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# THE ROLE OF BONE MARROW PROGENITOR CELLS IN ALLERGEN-INDUCED AIRWAY HYPERRESPONSIVENESS AND AIRWAY INFLAMMATION

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

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# THE ROLE OF THE BONE MARROW IN ASTHMA

DOCTOR OF PHILOSOPHY (1998) McMaster University

(Physiology and Pharmacology) Hamilton, Ontario

TITLE: The role of bone marrow progenitor cells in allergen-induced airway hyperresponsiveness and airway inflammation.

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ABSTRACT

Asthma is a disease characterised by reversible bronchoconstriction, airway hyperresponsiveness (AHR) and airway inflammation. Inhalation of allergen by sensitized subjects is an important cause of asthma, and results in an increase in the numbers of inflammatory cells, more specifically the eosinophil, in the airways.

An important aspect of allergic inflammatory responses is the induction of increases in inflammatory cell progenitors, which contribute to disease through the continued production of inflammatory cells. Higher numbers of circulating eosinophil/basophil progenitors are demonstrable in the blood of atopic subjects compared with normals, and in asthmatic subjects following allergen inhalation. In addition, there are increases in bone marrow progenitor cells following allergen inhalation in a canine model of allergen-induced AHR. Although these studies suggest that inflammatory cell progenitors in the blood arise from the bone marrow, this had not been shown in humans. The aim of this thesis was to examine the role of bone marrow inflammatory cell progenitors in allergen-induced AHR and airway inflammation, with respect to increased production and trafficking of these cells. In addition, the effect of therapeutic intervention with inhaled glucocorticosteroids was investigated.

We demonstrated, for the first time, that increases in bone marrow-derived eosinophil/basophil progenitors occurred 24 hours following allergen inhalation in mild

asthmatic subjects through up-regulation of the IL-5 receptor on progenitors, and that the degree of responsiveness of these cells was associated with the magnitude of the eosinophilic response in the airways. In addition, we demonstrated an increase in the trafficking of inflammatory cell progenitors, from the bone marrow through the circulation, into the airways, suggesting that the increased production of these cells can contribute to the ongoing inflammatory response. Finally, we showed that inhalation of the glucocorticosteroid, budesonide, was able to specifically suppress the baseline numbers of bone marrow inflammatory cell progenitors, but was unable to inhibit the allergen-induced increases in these cells.

In summary, the findings in this thesis suggest that inflammatory cell progenitors in the bone marrow are affected by inhalation of allergen, with increased production and trafficking to the airways. In addition, while budesonide may have an inhibitory effect on the steady state turnover of eosinophil/basophil progenitors, it has no effect on the allergen-driven events occurring in the bone marrow, suggesting that the inhibitory effects of budesonide inhalation on inflammation and airway responses may not be via inhibition of allergen-induced increases in eosinophil production.

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I would also like to thank the many people in the laboratory that helped out with my studies. Firstly to Dr.O'Byrne and Dr.Killian for supervising the clinical studies and secondly to Rick Watson whose expert clinical skills allowed the studies to run very smoothly and whose smooth talking convinced subjects to have their bone marrow taken! Many thanks also to Dr Gauvreau, Tracy and Karen for their help with sputum samples and bone marrow assays, and to Dr.Foley for taking our bone marrow samples and whose expertise persuaded our subjects to come back a second time! Thank you also to Dr Sehmi for sharing your

treasured FACS data - it was very much appreciated. My thanks also to Dr.Inman, Russ and Jennifer for all their hard work with the dog study - I could not have done it without you, and thanks again Mark for explaining those statistics for the umpteenth time.

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Finally, I would like to dedicate this thesis to the memory of a great man - Mr Peter Chopping, who inspired in me a love of science and research - Thanks "Chops" for everything.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ACh | Acetyl choline |
| AHR | Airway hyperresponsiveness |
| ANOVA | Analysis of variance |
| ATRA | All trans Retinoic acid |
| APAAP | Alkaline phosphatase anti-alkaline phosphatase |
| AP-1 | Activator protein-1 |
| BAL | Bronchoalveolar lavage |
| BALF | Bronchoalveolar lavage fluid |
| bFGF | Basic fibroblast growth factor |
| BFU-E | Burst forming unit-erythrocytes |
| BM | Bone marrow |
| BrdU | Bromo-deoxyuridine |
| BSA | Bovine serum albumin |
| CFU | Colony forming unit |
| CFU-GEMM | CFU-granulocyte/erythroid/macrophage/megakaryocyte |
| DPBS | Dulbeccos phosphate buffered saline |
| DR | Dual responder |
| Eo/B-CFU | Eosinophil/Basophil-CFU |

| | |
|---|---|
| ECP | Eosinophil cationic protein |
| EDN | Eosinophil-derived neurotoxin |
| EPO | Eosinophil peroxidase |
| FCS | Fetal calf serum |
| $FEV_1$ | Forced expiratory volume in 1 second |
| FITC | Fluorescein-5-isothiocyanate |
| FSC | Linear forward light scatter |
| G-CSF | Granulocyte-colony stimulating factor |
| GM-CSF | Granulocyte/macrophage-colony stimulating factor |
| GM-CFU | Granulocyte/macrophage-CFU |
| GR | Glucocorticoid receptor |
| HSC | Hemopoietic stem cell |
| IHC | Immunohistochemistry |
| IER | Isolated early responder |
| Ig-E | Immunoglobulin-E |
| IL | Interleukin |
| IL-R | Interleukin receptor |
| LAR | Late asthmatic responses |
| LIF | Leukemia inhibitory factor |
| $LTC_4$ | Leukotriene $C_4$ |

