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13. ABSTRACT (Maximum 200 Words)

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 adapted an animal model of asthma to our laboratory for studies of the effect of pulmonary allergen exposure on eosinophil progenitor cells (CFU-eo). These studies have revealed that CFU-eo numbers are elevated in the bone marrow of asthmatic mice following pulmonary allergen exposure. IL-5 is the primary cytokine that regulates eosinophil production and was originally thought to be synthesized exclusively by T lymphocytes. Cytokines such as IL-5, SCF, IL-4 and leukotrienes also influence eosinophil production during the onset of asthma and these cytokines originate from bone marrow stromal cells. We have determined that both stromal cells and T lymphocytes have a contributory role in both normal and accelerated eosinophil production noted in asthma. In addition, inflammatory mediators released from the lung alter stromal cell support of eosinophilopoiesis and this altered response may contribute to the chronic inflammation associate with long term asthma.

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Introduction

Asthma is the most common reason for hospitalization of children in both military and civilian hospitals[1-4]. In patients with asthma, pulmonary exposure to allergen results in bronchial hyperresponsiveness and airway inflammation mediated by eosinophils. Eosinophils are inflammatory cells, have limited life spans, and must be continually renewed from hematopoietic tissue. Exposure to allergen has also been correlated with systemic changes in hematopoietic function[5-9]. Our laboratory has demonstrated that initial exposure to allergen was associated with expansion of eosinophil progenitor cells, bone marrow eosinophilia, and accumulation of large numbers of eosinophils in both circulation and lung[10]. These bone marrow changes were regulated by a multi-step process with increased bone marrow output of eosinophils regulated by T cells. The increased CFU-eo production by athymic mice also demonstrates the critical importance of studying other regulatory mechanisms in the bone marrow, such as stromal cells. Data generated during this grant indicate that stromal cells may contribute to increased eosinophil production found during asthma. Our preliminary data generated during project years 01 through the 03 also suggest that stromal cells may also provide downregulatory signals during steady state eosinophil production and early in the development of asthma. 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.

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. *Due to mandated decrease in the final award, the second research objective was removed in post-award budget negotiation.*

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. In addition, we proposed to evaluate the effects of long-term allergen exposure on bone marrow eosinophilopoiesis (Research Objective 3).

A main focus of our research endeavors has been to study the effect of stromal cells on CFU-eo and eosinophil expansion. In Research Objective 1, one method proposed to evaluate this important hematopoietic function of stromal cells was to utilize diffusion chamber technology. After successfully determining best procedures for construction and insertion of diffusion chambers into mice in project years 1 and 2, studies in project year 3 were focused on determining stromal cell effects on CFU-eo proliferation. In both unsensitized and sensitized nude mice we repeatedly demonstrated a lack of CFU-eo growth in culture. Variations on timing of implantation of diffusion chamber compared to time of sensitizing dose of ovalbumin/alum and on length of time chambers were left implanted in the peritoneum (up to 7 days) did not alter these results. In addition, we eliminated the possibility of these results being induced by the stress of trypsinization of the chamber by utilization of a non-enzymatic cell dissociation fluid. These results mimic *in vitro* results obtained in which stromal cells were co-cultured with bone marrow cells in CFU-eo conditions. (Data not shown.) In these *in vitro* and now *in vivo* studies

we have determined that stromal cells unexpectedly inhibited CFU-eo formation completely during steady state eosinophilopoiesis.

Studies performed during project year one demonstrated that stromal cells functionally inhibited CFU-eo colony formation. These studies focused upon the inflammatory cytokine IL-1. IL-1 stimulated stromal cells appeared to increase production of a CFU-eo suppressive factor. Antibody inhibition studies determined that this stromal cell induced suppressive factor was IL-4. In addition, direct exposure of CFU-eo cultures to IL-4 resulted in a dose dependent suppression of CFU-eo colony formation. It was determined that T cells were not required for observed suppression of CFU-eo colony formation. We have hypothesized that inflammatory mediators released from the lung during asthma sensitization affect bone marrow hematopoietic function. We have previously demonstrated that IL-1 a systemically released inflammatory cytokine during asthma increases stromal cell production of IL-5. [12] This increase in stromal cell production of IL-5 is capable of supporting increased eosinophilopoiesis *in vitro*[12]. We now demonstrate that stromal cell production of Il-4 suppresses late eosinophilopoiesis and this suppression is enhanced by IL-1.

During project year 3, these studies have been taken to an *in vivo* mouse model. 100 μg IL-4 was injected i.p. at 24 and 48 hours prior to establishment of CFU-eo cultures. Saline was delivered i.p. to control animals. CFU-eo cultures were established with 7.5 x 10^5 bone marrow cells/ml, and 10 ng/ml IL-5. Statistical significance was determined utilizing an unpaired t-test. Significant differences from control values were recorded at 24 (*$p<0.01$), and 48 hours (**$p<0.007$) after IL-4 exposure. (See figure 1.)

