-dependent accumulation of lymphocytes leading to pulmonary Th2 inflammatory responses may represent a larger, more significant mechanism with implications for asthma patients.

Our larger hypothesis linking eosinophils, T cells, and allergic respiratory inflammation is that eosinophils mediate the recruitment of effector T cells by two apparently independent mechanisms: (a) activation and co-stimulation of T cells promoting proliferation and/or increased survival, and (b) pulmonary production of chemokines that promote the recruitment and/or accumulation of T cells in the lung. It is also noteworthy that these eosinophil-dependent mechanisms are not mutually exclusive, and each may contribute to the loss of Th2 immune responses and pulmonary pathologies in OVA-treated *PHIL* mice. We suggest that these dual func-

tions, as well as their requirement for a threshold level of eosinophils, are responsible for many of the difficulties associated with the interpretation of results from both earlier mouse-model studies of allergic inflammation and clinical studies of asthma patients that target eosinophils. For example, the partial eosinophil deficiency of IL-5^{-/-} mice after OVA sensitization/challenge (~90% reduction relative to wild-type mice) is likely only to attenuate and not abolish eosinophil-mediated effects on T cells. This, in part, may explain the differences in allergen-induced pulmonary inflammation that occurred in IL-5^{-/-} mice among different studies (e.g., reference 34 vs. reference 35). Indeed, a requirement for a threshold level of recruited eosinophils was suggested in studies examining the allergic respiratory inflammation in OVA-treated IL-5^{-/-} mice relative to OVA-treated IL-5/eotaxin-1 double-knockout (IL-5^{-/-}/eotaxin-1^{-/-}) animals. That is, although the partial loss of eosinophils failed to prevent OVA-induced inflammatory responses in IL-5^{-/-}, these responses were abolished in IL-5^{-/-}/eotaxin-1^{-/-} mice that had a complete ablation of pulmonary eosinophils (36). The demonstrated inability to completely eliminate lung tissue eosinophils among human subjects receiving anti-IL-5 therapeutics may also represent a confounding issue that has complicated conclusions from clinical studies of asthma patients (6, 37, 38). These observations suggest that together with potential activities on T cell proliferation/activation and destructive effector activities mediated by granule protein release, the additional capability of eosinophils to mediate effector T cell accumulation in the lung may provide yet another mechanism by which eosinophils initiate and maintain the immune/inflammatory responses associated with allergic asthma. Interestingly, two unrelated studies (12, 39) using another eosinophil-deficient strain of mice (i.e., Δ dblGATA-1 [40]) also provide relevant insights supporting the immunoregulatory roles of eosinophils. Fulkerson et al. (12) reported that microarray assessments of lung gene expression in *Aspergillus* allergen-challenged Δ dblGATA-1 mice (relative to allergen-challenged wild-type animals) showed that the absence of eosinophils was associated with a significant reduction in pulmonary Th2 gene expression (e.g., IL-4 and IL-13). In addition, Voehringer et al. (39), using Δ dblGATA eosinophil-deficient mice and a *Nocardia brasiliensis* mouse model of lung inflammation, concluded that eosinophils were not required for pulmonary T cell recruitment during the primary infection of mice. However, eosinophils in this study may be linked with a failure to activate/recruit CD4⁺ T cells to the lung and, in turn, inflammatory responses associated with secondary challenges with this parasite. Thus, data from both of these studies highlight a complexity of pulmonary immune responses but again imply a potentially dual role for eosinophils associated with the elaboration of secondary immune responses in the lung, leading to the proliferation/activation of antigen-specific T cells and/or subsequent recruitment of these T cells to the lung.

Finally, there is no reason to assume that either of the eosinophil-mediated effects on T cells outlined in this paper

are necessarily mutually exclusive, and each is likely to contribute to the induced pulmonary immune responses observed in eosinophil-sufficient wild-type mice. These eosinophil-dependent immunoregulatory mechanisms are also not likely to be limited to the lung and/or allergen-mediated inflammation. That is, the accumulation of tissue eosinophils in a variety of settings may represent provocative examples in which eosinophil-mediated expression of Th2 chemokines leads to the recruitment of effector T cell recruitment and the modulation of local tissue immune cascades. In particular, we suggest that the T cell-induced immune responses associated with acute rejection of transplanted organs (41), the onset/growth of tumors (42), the accumulation/differentiation of thymic T cell populations (43), and the immune modulation of the gastrointestinal tract mucosa (44) may ultimately each be causatively linked to tissue-specific recruitment of activated effector T cells by eosinophils accumulating at these sites.