## LIST OF ABBREVIATIONS (continued)

| | |
|---|---|
| MCP | Macrophage chemotactic peptide |
| M-CSF | Macrophage-colony stimulating factor |
| MBP | Major basic protein |
| Meg-CFU | Megakaryocyte-CFU |
| mRNA | messenger RNA |
| NAMC | Non adherent mononuclear cells |
| NF-κB | Nuclear factor-kappa B |
| PAF | Platelet activating factor |
| PBS | Phosphate buffered saline |
| $PC_{20}$ | Provocative concentration required to cause a 20% fall in $FEV_1$ |
| PE | Phycoerythrin |
| perCp | Peridinin chlorophyll protein |
| PFA | Paraformaldehyde |
| PLP | Periodate-lysine PFA |
| SCF | Stem cell factor |
| SEM | Standard error of the mean |
| SSC | Linear side-angle light scatter |
| Th2 | T-helper 2 |
| WBC | White blood cell count |

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# PREFACE

The following studies have been published or accepted for publication and form the basis of chapters two, three, four and five of this thesis:

**CHANGES IN BONE MARROW INFLAMMATORY CELL PROGENITORS AFTER INHALED ALLERGEN IN ASTHMATIC SUBJECTS.** Lorna J. Wood, Mark D. Inman, Richard M. Watson, Ronan Foley, Judah A. Denburg & Paul M. O'Byrne.
Published: American Journal of Respiratory Critical Care Medicine 1998; 157:99-105

**ALLERGEN-INDUCED INCREASES IN IL-5 RECEPTOR α-SUBUNIT EXPRESSION ON BONE MARROW-DERIVED CD34⁺ CELLS FROM ASTHMATIC SUBJECTS.** Roma Sehmi, Lorna J. Wood, Richard M. Watson, Ronan Foley, Qutayba A. Hamid, Paul M. O'Byrne & Judah A. Denburg.
Published: Journal of Clinical Investigation 1997; 100:2466-2475.

**ALLERGEN CHALLENGE INCREASES CELL TRAFFIC BETWEEN BONE MARROW AND LUNG.** Lorna J. Wood, Mark D. Inman, Judah A. Denburg & Paul M. O'Byrne.
Published: American Journal of Respiratory Cell and Molecular Biology 1998; 18:759-767.

**AN INHALED CORTICOSTEROID, BUDESONIDE, REDUCES BASELINE BUT NOT ALLERGEN-INDUCED INCREASES IN BONE MARROW INFLAMMATORY CELL PROGENITORS IN ASTHMATIC SUBJECTS.**
Lorna J. Wood, Roma Sehmi, Gail M. Gauvreau, Richard M. Watson, Ronan Foley, Judah A. Denburg & Paul M. O'Byrne.
American Journal of Respiratory Critical Care Medicine: In press

The clinical studies are the first studies completed by the Asthma Research Group in Hamilton, Canada, that involve the collection of bone marrow samples from human volunteers. These studies involved the input of many people in order to run smoothly and efficiently. Dr Paul O'Byrne provided the financial support, laboratory space, equipment and technical support, and in conjunction with Dr Kieran Killian supervised the clinical procedures performed on research subjects. Rick Watson carried out the clinical procedures, including the allergen and methacholine challenges, with the help of Dr Gail Gauvreau. Dr Gauvreau also supervised the collection and analysis of sputum samples with the technical help of Tracy Rerecich. Dr Ronan Foley performed the bone marrow aspiration procedures. Dr Judah Denburg and Dr Mark Inman offered considerable expertise.

The canine study also required the input of a number of people. Jennifer Wattie provided technical help with pre- and post-operative care of dogs and in the measurement of airway functions. Russ Ellis and Dr Mark Inman performed the bone marrow aspirations and bronchoalveolar lavages and offered invaluable advice on the study design.

I am first author on three of the papers which form the basis of this thesis. I was involved with the planning and design of all of the experiments and submitted the proposals that outlined the aims, methodology and sample analysis. In the human studies I was involved with the clinical procedures, and performed methacholine inhalation challenges and sputum induction, and I assisted with the bone marrow aspirations. I was responsible for subsequent analysis of the blood and bone marrow samples which included methyl cellulose tissue culture and cytochemical staining of cells. In the canine study, I assisted in all of the procedures

performed on the dogs, including general care, anaesthetization, airway measurements, and blood, bone marrow and BAL sampling. I performed pilot studies with BrdU infusion to establish the dose of BrdU, time course of sample collection, and to finalize the study design. I was responsible for all subsequent sample processing, and pilot studies were required to modify the immunohistochemical staining technique for BrdU positive cells. I collated the data and performed the statistical analyses for each study. The figures, tables and photos included in each paper are my own work, and I prepared the manuscripts for each paper.

I am also second author on one of the papers comprising the thesis. In this study I was responsible for the planning and design, and for the proposal outlining the aims and methodology. I was also involved in the clinical procedures as described above and was responsible for the preparation of bone marrow samples for the subsequent flow cytometric analysis. In collaboration with Dr Roma Sehmi, I was involved in the preparation of figures and statistical analyses, and provided input into the preparation of the manuscript for this study.

# CHAPTER 1

# INTRODUCTION

## ASTHMA

Asthma is a complex disease that is characterized by inflammation, reversible airway obstruction and increased airway hyperresponsiveness (AHR) to a number of different stimuli (Expert Panel Report, 1991). Because of its complexity, the formulation of a universally accepted definition for asthma has been difficult. However, the currently accepted definition recognizes asthma to be a chronic inflammatory disorder of the airways in which many cells play a role, in particular mast cells, eosinophils and T lymphocytes (Lemanske & Busse,1997). Asthmatic individuals suffer from recurrent episodes of wheezing, breathlessness, chest tightness and cough. These symptoms are associated with variable airway obstruction that can be reversed, either spontaneously or by treatment with inhaled bronchodilators, and are also associated with increases in AHR to stimuli such as histamine and methacholine.

