Regulation of Eosinophilopoiesis in a Murine Model of Asthma

Mary Beth Hogan, David N. Weissman, Ann F. Hubbs, Laura F. Gibson, Debra Piktel and Kenneth S. Landreth

J Immunol 2003; 171:2644-2651; doi: 10.4049/jimmunol.171.5.2644
http://www.jimmunol.org/content/171/5/2644

References
This article cites 31 articles, 11 of which you can access for free at:
http://www.jimmunol.org/content/171/5/2644.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Regulation of Eosinophilopoiesis in a Murine Model of Asthma

Mary Beth Hogan,²*†‡ David N. Weissman,§ Ann F. Hubbs,§ Laura F. Gibson,*†‡ Debra Piktel,†‡ and Kenneth S. Landreth*†‡

Eosinophilic inflammation plays a key role in tissue damage that characterizes asthma. Eosinophils are produced in bone marrow and recent observations in both mice and humans suggest that allergen exposure results in increased output of eosinophils from hemopoietic tissue in individuals with asthma. However, specific mechanisms that alter eosinophilopoiesis in this disease are poorly understood. The current study used a well-characterized murine animal model of asthma to evaluate alterations of eosinophil and eosinophil progenitor cells (CFU-eo) in mice during initial sensitization to allergen and to determine whether observed changes in either cell population were regulated by T lymphocytes. Following the first intranasal installation of OVA, we observed sequential temporal elevation of eosinophils in bone marrow, blood, and lung. In immunocompetent BALB/c mice, elevation of bone marrow eosinophils was accompanied by transient depletion of CFU-eo in that tissue. CFU-eo rebounded to elevated numbers before returning to normal baseline values following intranasal OVA exposure. In T cell-deficient BALB/c nude (BALB/cnu/nu) mice, CFU-eo were markedly elevated following allergen sensitization, in the absence of bone marrow or peripheral blood eosinophilia. These data suggest that eosinophilia of asthma results from alterations in two distinct hemopoietic regulatory mechanisms. Elevation of eosinophil progenitor cells in the bone marrow is T cell independent and likely results from altered bone marrow stromal cell function. Differentiation of eosinophil progenitor cells and phenotypic eosinophilia is T cell dependent and does not occur in athymic nude mice exposed to intranasal allergen. The Journal of Immunology, 2003, 171: 2644–2651.

Asthma is characterized by reversible airway hyperreactivity and progressive airway inflammation. In patients with asthma, this pulmonary reaction to inhaled allergen has been divided into early phase responses and late phase responses. The early phase response to inhaled allergen results in mast cell degranulation, release of vasoactive and bronchoconstrictive cytokines, restricted airflow, and wheezing (1). Mediators released by mast cells are chemotactic and initiate pulmonary infiltration of lymphocytes, neutrophils, and eosinophils following allergen exposure (2, 3). It is the accumulation of activated eosinophils during the late phase response to allergen exposure that ultimately results in progressive inflammatory tissue damage. In addition, pulmonary eosinophilia in response to allergen challenge is associated with elevated levels of eosinophil-derived cytokines in both the lung and peripheral blood (4, 5).

The eosinophilic inflammatory response is not limited to pulmonary tissue. Increased numbers of eosinophils have also been noted in bone marrow of atopic patients with asthma (6, 7). In a murine model of asthma, transient bone marrow eosinophilia was demonstrated following airway sensitization to OVA and following subsequent allergen challenge (8–10). In both cases, bone marrow eosinophilia was followed by peripheral blood and pulmonary eosinophilia (8, 9) and circulating eosinophils appeared to be newly produced cells emigrating from the bone marrow (11).

