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Defining a link with asthma in mice congenitally deficient in eosinophils

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24. Camel and llama V_HH domains share about 65% sequence identity with mouse and human V_H domains; however, several mutations in the canonical V_H interface region render the camelid V_HH domains more soluble than isolated human or murine V_H domains. Additionally, the long CDR3s in several of the camelid V_HH domains are folded back across this interface region, partially rescuing it from solvent exposure. The camelid CDR3 is sometimes anchored to the immunoglobulin core by formation of a disulfide bridge between CDR3 and CDR1 or FR2. Furthermore, some camelid V_HH domains can access recessed cavities, such as the HEL active-site cleft, by means of long CDR3 loops (*12*).
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28. The IgNAR CDR3 is 20 residues in length, spanning N84 to N103 (Gly-Leu-Gly-Val-Ala-Gly-Gly-Tyr-Cys-Asp-Tyr-Ala-Leu-Cys-Ser-Ser-Arg-Tyr-Ala-Glu).
29. Comparison of the IgNAR V domain structure with other antibody x-ray structures with the programs Dali and SSM (*27*) reveals similar root mean square deviations for the IgNAR V domain from V_α, V_L, and V_H domains of around 0.7 to 1.0 Å for 45 core Cα atoms.
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31. The IgNAR CDR1 has root mean square deviations from the cAb-Lys3 and cAb-RN05 CDR1s of 1.2 and 1.5 Å, respectively, for 15 Cα atoms (IgNAR residues N22 to N36).
32. Of the seven residues that differ between HEL and turkey egg-white lysozyme, two (Arg73→Lys73 and Asp101→Gly101) are found in the IgNAR-HEL interface. Arg^{L73} makes six van der Waals contacts, one hydrogen bond, and one salt bridge, and Asp^{L101} makes 17 van der Waals contacts and five hydrogen bonds in the interface with HEL. The camelid V_HH cAb-Lys3 also binds an epitope encompassing the recessed HEL active site, similarly using its CDR3 for access (*12*). Several murine antibodies to lysozyme (HyHEL10, HyHEL8, HyHEL26, and HyHEL63) recognize a common epitope around the active site, but do not penetrate this site as deeply as do cAb-Lys3 and IgNAR.
33. The camelid anti–ribonuclease A V_HH domain cAb-RN05 uses only CDR1 and CDR3 for binding antigen, whereas the camelid anticarbonic anhydrase V_HH domain cAb-CA05 uses CDR3 almost exclusively with only two van der Waals contacts to the antigen from CDR1. Similarly, the camelid anti–α-amylase V_HH domains AMB7 and AMD10 use all three CDR loops,

but CDR1 contributes only 5% of the total buried surface area on the antibody upon antigen binding.
34. The IgNAR-lysozyme interface is comparable in size to those seen in lysozyme-Fab (Fv) complexes that range from 538 to 829 Å^2 for the Fab and 540 to 831 Å^2 for the lysozyme (table S3). Similar interface sizes have also been observed for lysozyme complexes with camel V_HH domains. A total of 122 van der Waals contacts (table S2), eight hydrogen bonds, and three charged interactions are made between HEL and the IgNAR (table S4). The majority of the buried surface on the IgNAR V domain is contributed by CDR3 residues N85 to N89, N91, N93, N95 to N96, and N98 to N103 (75%), with the remainder by CDR1 N26 to N33 (Fig. 4). The HEL-IgNAR V region interface has a good shape-complementarity index of 0.70 (0.72 and 0.70 for crystal form 2) (*27*), with waters filling several cavities in the interface (fig. S4). A total of 14 water molecules contact both the IgNAR V domain and HEL, with 7 of these (waters 2, 4, 7, 8, 60, 114, and 266) sequestered from contact with external solvent.
35. Type I and type II sequences are 90% identical. IgNAR type II V domains have only four conserved cysteines, rather than the six or more found in type I, and tend toward smaller CDR3 lengths [9 to 18 amino acids (*26*)].
36. The structurally equivalent positions Lys^{N84} (IgNAR) or Lys^{96} (AMD10) correspond to positions Leu^{89} or His^{93} of conventional V domains.
37. IgNAR residues Asn^{N45}, Glu^{N46}, Ser^{N48}, Ser^{N50}, Lys^{N51}, Gly^{N62}, Ser^{N63}, and Lys^{N64} are under strong positive selection.
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40. Unexpectedly, a set of receptors (variable lymphocyte receptors) that may modulate immune recognition in lamprey has recently been identified. These receptors are arranged from leucine-rich repeats and may constitute a component of a primitive immune system in lampreys (*41*).
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Defining a Link with Asthma in Mice Congenitally Deficient in Eosinophils