Allergic (atopic) asthma is the most common form of the disease and results from the development of an Immunoglobulin-E (IgE)-mediated response to common aeroallergens. Repeated exposure to allergens results in the development of IgE antibodies and sensitization of susceptible individuals. Subsequent inhalation of the specific allergen leads to an allergic inflammatory response in the airways and the symptoms described above.

ALLERGEN INHALATION MODEL

In the early 1950's Herxheimer demonstrated that there were two distinct components in the response to inhaled allergen, which he termed the immediate and late reactions (Herxheimer, 1952). It is now recognized that allergen inhalation by sensitized subjects results in bronchoconstriction that develops within 10 minutes of the challenge, reaches a maximum by 30 minutes and usually resolves between 1 and 3 hours (Bentley *et al,* 1997). This is known as the immediate or early asthmatic reaction. In approximately 50% of adults (Booij-Noord *et al,* 1972) and 70% of children (Warner, 1976), a second late asthmatic reaction occurs, starting at 3-4 hours post challenge, is maximal at 6-12 hours and recovers by 24 hours.

Cockcroft *et al* (1977) demonstrated an increase in airway responsiveness to inhaled histamine and methacholine following inhaled allergen in subjects that develop late asthmatic responses. This increase in responsiveness occurred at 24 hours after the inhalation challenge and persisted for several days. In addition, he showed that subjects that developed only an isolated early response following inhaled allergen did not develop AHR.

Early and late asthmatic responses can be elicited by experimental challenge in the laboratory (O'Byrne *et al,* 1987). The most widely used method involves inhalation of doubling concentrations of allergen until the forced expiratory volume in 1 second ($FEV_1$) has fallen by 20% of its baseline value. The $FEV_1$ is then monitored every 10 minutes until 30 minutes, then every 15 minutes until 90 minutes, then again at 120 minutes and every hour for 8 to 12 hours.

Both early (Hill, 1981) and late (Kirby *et al,* 1986) asthmatic responses may be mediated by IgE. The early response is associated with the activation of pulmonary mast cells via cross-linking of high affinity IgE receptors by processed allergen. This leads to degranulation and the subsequent release of inflammatory mediators such as preformed histamine and tryptase, newly synthesised leukotrienes and thromboxanes, all of which have important bronchospasmogenic effects in addition to their stimulatory role in mucus secretion, vasodilation, microvascular leakage and chemotaxis (reviewed by Weersink *et al,* 1994). Release of chemotactic mediators by these and other cells may also be involved in the development of the late response. In addition, release of cytokines such as interleukin (IL)-3, IL-4, IL-5 and granulocyte-macrophage colony stimulating factor (GM-CSF) occurs during the early response, which have been implicated in the pathogenesis of the late phase reaction. IL-4 is central to the regulation of IgE synthesis (Romagnani *et al,* 1989) and IL-3, IL-5 and GM-CSF play a role in the recruitment, activation and prolonged survival of eosinophils and basophils (Rothenberg *et al,* 1988; Owen *et al,* 1987; Lopez *et al,* 1988).

It is now accepted that the development of the late response is not due solely to the effect of mast cell mediator release on airway smooth muscle. This is because, in contrast to the early asthmatic response, the late response is more prolonged and is not readily reversed by inhaled bronchodilators. Instead there is mounting evidence that the late asthmatic reaction is caused by an inflammatory response in the airway, with associated mucosal edema and secretions. In particular, it is the cellular component of this response that is thought to cause both the late phase bronchoconstriction and increases in airway responsiveness.

Several studies have demonstrated the presence of inflammatory cells, more specifically eosinophils, in both the circulation and the airways during the late response. Increased numbers of eosinophils have been detected in blood (Gibson *et al,* 1991), induced sputum (Pin *et al,* 1992), bronchoalveolar lavage fluid (BAL) (de Monchy *et al,* 1985; Rossi *et al,* 1991) and tissue biopsy specimens (Aalbers *et al,* 1993; Beasley *et al,* 1989) in association with the late response. The eosinophils are also in an activated state in asthma, as indicated by elevated levels of eosinophil cationic protein (ECP) in BAL fluid (Bousquet *et al,* 1991), and enhanced immunostaining of sputum eosinophils with the EG2 monoclonal antibody which recognises the cleaved and activated form of ECP (Gauvreau *et al,* 1996).

Increases in airway responsiveness have been shown to occur as early as 3 hours after allergen challenge (Durham *et al,* 1988) suggesting that the inflammatory response may be occurring well before the late response. In support of this, activated eosinophils have been demonstrated in BAL and submucosal biopsies at 3-4 hours following allergen challenge (Rossi *et al,* 1991; Aalbers *et al,* 1993). A hypothesis for the development of the late asthmatic response has been put forward by Bentley, in which T helper type-2 (Th2) $CD4^+$ T lymphocytes can respond directly to allergens encountered at mucosal surfaces (Bentley *et al,* 1997). Upon activation they secrete their characteristic array of cytokines (IL-3, IL-4, IL-5 and GM-CSF) which promote both Ig-E production, and the local recruitment and activation of eosinophils. However, this effect may not be solely due to T lymphocyte action since mast cells have also been shown to release a similar array of cytokines (Plaut *et al,* 1989). In addition, it is thought that $CD8^+$ T lymphocytes may afford protection against the

development of the late response since this population of cells was found to be increased in the BAL of asthmatic subjects who developed isolated early responses but not in those that developed more severe late asthmatic responses (Gonzalez *et al,* 1987).