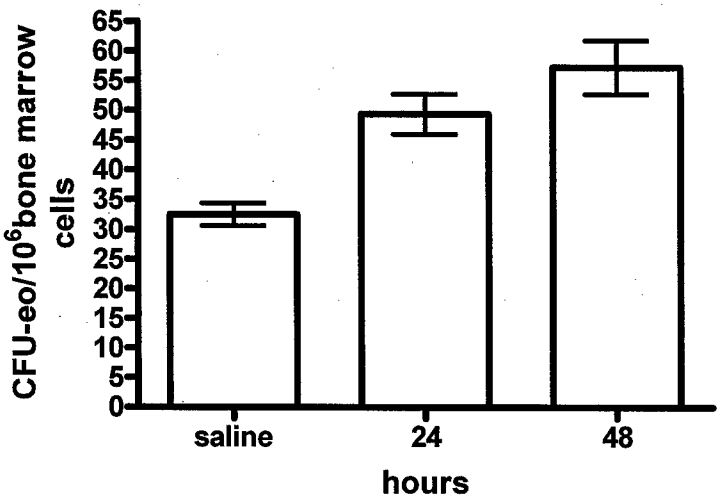

Figure 1

It appears from these experiments that IL-4 has a differential role during early and late eosinophilopoiesis; accelerating early eosinophilopoiesis while suppressing eosinophil differentiation to maturity. Figure 2 represents a new working model for the effect of IL-4 on eosinophilopoiesis. In this model IL-4 supports early eosinophil progenitor proliferation while suppressing late maturation of progenitors into eosinophils. Our laboratory has previously demonstrated this differential role in regulation of lymphopoiesis for IL-4.[12] During lymphocyte

maturation, IL-4 inhibited pre-B cell formation while accelerating B cell differentiation to maturity. These studies were selected for presentation at the American Academy of Allergy, Asthma and Immunology Annual Meeting, March 2005. A manuscript outlining these findings is currently being prepared for publication.

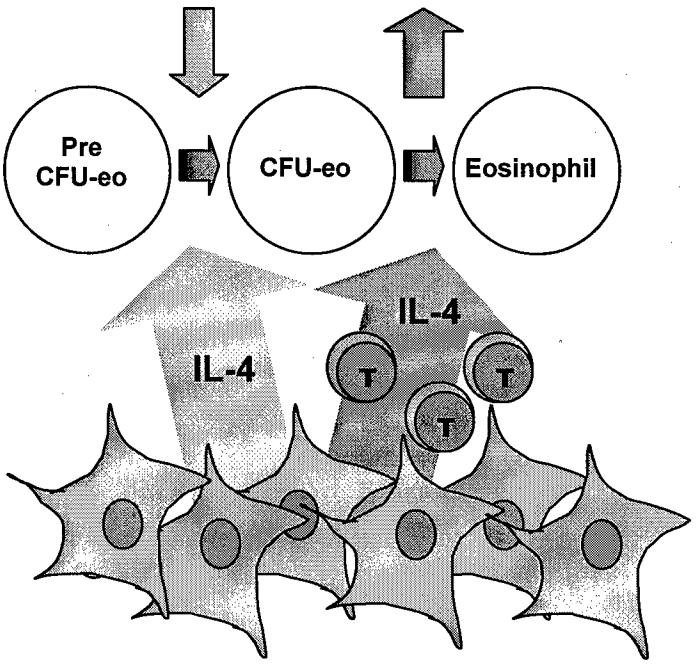

Figure 2

The early phase of a prototypic asthmatic response results from specific IgE activation by allergen and release of mast cell products with ensuing degranulation. Mast cells release a cascade of cytokines, which initiate the immediate phase of bronchial hyperreactivity, including leukotrienes.[13] Release of leukotrienes is directly responsible for eosinophil migration[14] and vascular permeability[15] in episodic pulmonary inflammation associated with asthma. The role of leukotrienes in observed alterations of eosinophilopoiesis has not been investigated. CysLT1 receptors are present on both hematopoietic progenitor cells and T lymphocytes in that tissue. These studies suggest that leukotrienes may be involved in both progenitor cell expansion and mature cell formation in the bone marrow. However, virtually nothing is known about the role of cysteinyl leukotrienes or their receptors in early hematopoietic production of eosinophils in the bone marrow. In these preliminary experiments the role of leukotrienes in proliferation and differentiation of eosinophil progenitor cells will be determined.

In these studies, balb/c bone marrow and T cell deficient bone marrow from athymic mice were exposed to suboptimal amounts of IL-5 in addition to cysteinyl leukotrienese LTD_4 and LTE_4. Use of T cell deficient bone marrow allowed us to determine whether the effect of leukotriene exposure on late eosinophil maturation was mediated indirectly through T cells or were directly affecting eosinophil colony formation. Preliminary data demonstrated that LTE_4 has an effect upon CFU-eo development in both balb/c and T cell deficient bone marrow indicating that LTE_4 is acting directly upon CFU-eo progenitor cells to increase eosinophil maturation. (Figure 3.)