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Eosinophils are often dominant inflammatory cells present in the lungs of asthma patients. Nonetheless, the role of these leukocytes remains poorly understood. We have created a transgenic line of mice (PHIL) that are specifically devoid of eosinophils, but otherwise have a full complement of hematopoietically derived cells. Allergen challenge of PHIL mice demonstrated that eosinophils were required for pulmonary mucus accumulation and the airway hyperresponsiveness associated with asthma. The development of an eosinophil-less mouse now permits an unambiguous assessment of a number of human diseases that have been linked to this granulocyte, including allergic diseases, parasite infections, and tumorigenesis.

The underlying features of asthma display a marked heterogeneity (*1*, *2*), yet the presence of eosinophils in the airway lumen and lung tissue has been recognized even in the earliest studies (*3*) and is often regarded as a defining feature of this disease (*4*, *5*). Moreover, the recruitment of eosinophils occurs in animal models of allergen-mediated respiratory inflammation; in particular, mouse models have offered unique opportunities with which to examine detailed pathologic features of this disease. However, the availability of clinical studies and numerous mouse models of asthma have not led to an unambiguous description of eosinophil effector functions in asthma, and questions remain as to the specific role(s), if any, of these leukocytes (*6*).

A line of mice devoid of eosinophils was created to test hypotheses that link eosinophils and asthma-related pathogenesis. Transgenic mice devoid of eosinophils were created by lineage-specific expression of a cytocidal protein

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with a promoter fragment identified from studies of secondary granule protein genes expressed in mouse eosinophils (7–11). A candidate promoter from the gene for eosinophil peroxidase (EPO) was selected on the basis of transfection studies with EPO promoter–luciferase reporter constructs in the eosinophilic cell line AML14.3D10 (12). These studies revealed that upstream sequences from the mouse EPO gene were capable of supporting high-level expression that was unique to eosinophil lineage–committed cells (fig. S1). In addition to mouse EPO-derived sequences, the transgenic construct developed included the diphtheria toxin A (DTA) chain open reading frame (13). The cytocidal character of diphtheria toxin is mediated by the DTA chain (the B chain provides entry into eukaryotic cells) through the catalytic degradation of elongation factor-2 and the subsequent collapse of protein synthesis (14).

Assessment of circulating leukocytes (15) in the resulting EPO-DTA transgenic (PHIL) mice demonstrated that these animals were devoid of eosinophils but otherwise have a full complement of hematopoietically derived cells (Fig. 1A). An examination of splenic lymphoid cells (15) revealed normal numbers of B cells, T cells, and the T lymphocyte $CD4^+$ and $CD8^+$ subtypes (Fig. 1B). Assessments of lung sections and peritoneal cavity exudates from PHIL mice revealed wild-type levels of mast cells (fig. S2, A and B). Moreover, circulating basophils were identified in peripheral blood from PHIL mice (fig. S2C), demonstrating that even a leukocyte lineage sharing a direct common precursor with the eosinophil lineage was unaffected. The specific ablation of eosinophils in PHIL mice also occurred with no effects on either erythropoiesis or the production of platelets (Fig. 1C). A nominal elevation of total white blood cell counts was consistently observed in PHIL mice relative to negative littermates. This increase, however, was not specific to any one cell type and did not elevate circulating cell numbers beyond the normal observable range in wild-type mice.