EOSINOPHILS

Eosinophils are specialised bone marrow-derived leukocytes approximately 8μm in diameter and are characterized by membrane bound specific granules (reviewed by Wardlaw, 1994). These granules contain a core comprised of major basic protein (MBP) which is surrounded by an electron dense matrix containing three other basic proteins, ECP, eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN). Eosinophils differentiate and mature in the bone marrow and are released into the peripheral circulation before migrating into the tissues. As previously discussed there are increased numbers of eosinophils in the circulation and airways following allergen inhalation. This selective recruitment of eosinophils from the bone marrow into local tissue sites involves a multistep process that includes eosinophil differentiation, endothelial adhesion, chemotaxis and survival (Leung, 1997). IL-5 plays a critical role in eosinophil differentiation (see below), activation and survival (Weller, 1992) while C-C chemokines such as eotaxin, RANTES, monocyte chemotactic peptide-2 (MCP-2), MCP-3 and MCP-4 play a role in recruitment of eosinophils from the circulation into the airways (Stellato *et al,* 1997). Eotaxin is a specific chemoattractant for eosinophils (Garcia-Zepeda *et al,* 1996) while other C-C chemokines are also chemotactic for monocytes and lymphocytes.

Eosinophils may contribute to airway narrowing and increases in airway responsiveness by the release of newly formed mediators and granule-derived toxic basic proteins. The eosinophil produces large amounts of leukotriene $C_4$ ($LTC_4$) and platelet activating factor (PAF) ( Shaw *et al,* 1985; Jouvin-Marche *et al,* 1984). PAF can induce both acute and persistent increases in non-specific airways responsiveness (Page, 1992), and can activate eosinophils resulting in the release of basic proteins. MBP has been shown to damage the upper respiratory epithelium in an *in vitro* system, and it can cause AHR and smooth muscle contraction in primates (Gleich *et al,* 1979; Gundel *et al,* 1991). The eosinophil therefore plays an important role in the development of the late asthmatic response and associated airway inflammation in response to inhaled allergen.

Inhaled glucocorticosteroids are known to inhibit both allergen-induced late responses and AHR (Booij-Noord *et al,* 1971; Cockcroft *et al,* 1987; Boner *et al,* 1995), and to attenuate allergen-induced increases in blood eosinophils and sputum total and activated eosinophils (Gauvreau *et al,* 1996). In addition, glucocorticosteroids can inhibit eosinophil chemotaxis (Warringa *et al* 1993; Altman *et al,* 1981; Taborda-Barata *et al,* 1996), eosinophil survival (Lamas *et al,* 1991; Wallen *et al,* 1991; Cox *et al* 1991) and the production of both cytokines and chemokines from airway epithelium (Devalia *et al,* 1994; Sousa *et al,* 1993). Finally, glucocorticosteroids can act systemically by decreasing circulating eosinophils (Gauvreau *et al,* 1996; Evans *et al,* 1993) and inhibiting their release from the bone marrow (Gibson *et al,* 1991).

CANINE MODEL OF ALLERGEN-INDUCED AIRWAY HYPERRESPONSIVENESS

Inhalation of an extract of the pig nematode, Ascaris *suum,* by dogs that are naturally sensitized to this allergen, results in an increase in pulmonary resistance which reaches a maximum 3 to 10 minutes after challenge and resolves within one hour (Gold *et al*, 1972). This bronchoconstriction is thought to be due to cross-linking of Ig-E antibodies on mast cells that are specific for Toxacara *canis*, a parasitic nematode commonly found in dogs and which cross-react with the pig antigen (Patterson *et al*, 1974). Subsequent studies have shown several mediators to be involved, including histamine (Gold *et al*, 1977), prostaglandins (Kleeberger *et al*, 1986) and thromboxanes (Kleeberger *et al,* 1987), in a manner similar to that seen during the early bronchoconstrictor response in humans. The development of a late bronchoconstrictor response in dogs has been more difficult to demonstrate. Late bronchoconstrictor responses have been observed following inhalation of Ascaris by dogs pretreated with metyrapone (a cortisol synthesis inhibitor)(Sasaki *et al*, 1987) and after local installation of Ascaris in the peripheral airways (Turner & Spannhake, 1990), but not in untreated dogs after whole lung Ascaris inhalation (Stevens *et al*, 1989). Several studies have reported increases in AHR of skin test positive dogs after Ascaris inhalation (Kannan *et al*, 1986; Stevens *et al*, 1989; Woolley *et al*, 1994[a]; Inman *et al*, 1996[a]). AHR develops as early as one hour after inhalation (Kannan *et al*, 1986) and can persist for at least one week (Stevens *et al*, 1989). However, AHR only develops in about 50% of skin test positive dogs (Stevens *et al*, 1989) indicating the need for a screening allergen challenge when selecting dogs for use in this model. The airway inflammation that

develops in this model is predominantly due to an increase in neutrophils (Oostveen *et al*, 1986; Woolley *et al*, 1994[b]; Inman *et al*, 1996[a]). Increases in airway eosinophils have also been demonstrated following Ascaris challenge (Woolley *et al*, 1995; Inman *et al*, 1996[a]). However, these increases tend to be restricted to those dogs that do not develop AHR and occur only in a small number of dogs. The number of airway eosinophils and their activation state *before* Ascaris inhalation has been examined and shown to be higher in those dogs that went on to develop AHR than those that did not, suggesting that eosinophils may be involved early in the development of AHR (Woolley *et al*, 1995). Thus the contribution of eosinophils to the airway pathology in this model remains unclear. The influx of neutrophils, however, is more profound and these cells may be more important than the eosinophil in contributing to the development of AHR in dogs.

Both eosinophils and neutrophils are produced in the bone marrow from stem cell precursors and the mechanisms underlying inflammatory cell differentiation constitutes a potential target for the development of an anti-asthma therapy, since a selective inhibition of the production of these inflammatory cells would have a profound effect on the events occurring in the airways..

## INFLAMMATORY CELL HEMOPOIESIS

### GENERAL HEMOPOIESIS

In the following section, some general concepts of hemopoiesis are covered, but an extensive review of this area is beyond the scope of this chapter.