LTD_4 had no demonstrable effect on late eosinophil maturation. These studies have been repeated to verify observed experimental effects.

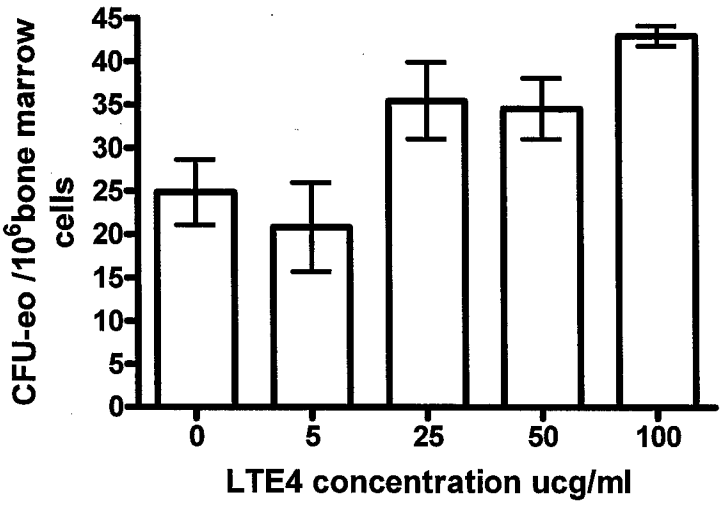

Figure 3

We also performed several experiments to determine whether bone marrow stromal cells were a potential source of locally produced cysteinyl leukotrienes. Our original experimental design was to collect conditioned media from bone marrow stromal cells. Bone marrow stromal cells are an important producer of hematopoietic mediators to stem cells and other cell lineages in the bone marrow. Hematopoietic cells and stromal cells are in direct contact in the bone marrow which obviates the need for high quantity production of mediators by stromal cells. Anticipating low level production of cysteinyl leukotrienes by bone marrow cells, conditioned media was collected, concentrated and utilized in a cysteinyl leukotriene specific ELISA. However, elevated background readings from control samples utilized in the ELISA have prevented accurate determination of cysteinyl leukotriene production by stromal cells. Experiments were performed to decrease this background reading including employing cysteinyl leukotriene extraction across a C18 reverse phase column, removal of all fetal calf serum from culture media, and utilizing several different culture medias including a dye free RPMI media and varying the amount of concentrated and unconcentrated conditioned media across the C18 column. These experiments failed to remove the elevated background reading on the ELISA.

In previous assays of IL-5 production by stromal cells[11], we determined intracellular quantities of IL-5 by applying GolgiStop to stromal cells and then lysing stromal cells with a lysate buffer and utilizing the resulting supernatant in the ELISA. However, we have also determined that the lysate buffer also interferes with the cysteinyl leukotriene ELISA efficiency. To circumvent this difficulty we have lysed stromal cells by sonication, centrifuged the resultant lysate to remove extracellular membrane debris and applied the supernatant to the ELISA plate. We have obtained reproducible data by this method which demonstrates that bone marrow stromal cells do produce cysteinyl leukotrienes, and that this production of cysteinyl leukotrienes is not inducible or suppressible by the inflammatory cytokine IL-1. (Figure 4.) To determine if this stromal cell production is alterable by inflammatory mediators we have also exposed bone marrow stromal cells to conditioned media derived from concavalan A stimulated splenic cells (a source of T cell secreted cytokines). In these studies no effect on cysteinyl leukotriene production was noted by

concavlan A stimulated splenic cell conditioned media. (data not shown) It appears that cysteinyl leukotriene production by bone marrow stromal cells may be constitutive in nature.