The loss of eosinophils in PHIL mice was nearly absolute, with only an occasional eosinophil identified in surveys of blood films from 1 of 20 animals examined. This eosinophil deficiency is lifelong and a Mendelian inheritable trait of the line. The specificity of the eosinophil deficiency in PHIL mice was achieved through a cross with interleukin (IL)–5 transgenic animals (16). These IL-5 transgenic mice have circulating eosinophil levels that, in some cases, exceed 100,000 per mm^3 of blood, representing ~50% of all white blood cells. Analyses of blood from double transgenic animals (i.e., mice carrying both the DTA and IL-5 transgenes) again revealed a complete absence of eosinophils (Fig. 1D).

The eosinophil-deficient character of PHIL mice was extended further by immunohistochemistry with antibodies specific for Major Basic Protein (MBP) (15, 17, 18). Tissues with abundant resident populations of eosinophils (i.e., bone marrow, uterus, small
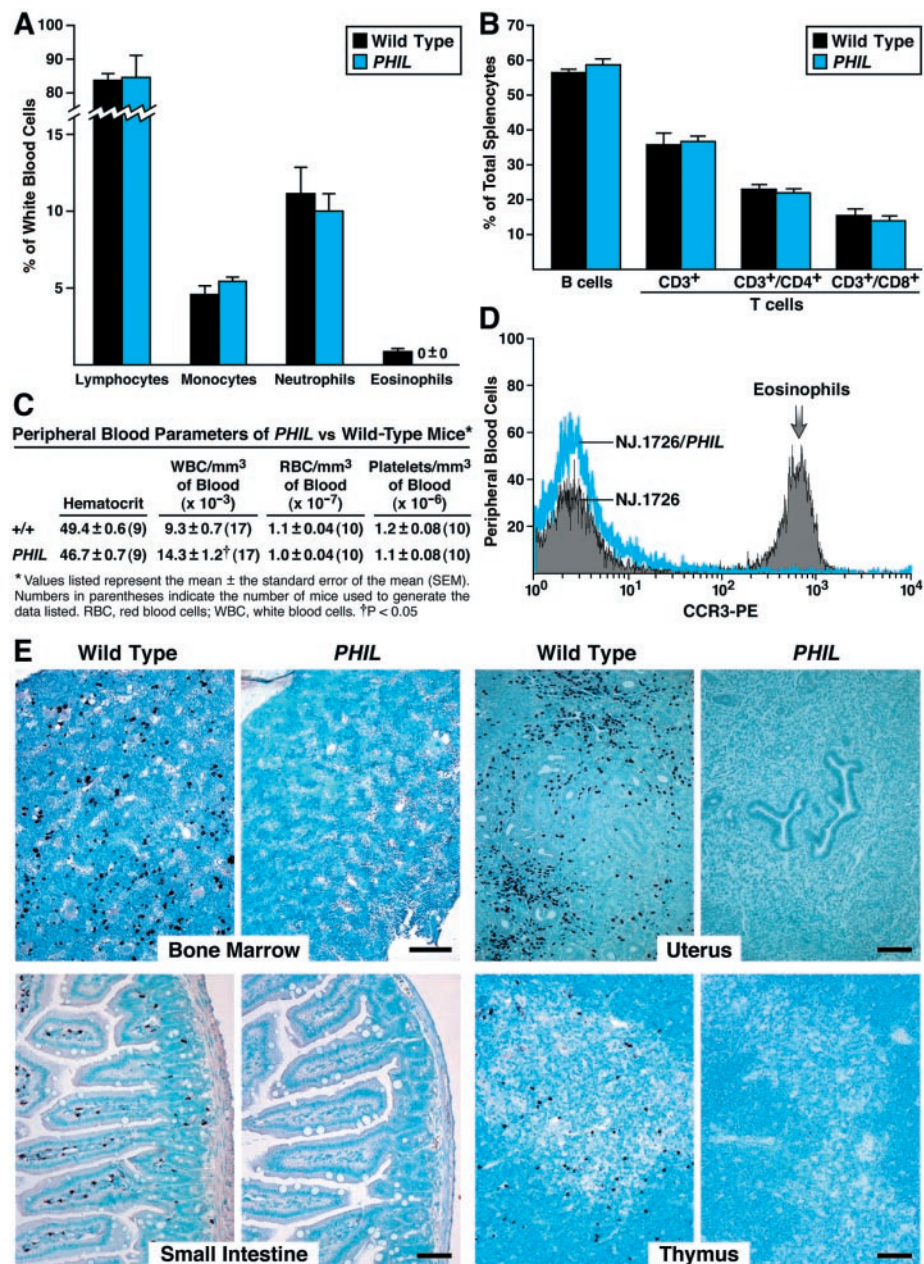
Fig. 1. The eosinophil deficiency of PHIL mice is specific and definitive. (**A**) Peripheral blood of PHIL mice is devoid of eosinophils without effects on the composition of other leukocytes (mean ± SE, n = 17 animals per group). (**B**) The targeted loss of eosinophils had no effects on lymphocyte subtypes (n = 5 animals per group). (**C**) The specific ablation of eosinophil lineage–committed cells had no additional effects on other hematopoietic parameters, although a nonspecific marginal increase in the steady-state levels of total circulating white blood cells was observed. (**D**) Fluorescence-activated cell sorting analyses demonstrated that the marked blood eosinophilia (i.e., the presence of $CCR3^+$ cells) of the IL-5 transgenic line NJ.1726 (16) was completely abolished in NJ.1726/PHIL double transgenic mice. PE, phycoerythrin. (**E**) Immunohistochemistry (dark purple–stained cells) with eosinophil-specific rabbit polyclonal antisera to MBP demonstrates that tissues or organs with prominent resident populations of eosinophils at baseline in wild-type mice were devoid of these granulocytes in PHIL mice. Scale bar, 100 μm.