The earliest recognisable inflammatory cell precursor in the bone marrow is the hemopoietic stem cell (HSC) and these cells are defined by their capacity for long term self renewal and by their ability to differentiate along multiple lineage pathways (Ogawa 1994). The generation of mature cells from these pluripotent HSC involves the highly regulated progression through successive stages involving commitment to a specific cell lineage, terminal differentiation of lineage restricted progenitors and growth arrest (Bedi & Sharkis, 1995). The majority of HSC are thought to be dormant ($G_0$) in the cell cycle in the steady state with only a few cells supplying all of the hemopoietic cells at a given time. The mechanisms controlling the decision of an HSC to self renew versus differentiate are unknown but various theories have been proposed to explain the differentiation and commitment of these multipotent progenitors. The stochastic model proposes that multipotent HSC are intrinsically and randomly committed to differentiation and lineage restriction, and thus the expression of the mature phenotype is not dependent upon external, inducing stimuli (Till *et al*, 1964; Humphries *et al*, 1981; Nakahata *et al*, 1982[a]; Nakahata & Ogawa, 1982[b]). The inductive model argues that lineage selection and differentiation are dependent upon external stimuli such as hemopoietic cytokines and the cellular microenvironment, which affect gene transcription (Trentin, 1970; VanZant *et al*, 1979; Dexter *et al*, 1990; Metcalf, 1991). *In vitro* cultures of HSC require hemopoietic cytokines to prevent apoptosis (programmed cell death) and maintain survival (Williams *et al*, 1990), which has made it difficult to study the ability of HSCs to differentiate without the presence of growth factors. However, recent work has shown that introduction of the *bcl-2* gene

(which suppresses apoptosis) into a multipotent hemopoietic cell line, results in multi-lineage differentiation of these cells in the absence of growth factors (Fairbairn *et al*, 1993). This study suggests that growth factors are not essential for differentiation, thus favouring the stochastic model. It is also possible that a hybrid of these two proposals exist where stochastic processes determine the preferred differentiation patterns in each daughter cell but exposure to a particular microenvironment and/or cytokine pattern, may re-enforce or override the selection (Inman *et al*, 1998).

Whatever the mechanisms of self-renewal and differentiation, it is known that survival and proliferation of progenitor cells is regulated by cytokines. Cytokines exert their effects in concert on primitive progenitor cells (synergistic early-acting factors) and continue to influence progenitor expansion (intermediate-acting lineage-non-specific factors). Finally one or two factors influence progenitor maturation along specific lineages (late-acting lineage-specific factors). It has been shown that IL-6, granulocyte-colony stimulating factor (G-CSF), IL-1, IL-11, IL-12, leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), stem cell factor (SCF) are early acting cytokines (reviewed by Abboud & Liesveld, 1995). These factors are thought to act by triggering cycling of dormant progenitors. IL-3, GM-CSF and IL-4 are considered to be intermediate acting lineage non-specific cytokines and appear to support the proliferation of multipotential progenitors *after* they have exited from $G_0$. Finally, erythropoietin, macrophage-colony stimulating factor (M-CSF), G-CSF and IL-5 are considered to be lineage specific cytokines which support proliferation and maturation of committed progenitors only. The role of IL-3, GM-CSF and IL-5 in myeloid

cell production is discussed in further detail below.

Progenitors for various lineages were originally identified and defined using colony forming assays performed in semi-solid media such as agar or methylcellulose (Pluznik & Sachs, 1965; Bradley & Metcalf, 1966). These assays are based on the culture of cell populations containing lineage-specific hemopoietic progenitors in the presence of hemopoietic support media, which allows commitment to a given differentiation pathway. The lineage-specific colonies for a given number of cultured cells are, by definition, colony forming units (CFU), each representing the progeny of a single progenitor cell under standard plating conditions. The various CFU that can be identified this way are erythroid (Burst forming unit-erythroid - BFU-E), megakaryocytic (Meg-CFU), granulocyte/macrophage (GM-CFU), eosinophil/basophil (Eo/B-CFU) and lymphocyte-CFU (pre-B- and Pre-T), and identification is based on a combination of distinct morphological characteristics and immunohistochemical staining. More recently, flow cytometry has been used to identify and isolate progenitors based on the expression of cell surface markers, more specifically the CD34 antigen. This antigen is a monomeric, transmembrane, O-sialylated glycophosphoprotein, whose expression within the hemopoietic system is restricted to early lymphohemopoieic stem cells and progenitors (Civin *et al*, 1984; Katz *et al*, 1985; Sutherland & Eating,1992). CD34 may play an important role in progenitor cell adhesion and activation within the bone marrow stromal compartment, in a process involving carbohydrate interaction (Majdic *et al*, 1994). Studies of progenitor cell surface markers have shown a close correlation between $CD34^+$ cell numbers and total colony forming units (Eo/B and GM-

CFU) in cultures of progenitor cells with IL-5 and GM-CSF (Sehmi *et al*, 1996[a])

Single cell cloning experiments have revealed that $CD34^{+}$ cells co-express varying levels of distinct surface markers at specific differentiative stages in the hemopoietic pathway (Civin *et al*, 1989). The earliest progenitor cells bear $CD34^{high}$, Thy-1/CDw90 (Baum *et al*, 1992) and A83/CDw109 (Murray *et al*, 1994) antigens, and later acquire *c-kit* (the proto-oncogene cell product which binds SCF) (Strobl *et al*, 1992), $CD38^{low}$ (Hao *et al*, 1995), and $HLA\text{-}DR^{low}$ (Saeland *et al*, 1992). Myeloid-lineage committed progenitors are characterised as $CD34^{low}$ $CD33^{high}$ cells (Pierelli *et al*, 1993) while B-lineage and T-lineage precursors bear $CD34^{low}$ $CD19^{high}$ cells and $CD34^{low}$ $CD7^{high}$, respectively (Ryan *et al*, 1986; Mossalayi *et al*, 1990).