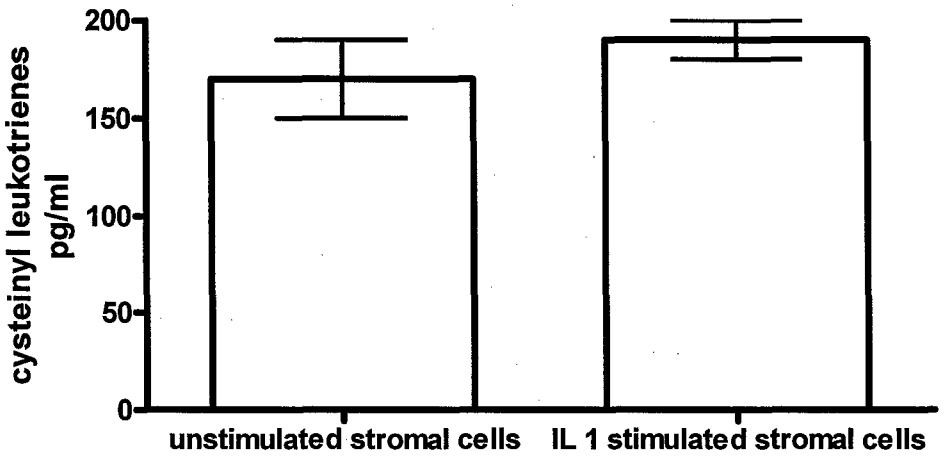

Figure 4

These studies demonstrating that bone marrow stromal cells are capable of producing cysteinyl leukotrienes are novel. To determine the source and role of cysteinyl leukotriene production in the bone marrow during onset of asthma, we are currently performing studies *in vivo*. We are currently utilizing the cysteinyl leukotriene receptor antagonist; montelukast to observe the effect of decreased cysteinyl leukotrienes on eosinophilopoiesis during the onset of asthma. These studies will be performed in both balb/c and athymic nude mice to also the primary mechanism of cysteinyl leukotriene production (bone marrow stromal cell or T cell derived) in our murine model of asthma. At the completion of these studies on the role and source of cysteinyl leukotrienes in the accelerated eosinophilopoiesis of asthma, a manuscript will be prepared for publication.

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. We have attempted to determine which cytokine signals are responsible for the accelerated eosinophilopoiesis noted during asthma. In project year one, we have determined that IL-5 is not responsible for increased CFU-eo production in the bone marrow during asthma sensitization. This work has now been published in the *Journal of Immunology*[10]. We have also validated through repetitive experimentation that GM-CSF, a cytokine known to support eosinophilopoiesis, is not responsible for the increased CFU-eo production noted in our model. These studies were selected for presentation at the American Academy of Allergy, Asthma and Immunology Annual Meeting, March 2005.

We hypothesized that accelerated CFU-eo production may result from cytokine stimulation of accelerated stem cell production in the bone marrow. Stem cell factor (SCF) is stimulatory of bone marrow stem cell proliferation. Therefore, we chose to investigate the effects of SCF on early eosinophil progenitor production. In this *in vivo* study, athymic nude mice were sensitized to ovalbumin. Anti-murine SCF antibody or its isotype control antibody was administered to

mice on days 9-12 of the sensitization period. These studies have now been replicated and confirmed. These *in vivo* data suggest that SCF may be responsible for increases in CFU-eo production noted during asthma sensitization. We have also performed in vitro studies investigating the effect of SCF on late eosinophilopoiesis. These experiments have been replicated and demonstrate that SCF also accelerates late eosinophil maturation in the bone marrow. These studies were selected for presentation at the American Academy of Allergy, Asthma and Immunology Annual Meeting, March 2005. A figure of our model of accelerated eosinophilopoiesis is noted in figure 5.

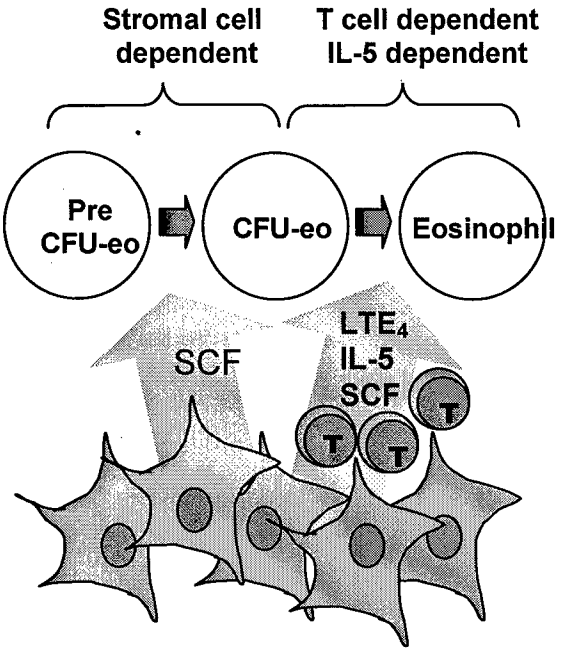

Figure 5

We have also proposed to determine the role of T cells in the accelerated eosinophilopoiesis noted during asthma (Research Objective 1). In project year one, we determined that T cells are critical to the development of the accelerated mature eosinophilopoiesis noted during asthma. These data were published in the Journal of Immunology[10]. We have undertaken studies in project year three to identify the mechanism in which T cells perform this critical function in supporting eosinophilopoiesis during asthma.

In our initial set of experiments, wild type (euthymic) Balb/c +/+ mice were sensitized to ovalbumin by intranasal exposure using a regimen established to induce asthma in this experimental model. Specifically, mice were first exposed to allergen by intraperitoneal (IP) administration of ovalbumin co-precipitated with aluminum potassium sulfate (alum) on day 0. Mice were exposed to allergen a second time on day 10 with a second IP exposure and simultaneous intranasal (IN) administration of ovalbumin. Spleens from these animals were collected and T cells isolated using a well-established streptavidin microbead technique. 10^7 enriched T cells were injected IP to naïve athymic Balb/c nu$^-$/nu$^-$ mice. At 96 and 120 hours, CFU-eo and absolute eosinophils were enumerated. Our results indicated that at 96 and 120 hours following adoptive cell transfer, CFU-eo were significantly reduced in the bone marrow of

Balb/c nu⁻/nu⁻ mice as compared to athymic mice which did not receive an adoptive transfer of sensitized T cells. (Data not shown.) This work has now been replicated during project year 3.

