Award Number: DAMD17-02-1-0203

TITLE: Bone Marrow Function in Development of Childhood Asthma

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REPORT DATE: March 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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**Bone Marrow Function in Development of Childhood Asthma**

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**SYMPATHETIC AGENT EXPLANATION:**
No other information provided.

**DISTRIBUTION / AVAILABILITY STATEMENT:**
Approved for Public Release; Distribution Unlimited

**ABSTRACT:**
Asthma is the most common reason for hospitalization of children in both military and civilian hospitals. In children with asthma, pulmonary exposure to allergen results in damage to bronchioles by invasion of eosinophils. Eosinophils are inflammatory cells, have limited life spans, and must be continually renewed from hematopoietic tissue. We have adapted an animal model of asthma for studies of the effect of pulmonary allergen exposure on eosinophil progenitor cells (CFU-eo). These studies previously revealed that CFU-eo numbers were elevated in the bone marrow of asthmatic mice following pulmonary allergen exposure. IL-5 is the primary cytokine that regulates eosinophil production and we demonstrated that fibroblastic bone marrow stromal cells produce IL-5 and that stromal cells regulate eosinophil production in vitro. However, the relative role of bone marrow stromal cells and T lymphocytes in eosinophilia that accompanies chronic asthma has not been investigated. The primary objective of this study is to determine the role of stromal cells in normal and asthmatic eosinophil production and the extent to which inflammatory mediators released in asthma affect stromal cell function. Data presented in this report document our progress to date in this investigation and present a refined working hypothesis of regulation of altered eosinophil production in onset of disease. Better understanding of cellular and molecular mechanisms involved in initiation and progression of disease is essential for design of more effective intervention strategies to interrupt growing incidence of this disease.

**SUBJECT TERMS:**
bone marrow, asthma, hematopoiesis, eosinophil, cytokine
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Introduction

Asthma is the most common reason for hospitalization of children in both military and civilian hospitals. In children with asthma, pulmonary exposure to allergen results in damage to bronchioles by invasion of eosinophils. Eosinophils are inflammatory cells, have limited life spans, and must be continually renewed from hematopoietic tissue. In the studies described here, we have utilized an animal model of asthma adapted to our laboratory for studies of the effect of pulmonary allergen exposure on expansion of eosinophil progenitor cells (CFU-ec) in the bone marrow. The bone marrow is a complex anatomic structure composed of immature hematopoietic cells, fibroblastic stromal cells, and functionally mature lymphocytes. From preliminary data included in the original application, we proposed that expansion of eosinophil progenitor cells and subsequent maturation to eosinophils may be regulated by separable mechanisms. Our proposal centered upon determining the role of both bone marrow stromal cells and T lymphocytes on normal eosinophilopoiesis and the extent to which each of these cell types participates in exaggerated production of eosinophils during development of asthma. These studies focused on changes in bone marrow function that occurred early in the development of asthma (or during the period of allergen sensitization). These studies take on increased importance because little is actually known about normal regulation of hematopoiesis or the possibility that systemic inflammatory responses may alter these mechanisms. Our studies have been accelerated by the use of congenitally T cell deficient nude mice. We found that following intranasal exposure to allergen, there was significant CFU-ec expansion in athymic nude mice without a corresponding bone marrow eosinophilia. These data indicate the vital role of T lymphocytes in the final differentiation of mature functional eosinophils and the fact that CFU-ec expansion was regulated cells other than T cells.

Body

Original Aims.

Research Objective 1: To determine cellular mechanisms that regulate bone marrow eosinophilia following allergen challenge. In our initial attempt to dissect regulation of eosinophil development in the bone marrow, we found that bone marrow stromal cells produce IL-5 and supported eosinophil differentiation in vitro. IL-5 production by bone marrow stromal cells was upregulated by exposure to IL-1β and this correlated with increased eosinophil differentiation in vitro. However, other investigators have documented IL-5 production by CD3+ T lymphocytes in the bone marrow. Experiments in this specific aim will utilize T cell deficient nude mice to determine the role of bone marrow stromal cells and T lymphocytes in eosinophil progenitor cell expansion and differentiation that lead to bone marrow eosinophilia.

Research Objective 2. To determine the effect of inflammatory mediators associated with asthma on stromal cell function. Previous experiments from this laboratory revealed that exposure of stromal cells to IL-1 and IL-4 resulted in failure of their ability to support early events in B lymphocyte development. In this specific aim we will determine the effect of inflammatory mediators that are systemically elevated in asthma on stromal cell cytokine production and function. Specifically, we will investigate stromal cell support of myeloid and lymphoid progenitor expansion.

Research Objective 3: To determine the kinetics of altered bone marrow cell function in asthma. The duration of altered hematopoietic cell production following pulmonary allergen...
exposure is not known. This question is pertinent to the sensitization and subsequent development of childhood asthma. Establishing the kinetics of this response will be particularly important in understanding whether the bone marrow response changes with repeated exposure to allergen. Experiments in this specific aim are designed to determine the durability of altered hematopoiesis following single or repeated pulmonary exposure to allergen.

Statement Of Work (Revised 12/31/01)

Project Year 01: In the first year of this project, we will initiate the in vitro and in vivo studies described in Research Objective 1. Although our laboratory is experienced in rodent surgery and we have an attending veterinarian consulting on this aspect of the project, it is expected that development and conduct of the diffusion chamber experiments will require a total of 30 months and will extend through the second year of the project and be concluded in Project Year 03. Completed studies will be presented at appropriate scientific meetings and prepared for publication in refereed journals.

Project Year 02: In vitro studies initiated in Project Year 01 (Research Objective 1) will continue throughout Project Year 02. We will initiate studies proposed in Research Objective 3 that focus on the durability of effects of repeated in vivo allergen dosing regimens on bone marrow function. Completed studies will be presented at appropriate scientific meetings and prepared for publication in refereed journals.

Project Year 03: During Project Year 03, we will complete remaining in vivo diffusion chamber studies described in Research Objective 1. We will complete studies of long-term allergen exposure and evaluate bone marrow transplantation studies proposed in Research Objective 3. We will repeat studies in each Research Objective 1 and Research Objective 3 as necessary to complete and appropriately document this project in published literature. Completed studies will be presented at appropriate scientific meetings and publications prepared for refereed journals.