MYELOID CELL PRODUCTION

Granulocytes, more specifically eosinophils and neutrophils, are derived from a common myeloid progenitor known as the CFU-GEMM (colony-forming unit-granulocyte/erythroid/macrophage/megakaryocyte) (Figure 1), which can further differentiate to GM-CFU and Eo/B-CFU. These two progenitor types are the earliest recognisable progenitors that can be identified using semi-solid culture techniques. Flow cytometric analysis has characterized GM-CFU as being $CD34^{+}$, $CD33^{+}$, $CD13^{+}$, $CD45RA^{+}$ and myeloperoxidase positive (Strobl *et al*, 1993). The phenotype of Eo/B-CFU still requires clarification although preliminary studies using cultures of the human myeloid leukemic cell line, HL60, and methylcellulose culture systems suggest that the immunophenotype of an

intermediate stage eosinophil/basophil progenitor is $CD34^+CD35^+$ (Wong *et al*, 1996). The first recognisable cell by morphological analysis is the myeloblast which can further develop through a series of promyelocytes, myelocytes, metamyelocytes, and band cells until they become mature cells (Figure 1). Myeloblasts, promyelocytes and myelocytes are all mitotic while metamyelocytes, band cells and mature cells constitute the post mitotic compartment of the bone marrow.

The mechanisms of lineage commitment towards granulopoiesis, and the role that cytokines play in this process are still incompletely understood. At least four different cytokines are known to induce the differentiation of GM-CFU into mature neutrophils, macrophages or both (Metcalf, 1989). These include IL-3, GM-CSF, G-CSF and M-CSF. There is no significant homology between these glycoproteins and their pleiotropic activity is due to their ligation of specific cell receptors that share common subunits. Molecular cloning of human cytokine receptors has revealed that the IL-3 receptor (IL-3R), GM-CSFR and IL-5R are uniquely composed of heterodimeric structures consisting of a distinct α-subunit that binds the cognate cytokine with low affinity, and a common, shared β-subunit. The β-subunit itself does not bind ligand but forms high affinity binding sites in association with the α-subunit and activates specific signal transduction pathways (Murata *et al*, 1992; Tavernier *et al*, 1991). *In vitro* studies of murine embryonic stem cells and embryoid bodies have demonstrated a temporal, co-ordinated expression of growth factor genes during cell maturation (McClanahan *et al*, 1993). Certain genes such as c-*kit* (SCFR) and the α-subunits of the growth factor receptor genes are constitutively expressed during development of these