C Peripheral Blood Parameters of PHIL vs Wild-Type Mice*

	Hematocrit	WBC/mm³ of Blood (x 10⁻³)	RBC/mm³ of Blood (x 10⁻⁷)	Platelets/mm³ of Blood (x 10⁻⁶)
+/+	49.4±0.6(9)	9.3±0.7(17)	1.1±0.04(10)	1.2±0.08(10)
PHIL	46.7±0.7(9)	14.3±1.2†(17)	1.0±0.04(10)	1.1±0.08(10)

* Values listed represent the mean ± the standard error of the mean (SEM). Numbers in parentheses indicate the number of mice used to generate the data listed. RBC, red blood cells; WBC, white blood cells. †P < 0.05.

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intestines, and thymus) in wild-type animals were shown to be devoid of these granulocytes in PHIL mice (Fig. 1E).

PHIL mice were subjected to an acute allergen sensitization/aerosol challenge model of asthma (15) to determine if the presence of eosinophils was causatively linked to the development of disease symptoms. Whereas wild-type mice sensitized/aerosol challenged with chicken ovalbumin (OVA) developed a significant airway eosinophilia [~50% of bronchoalveolar lavage fluid (BAL) cells], PHIL mice were essentially devoid of eosinophils, with only trace numbers (<0.5%) of eosinophils identifiable in the BAL of one of four OVA-treated animals (Fig. 2A). The loss of eosinophils from the lungs of OVA-treated PHIL mice also extended to tissue-infiltrating cells. Specifically, the lungs of OVA-treated PHIL mice were devoid of eosinophils, unlike the significant tissue eosinophilia that occurred in the areas surrounding the central airways (peribronchial) and the vasculature (perivascular) of OVA-treated wild-type animals (Fig. 2, B to D, and fig. S3). Examination of blood films and bone marrow smears from OVA-treated PHIL animals again revealed only an occasional eosinophil in a fraction of the animals examined.