The aim of the present study was to better define the temporal sequence of events that lead to bone marrow eosinophilia following initial airway exposure to allergen in this animal model and to determine cellular mechanisms that regulate altered eosinophil production in response to allergen exposure. Following the initial sensitizing airway exposure to OVA, we observed sequential eosinophilia in bone marrow, peripheral blood, and lungs of mice. Eosinophil progenitor cells (CFU-eo)² in the bone marrow were initially depleted in the bone marrow of mice exposed to allergen, followed by rebound in CFU-eo numbers to greater than baseline values before returning to the level found in untreated controls. To determine the requirement for T lymphocytes in this bone marrow response to initial allergen exposure, T cell-deficient BALB/c nude mice were evaluated using the same exposure regimen. In nude mice, CFU-eo were markedly increased immediately following allergen sensitization, in the absence of detectable eosinophilia in bone marrow or peripheral blood. These findings confirm the importance of T lymphocyte function in bone marrow and pulmonary eosinophilia of asthma, but reveal that altered kinetics of eosinophil progenitor cells in the bone marrow is T cell independent and likely due to altered bone marrow stromal cell function in response to allergen exposure.

Materials and Methods

Mice

Four- to 6-wk-old, female, BALB/cnu/nu or athymic BALB/cnu/nu (nude) mice were obtained from Taconic Laboratories (Germantown, NY). All mice were housed in autoclaved microisolator cages (Lab Products, Maywood, NJ) and autoclaved food and acidified water (pH 2.8) were provided.

Abbreviations used in this paper: CFU-eo, CFU eosinophil; i.n., intranasal; BAL, bronchoalveolar lavage.
ad libitum. A 12-h light-dark cycle was provided. All procedures were approved by the West Virginia University Animal Care and Use Committee that follows the Guide for the Care and Use of Laboratory Animals.

**Allergen sensitization**

Pulmonary sensitization to OVA has been previously described in detail (8). Briefly, in each experiment at least four mice were injected i.p. with 100 mg/kg OVA (Sigma-Aldrich, St. Louis, MO) suspended in a saturated solution of aluminum potassium sulfate (alum; Sigma-Aldrich) in sterile distilled water on day 0. For i.p. injections, OVA (0.5 mg/ml) was suspended in 10 ml of endotoxin-free 0.9% saline and equal volumes of washing solutions of OVA and alum mixed, adjusted to pH 6.5, and allowed to precipitate for 30 min. The precipitate was centrifuged at 1800 rpm at room temperature, supernatant was removed, and precipitate was resuspended in 10 ml of endotoxin-free saline (8). On day 10, mice were exposed to 25 μl of OVA-Au biodotter. PBS (500 μl of PBS) was injected and withdrawn from the lung using a tuberculin syringe. This procedure was repeated five times. A white blood cell count of BAL fluid was evaluated microscopically by hemocytometer. Cells were then cyt centrifuged onto clean glass slides and stained with May-Grünewald-Giemsa (Sigma-Aldrich) for enumeration of eosinophils.

**Bone marrow and peripheral blood**

Mice were euthanized by CO₂ asphyxiation, the peritoneal cavity was opened, and peripheral blood was obtained directly from the inferior vena cava using a heparinized tuberculin syringe. Total white blood cell counts were obtained using a Coulter counter and peripheral blood smears were made to establish a differential white blood cell count. Bone marrow was obtained by flushing femora with αMEM (Life Technologies, Gaithersburg, MD) supplemented with 1% FCS (Summit Biotechnology, Fort Collins, CO) using a syringe fitted with a 23-gauge needle. Total white blood cell counts were evaluated microscopically using a hemocytometer. Bone marrow (10³) or peripheral blood cells were cytocentrifuged onto clean glass slides and stained with May-Grünewald-Giemsa (Sigma-Aldrich) for enumeration of eosinophils.

**Bronchoalveolar lavage (BAL)**

Mice were euthanized by CO₂ asphyxiation, the peritoneal cavity was opened, and the trachea was exposed. The trachea was cannulated with a 22-gauge TB needle. PBS (500 μl of PBS) was injected and withdrawn from the lung using a tuberculin syringe. This procedure was repeated five times. A white blood cell count of BAL fluid was evaluated microscopically by hemocytometer. Cells were then cyt centrifuged onto clean glass slides and stained with May-Grünewald-Giemsa stain and cell differential counts were obtained. After BAL, lungs were inflated with 1 ml of 10% neutral-buffered Formalin (Sigma-Aldrich). Lungs were embedded in paraffin, sectioned, and stained with H&E and 0.5% chromatrope 2R for identification of eosinophils.