MATERIALS AND METHODS

Mice. All studies were performed with either C57BL/6J wild-type or eosinophil-deficient *PHIL* mice (5) crossed to C57BL/6J for >10 generations. C57BL/6J mice and TCR OVA-transgenic animals on a C57BL/6J background (OT-II) were purchased from the Jackson Laboratory. The mice were maintained in ventilated microisolator cages housed in the specific pathogen-free animal facility at the Mayo Clinic Arizona. The sentinel cages within the animal colony surveyed negative for the presence of known mouse pathogens. Protocols and studies involving animals were performed in accordance with National Institutes of Health and Mayo Foundation institutional guidelines.

Acute OVA sensitization and challenge protocols. Mice were sensitized and challenged with OVA as previously described (5) and outlined in Fig. S1 A. The kinetics of leukocyte recruitment to the lungs of sensitized and challenged mice were performed as previously described (33) and outlined in Fig. S1 B. Studies involving adoptive transfer of T cells derived from OVA-specific TCR transgenic mice (OT-II cells) were performed using a modification of a protocol previously described (45) and outlined in Fig. 4 A. Specifically, on day 0 of this protocol, either PBS or 1.5×10^7 in vitro Th2-polarized effector T cells (OT-II) were injected i.v. into OVA-naïve wild-type or *PHIL* mice. Within 3 h of i.v. OT-II cell transfer, the mice were gently anesthetized and received an i.n. administration of 20 μ g OVA (1 μ g/ μ l in saline). Mice were additionally challenged by i.n. administration of OVA on days 1 and 2 of this protocol (i.e., mice were OVA challenged via the nasal passages on three consecutive days). In some groups of mice, 10^7 eosinophils in 20 μ l of saline were adoptively transferred (control animals received saline vehicle alone) via the trachea (i.t.) on days 0, 1, 2, and 3 of this protocol, and within 1 h after OVA administration on the allergen-challenge days. OVA-induced responses in these recipient mice were assessed on day 4.

Collection and differentials of BAL fluid-derived cells. BAL assessments were completed as described previously (5, 46).

Histology. Histopathologic changes of the airways were assessed as described previously (5). 4- μ m formalin-fixed, paraffin-embedded sections of mouse lungs were stained for epithelial cell mucin accumulation using periodic acid Schiff or, for eosinophils, rabbit polyclonal anti-mouse eosinophil MBP antiserum (46).

Splenocyte, lymph node, and lung cell isolation. Total pulmonary leukocytes were recovered from lungs perfused with PBS-EDTA before removal, placed in complete media with 175 U/ml collagenase IV, and

digested for 45 min at 37°C, as described previously (47). Single-cell suspensions of lung, spleens, and caudal mediastinal lymph nodes (regional draining 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. Red blood cells were lysed by ammonium chloride (PharmLyse; BD Biosciences). Viability of the cells was >95%, as determined by Trypan blue exclusion. Cell counts were completed after the last wash before staining for flow cytometry.

Cytokine assays. Mouse IL-4, IL-5, IL-13, MDC, and TARC levels were assessed using immunoassay kits (R&D Systems) according to the manufacturer's instructions. The limits of detection for each cytokine assay were 5–10 pg/ml.

Splenocyte culture. Single-cell suspensions were obtained from sensitized and sensitized/challenged *PHIL* and wild-type mice using the protocol shown in Fig. S1 A. 10^6 cells/well of splenocytes were cultured in a 96-well plate with either media alone, with 200 μ g/ml OVA (Sigma-Aldrich), or with plates precoated with anti-CD3 and -CD28 (BD Biosciences) for 96 h. Supernatants were analyzed by ELISA.

Flow cytometry analysis. Single-cell suspensions were stained for 25 min on ice with cell type-specific antibodies after blockade of Fc receptors using 1 μ g/ μ l of Fc blocker (CD16/32; BD Biosciences). Antibodies used for staining specific cell types were obtained from BD Biosciences and include the T cell subtype-specific antibodies CD3, CD4, CD8, and CD62L, and the NK/NKT cell-specific marker NK1.1. Additional antibodies representing other T cell markers (i.e., CD44 and CD25) were purchased from eBioscience. Propidium iodide staining was used to identify and “gate out” dead cells. Flow cytometry was performed on a cytofluorimeter (FACScan; Becton Dickinson). Data acquisition and analysis were performed using CellQuest Pro (Becton Dickinson) and Summit (version 4.3; Dako) software.