The targeted ablation of eosinophils had significant effects on allergen-induced pulmonary pathology, suggesting a causative role for these granulocytes. Overall, OVA-induced histopathology in PHIL mice was attenuated relative to OVA-treated wild-type littermates. This lack of pathology was manifested by the reduced airway epithelial hypertrophy in OVA-treated PHIL mice (Fig. 2, B and C). In addition, assessment of airway mucins by periodic acid–Schiff (PAS) staining (15) demonstrated that OVA-induced goblet cell metaplasia/mucus accumulation (GM/MA) in PHIL mice was significantly reduced (Fig. 3, A to C). A quantitative assessment of the staining in these tissue sections revealed a 68% reduction of PAS staining in PHIL mice relative to OVA-treated wild-type animals (Fig. 3D). However, the GM/MA observed in OVA-treated PHIL mice was still significant when compared to allergen-naive animals. This observation suggests that eosinophils contribute to, and are necessary for, the levels of pathology observed in wild-type mice, but that they are not alone sufficient to account for these wild-type levels. That is, both eosinophil-dependent and -independent mechanisms exist in the lung that elicit GM/MA after allergen challenge.

The association between allergen-induced pulmonary eosinophilia and the development of lung dysfunction in both asthma patients and mouse models has been, at best, a collection of confusing and often contradictory observations. The lack of symptom improvement in asthma patients after administration of antibodies to IL-5 exemplifies the ambiguous character of clinical studies that attempt to ablate eosinophils (6). Mouse models purporting to ablate eosinophils
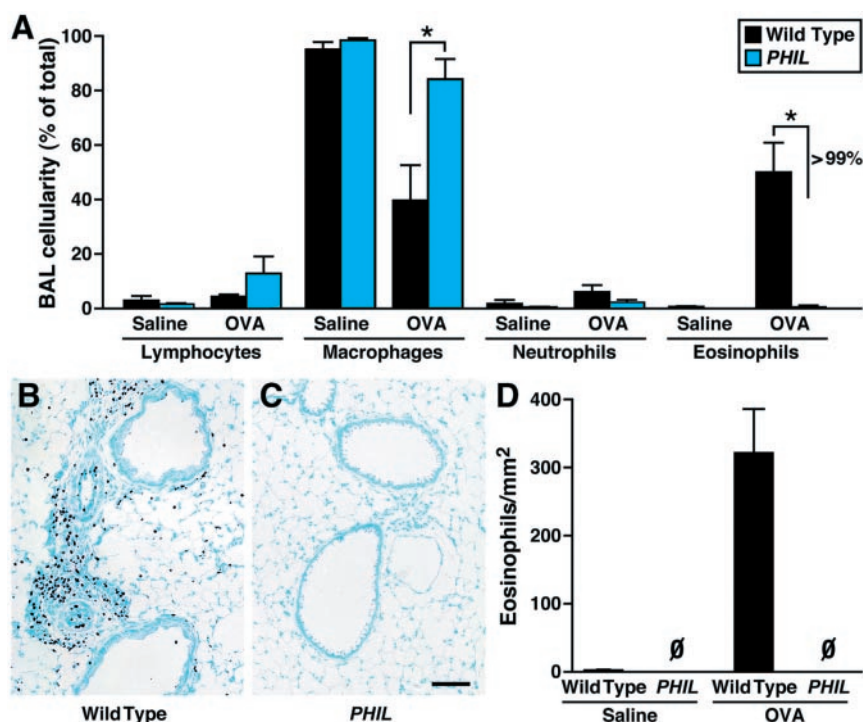
Fig. 2. The pulmonary eosinophilia associated with OVA sensitization/aerosol challenge was abolished in PHIL mice. (**A**) OVA-induced eosinophilia of the airway lumen was lost (decreased by >99%) in PHIL mice (mean $\pm$ SE, $n = 5$ animals per group). *, $P < 0.001$. (**B** and **C**) Assessments of infiltrating eosinophils in (**B**) wild-type and (**C**) PHIL mice by immunohistochemistry (dark purple–stained cells) with rabbit polyclonal antisera to mouse MBP revealed that OVA-induced accumulation of eosinophils was also extinguished in PHIL mice. Scale bar, 100 μm. (**D**) Quantitative assessments of the number of eosinophils infiltrating peribronchial areas (i.e., eosinophils per mm^2) demonstrated that OVA-treated PHIL mice were devoid of tissue eosinophils (mean $\pm$ SE, $n = 5$ animals per group). Ø indicates the absence of eosinophils in any of the sections of any of the mice in the cohort examined.
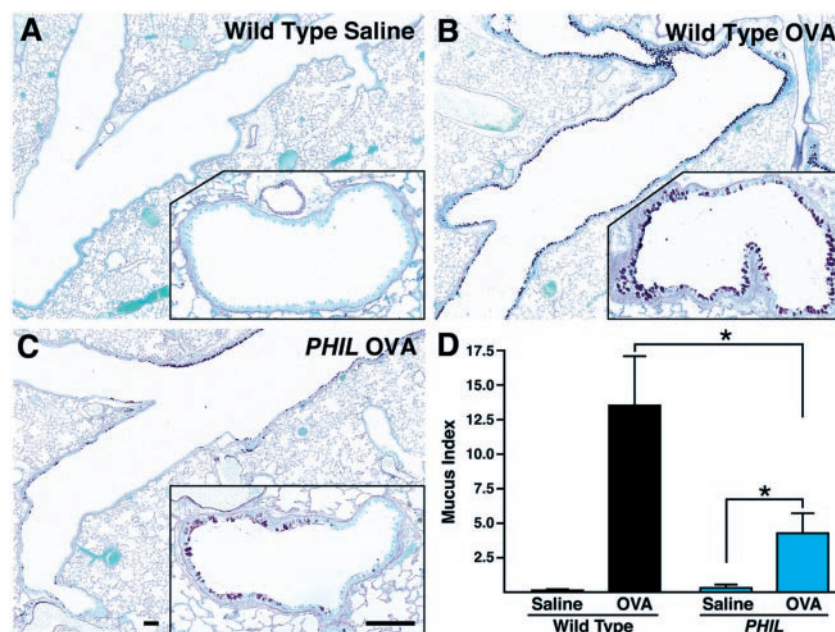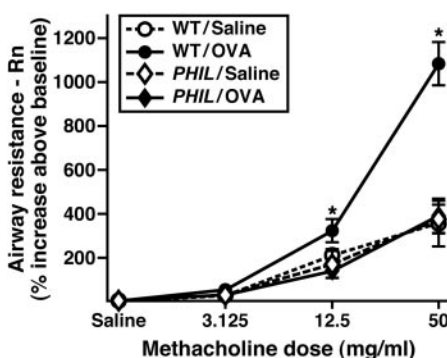