Progress Report

In our statement of work, we proposed initiating studies that were to determine the cellular mechanisms, which regulate bone marrow eosinophilia following allergen challenge (Research Objective 1). These studies focused on the relative roles of bone marrow stromal cells and bone marrow T cells in regulating progenitor cell expansion and expression of eosinophilia following allergen exposure and encompassed both in vivo and in vitro approaches.

Our initial studies were to determine the kinetics of eosinophil progenitor cell expansion in the bone marrow of athymic mice following exposure to allergen. Experiments performed demonstrated that athymic nude mice had significant CFU-eo expansion, which peaks four days following initial intranasal sensitization with ovalbumin and returns to baseline by day 18. Eosinophil numbers in nude mice were elevated 5 days after the initial intranasal exposure to ovalbumin, but this elevation was minor as compared to eosinophilia in matched allergen sensitized normal balb/c mice (Figure 1). These findings indicate that CFU-eo expansion occurs independent of T cell activation following allergen exposure. These findings are detailed in a manuscript submitted to the Journal of Immunology, which is now under review and in Abstract #1. (See Appendix materials).
Previous studies in this laboratory indicated that bone marrow stromal cells supported eosinophil differentiation in vitro through IL-5 production\(^1\). Stromal cell IL-5 production was capable of being upregulated by an inflammatory cytokine associated with asthma, IL-1. Relevance of stromal cell support of elevated CFU-eo production by increased IL-5 production was investigated utilizing the in vivo athymic mouse model developed for this grant. In this study, anti-IL-5 antibody TRFK-5 was administered to athymic mice and CFU-eo cultured 3 days after the initial intranasal delivery of ovalbumin. Anti-IL-5 antibody failed to prevent CFU-eo expansion in this model (Figure 2). These results suggest that stromal cell support of CFU-eo expansion is not due to increased IL-5.
FIGURE 2

These studies identified substantial CFU-eo expansion in athymic nude mice without progression to eosinophilia and indicated that T lymphocytes are required for eosinophil maturation, but are not required for CFU-eo expansion during sensitization phase of asthma. However, baseline numbers of T cells in the bone marrow indicate a frequency of T cells to be approximately 1-3% of the total bone marrow. Interest in the sensitization phase of asthma lead us to investigate the possibility of T cell emigration to the bone marrow during sensitization contributing to marked bone marrow eosinophilia. Experiments were designed to document both the number of T cells and of total IL-5 producing cells in the bone marrow over the course of allergen sensitization. Bone marrow T cells or IL-5 producing cells were not elevated in BALB/c mice during allergen sensitization (Figures 3 and 4 on pages 8 and 9 of this document). This is in contrast to observations by other investigators during a mature asthma response (after allergen challenge)²³. These studies were selected for presentation at the American Academy of Allergy, Asthma and Immunology Annual Meeting, March 2003 (Abstract #2 in Appendix Materials).

We have initiated studies of the effect of stromal cells on CFU-eo and eosinophil expansion by employing diffusion chambers. Preliminary studies developing the technology of utilizing these diffusion chambers in our athymic mouse model were begun. Initial studies of surgically implanting diffusion chambers without an accompanying inflammatory response were successful. (Data not shown.) The original protocol for sensitization of mice required a second IP dose of ova/alum at the time of the initial intranasal dosing (day 10). In order to minimize inflammation at the time of diffusion chamber insertion, alternate methods of allergen sensitization were investigated to avoid IP delivery of ova/alum at the time of diffusion chamber insertion. We found that intranasal delivery of ovalbumin alone is similar to a dual approach of IP ova/alum administration along with IN delivery of ova (data not shown).
FIGURE 3
We also initiated the *in vivo* experiments planned in Research Objective #1. Experiments were performed to determine the effect of the diffusion chamber apparatus on cell viability. Bone marrow cells were injected into diffusion chambers suspended in standard tissue culture medium and chambers submerged in tissue culture medium in 100mm tissue culture dishes and incubated for 24 hours. The end of this culture period, cells were removed and survival and viability established using trypan blue exclusion. The number of CFU-eo injected into the chamber and the number surviving was determined by placing cells in 2% methylcellulose with IL-5 and enumerating colonies formed during 6 days of culture. These studies evaluated cells culture with IL-5 (10ng/ml) alone, cells cultured with IL-5 and the ring apparatus, and cells cultured with IL-5 and the glue used to construct the chambers. The viability of cultured bone marrow cells at 24 hours was 91% and this viability was not affected by the ring apparatus alone (90%). However, cells exposed to the glue used to construct the ring resulted in a significant cell viability (67%) and that loss of cells could only be partially reversed by washing the glued rings with PBS. However, the number of CFUeo in these chambers was unaffected by the washed ring apparatus despite changes in total bone marrow cell viability. (Figure 5).
Ultimate recovery of CFU-eo/eosinophils from the implanted diffusion chamber would require enzymatic dissociation of clotted cells. We have evaluated separation methods for removing CFU-eo from S10 stromal cell layers by both enzymatic (trypsin and pronase) or non-enzymatic methods, cell dissociation fluid (Sigma, St. Louis, MO). Endpoint of exposure to dissociation fluid was cell suspension of adherent cells. CFUeo number was determined for each condition. No CFU-eo were cultured from any treatment condition. Timed exposure to bone marrow cells (5 minute exposure) was then evaluated. Trypsin exposed bone marrow cells were 89% viable after 5 minutes. Pronase exposed cultures were only 53% viable. CFUeo recovery was comparable to control cultures with use of trypsin. (Figure 6).

![Effect of cell dissociation on CFU-eo](image)

FIGURE 6

We are currently evaluating whether heat sealing of diffusion chambers will be optimal to preserve cell viability in these chambers. We have implanted our first set of chambers in mice and the number of surviving CFU-eo over three days is being determined as described. These studies will also determine the optimal cell number to be used in these chambers for in vivo studies.