We previously performed preliminary experiments to determine the effect of the addition of ovalbumin sensitized T cells to athymic nude mice. In order to facilitate *in vitro* studies of sensitized T cells into athymic nude mice, a dose ranging study was performed. Balb/c +/+ mice were treated with allergen on days 0 (IP), and 10 (IP and IN) as before. Spleens were then removed from sensitized animals and T cells enriched. Graded numbers of enriched Balb/c T lymphocytes (from 2 x 10^6 through 4 X 10^6 T cells) were co-cultured with 20 X 10^6 athymic Balb/c nu⁻/nu⁻ bone marrow cells *in vitro*. At 24 hours, CFU-eo and absolute numbers of eosinophils in the bone marrow were enumerated. The *in vitro* data suggested that there were no statistically significant differences in CFU-eo or eosinophil numbers between groups and 2 X 10^6 sensitized T cells were adopted for all subsequent co-culture experiments. (Data not shown.)

Studies were also performed to determine the time course of CFU-eo suppression by adoptively transferred T cells. In subsequent experiments, spleens were removed from Balb/c +/+ mice and T cells enriched as before. 10^7 enriched T cells were injected IP to athymic Balb/c nu⁻/nu⁻ mice. Bone marrow was then harvested at 24, 48, and 96 hours and CFU-eo and eosinophils enumerated. The results demonstrated that CFU-eo numbers were significantly decreased in a time-dependent manner over 96 hours following adoptive T cell transfer with greatest decreases recorded at 96 hours. There appeared to be no statistically significant differences between groups with respect to eosinophil numbers. (Figure 6.)

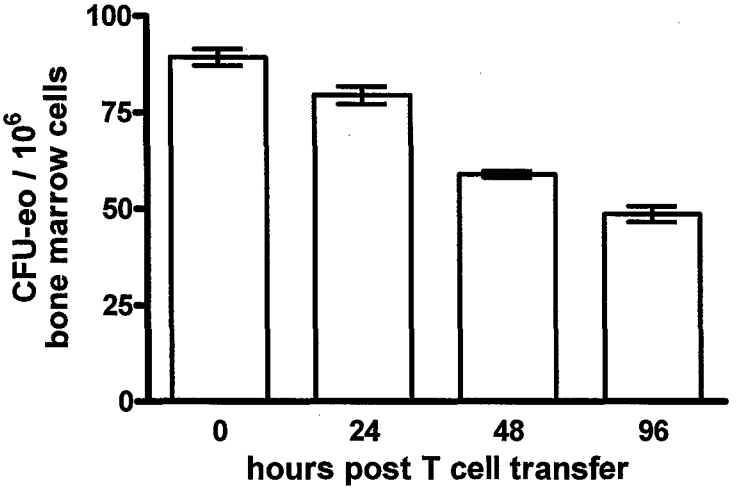

Figure 6

Studies were also performed to determine if sensitization of T cells to ovalbumin was specifically responsible for observed suppression of CFU-eo in co-culture experiments. In this experiment, spleens were removed from unsensitized balb/c +/+ mice and T cells enriched. Enriched T cells were co-cultured with bone marrow cells from Balb/c nu⁻/nu⁻ mice as previously described. At 24 hours, CFU-eo and eosinophils were enumerated. The results suggest that at 24 hours, the addition of unsensitized T cells to athymic mice bone marrow significantly reduced CFU-eo. Eosinophil numbers were found to be increased at this time.

These studies as a whole demonstrate the importance of functioning T cells on eosinophilopoiesis in the bone marrow. T cells appear to suppress CFU-eo formation whether they are sensitized to allergen or not. This effect appears to be early during the development of asthma and is consistent with previous findings that athymic nude mice have an increased number of CFU-eo in the bone marrow at baseline compared to balb/c mice. In addition, balb/c mice develop an initial depression in CFU-eo numbers on day 13, before the noted acceleration in CFU-eo numbers on day 16.[10] In addition, this *in vitro* work also supports previously published data that T cells are required for the accelerated mature eosinophil numbers seen during asthma.[10] Work is ongoing to complete these experiments and develop a manuscript for publication.