**ELISA**

Murine anti-OVA IgE Ab was detected in plasma samples using an IgE capture ELISA. The following reagents were used, with appropriate washing between incubations: monoclonal anti-mouse IgE (BD Pharmingen, San Diego, CA), PBS/1% skim milk; plasma samples diluted 2-fold over a range from 1/50 to 1/5200; OVA (25 μg/ml; Sigma-Aldrich), rabbit anti-OVA-HRP conjugate (Rockland Immunobiochemicals, Gilbertsville, PA), and tetramethylbenzidine substrate solution (Kirkegaard & Perry, Gaithersburg, MD). After incubation for 30 min at room temperature, reactions were stopped (Tetramethylbenzidine Stop Solution: Kirkegaard & Perry) and color development evaluated as OD₄₅₀ using an automated plate reader. Specific IgE levels are reported as the reciprocal titer yielding an OD₄₅₀ greater than two times background. A positive titer was defined as >1:12.

**Results**

Effect of allergen sensitization on eosinophil populations in the bone marrow

In all experiments, mice received an initial i.p. exposure to OVA (day 0) followed by i.n. exposure to the same allergen on day 10 as described in Materials and Methods. This allergen exposure regimen did not result in altered numbers of total nucleated bone marrow cells in any of the experiments presented (data not shown). On the other hand, we consistently noted significant depression of the number of eosinophil progenitor cells, or CFU-eo, 3 days following i.n. installation of allergen (day 13, Fig. 1). This initial depression of bone marrow CFU-eo was accompanied by significant elevation of bone marrow eosinophils (Fig. 2). CFU-eo numbers in bone marrow of allergen-exposed mice rebounded to greater than control values on day 17 (Fig. 1) and returned to baseline values by day 19. Bone marrow eosinophilia in allergen-exposed mice resolved to control values by day 17 (Fig. 2).

In the experimental protocol previously used to establish OVA allergen sensitivity in mice (8), the initial i.n. exposure to OVA on day 10 was accompanied by a second i.p. exposure to the same allergen coprecipitated with aluminum potassium sulfate. To determine whether observed alterations of CFU-eo following allergen sensitization were due to the i.n. deposition of OVA or to the
accompanying i.p. exposure, we compared the effect of the traditional exposure regimen to one which utilized i.n. exposure to OVA in the absence of a second i.p. treatment. As shown in Fig. 3, i.n. exposure and i.n. exposure combined with an i.p. exposure to OVA were equally effective in stimulating the observed drop in bone marrow CFU-eo on day 13 of the exposure regimen.

Effect of allergen sensitization on peripheral blood and pulmonary eosinophils

In mice receiving an initial i.n. exposure to OVA, peripheral blood eosinophilia was not observed until 5 days following i.n. allergen exposure (Fig. 4, day 15) and eosinophilia was not resolved by day 19. Leukocytes were elevated in BAL fluid obtained from these mice on days 11 and 17 as compared with control mice, with significant elevations in neutrophils (day 11, data not shown), eosinophils (days 15–19, Fig. 5), and macrophages (days 11–17, data not shown). Histopathology of lung tissue samples obtained from saline control mice did not reveal detectable infiltration of inflammatory cells (Fig. 6). However, OVA-exposed mice developed substantial eosinophilic alveolar inflammation (Fig. 6). Histologic evaluation revealed bronchial changes in mice exposed to i.n. OVA, including secretory cell hypertrophy and hyperplasia (Fig. 7). Eosinophilic infiltration was consistently observed in perivascular spaces of the lung (Fig. 7).

Effect of allergen sensitization on bone marrow eosinophil populations in T cell-deficient mice

To determine the requirement for T lymphocytes in observed alterations of bone marrow CFU-eo following allergen sensitization, athymic nude mice were exposed to OVA using exactly the same protocol described for wild-type BALB/c mice (Fig. 8). Unlike observations in euthymic BALB/c mice, BALB/c nude mice had
significantly elevated numbers of bone marrow CFU-eo immediately following i.n. allergen exposure (Fig. 8) and CFU-eo remained elevated in athymic mice on day 16.