Research objective 1 also sought to determine the capacity of stromal cells to support CFU-eo expansion utilizing in vitro methods. These experiments were to be performed utilizing S10 stromal cells co-cultured with nonadherent bone marrow cells. In addition, subsequent investigation of the role of stromal cell adhesion molecules in the expansion of CFU-eo was proposed. Initial studies in this set of experiments determined optimal methods to achieve dissociation of adherent co-cultured S10 stromal cells and bone marrow cells into a single cell suspension for evaluation in CFU-eo cultures. Despite various methods of enzymatic dissociation (see above), and subsequent passage of the suspension over a G10 column (to remove adherent cells), or allowing adherent cells to stick to a plastic tissue culture plate prior to use in a CFU-eo assay, no CFU-eo colonies were recovered when these cell layers were dissociated. We also cultured S10 stromal cells in tissue cultures dishes prior to plating CFU-eo in the same plates. S10 cells were allowed to expand to confluence, media was removed and
bone marrow cells suspended in methylcellulose with IL-5 were overlaid. Interestingly, we again recorded no CFU-eo colonies formation. This repeated observation led to hypothesize that bone marrow stromal cells may functionally inhibit CFU-eo formation, and that observed expansion following allergen exposure in vivo may be the result of loss of this suppression rather than elevated production of stimulatory factors.

In order to test this new hypothesis, conditioned medium was obtained from S10 bone marrow cells. As described in research objective 1, the effects of inflammatory cytokines IL-1 and IL-4 on stromal cell function in support or inhibition of CFU-eo cells was also investigated. Here inflammatory cytokines IL-1 (50 ng/ml) and IL-4 (10 ng/ml) were cultured with S10 cells for 24 hours and the cytokines removed by extensive washing with PBS. Medium was replaced for 72 hours and ‘conditioned medium’ collected and concentrated. As shown in Figure 7, stromal cell conditioned medium was inhibitory to CFU-eo colony formation and this effect was intensified by prior exposure of S10 to inflammatory cytokines. In addition, experiments were performed to determine if this inhibitory effect of stromal cell conditioned media was lost during dialysis (eg: was the result of toxic small molecular weight contaminants such as thymidine). Figure 8 demonstrates that the inhibitory effect of stromal cell conditioned medium was not due to compounds under 10,000 daltons. Inhibition of CFU-eo by stromal cell conditioned medium was also shown to be dose responsive (Figure 9).

**Comparison of IL-1, IL-4 stimulated S10 Conditioned media**

![Graph showing comparison of IL-1, IL-4 stimulated S10 Conditioned media]

**FIGURE 7**

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page 11
Effect of dialyzing S10 CM on CFUeo

![Bar graph showing CFUeo/10^6 bone marrow cells for different conditions: nl CFUeo, S10 CM, and S10 CM dialyzed.]

**FIGURE 8**

Dose response curve to S10 conditioned media

![Bar graph showing CFUeo/plate for different concentrations: onc. regular medi, 5% CM, 10% CM, 25% CM, and 50% CM.]

**FIGURE 9**
As proposed in research objective 1, the direct effect of these inflammatory cytokines on CFU-eo numbers was investigated. IL-4 (100 ng/ml) was cultured with bone marrow cells and IL-5 (10ng/ml). IL-4 inhibited the formation of CFU-eo (Figure 10), and this effect is recoverable when 300ng/ml anti-IL-4 antibody, is also placed in the culture (Figure 10). Interestingly, IL-1 was found to have no effect on CFU-eo progenitor numbers (data not shown). The direct effect of several other hematopoietic cytokines was also investigated. IL-6 was found to not effect CFU-eo progenitor numbers (data not shown). However, TGF-β was found to profoundly inhibit CFU-eo progenitor cell numbers.

**FIGURE 10**

Experiments were designed to determine whether any of a battery of known cytokines were contributing to the suppression of CFU-eo formation observed in stromal cell cultures. Anti-IL-4 antibody was added to cultures containing conditioned medium, IL-1 stimulated S10 conditioned medium, or IL-4 stimulated S10 conditioned medium. As shown in Figure 11, CFU-eo cultures established with anti-IL-4 antibody in the presence of conditioned media reversed this inhibitory effect. Studies are currently underway to evaluate the possibility that stromal cells also produce TGF-β, which we have shown, is capable of inhibiting CFU-eo progenitor expansion.
Effect of anti IL-4 antibody on S10 conditioned medium

Effect of anti IL-4 antibody on IL-1 stimulated S10 conditioned medium

Effect of anti IL-4 antibody on IL-4 stimulated S10 conditioned medium

FIGURE 11
We had initially proposed to evaluate whether T cells are required for CFU-eo expansion. Studies performed in athymic nude mice suggest that T cells are not required for CFU-eo expansion. However, in light of the observation that stromal cell conditioned media inhibits CFU-eo progenitor cells, we sought to determine if T cells are indirectly involved in CFU-eo inhibition. Studies with conditioned media were repeated utilizing T cell deficient bone marrow obtained from athymic nude mice. Results from these studies demonstrate that the absence of T cells in the CFU-eo assay does not prevent inhibition of eosinophil progenitor numbers (Figure 12).

**FIGURE 12**
Key Research Accomplishments

- Eosinophil progenitor cell expansion is regulated by a T-cell independent mechanism.
- Eosinophil progenitor expansion is not regulated by IL-5.
- Bone marrow T cell numbers remain stable during allergen sensitization.
- IL-5 producing cell numbers in the bone marrow are unchanged after sensitization.
- Surgical implantation of diffusion chambers is achieved without significant accompanying inflammation.
- Determined that diffusion chamber apparatus does not affect CFU-eo numbers in culture.
- Determined that removal of cells from diffusion chamber will be accomplished by trypsinization.
- Stromal cells inhibit CFU-eo formation.
- Stromal cell conditioned media inhibits CFU-eo formation in a dose dependent fashion.
- Exposure of stromal cells to inflammatory cytokines IL-1 and IL-4 intensifies CFU-eo inhibition.
- IL-4 and TGF-β directly inhibit CFU-eo progenitor cell formation.
- There is no direct effect of IL-1 or IL-6 on CFU-eo progenitor cells.
- Stromal cell conditioned media inhibition of CFU-eo is reversed by anti-IL-4 antibody.
- Bone marrow T cells are not indirectly involved in CFU-eo inhibition by stromal cells conditioned medium.

Reportable Outcomes

Publications resulting from this award.


Abstracts presented.


Hogan MB, Weissman DN, Gibson LF, Piktel D, Welch J, Landreth KS. Role of bone marrow T cells in eosinophil production of asthma. AAAAI National meeting, Denver, CO. March 2003.