In research object 3, we proposed to determine the durability of the bone marrow response in asthma by establishing a repetitive challenge model more close mimicking chronic repetitive exposure to allergen in childhood asthma. In year two we initiated repetitive challenge studies as proposed in balb/c mice. Exposure period was weekly over 2 months. In these studies, no evidence of chronic altered production of eosinophil progenitors or mature eosinophils was demonstrated. However, a publication by Shinagawa and Kojima[18] suggested that murine strain difference may be responsible for these findings. A/J mice were demonstrated in this study to have asthma features consistent with chronic asthma in humans. These findings included airway wall thickening, and persistent airway hyperreactivity[18]. The contribution of bone marrow eosinophil production to the development of these chronic asthma changes was not determined. We have recently initiated studies utilizing Shinagawa's published methods in A/J mice, which are focused on answering whether bone marrow eosinophilopoiesis is altered during chronic asthma. Balb/c and A/J mice were exposed to IN ova or saline 3 times per week for 12 weeks. Initial morphometric studies have shown that ova exposed A/J mice developed eosinophilia, membrane thickening and goblet cell hyperplasia consistent with chronic asthma. (Data not shown). Bone marrow studies revealed that eosinophilia existed in ova exposed A/J mice, but not saline exposed mice ($p<0.04$). (Figure 7). However, no effect on CFU-eo numbers was seen in A/J mice (Data not shown).

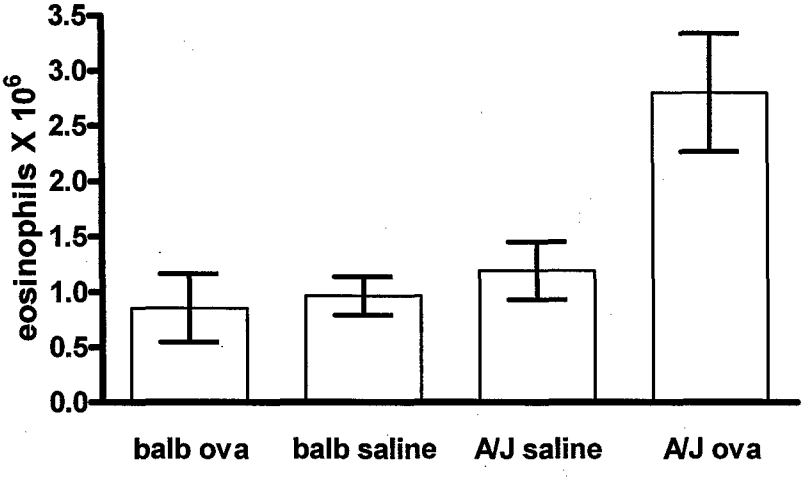

Figure 7

Over the past 6 months, we experienced difficulty repeating experiments that had been consistent over the first three years of this grant. After conducting an exhaustive battery of experiments designed to systematically check our results, we were notified by our attending veterinarian, Dr. Linton (Director of the office of Laboratory Animal Resources), that our mouse colony had been unexpectedly infected with parvovirus. Parvovirus is known to infect and later the function of both hematopoietic bone marrow cells and the stromal cells that have been the focus of this study. This infection has delayed our completion of experiments designed to replicate the proposed chronic asthma model. In order to compete these studies, we found it necessary to request a 'no-cost extension' for our project to institute infection control measures for our mouse colony, repeat essential experiments, and validate our results prior to publication. The need for this no-cost extension was supported by Dr. Linton and granted for this reason.

Key Research Accomplishments: Year 1

- 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.

Key Research Accomplishments: Year 2

- Determined best method of diffusion chamber construction to enhance CFU-eo viability.
- Determined optimal cell number to inject into diffusion chambers to enhance CFU-eo recovery and viability.
- Determined utilizing diffusion chamber technology that stromal cells *in vivo* suppress CFU-eo colony formation.
- Confirmed suppressive effect of stromal cells on CFU-eo formation is in part due to IL-4.
- Confirmed suppressive effect of IL-4 on CFU-eo does not require T cells.
- Determined that stromal cells are capable of producing IL-4 protein.
- Determined that stromal cells are capable of increasing IL-4 protein production after stimulation with inflammatory cytokines present in asthma, such as IL-1.
- Determined that substance P directly inhibits CFU-eo formation *in vitro*.

- Determined that neurokinin A directly inhibits CFU-eo formation *in vitro*.
- Determined that administration of substance P receptor antagonist in vivo, results in increased CFU-eo numbers, which reflects the role of substance P as a potential inhibitor of CFU-eo colony formation.
- Determined that GM-CSF does not contribute to accelerated CFU-eo production during asthma sensitization.
- Determined that SCF is partially responsible for accelerated CFU-eo production noted *in vivo* during asthma sensitization.
- Determined that SCF is synergistic with IL-5 in accelerating mature eosinophil production.
- Determined that reconstitution of athymic nude mice with wild type T cells results in suppressed CFU-eo numbers *in vivo*.
- Determined that reconstitution of athymic nude mouse bone marrow with wild type T cells suppresses CFU-eo numbers *in vitro*.
- Determined that balb/c mice do not have functional alteration in bone marrow eosinophilopoiesis in a long-term allergen challenge model.