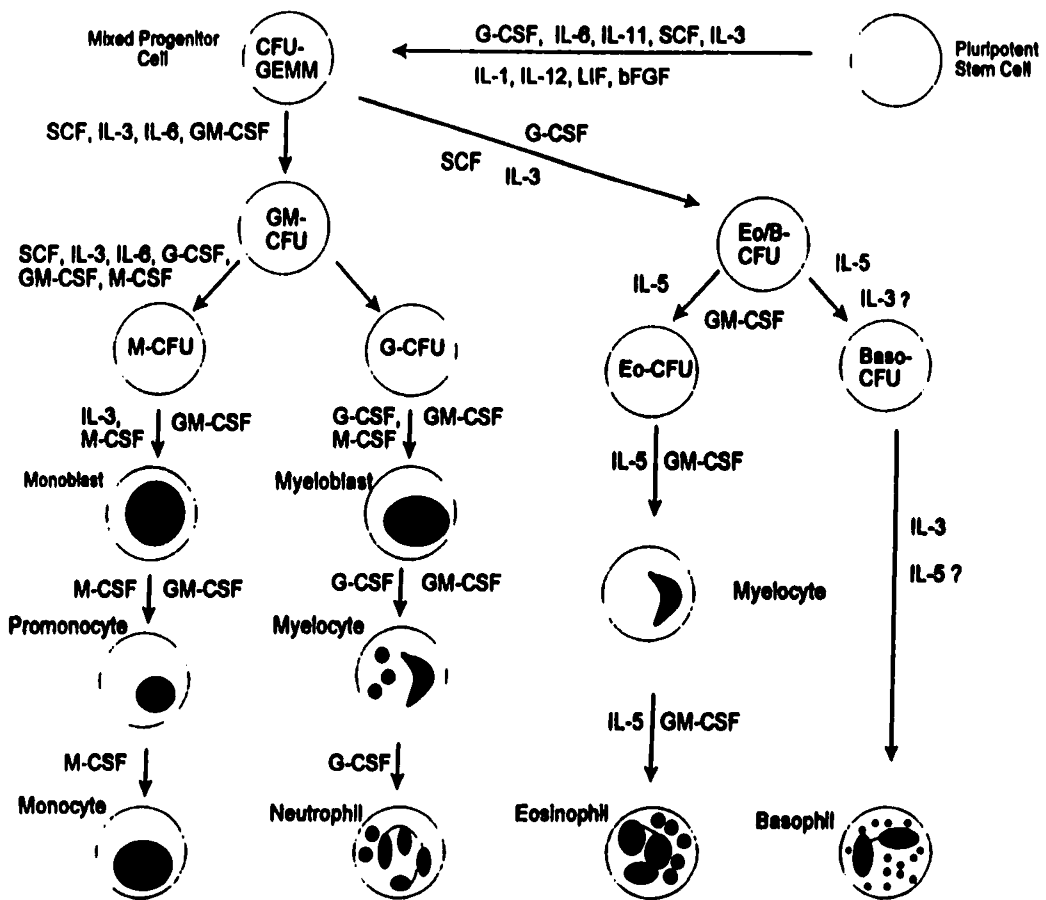


Figure 1.1 Hemopoietic differentiation pathways of myeloid inflammatory cells

cells, while the common β-subunit of the IL-3/IL-5/GM-CSF receptors, as well as SCF are induced early. In contrast, M-CSFR (c-*fms*), G-CSFR and CD34 are induced later, at an intermediate stage of development. Recent studies of human bone marrow and peripheral blood cells have shown that high levels of the α-subunit of IL-3R are expressed on primitive $CD34^+$ cells and this is attenuated as the cells become committed to the myeloid lineage ($CD34^+$ $CD33^+$) (Kurata *et al*, 1995). In contrast, the α-subunit of the GM-CSFR is undetectable on $CD34^+$ $CD33^-$ cells but is upregulated on $CD34^+$ $CD33^{low/high}$ cells. Augmented expression of IL-5R-α mRNA has been detected on a subline of AML14 cell line that spontaneously differentiate to eosinophil myelocytes (Paul *et al*, 1995). Treatment with all trans retinoic acid (ATRA), which causes a switch in differentiation towards neutrophils, causes a downregulation of IL-5R-α mRNA, suggesting a lineage-specific association of IL-5R expression and the development of eosinophils (Paul *et al*, 1995).

IL-3 and GM-CSF can both induce the production of neutrophils or macrophages from GM-CFU wheras M-CSF promotes the preferential generation of macrophages, and G-CSF produces neutrophils (Bedi & Sharkis, 1995). IL-3 and GM-CSF appear to act dominantly in a concentration-dependent manner to suppress M-CSFR expression on their target cells (Gliniak & Rohrschneider, 1990). At low concentrations of GM-CSF, macrophage colony formation is favoured due to relatively high expression of M-CSFR wheras at higher concentrations of GM-CSF, mixed colony formation is preferred due to lower levels of M-CSFR expression (Caracciolo et al, 1987). G-CSF supports the proliferation and differentiation of neutrophils from GM-CFU and these functions have been

shown to be regulated by different regions of the G-CSFR (Fukunaga *et al*, 1993). The N-terminal region transduces the proliferation signal wheras the C-terminal region transduces the differentiation signal.

Eosinophils and basophils share a common late-stage progenitor designated Eo/B-CFU (Denburg *et al*, 1985[a]) and commitment to the eosinophil and basophil lineages appears to be regulated by IL-3, IL-5 and GM-CSF (Denburg, 1990; Denburg *et al*, 1992) (Figure 1). Eosinophil and basophil differentiation is specifically supported by IL-5, which acts on a late-stage progenitor (Sanderson *et al*, 1985; Clutterbuck & Sanderson, 1988) and while there is some evidence that IL-3 alone may support differentiation along the basophil lineage (Valent *et al*, 1989), this cytokine has not been shown to be as specific as IL-5. Several growth factors are thought to act on early eosinophil progenitors in a synergistic fashion, such as G-CSF and SCF, that are not specific for this lineage (Ema *et al*, 1990; Kobayashi, 1993). IL-3 but not IL-5 can induce colony formation from $CD34^{+}CD33^{-}$ progenitors but when these cells are grown first in IL-3 followed by IL-5, eosinophil colonies emerge (Ema *et al*, 1990).

Thus IL-5 is unique in its ability to promote the terminal differentiation and maturation of eosinophil/basophil committed lineages (Clutterbuck *et al*, 1989). In addition, IL-5 prolongs eosinophil survival by delaying apoptotic death (Yamaguchi *et al*, 1988 & 1992), primes and stimulates eosinophil chemotactic activity (Sehmi *et al*, 1992), increases eosinophil adhesion to endothelial cells (Walsh *et al*, 1990) and enhances eosinophil effector function (Yamaguchi *et al*, 1988; Lopez *et al*, 1988). In mice that over-express the IL-5

transgene, IL-5 has been shown to be the predominant regulator of eosinophilia (Dent *et al*, 1990). However, the absence of any eosinophil-mediated tissue damage suggests that additional factors, such as eotaxin, may be required to activate these cells.

EVIDENCE FOR BONE MARROW PROGENITOR ACTIVITY IN ALLERGIC DISEASE

A large number of studies in both human subjects and animal models have shown that hemopoietic progenitors are raised in allergic disease and support the view that activation of specific hemopoietic pathways within the bone marrow may be contributing to the ongoing airway inflammation.

The first observation of an association between allergic disease and increased numbers of inflammatory cell progenitors was made using colony assays of peripheral blood from atopic and non-atopic individuals. Atopics demonstrated greater numbers of Eo/B-CFU than controls (Denburg *et al*, 1985[b]; Otsuka *et al*, 1986[a]). In addition, increased numbers of Eo/B-CFU were observed in atopic subjects when active disease was compared with quiescent atopic disease, suggesting a link between disease severity and progenitor numbers (Otsuka *et al*, 1986[a] & 1986[b]). Furthermore, increased numbers of both IL-5-responsive eosinophil progenitors and $CD34^+$ hemopoietic progenitor cells can be demonstrated in the blood of atopics compared with normal control subjects (Sehmi *et al*, 1996[a]). Interestingly, within the group of asymptomatic atopics, the highest levels of $CD34^+$ cell numbers were detected in subjects who had high serum IgE levels and were skin prick test-positive for a number of aero-allergens. This suggests that continual exposure to low levels of these

allergens may have contributed to mild subclinical inflammation resulting in the elevation of blood progenitor numbers.