Conclusions:

Our interest in childhood asthma has led us to investigate events in eosinophilopoiesis during the sensitization phase of asthma. This investigation has led us to propose that eosinophilopoiesis is regulated in a step-wise manner by bone marrow stromal cells and T lymphocytes (Figure 13). This hypothesis is the basis for the design of experiments to be done in the second year of this grant.
Experiments to be completed in the next year of this grant will be focused on understanding both the downregulation of CFU-eo generation observed in the presence of stromal cells and the significant increases in CFU-eo production during both steady state hematopoiesis and during the initiation of asthma. We will continue studies determining aimed at identifying CFU-eo downregulatory cytokines produced by stromal cells. In addition, studies will be completed determining if IL-5 is required for CFU-eo expansion. Investigation of alternate cytokine signals that accelerate CFU-eo expansion are also planned. We will complete the initial evaluation of diffusion chamber implantation in vivo, and complete planned experiments with this model. In Project Year 02 we will begin to evaluate the effect of repeated administration of allergen on eosinophil production in the bone marrow as described in Research Objective #3.

Currently there are no long-term options for intervening in the process of allergen sensitization and development of childhood asthma. Studies proposed in this grant will determine the regulatory mechanisms of bone marrow eosinophil production at both steady state and as altered in the disease state of asthma. Special emphasis on investigating the role of both bone marrow stromal cells and T cells in eosinophilopoiesis is ongoing. Preliminary data suggest that T cells may be critical to eosinophil maturation. In addition, ongoing studies suggest that stromal cells may provide key downregulatory signals controlling eosinophil production under normal and inflammatory conditions. Elucidation of the normal downregulatory mechanisms of eosinophil production may lead to strategies for childhood asthma that ultimately inhibit disease development or progression.

References:


Appendices

1. Abstract #1


2. Abstract #2

Hogan MB, Weissman DN, Gibson LF, Piktel D, Welch J, Landreth KS. Role of bone marrow T cells in eosinophil production of asthma. AAAAI National meeting, Denver, CO. March 2003

3. Abstract #3


4. Submitted Manuscript

Abstract

American Academy of Allergy Asthma and Immunology Annual Meeting
New York, NY, March 2002

Bone marrow CFU-eosinophil (CFU-eo) production in a murine asthma model. Hogan MB, Weissman DN, Zhuang ZZ, Landreth KS. West Virginia University School of Medicine, CDC-NIOSH-HELD, Morgantown, WV

Purpose: Although it has been demonstrated that murine experimental asthma is associated with increased bone marrow eosinophil production, its effects on levels of eosinophil precursors (CFU-eo) have not been as well documented. We hypothesized that a systemic and pulmonary sensitization protocol sufficient to induce murine experimental asthma would also expand bone marrow CFU-eo populations and that the bone marrow CFU-eo response would be dependent on T cells.

Methods: 6 week old Balb/c mice and athymic nude (nu/nu) mice as follows: 100 μg ovalbumin/0.5mg aluminum potassium sulfate IP on days 0 and 10; and 100 μg ovalbumin IN on day 10. Control mice were treated with saline IP on days 0 and 10 and saline IN on day 10. Mice were sacrificed for evaluation of bone marrow CFU-eo, bone marrow eosinophils, peripheral blood eosinophils and bronchoalveolar lavage (BAL) eosinophils on days 10-19. Bone marrow CFU-eo assays were performed in methylcellulose with or without IL-5. Bone marrow and peripheral blood eosinophil numbers were enumerated after cytoospin slides were stained with May-Grünwald-Giemsa stain.

Results: Ovalbumin sensitized Balb/c mice had significantly decreased bone marrow CFU-eo numbers relative to control mice on days 13-15 and were significantly increased relative to control mice on days 16-18 (p<0.05). Bone marrow eosinophil numbers were significantly increased on days 13-15 (p<0.05); and BAL eosinophils were increased on day 12-16. Ovalbumin-sensitized nude mice studied at day 13 and 16 were markedly different from Balb/c mice. At days 13 and 16, bone marrow CFU-eo numbers in ovalbumin treated nu/nu mice significantly exceeded both nude mouse saline controls and similarly ovalbumin sensitized Balb/c mice (p<0.01). Despite their prominent bone marrow CFU-eo responses, nude mice did not have significant changes in mature eosinophil numbers following sensitization to ovalbumin.

Conclusions: In normal wild-type Balb/c mice, a systemic and pulmonary sensitization protocol sufficient to induce experimental asthma is associated with a marked mature eosinophil response. This response transiently depletes bone marrow CFU-eo numbers followed by a detectable rebound. In contrast, T cell deficient nude mice mount a bone marrow CFU-eo response to sensitization but cannot convert CFU-eo precursors into mature eosinophils. These findings suggest that in murine experimental asthma, generation of bone marrow eosinophil precursors does not depend on T cells. However, generation of mature eosinophils from these progenitor cells is T cell dependent.

This research is supported by DOD Grant DAMD17-02-1-0203.
Abstract

American Academy of Allergy Asthma and Immunology Annual Meeting
Denver, CO, March 6-13, 2003

Role of Bone Marrow T Cells in Eosinophil Production.

MB Hogan, DN Weissman, LF Gibson, D Piktel, J Welch, KS Landreth, West Virginia University School of Medicine and NIOSH-HELD, Morgantown, WV.

Abstract:

Rationale: Eosinophil progenitors (CFU-eo) and mature eosinophils are increased in bone marrow by allergen sensitization. We hypothesized that these changes were mediated by expansion of bone marrow T cell populations with increased production of IL-5 by these cells.

Methods: 6 week old Balb/c mice were sensitized to ovalbumin according to the following schedule: Day 0: intraperitoneal (IP) ovalbumin (OVA)/alum; Day 10: IP OVA/alum + intranasal OVA. Control mice received normal saline. On days 10-18, the following cell types were enumerated in bone marrow: eosinophils by light microscopy; and CD3+, CD4+, and CD8+ lymphocytes by flow cytometry. In addition, numbers of bone marrow IL-5 secreting cells were enumerated by ELISPOT.

Results: Marked increases in bone marrow eosinophils were noted in OVA but not saline treated mice. In contrast, no significant differences were noted between groups in bone marrow CD3+, CD4+ or CD8+ lymphocytes; or in numbers of IL-5 secreting bone marrow cells.