Key Research Accomplishments: Year 3

- Verified that stromal cells suppress CFU-eo colony formation *in vivo*. Variation in procedures followed during implantation of diffusion chambers and post removal of chamber do not affect these results.
- IL-4 stimulates early eosinophilopoiesis by increasing CFU-eo numbers *in vivo*.
- Determined that bone marrow stromal cells produce cysteinyl leukotrienes.
- Stimulation of bone marrow stromal cells by inflammatory cytokine IL-1 or conditioned medium derived from splenic cell stimulation by concavalin A does not alter bone marrow production of cysteinyl leukotrienes.
- Determined that CFU-eo colony formation is increased in the presence of cysteinyl leukotrienes.
- Validated that GM-CSF is not the cytokine responsible for increased CFU-eo formation during allergen sensitization.
- Validated that SCF increases CFU-eo formation early in the response to allergen during the onset of asthma.
- Determined that T cells suppress CFU-eo formation after adoptive transfer of balb/c T cells into athymic nude mice.
- Determined that T cell suppression of CFU-eo occurs within the first 4 days after adoptive transfer regardless of whether T cells are sensitized to allergen or not.
- Determined that athymic nude mice are capable of developing increased mature eosinophil numbers after adoptive T cell transfer.
- Determined best procedure for *in vitro* T cell co-culture experiments.
- Validated that T cells will suppress CFU-eo colony formation during *in vitro* co-culture experiments.
- Determined that chronically allergen exposed A/J mice (as opposed to balb/c mice) develop pulmonary eosinophilia, thickened basement membranes and pulmonary goblet cell hyperplasia consistent with chronic asthma as seen in humans.

- Determined that bone marrow eosinophilia contributes to the development of chronic asthma in genetically predisposed mice (A/J strain), as opposed to balb/c mice which do not develop chronic asthma and do not have bone marrow eosinophilia.

Reportable Outcomes

Publications resulting from this award.

Hogan MB, Weissman DN, Hubbs AF, Landreth KS. Regulation of eosinophilopoiesis in a murine model of asthma. J Immunol 2003 171:2644-51.

Landreth, K. S. and S.V.M. Dodson. 2004. Development of the rodent immune response. Developmental Immunotoxicology. Ed. S. Holladay. New York: CRC Press. p.3-20

Barnett, J. L.F. Gibson,, K.S. Landreth. 2004. Deveopmental immunotoxicity of chlordane. Developmental Immunotoxicology. Ed. S. Holladay. New York: CRC Press. p.153-168.

Holsapple, M.P., L.J. West, and K.S. Landreth. 2004. Species comparison of anatomical and functional immune system development. IN: Role of Juvenile Studies in Assessment of Pediatric Safety. Published by International Life Sciences Institute.

Abstracts presented.

Hogan MB, Weissman DN, Zhuang ZZ, Landreth KS Bone marrow CFU-eosinophil (CFU-eo) production in a murine asthma model. American Academy of Allergy Asthma and Immunology Annual Meeting New York, NY, March 2002

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.

KS Landreth, D Piktel, LF Gibson, DN Weissman, J Welch, MB Hogan. Regulation of eosinophilopoiesis by stromal cells is modulated by inflammatory cytokines. International Society of Experimental Hematology Annual Meeting. Paris, France, July 5-8 2003

Hogan MB, Landreth KS. Bone Marrow Function in Development of Childhood Asthma. Peer Reviewed Medical Research Program (PRMRP) 2003 Programmatic Review Meeting, August, 2003.

Hogan MB, Piktel D, Simpson R, Gibson LF, Welch JE, Landreth KS. Suppression of CFU-eo Formation by Bone Marrow Stromal Cells and IL-4. AAAAI National Meeting, San Francisco, CA, March 2004.

Landreth KS, Hogan MB, Gibson LF, Weissman DN, Piktel D. Altered bone marrow function associated with development of asthma. Department of Defense Peer Reviewed Medical Research Program Investigators Meeting. Puerto Rico, USVI .

Hogan MB, Piktel D, Landreth KS. Cytokine regulation of early eosinophilopoiesis. Presented, AAAAI 2005 National meeting, San Antonio, TX .

Invited Presentations.

The role of the bone marrow in the onset of asthma. Department of Pediatrics Grand Rounds. WVU, May 21, 2003.

The role of the bone marrow in the onset of asthma. Pediatric Allergy/Immunology Basic Science Seminar. Northwestern University, Chicago, IL. August 21, 2003

The role of the bone marrow in the onset of asthma. Department of Pediatrics Grand Rounds. Children's Memorial Hospital, Chicago, IL August 22, 2003.

The role of the bone marrow in the onset of asthma. Allergy/Immunology Seminar. Rush Presbyterian Hospital, University of Chicago, Chicago, IL August 22, 2003.

The role of the bone marrow in the onset of asthma. West Virginia Chapter, American Lung Association Annual Meeting. Morgantown, WV, October 2, 2003

Pending Future Funding.

Department of Defense. Peer Reviewed Medical Research Program. Role of airway irritants in development of asthma in children. **Co-Principle Investigator** (Hogan, Mary Beth), (Principle Investigator, Kenneth S. Landreth). Direct Costs. $1,000,000. Submitted.