Fig. 3. The specific loss of eosinophils in PHIL mice resulted in a significant reduction in OVA-induced GM/MA. Representative lung sections after PAS staining are shown for (**A**) saline control and (**B**) OVA sensitized/OVA aerosol challenged wild-type mice in comparison to (**C**) OVA sensitized/OVA aerosol challenged PHIL mice. The sections of each panel show early-branching central conducting airways, whereas the insets show smaller, more distal bronchioles. Scale bars, 100 μm. (**D**) Quantitative assessments of airway epithelial mucus content showed a marked decrease (relative to wild type) in PHIL mice (mean $\pm$ SE, 5 to 10 animals per group). All evaluations of histopathology were performed in duplicate as independent observer-blinded assessments. *, $P < 0.05$.

are also ambiguous, as they either do not completely eliminate pulmonary eosinophils or they elicit the loss of eosinophils by mechanisms that do not differentiate between effects on eosinophils and other potentially important cellular targets (19–22). However, measurements of lung function after OVA sensitization/aerosol challenge of PHIL mice (15) showed that methacholine-induced airway hyperresponsiveness was dependent on the presence of eosinophils (Fig. 4). Moreover, the specific loss of eosinophils also led to improvement of other pulmonary function parameters associated with the distal regions of the lung (fig. S4).