Conclusions: Bone marrow eosinophil responses to allergen exposure are not mediated by expansion of bone marrow T cell populations or increased numbers of IL-5 secreting bone marrow cells.

Funding: This research is supported by DOD Grant DAMD17-02-1-0203 and an American Lung Association Grant ALA-CI-017-N
Abstract

International Society of Experimental Hematology Annual Meeting
Paris, July 5-8 2003

Regulation of eosinophilopoiesis by stromal cells is modulated by inflammatory cytokines.

KS Landreth, D Piktel, LF Gibson, DN Weissman, J Welch, MB Hogan, West Virginia University School of Medicine and NIOSH-HELD, Morgantown, WV.

Hematopoiesis is regulated by local cytokine production and by circulating hormones and cytokines. Our laboratory focused on mechanisms that regulate eosinophilopoiesis in normal mice and alterations of eosinophilopoiesis that accompany onset of inflammatory airway disease. BALB/c^+/+ mice were sensitized to ovalbumin (OVA) using a standard protocol for induction of asthma. Following intranasal installation of OVA, mice developed initial depletion and subsequent rebound of CFU-eo, followed by eosinophilia in bone marrow, peripheral blood, and lung. Athymic BALB/c^nu/nu exhibited even more dramatic expansion of CFU-eo following treatment, but not eosinophilia. These data led to the working hypothesis that eosinophilopoiesis is regulated by both stromal cells and T lymphocytes. In the absence of T lymphocytes, pulmonary inflammation and release of inflammatory cytokines led to expansion of CFU-eo in bone marrow in vivo. Expansion of CFU-eo was also observed in vitro when stromal cells were exposed to IL-1α. However, when normal bone marrow was incubated with stromal cell line S10, or exposed to conditioned medium from S10 cells in vitro, CFU-eo failed to differentiate to form eosinophil colonies in the presence of IL-5. Using antibody blockade, inhibition of CFU-eo proliferation and differentiation in these experiments was attributed to IL-4. Exposure of stromal cells to rIL-1α resulted in increased production of both IL-4 and IL-5. These data confirm that production of CFU-eo in bone marrow is regulated by stromal cells and that CFU-eo expansion is accelerated by inflammatory cytokines. However, stromal cells inhibit further differentiation of CFU-eo and maintain normally low output of mature eosinophils. Allergen stimulation results in increased pools of CFU-eo and increased CFU-eo differentiation that is T lymphocyte and IL-5 dependent. These data have been used to construct a model of regulation of eosinophilopoiesis in which antigen stimulation and inflammation serve to regulate the tempo of eosinophil production in the bone marrow.

This research is supported by DOD Grant DAMD17-02-1-0203 and an American Lung Association Grant ALA-CI-017-N.
Regulation of eosinophilopoiesis in a murine model of asthma

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Running Title: Regulation of Eosinophilopoiesis in Asthma

Key words: eosinophils, hematopoiesis, stromal cells, inflammation, lung

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Abstract

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 hematopoietic tissue in individuals with asthma. However, specific mechanisms that alter eosinophilopoiesis in this disease are poorly understood. The current study utilized 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 ovalbumin, 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 ovalbumin exposure. In T cell deficient BALB/c nude (BALB/c\textsuperscript{nu/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 hematopoietic 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.
Introduction

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. Mediators released by mast cells are chemotactic and initiate pulmonary infiltration of lymphocytes, neutrophils and eosinophils following allergen exposure. 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.

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. In a murine model of asthma, transient bone marrow eosinophilia was demonstrated following airway sensitization to ovalbumin, and following subsequent allergen challenge. In both cases, bone marrow eosinophilia was followed by peripheral blood and pulmonary eosinophilia and circulating eosinophils appeared to be newly produced cells emigrating from the bone marrow.

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 ovalbumin, we observed sequential eosinophilia in bone marrow, peripheral blood, and lungs of mice. Eosinophil progenitor cells (CFU-eo) 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. In order 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, blood, or pulmonary tissue. 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 six week old, female, BALB/c\(^{+/+}\) or athymic BALB/c\(^{nu/nu}\) (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 ad libitum. A 12 hour 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 ovalbumin (ova) has been previously described in detail\(^8\). Briefly, in each experiment at least four mice were injected intraperitoneally (IP) with 100mg/kg ovalbumin (Sigma Chemical Co., St. Louis, MO) suspended in a saturated solution of aluminum potassium sulfate (alum, Sigma Chemical Co.) in sterile distilled water on days 0 and 10.\(^8\) For IP injections, ovalbumin (5mg/ml) was suspended in 10 ml of endotoxin free 0.9% saline and equal volumes of working solutions of ovalbumin and alum mixed; adjusted to pH 6.5, and allowed to precipitate for 30 minutes. The precipitate was centrifuged at 1800 rpm at room temperature, supernatant removed and precipitate resuspended in 10 ml endotoxin free saline. On day 10, mice were exposed to 25\(\mu\)l ovalbumin dissolved in endotoxin-free sterile saline delivered into the lung by intranasal (IN) deposition under ketamine anesthesia. In all experiments, control mice were handled identically and administered saline IP and IN on the same schedule.
**Bone marrow and peripheral blood.** Mice were euthanized by CO₂ asphyxiation, the peritoneal cavity opened, and peripheral blood obtained directly from the inferior vena cava using a heparinized tuberculin syringe. Total white blood cell counts were obtained utilizing a Coulter counter and peripheral blood smears made to establish a differential white cell count. Bone marrow was obtained by flushing femora with α-Minimal Essential Medium (Gibco, Gathersburg, MD) supplemented with 1% fetal calf serum (Summitt Biotechnology, Fort Collins, CO) using a syringe fitted with a 23-gauge needle. Total white cell counts were evaluated microscopically using a hemocytometer. 10⁵ bone marrow or peripheral blood cells were cytocentrifuged onto cleaned glass slides and stained with May-Grünwald-Giemsa stain (Sigma Chemical Co., St. Louis, MO) for enumeration of eosinophils.

**CFU-eo cultures.** Eosinophil progenitors were evaluated using standard in vitro colony forming cell assays (CFU-eo). CFU-eo were established with 7.5 x 10⁵ bone marrow cells/ml suspended in Methocult M3234 (Stem Cell Technologies, Vancouver, Canada) with or without 10 ng/ml IL-5 (Biosource International, La Jolla, CA). Colonies of greater than 50 cells were counted after 7 days under a stereomicroscope and colony numbers corrected to absolute values. Colonies were picked, cytocentrifuged and stained with May-Grünwald-Giemsa stain to verify the presence of eosinophils.