To determine the duration of this elevation of CFU-eo following allergen exposure in athymic mice, a second series of experiments enumerated CFU-eo through day 19 of the experimental protocol. The elevation of CFU-eo in the bone marrow of nude mice resolved to baseline numbers 8 days following i.n. exposure to allergen or experimental day 19 (data not presented).

Athymic BALB/c<sup>nu/nu</sup> mice and euthymic BALB/c<sup>/H11001</sup>/H11001 mice did not differ in numbers of bone marrow eosinophils before treatment. However, at 3 days following i.n. exposure to allergen (experimental day 13), the number of bone marrow eosinophils rose dramatically in BALB/c<sup>/H11001</sup> mice but remained unchanged in BALB/c<sup>nu/nu</sup> mice (Fig. 8). No differences were found in the total number of nucleated cells in bone marrow of euthymic BALB/c or athymic nude BALB/c mice throughout the experiment (data not shown).

**Effect of allergen sensitization on serum levels of anti-OVA IgE**

Wild-type BALB/c mice developed anti-OVA IgE Abs over the course of allergen sensitization. On day 11, 30% of BALB/c mice had detectable elevations of IgE and by day 13, 83% had developed OVA-specific IgE Ab. By day 15, all BALB/c mice tested had detectable circulating levels of anti-OVA IgE Abs. None of the athymic BALB/c nude mice in this study developed detectable anti-OVA IgE Abs (observations made on days 13 and 16).

**Role of IL-5 in CFU-eo expansion in BALB/c<sup>nu/nu</sup> mice**

It was important to determine whether CFU-eo expansion in nude mice was due to IL-5 produced by cells other than T cells. We determined the number of IL-5-secreting cells in the bone marrow of euthymic and athymic BALB/c mice using ELISPOT analysis to capture IL-5 secreted from individual cells. As shown in Fig. 9, IL-5-producing cells were detected in the bone marrow of both mouse strains; however, there were significantly more IL-5-secreting cells in the bone marrow of wild-type mice as compared with age- and sex-matched nude mice. To determine differences in total IL-5-secreting cells between these mice, cells were also stimulated...
with PMA and ionomycin before evaluation in the ELISPOT assay. There was a statistically significant increase in the number of IL-5-producing cells in both euthymic and athymic mice following stimulation with PMA; however, differences between nude and wild-type BALB/c mice continued to be detectable (data not presented).

To determine the effect of IL-5 on expansion of bone marrow CFU-eo, nude mice were treated with saturating concentrations of neutralizing Ab to IL-5 in vivo (50 μg/day i.p.) during i.n. exposure to allergen and evaluated 4 days later. TRFK-5 anti-IL-5 Ab treatment did not alter expansion of CFU-eo in nude mice exposed to i.n. allergen (Fig. 10). However, when the same batch of TRFK-5 Ab was added to in vitro bone marrow cultures, it completely neutralized IL-5-mediated formation of CFU-eo colonies (Fig. 11).

Discussion
Development of asthma in humans or mice is characterized by pulmonary eosinophilia and progressive tissue damage caused by eosinophilic inflammation. Eosinophils are produced in the bone marrow of mammals and recent observations in both mice and humans suggest that pulmonary allergen exposure results in both increased output of eosinophils from hemopoietic tissues and increased migration of these cells to the lung. These observations suggest that alterations of bone marrow function in response to allergen exposure may be a primary factor in understanding progression of asthmatic disease. The purpose of the present study was to use an established animal model of asthma to evaluate alterations of bone marrow function that accompany allergen sensitization and to determine hemopoietic regulatory mechanisms that are affected by pulmonary allergen exposure. These studies revealed that the population dynamics of eosinophil progenitor cells in the bone marrow is altered following the initial i.n. exposure to allergen. These changes in eosinophiloipoiesis preceded development of allergen-specific IgE and were, in part, independent of T cell function. Taken together with previous data from this and other laboratories, these studies suggest a working model of bone marrow response to allergen in which bone marrow stromal cells and T lymphocytes act in concert to initiate eosinophilia of asthma (Fig. 12).