The lack of observable phenotypes in knockout mice deficient for the abundant secondary granule proteins MBP-1 (18) and EPO (17) suggests that activities other than degranulation, including antigen presentation (23), the release of small molecule mediators of inflammation [e.g., the synthesis and release of eicosanoid mediators of inflammation (24)], and immune regulation of the pulmonary microenvironment through either modulations of T cell activities (21) or eosinophil-derived cytokine and/or chemokine expression (25) are likely to be the relevant effector functions. Eosinophil-derived cytokine and/or chemokine expression, in particular, is noteworthy as it may account for the chronic and seemingly self-sustaining character of allergic pulmonary inflammation, which often leads to lung remodeling events (26, 27). Significant decreases of Th2 cytokine levels in BAL of OVA-treated PHIL mice (28) lend support to this hypothesis and suggest that a prominent eosinophil effector function in the lung is localized immune regulation.

This study shows that eosinophil activities are important contributory factors leading to

symptoms that are classically defined as hallmark features of asthma. More importantly, these data provide validation of earlier studies that independently concluded that a causative link exists between eosinophils and allergic pulmonary pathologies (22, 29). The dependency of allergen-induced pulmonary pathologies on eosinophils suggests that these granulocytes participate at a significant level in underlying inflammatory responses. Regardless of the ultimate definition of the causative activities mediated by eosinophils, the challenge of future studies will be to develop confirmatory clinical studies to unambiguously define the role(s) and extent of eosinophil effector functions in asthma patients. The results of such studies will not only widen our understanding of the principle causes of asthma, but are also likely to lead to targeted therapeutic approaches previously dismissed and/or overlooked.

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30. We wish to acknowledge the kind gift of the gene encoding DTA by I. Maxwell. We also thank the Mayo Clinic Arizona Core Facilities (L. Barbarisi, T. Brehm-Gibson, S. Savarirayan, M. Ruona, B. Schimeck, J. Caplette, and J. Protheroe) and our administrative staff (L. Mardel, J. Ford, and P. McGarry). Supported by the Mayo Foundation; an American Heart Association grant to J.J.L. (no. 045580Z); grants from NIH to J.J.L. (no. HL065228), N.A.L. (no. HL058723), S.J.A. (nos. AI033043 and AI025230), and C.G.I. (nos. NCRR-COBRE P20RR15557, P01-HL67004, and HL-EB67273); and by NIH postdoctoral fellowships to MM.P.M. (no. HL10105) and S.A.C. (no. AR08545).

Fig. 4. In the absence of eosinophils, OVA-induced airway hyperresponsiveness does not develop. Lung function was assessed as airway resistance (Rn) in response to aerosolized methacholine, in saline-treated (WT/Saline) and OVA sensitized/OVA aerosol challenged (WT/OVA) wild-type mice in comparison to saline-treated (PHIL/Saline) and OVA sensitized/OVA aerosol challenged (PHIL/OVA) PHIL mice (n = 5 to 10 animals per group). Asterisks indicate a significant difference ($P < 0.01$) between WT/OVA and either WT/Saline, PHIL/Saline, or PHIL/OVA mice.

A Critical Role for Eosinophils in Allergic Airways Remodeling

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Features of chronic asthma include airway hyperresponsiveness, inflammatory infiltrates, and structural changes in the airways, termed remodeling. The contribution of eosinophils, cells associated with asthma and allergy, remains to be established. We show that in mice with a total ablation of the eosinophil lineage, increases in airway hyperresponsiveness and mucus secretion were similar to those observed in wild-type mice, but eosinophil-deficient mice were significantly protected from peribronchiolar collagen deposition and increases in airway smooth muscle. These data suggest that eosinophils contribute substantially to airway remodeling but are not obligatory for allergen-induced lung dysfunction, and support an important role for eosinophil-targeted therapies in chronic asthma.

Since its discovery by Paul Erlich in 1879, there has been a wealth of information documenting the association between eosinophils and parasitic or allergic diseases (1). The role of eosinophils in allergic disease remains controversial. Although T helper cell 2 (T_H2) lymphocytes are thought to drive asthmatic

responses, increasing evidence suggests that eosinophils are associated with development of lung dysfunction and subsequent immunopathology (2–4).

Asthma is a chronic disease characterized by airway hyperresponsiveness (AHR), airway inflammation, and reversible airway ob-

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