**Bronchoalveolar lavage (BAL).** Mice were euthanized by CO₂ asphyxiation, the peritoneal cavity opened, and trachea exposed. The trachea was cannulated with a 22g IV catheter. Phosphate buffered saline (500 µl PBS) was injected and withdrawn from
the lung utilizing a tuberculin syringe. This procedure was repeated five times. A white cell count of BAL fluid was evaluated microscopically by hemocytometer. Cells were then cytocentrifuged onto clean glass slides and stained with May-Grünwald-Giemsa stain and cell differential counts obtained. After BAL, lungs were inflated with 1 ml 10% neutral buffered formalin (Sigma Chemical Co.). Lungs were imbedded in paraffin, sectioned and stained with hematoxylin and eosin and 0.5% chromatrope 2R for identification of eosinophils.

**ELISA.** Murine anti-OVA IgE antibody was detected in plasma samples using an IgE-capture ELISA. The following reagents were used, with appropriate washing between incubations: monoclonal anti-mouse IgE (Pharmins, San Diego, CA); PBS/1% skim milk; plasma samples diluted 2-fold over a range from 1:50 to 1:3200; ovalbumin (25 micrograms/ml; Sigma Chemical Co., St. Louis, MO); rabbit anti-ovalbumin-HRP conjugate (Rockland Immunochemicals, Gilbertsville, PA); and TMB substrate solution (Kirkegaard and Perry, Gaithersburg, MD). After incubation for 30 minutes at room temperature, reactions were stopped (TMB Stop Solution; Kirkegaard and Perry) and color development evaluated as OD-450 using an automated plate reader. Specific IgE levels are reported as the reciprocal titer yielding an OD-450 greater than two times background. A positive titer was defined as greater than 1:2.

**ELISpot.** Millipore Multiscreen-IP plates (Millipore, Bedford, MA) were coated with 50μL/well of 10μg/mL solution TRFK-5 antibody (Mabtech Inc., Cincinnati, OH) diluted in coating buffer (0.1M Na₂CO₃, 0.1M NaHCO₃, pH9.6). Plates were incubated
overnight at 4°C in a moist chamber, washed 3X with 200µL sterile PBS, and blocked by addition of 100µL/well αMEM containing 10% FCS for 1 hour at room temperature. Medium was then removed from wells and 2x10⁵ bone marrow cells in 100µl medium added. Cells were incubated overnight at 37°C, culture medium removed, and wells washed 6 times with 0.05% Tween in PBS (PBS-Tween; Sigma, St. Louis, MO). 1ug/mL TRFK-4 anti-IL-5 antibody (Mabtech) was diluted in 0.5%BSA, 0.05% Tween in PBS and 100µL /well incubated for 2 hours at room temperature. Plates were then washed six times with PBS-Tween, allowing 15 minutes incubation at room temperature with each PBS-Tween wash. 100µL/well Vectastain Elite (Vector Labs Inc., Burlingame, CA) was added to all wells, incubated for 1 hour at room temperature, and washed a final time with PBS-Tween and three washes with normal PBS. 100µL/well of Vector VIP Substrate Kit for Peroxidase (Vector Laboratories, Inc.) was added and the plate developed until spots were visualized. At the termination of development, plates were rinsed 5 minutes with deionized water and air dried overnight. Spots were counted utilizing Optimas Imaging Software (MediaCyberneics, Carlsbad, CA).

In vivo antibody suppression of IL-5 expression. IL-5 production was experimentally blocked in BALB/cnu/nu mice by administering 50µg anti-IL-5 monomoclonal antibody TRFK-5 by intraperitoneal injection one day prior to intranasal exposure to allergen (experimental day 9). Intraperitoneal injection of TRFK-5 was repeated daily for three days following the initial intranasal allergen exposure. On experimental day 14, mice were euthanized and bone marrow collected to determine number of CFU-eo as described above.
**Statistical analysis.** Unless otherwise indicated, all data in this study were analyzed using a one-way ANOVA and the Student Newman Keuls post test was used to evaluate difference among experimental means. All statistical analysis was performed using GraphPad InStat Software (San Diego, CA).
Results

Effect of allergen sensitization on eosinophil populations in the bone marrow. In all experiments, mice received an initial intraperitoneal (IP) exposure to ovalbumin (day 0) followed by intranasal exposure to the same allergen on day 10 as described in the 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, three days following intranasal installation of allergen (day 13, Figure 1). This initial depression of bone marrow CFU-eo was accompanied by significant elevation of bone marrow eosinophils (Figure 2). CFU-eo numbers in bone marrow of allergen exposed mice rebounded to greater than control values on day 17 (Figure 1), and returned to baseline values by day 19. Bone marrow eosinophilia in allergen exposed mice resolved to control values by day 17 (Figure 2).

In the experimental protocol previously used to establish ovalbumin allergen sensitivity in mice\(^9\), the initial intranasal exposure to ovalbumin on day 10 was accompanied by a second IP exposure to the same allergen co-precipitated with aluminum potassium sulfate. In order to determine whether observed alterations of CFU-eo following allergen sensitization were due to the intranasal deposition of ovalbumin or to the accompanying IP exposure, we compared the effect of the traditional exposure regimen to one which utilized intranasal exposure to ovalbumin in the absence of a second IP treatment. As shown in Figure 3, intranasal exposure and intranasal exposure combined with an IP
exposure to ovalbumin 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 intranasal exposure to ovalbumin, peripheral blood eosinophilia was not observed until 5 days following intranasal allergen exposure (Figure 4, day 15) and eosinophilia was not resolved by day 19. Leukocytes were elevated in bronchoalveolar lavage (BAL) fluid obtained from these mice on days 11 and 17 as compared to control mice, with significant elevations in neutrophils (day 11, data not shown), eosinophils (days 15-19, Figure 5), and macrophages (day 11-17, data not shown). Histopathology of lung tissue samples obtained from saline control mice did not reveal detectable infiltration of inflammatory cells (Figure 6). However, ovalbumin exposed mice developed substantial eosinophilic alveolar inflammation (Figure 6). Histologic evaluation revealed bronchial changes in mice exposed to intranasal ovalbumin, including secretory cell hypertrophy and hyperplasia (Figure 7). Eosinophilic infiltration was consistently observed in perivascular spaces of the lung (Figure 7).