Other laboratories have described altered bone marrow function in response to pulmonary allergen challenge in mice, dogs, and humans (6, 12–16). These studies have largely focused on the response of bone marrow in later stages of asthmatic eosinophilia following development of allergen-specific IgE and the potential role of T cells in alterations of bone marrow function (9, 12–15). We have now evaluated eosinophil development in bone marrow early in the development of asthma and describe a characteristic temporal alteration of eosinophiloipoiesis that resulted in increased eosinophil output at this early stage of disease. Following the first i.n. installation of allergen in BALB/c mice, the bone marrow eosinophil compartment expanded rapidly and was significantly different from control animals within 72 h following allergen exposure. This increase in bone marrow eosinophils was transient and returned to normal values 7 days following pulsed allergen exposure (Fig. 2). This pattern of bone marrow eosinophilia following allergen sensitization is in general agreement with previous studies (10); however, unlike previous reports, we did not detect differences in overall bone marrow cellularity following allergen exposure at any of the time points tested. Bone marrow eosinophilia

FIGURE 7. Photomicrograph of pulmonary eosinophil infiltration. Infiltration of the perivascular space by a population of inflammatory cells principally comprised of eosinophils. Bar, 20 μm.
...was followed by peripheral blood (Fig. 4) and pulmonary (Fig. 5) eosinophilia on day 5 following exposure, suggesting a plausible temporal sequence of events leading to accumulation of eosinophils in the lung during onset of disease.

Of particular interest to our laboratory, eosinophil progenitor cells (CFU-eo) declined during the first 3 days following the initial i.n. installation of allergen, then rebounded to significantly greater than normal numbers for a period of 48 h before returning to control levels (Fig. 1). This pattern of perturbation of hemopoietic progenitor cells has been previously documented in erythropoietic recovery following exposure to hyperbaric conditions (17) and in myeloid progenitors following chemotherapy (18). In both cases, increased demand for end cells resulted in initial depletion, followed by rebound of specific hemopoietic progenitor cells and data presented here suggests that perturbations of eosinophilopoiesis in the bone marrow follows a similar sequence of events. It is interesting to note that, although nasal exposure to allergen in these studies was characterized by pulmonary neutrophilia, no differences in granulocyte-macrophage progenitors (CFU-GM, data not shown) were detected during these early phases of pulmonary allergen exposure in any of the experiments reported here. These observations suggest that increased pulmonary immigration of neutrophils may be more due to redistribution of cells from circulation than altered bone marrow production.

In previous studies, we identified a role for bone marrow stromal cells in regulation of eosinophil production in the bone marrow. However, the relative contribution of stromal cells and T lymphocytes to bone marrow response to allergen has remained unclear. In the studies reported here, we determined the role of T cells in altered bone marrow function by repeating these experiments in T cell-deficient nude mice. In the absence of T lymphocytes, bone marrow eosinophilia did not result from allergen exposure. However, eosinophil progenitor cells (CFU-eo) were dramatically elevated, and this elevation occurred earlier in nude mice than in fully...
immunocompetent mice. These studies suggest two distinct regulatory processes; with expansion of eosinophil progenitor cells following pulmonary allergen exposure being T cell independent and subsequent proliferation and maturation of expanded progenitor cells to form functional eosinophils being T lymphocyte dependent.

The role of T lymphocytes in development of asthma is well documented. CD4+ T cells contribute to inflammatory changes observed in lung following pulmonary allergen challenge (19) and both Th1 and Th2 cells participate in this process (20). CD8+ T cells have also been implicated in the development of airway hyperresponsiveness associated with asthma (21, 22) and this role for T cells appears to be independent of production of specific IgE-mediated Ab responses (23-25).