Isolation and polarization of OT-II cells. Naïve OT-II cells were isolated from splenocytes and lymph nodes of 5–12-wk-old mice by two rounds of positive selection using magnetic beads coupled to the T cell-specific marker CD90⁺ (Thy1.2; MACS [Miltenyi Biotec]). 0.5×10^6 purified CD90⁺ T cells/ml were differentiated to the Th2 effector phenotype by incubating the cells in RPMI 1640 media (supplemented with 15% FBS [HyClone Technologies], 2×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, $1 \times$ nonessential amino acids, 10 μ g/ml penicillin, 10 μ g/ml streptomycin, and 2 mM L-glutamine; all from Invitrogen) in the presence of 50 μ g/ml OVA 323–339 peptide, 8 ng/ml mouse IL-4 (R&D Systems), 5 μ g/ml anti-IL-12 mAb (C17.8; BD Biosciences), and 5 μ g/ml anti-IFN- γ (XMG1.2; BD Biosciences). Irradiated (30 Gy) splenic APCs (depleted of CD 90⁺ cells) were also added to these cultures (2×10^6 cells/ml). After 2 d of culture, cells were given 10 ng/ml IL-2 (R&D Systems), and on days 4 and 6 cells were given fresh media with 10 ng/ml of IL-2. After culture for a total of 7 d, the recovered Th2-polarized effector T cells were used for i.v. injection (1.5×10^7 cells/mouse) into recipient mice. In some experiments, cells were labeled with CFSE, as described by the manufacturer (Invitrogen), before injection. FACS analysis reveals >98% of cells using this method are CD3⁺, with a composition of ~85% CD4⁺ and ~15% CD8⁺. Trypan blue exclusion demonstrated >98% cell viability. Th2 polarization of this cell population was confirmed by ELISA assessments for the presence of the Th2 cytokines (i.e., IL-4, IL-5, and IL-13) of 7-d cell-culture supernatants.

Isolation of mouse eosinophils. Eosinophils were isolated from IL-5-expressing transgenic mice (NJ.1638) (30), as previously described. In brief, peripheral blood pooled from four mice was layered onto single-step Percoll gradients. Eosinophils from the buffy coat at the blood-Percoll interface were subsequently isolated by removing the contaminating lymphocytes using MACS with magnetic beads conjugated with CD45R/B220 (B cells) and CD90/Thy1.2 (T cells), according to the manufacturer's recommendations.

Visual examination of DiffQuick-stained (Dade Behring Inc.) cytospin preparations and by flow cytometry analysis with staining for anti-CCR3 (R&D Systems) revealed that the purity of these eosinophil preparations was >99%.

i.t. transfer of eosinophils. The adoptive transfer of eosinophils and/or vehicle control (saline) into the lungs of mice was accomplished in subject animals lightly anesthetized with 1% isoflurane before i.t. instillation, as described previously (30). The transfer of eosinophils (or saline vehicle) was completed 1 h after i.n. administration of OVA on days 0, 1, and 2, and at the same time of day without OVA on protocol day 3 (Fig. 4 A).

Antibody neutralization of MDC and TARC. Mice were treated with antibodies to MDC and TARC with minor modifications of a protocol described previously (48). Specifically, 1 h before the OVA challenges, the *PHIL* mice received an i.p. injection of either control polyclonal goat IgG antibody or 20 µg each of polyclonal IgG goat anti-TARC and anti-MDC (R&D Systems) on days 0, 1, 2, and 3 of the protocol outlined in Fig. 4 A.

Statistical analysis. All data were derived from at least three independent experiments, each with cohort sizes of two to five mice. Data presented are the means \pm SE. Statistical analyses were performed using the Student's *t* test with differences between means considered significant when *P* < 0.05.

Online supplemental material. Fig. S1 presents schematic timelines of the acute OVA sensitization/aerosol challenge protocols used to elicit allergic respiratory inflammation, including the recruitment/accumulation of eosinophils and lymphocytes to the lung. Fig. S2 demonstrates that OT-II cells displayed the same proliferative capacity after adoptive transfer into eosinophil-sufficient wild-type and *PHIL* mice. Fig. S3 shows that OT-II cells displayed the same T cell surface phenotype after adoptive transfer into eosinophil-sufficient wild-type and *PHIL* mice. Fig. S4 demonstrates that OVA provocation of *PHIL* mice after adoptive transfer of both OT-II cells and eosinophils resulted in the development of a BAL eosinophilia and proinflammatory aggregates in the peribronchial regions of the lung. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20071840/DC1>.

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