*Effect of allergen sensitization on bone marrow eosinophil populations in T cell deficient mice.* In order to determine the requirement for T lymphocytes in observed alterations of bone marrow CFU-eo following allergen sensitization, athymic nude mice were exposed to ovalbumin using exactly the same protocol described for wild-type BALB/c mice. Unlike observations in euthymic BALB/c mice, BALB/c nude mice had significantly elevated numbers of bone marrow CFU-eo immediately following intranasal
allergen exposure (Figure 8) and CFU-eo remained elevated in athymic mice on day 16. In a second series of experiments, CFU-eo were enumerated through day 19. The elevation of CFU-eo in the bone marrow of nude mice resolved to baseline numbers by 8 days following intranasal exposure to allergen (experimental day 18; data not shown) and did not remain elevated.

Athymic BALB/c<sup>nu/nu</sup> mice and euthymic BALB/c<sup>+/+</sup> mice did not differ in numbers of bone marrow eosinophils prior to treatment. However, at three days following intranasal exposure to allergen (experimental day 13), the number of bone marrow eosinophils rose dramatically in BALB/c<sup>+/+</sup> mice but remained unchanged in BALB/c<sup>nu/nu</sup> mice (Figure 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-ovalbumin IgE.** Wild type BALB/c mice developed anti-ovalbumin IgE antibodies 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 ovalbumin specific IgE antibody. By day 15, all BALB/c mice tested had detectable circulating levels of anti-ovalbumin IgE antibodies. None of the athymic BALB/c nude in this study developed detectable anti-ovalbumin IgE antibodies (observations made on days 13 and 16).
Role of IL-5 in CFU-eo expansion in BALB/c\textsuperscript{nu/nu} 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 Figure 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 to age and sex matched nude mice. In order to determine differences in total IL-5 secreting cells between these mice, cells were also stimulated with PMA and ionomycin prior to 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).

Nude mice were also treated with neutralizing antibody to IL-5 in vivo (50\mu g/day IP) at the time of intranasal exposure to allergen and evaluated 4 days later to determine effects on expansion of bone marrow CFU-eo. TRFK-5 anti-IL-5 antibody treatment did not alter expansion of CFU-eo in nude mice exposed to intranasal allergen (Figure 10). However, when the same batch of TRFK-5 antibody was added to \textit{in vitro} bone marrow cultures, it completely neutralized IL-5 mediated formation of CFU-eo colonies (Figure 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 hematopoietic 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 utilize an established animal model of asthma to evaluate alterations of bone marrow function that accompany allergen sensitization and to determine hematopoietic 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 intranasal exposure to allergen. These changes in eosinophilopoiesis 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 (Figure 12).

Other laboratories have described altered bone marrow function in response to pulmonary allergen challenge in mice, dogs, and humans.6,8,16 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 eosinophilopoiesis that resulted in increased eosinophil output at this early stage of disease. Following the first intranasal installation of allergen in BALB/c mice, the bone marrow eosinophil compartment expanded rapidly and was significantly different from control animals within 72 hours following allergen exposure. This increase in bone marrow eosinophils was transient and returned to normal values 7 days following pulsed allergen exposure (Figure 2). This pattern of bone marrow eosinophilia following allergen sensitization is in general agreement with previous studies\(^\text{10}\), 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 was followed by peripheral blood (Figure 4) and pulmonary (Figure 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 three days following the initial intranasal installation of allergen, then rebounded to significantly greater than normal numbers for a period of 48 hours before returning to control levels (Figure 1). This pattern of perturbation of hematopoietic progenitor cells has been previously documented in erythropoietic recovery following exposure to hyperbaric conditions\(^\text{17}\) and in myeloid progenitors following chemotherapy\(^\text{18}\). In both cases, increased demand for end cells resulted in initial depletion, and then rebound of specific hematopoietic progenitor cells and the data presented here suggests that eosinophilopoiesis in the bone marrow is regulated in a similar manner. 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) 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 in 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 challenge²² and both Th1 and Th2 cells participate in this process¹⁹. CD8⁺ T cells have also been implicated in the development of airway hyperresponsiveness
associated with asthma\textsuperscript{20-21} and this role for T cells appears to be independent of production of specific IgE mediated antibody responses\textsuperscript{23-25}.

IL-5 is a critical cytokine in development of eosinophils\textsuperscript{25} and previous studies have concluded that IL-5 detected in the marrow is produced by T lymphocytes\textsuperscript{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\textsuperscript{32}. 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\textsuperscript{32} in the bone marrow microenvironment, the observation that nude mice treated daily with a neutralizing antibody to IL-5 did not have altered expansion of CFU-eo following allergen exposure suggests that IL-5 may not be the cytokine responsible for CFU-eo expansion in consistently observed following this treatment. 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 (Figure __). 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 there was little difference in the amount of IL5 produced per cell in normal and nude mice.

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 ovalbumin specific IgE antibody in both euthymic and athymic mice. Alterations of CFU-eo were documented in immunocompetent BALB/c mice on day 13, a time at which only 30% of animals had detectable ovalbumin specific IgE antibody.

The finding that eosinophil progenitor proliferation and subsequent eosinophil differentiation are regulated by separable mechanisms is consistent with data for other developing hematopoietic cell lineages. We previously reported that early development of B lymphoid progenitors was T cell independent and required the presence of bone marrow stromal cells27, 28. 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.29, 30 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 hematopoiesis is not well understood. We previously demonstrated that elevated levels of both IL-1 and IL-4 altered bone marrow stromal cell function and production of B lymphocytes in that tissue.\textsuperscript{30} Our recent work has extended that observation to eosinophilopoiesis. Bone marrow stromal cells produce the primary eosinophilic cytokine, IL-5, and increased IL-5 abundance in stromal cells is correlated with increased eosinophil production increased when stromal cells were exposed \textit{in vitro} to rIL-1.\textsuperscript{32} The present study strongly suggests that stromal cells also regulate eosinophil progenitor cell expansion in the bone marrow in response to airway inflammation. Taken together, these studies support the hypothesis that systemic release of inflammatory mediators following tissue specific immune responses, may serve as a primary regulatory stimulus for altered hematopoietic responses to immune insult, including allergen exposure in the lung.
Acknowledgements

The authors acknowledge ZhenZhen Zuang and Debra Pikel for technical expertise in conducting these studies.
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Footnotes

1 Funded in part by Grants DAMD17-02-1-0203 from the Department of Defense, ALA-CI-017-N from the American Lung Association, and a Research Development Grant from the West Virginia University Research Corporation.