Research Personnel:
Mary Beth Hogan, MD: Principal Investigator
Kenneth S. Landreth, PhD: Co-Principal Investigator
Debra A. Piktel, BS: Research Assistant III

Conclusions:

Asthma is a complex disease in which multiple mediators and cell types contribute to the pathogenesis of airway compromise. It has been recently appreciated that asthma also has systemic effects upon bone marrow regulation of hematopoiesis, in particular eosinophilopoiesis. The bone marrow environment consists of hematopoietic cells, stromal cells, mature end cells and T lymphocytes. Inflammatory mediators generated and released from pulmonary tissue, and potentially produced locally in the bone marrow during the development of asthma have the potential to exert regulatory control on bone marrow cells. In our own studies, numbers of eosinophil progenitor cells (CFU-eo) were found to be initially depleted in the bone marrow, followed by a transient rebound to supra-normal levels. This rise in numbers of CFU-eo following allergen stimulation appeared to be regulated by stromal cells.

In the experiments presented here, we demonstrate that stromal cells from untreated mice actually secrete cytokines that inhibit eosinophil production. This inhibitory function is accelerated by exposure to IL-1, an inflammatory cytokine released systemically following allergen exposure in *vivo*. These data suggest that the decline of bone marrow CFU-eo that

follows allergen stimulation may be due to increased suppression of eosinophilopoiesis rather than loss due to increased demand for mature eosinophils as previously reported from our laboratory. Subsequent rebound of CFU-eo and eosinophils, which has been observed following allergen exposure, is likely due to normal feedback mechanisms that regulate eosinophil homeostasis.

Our interest in childhood asthma has led us to investigate events in eosinophilopoiesis during the initial phase of the development of asthma and in events contributing to the development of chronic 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. Experiments to be completed during the next year of this grant (no-cost extension) will be focused on replicating experiments performed during the first 3 years of the grant, but delayed due to parvovirus infection of our mouse colony. In particular, we will focus attention on the effect of cysteinyl leukotrienes on the acceleration of eosinophilopoiesis during asthma, replication of studies outlining the down regulatory signals sent by T cells to decrease CFU-eo number, and complete work on our chronic asthma model. We will also prepare these works for submission for publication once completed.

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 the role of T cells in eosinophilopoiesis may be complex, with T cells providing downregulatory signals early in eosinophil progenitor formation, but providing signals accelerating mature eosinophil production. In addition, ongoing studies suggest that stromal cells may provide key downregulatory signals, such as IL-4, which control eosinophil production under normal and inflammatory conditions. Stromal cells and T cells may also contribute to the accelerated eosinophilopoiesis found during asthma. Studies demonstrating that IL-5, derived from both stromal cells and T cells are responsible for accelerated mature eosinophilopoiesis. In now also is apparent that both of the cells can contribute to the increase number of immature eosinophils found during the onset of asthma in the form of SCF and cysteinyl leukotrienes. Preliminary data suggests that bone marrow eosinophilia is also a contributory factor to the development of chronic asthma. Elucidation of the normal downregulatory mechanisms of eosinophil production may lead to strategies for childhood asthma that ultimately inhibit disease development or progression.

References:

1. P.A. Simon et. al. J Asthma **40**,535 (2003).

2. E. Chrischilles E et. al. J Allergy Clin Immunol **113**,66 (2004).

3. A. Roth et. al. Ann Allergy. **71**, 533 (1993).

4. Y. Chen et. al. Pediatr Pulmonol **36**,22 (2003).

5. M. D. Inman et. al. Am J Respir Cell Mol Biol **21**, 473 (1999).

6. E. M. Minshall et. al. Am J Respir Crit Care Med **158**, 951 (1998).

7. L. J. Wood et. al. Am J Respir Crit Care Med **166**, 883 (2002).

8. K. Zeibecoglou et. al. J Allergy Clin Immunol **103**, 99 (1999).

9. Y. Ohkawara et. al. Am J Respir Cell Mol Biol **16**, 510 (1997).

10. M.B. Hogan et. al. J Immunol **171**, 2644 (2003).

11. M. B. Hogan et. al. J Allergy Clin Immunol **106**, 329 (2000). (Editors Choice Selection).

12. L. G. Billips et. al. Blood **75**, 611 (1990).

13. D. D. Metcalfe et. al. Physiological Reviews **77**, 1033 (1997).

14. E. Pizzichini et. al. Eur Respr J **14**, 14 (1999).

15. M. A. Kaliner J Respir Dis **135**, S6-9 (1987).

16. D. J. Figueroa et. al. Am J Respir Crit Care Med **163**, 226 (2001).

17 D. Ihaku et. al. J Allergy Clin Immunol **104**, 1147 (1999).

18. K. Shinagawa et. al. Am J Respir Crit Care Med. **168**, 959 (2003).