IL-5 is a critical cytokine in development of eosinophils (26) and previous studies have concluded that IL-5 detected in the marrow is produced by T lymphocytes (11, 14) Previous studies from our laboratory documented that bone marrow stromal cells also produce IL-5 and potentially regulate steady-state production of eosinophils in the absence of asthmatic disease (27). This hypothesis is supported by the presence of normal numbers of eosinophils in athymic nude mice in the present study. However, although we have shown that IL-5 mRNA and protein in stromal cells is elevated by exposure to IL-1, an inflammatory mediator associated with asthma, eosinophil production was not altered by pulmonary allergen exposure in T cell-deficient mice. These data suggest that regulation of both the progenitor cell compartment and phenotypic maturation to functional end cells may be multifactorial and more complex than previously described.

The finding that CFU-eo were increased following allergen challenge in the absence of T cells suggests that the primary role of stromal cells may be in regulation of the compartment size of eosinophil progenitor cells (CFU-eo) in response to pulmonary inflammation. Although stromal cells produce IL-5 (27) in the bone marrow microenvironment, the observation that observed expansion of CFU-eo in nude mice following sensitization to OVA was not affected by daily administration of a neutralizing Ab to IL-5 suggests that IL-5 is likely not to be the cytokine primarily responsible for CFU-eo expansion in response to allergen exposure. We also noted that nude mice had little alteration of eosinophil output, even though cells other than T cells produce IL-5 in these mice (Fig. 9). This failure of IL-5 production to stimulate increased numbers of eosinophils may be due to the relative levels of IL-5 released by T lymphocytes and stromal cells, the sequestration of cytokine on stromal cell surfaces, or the presence of inhibitors of cell differentiation known to be produced by bone marrow stromal cells. Surprisingly, we noted in ELISPOT assays that the amount of IL-5 captured on plates did not differ between normal and nude mice and, therefore, there is no evidence for a difference in the amount of IL-5 produced per cell in these mice (data not presented).

These experiments confirm that allergen-specific IgE is not required for the bone marrow CFU-eo response to allergen during sensitization. Changes in bone marrow CFU-eo populations occurred in the absence of detectable OVA-specific IgE Ab in athymic mice. In addition, maximal alteration of CFU-eo was documented in immunocompetent BALB/c mice on day 13, a time at which only 30% of animals had detectable OVA-specific IgE Ab.

The finding that eosinophil progenitor proliferation and subsequent eosinophil differentiation are regulated by separable mechanisms is consistent with data for other developing hemopoietic cell lineages. We previously reported that early development of B lymphoid progenitors was T cell independent and required the presence of bone marrow stromal cells (28-31). However, differentiation of pre-B cells in the bone marrow to form functional B lymphocytes depended on the presence of IL-4, a T cell-derived cytokine (28, 32). The present study presents a similar working hypothesis for the production of eosinophils in the bone marrow and suggests that stromal cell regulation of eosinophil progenitor cell expansion is independent of both T cells and IL-5 production. Defining the identity of cytokines and cellular interactions which regulate early events in this lineage will be essential to understanding the role of bone marrow in the allergic response to allergen.

The role of tissue inflammation in regulation of hemopoiesis is not well understood. We previously demonstrated that elevated levels of IL-1 or IL-4 altered bone marrow stromal cell function and production of B lymphocytes in that tissue (32). Our recent work has extended that observation to eosinophilopoiesis. Bone marrow stromal cells produce the primary eosinophilopoietic cytokine, IL-5, and IL-5 abundance in stromal cells increased when stromal cells were exposed in vitro to rIL-1 (27). This increase in IL-5 production by stromal cells was shown to be correlated with increased eosinophil production in vitro. However, the present study strongly suggests that stromal cells regulate eosinophil progenitor cell expansion in the bone marrow by an IL-5-independent mechanism and that this regulatory function is also elevated in response to airway inflammation. Taken together, these studies
support the hypothesis that systemic release of inflammatory mediators may serve as a primary regulatory stimulus for altered hematopoietic response to immune insult, including alterations of bone marrow function known to result from pulmonary allergen exposure.

Acknowledgments

We acknowledge ZhenZhen Zuang for technical expertise in conducting these studies.

References