2 Address correspondence to: Mary Beth Hogan, M.D., Department of Pediatrics, P.O. Box 9214, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, WV 26505-9214.
Figure Legends

Figure 1. Kinetics of eosinophil progenitor cells (CFU-eo) during allergen sensitization. Mice were exposed to ovalbumin and alum by IP injection on days 0 and 10. Intranasal ovalbumin was delivered on day 10 under light anesthesia. Bone marrow CFU-eo were evaluated as described by incubating $5 \times 10^5$ bone marrow cells in methylcellulose for 7 days in the presence or absence of 10 ng/ml recombinant mouse IL-5. Data presented are the means ± SEM of 3 independent observations. Statistically significance was determined utilizing Student Newman Kuels comparison testing. Significant differences from control values were recorded on days 17 and 18, (p<0.001).

Figure 2. Kinetics of bone marrow eosinophils during allergen sensitization. Bone marrow cells were cytocentrifuged onto clean glass slides and stained with May-Grünwald-Giemsa. A minimum of 200 bone marrow cells were counted under high-power light microscopy and eosinophil number determined for each sample. Data presented are the means ± SEM of 3 independent observations. Statistically significance was determined utilizing Student Newman Kuels comparison testing. A statistically significant change occurred on day 13 (p<0.001) and day 15 (p<0.01).

Figure 3. Effect of route of allergen exposure on bone marrow CFU-eo. Mice were exposed to ovalbumin and alum by IP injection on days 0. On day 10, mice received either intranasal ovalbumin delivered under light anesthesia or intranasal ovalbumin accompanied by a second IP treatment with ovalbumin and alum and were compared to
untreated controls. Bone marrow CFU-eo were evaluated as described by incubating 5x10^5 bone marrow cells in methylcellulose for 7 days in the presence or absence of 10 ng/ml recombinant mouse IL-5. Data presented are the means ± SEM of 3 independent observations. Statistically significance was determined utilizing Student Newman Kuels comparison testing. Significant differences from control values were recorded on days 17 and 18, (p<0.001).

Figure 4. Kinetics of peripheral blood eosinophils during allergen sensitization. Peripheral blood was obtained from each animal and a total white count established utilizing a coulter counter. Peripheral smears are stained with May-Grünwald-Giemsa and a minimum of 200 white cells counted. Data presented are the means ± SEM of 3 independent observations. Statistically significance was determined utilizing Student Newman Kuels comparison testing. Statistically significant changes occurred at days 15 (p<0.001), 17 (p<0.01), and 19 (p<0.001).

Figure 5. Kinetics of bronchoalveolar lavage eosinophils during allergen sensitization. The trachea of mice was cannulated and bronchoalveolar lavage performed as described. White cells were counted under visual microscopy utilizing a hemocytometer. Lavage fluid smears are stained with May-Grünwald-Giemsa and a minimum of 200 white cells counted. Data presented are the means ± SEM of 3 independent observations and are representative of three identical experiments. Statistically significance was determined
utilizing ANOVA and Kruskal-Wallis testing. Statistically significant differences were found on day 17 (p<0.05).

**Figure 6.** Comparison of pulmonary inflammation between control and ovalbumin sensitized mice. 
A. Bronchiole and perivascular space in a representative control mouse  
B. Bronchiole and perivascular space in an ovalbumin sensitized mouse.  
C. Higher magnification of secretory cell hypertrophy and hyperplasia in an ovalbumin sensitized mouse.  

**Figure 7.** Photomicrograph of pulmonary eosinophil infiltration. Infiltration of the perivascular space by a population of inflammatory cells principally comprised of eosinophils. Bar = 20 microns.

**Figure 8.** Comparison of kinetics of bone marrow CFU-eo and eosinophils in euthymic and athymic BALB/c mice during allergen sensitization. Euthymic BALB/c^{+/+} or athymic BALB/c^{nu/nu} (nude) mice were treated with ovalbumin and alum as described. Bone marrow CFU-eo and eosinophil numbers were enumerated as described. Data presented are the means ± SEM of 3 independent observations and are representative of three identical experiments. Statistically significance was determined utilizing Student Newman Kuels comparison testing. Bone marrow CFU-eo were significantly different between euthymic and athymic nude BALB/c mice on day 13 (p<0.001) and day 16.
(p<0.01). Bone marrow eosinophil numbers were significantly different between euthymic and athymic nude BALB/c mice on days 13 (p<0.001) and (p<0.01).
Figure 1

[Graph showing the number of CFUe/10^-6 bone marrow cells over days 0 to 20. The graph peaks around day 16.]
Figure 2

eosinophils x 10^{-6}/femurs

Days
Figure 3

CFUeo/10⁶ BM cells

no rx | ip/in | in only

* | *
Figure 5
Figure 8

Graph showing CFU-eo and Eosinophils over days.

- **CFU-eo**
  - **nude** (dashed line)
  - **balb** (solid line)

- **Eosinophils**
  - **x 10^6/femurs**

Days: 0, 10, 11, 12, 13, 14, 15, 16
Figure 9

[Bar graph showing IL-5 producing cells per 10^6 bone marrow cells for BALB/c +/+ and BALB/c nu/nu strains. The graph indicates a significantly higher number of IL-5 producing cells in BALB/c +/+ compared to BALB/c nu/nu with a double asterisk (* *) symbol indicating statistical significance.]
Figure 10

![Bar graph showing CFU-eo/10^6 bone marrow cells with treatment groups: no treatment, isotype, and TRFK5.](image)
Figure 11

- CFU-eo/10^6 bone marrow cells
- no treatment
- isotype
- TRFK5

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Figure 12

Stromal cell dependent

Pre CFU-eo \(\rightarrow\) CFU-eo \(\rightarrow\) Eosinophil

T cell dependent IL-5 dependent

? \(\rightarrow\) IL-5