The link between disease severity and progenitor numbers was further investigated by observing the effect of allergen challenge both *in vitro* and *in vivo* on progenitor cell numbers in peripheral blood. Greater numbers of Eo/B-CFU were grown from the blood of atopics in the presence of antigen-stimulated lymphomononuclear cell conditioned medium, indicating the generation of a hemopoietic signal following *in vitro* allergen challenge (Ohnishi *et al*, 1988). *In vivo* studies in atopic subjects have shown that there are fluctuating numbers of Eo/B-CFU during seasonal exposure to allergen (Otsuka *et al*, 1986[b]; Linden *et al*, 1994). These findings were followed by observations of selective increases in Eo/B-CFU, but not GM-CFU, numbers during exacerbations of asthma after corticosteroid withdrawal (Gibson *et al*, 1990). Resolution of the exacerbation with inhaled beclomethasone resulted in significant falls of eosinophil progenitors. In atopic asthmatics following allergen inhalation challenge, a significant increase in blood Eo/B-CFU, but not GM-CFU, was observed 24 hours post-allergen (Gibson *et al*, 1991). These changes were only detected in subjects who developed late asthmatic responses. The link between disease severity and progenitor cell numbers was again evident since the increase in Eo/B-CFU was significantly negatively correlated with the degree of AHR measured at the time. Finally, flow cytometric analysis has shown that a significant increase in the number of circulating $CD34^+$ cells can be detected in asthmatic subjects 24 hours following allergen challenge (Sehmi *et al*, 1996[b]). In agreement with the colony forming assays, these increases in $CD34^+$ cells were only

detected in asthmatics who developed a late bronchoconstrictor response, and not in isolated early responders.

The first direct evidence for the involvement of the bone marrow-derived progenitor cells in allergen-driven airway responses was demonstrated using the canine model of allergen-induced AHR. Studies using this model have shown that bone marrow-derived GM-CFU are significantly increased 24 hours following allergen inhalation compared with a diluent challenge (Woolley *et al*, 1994[a]), a result consistent with the neutrophilic airway inflammation seen in this model. In the same study, treatment of these dogs with the inhaled corticosteroid, budesonide, attenuated the allergen-induced increases in bone marrow granulocyte progenitors (GM-CFU). Further studies have shown that the increase in bone marrow GM-CFU is associated with the production of a serum hemopoietic factor produced following allergen challenge (Inman *et al*, 1996[a] & 1996[b]). Due to the lack, until recently (McSweeney *et al*, 1996 & 1998), of available dog antibodies to progenitor cell surface antigens, such as CD34, it has not yet been possible to confirm these studies using surface marker assays.

In humans, higher numbers of bone marrow progenitors have been demonstrated in subjects where analysis of bone marrow samples from patients undergoing thoracotomy for cardiac surgery demonstrated significantly greater numbers of $CD34^+$ progenitor cells in atopics compared with non-atopic patients (Sehmi *et al*, 1996[a]).

Based on these studies, it is clear that human allergic disease is associated with increased progenitor levels both in the circulation and possibly in the bone marrow. Whether

increases in bone marrow progenitors occur following allergen inhalation in human subjects, remains to be established and is the main focus of this thesis.

## INFLAMMATORY CELL TRAFFICKING

In addition to the increased production of inflammatory progenitor cells, the presence of these cells in the circulation, particularly after an allergen challenge, suggest that they leave the bone marrow and it is possible that they are contributing to the ongoing airway pathology by specific homing to the site of inflammation. Little is known about both the release and trafficking of these cells and their progeny in response to an allergic stimulus or the role that they play once they reach the airways. Several studies in animal models have demonstrated decreases in the maturation and kinetics of release of neutrophils in response to inflammation. Studies in dogs have shown that an induced sterile uterine inflammation results in the appearance of tritiated thymidine-labelled cells in the blood at 16 hours compared with 40 hours in control dogs (Cronkite, 1988) while infusion of starch into the peritoneal cavity of dogs causes a rapid rise in tritiated thymidine-labeled cells in the blood within 10 hours following infusion (Chikkappa *et al*, 1977). In addition, the induction of pneumococcal pneumonia in dogs has been shown to increase the rate of production of neutrophils from their precursors, shortening their maturation time, and decreasing their storage time in the marrow, with release of mature and immature neutrophils into the circulation (Marsh *et al*, 1967).

The development of a monoclonal antibody to the thymidine analogue 5'-bromo-2'-

deoxyuridine (BrdU) (Gratzner, 1982) has been a major development in studies of cell kinetics. BrdU is incorporated into the nuclei of S-phase cells (Goz, 1978), thus labeling dividing cells, and can be identified by immunohistochemical staining with anti-BrdU antibody (Bicknell *et al*, 1994; Terashima *et al*, 1996). BrdU has been used in rabbits where, after the introduction of *Streptococcus pneumoniae* in the lung, the transit times of BrdU-labeled neutrophils through the proliferating and non-proliferating pools in the bone marrow were shown to be considerably shortened (Terashima *et al*, 1996). It is therefore possible to use this technology to examine the proliferation, release and trafficking of bone marrow progenitor cells, through the circulation and into the airways in the canine model of allergen-induced AHR.

The aim of this thesis is to examine the role of bone marrow inflammatory cell progenitors in allergen-induced AHR and airway inflammation with respect to the following hypotheses;

**Overall Hypothesis:**

Inhalation of allergen by asthmatics is associated with increases in bone marrow inflammatory cell progenitor cells, which contribute to the ongoing airway inflammation.

**Specific Hypotheses:**

1. Allergen inhalation will be associated with an increase in the number of eosinophil progenitor cells in the bone marrow of asthmatic subjects.
2. The quality and quantity of changes in bone marrow eosinophil progenitors differs in subjects with different degrees of airway inflammation.

3. Trafficking of bone marrow progenitors from the bone marrow to the lung occurs following allergen inhalation in dogs with allergen-induced AHR.
4. Modulation of allergen-induced airway responses by inhaled corticosteroids is associated with a reduction in the numbers of bone marrow eosinophil progenitors

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# CHAPTER 2

# CHANGES IN BONE MARROW INFLAMMATORY CELL PROGENITORS AFTER INHALED ALLERGEN IN ASTHMATIC SUBJECTS

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Lorna Wood's contribution:

Experimental design
Collection of clinical data
Processing of laboratory samples
Identification and quantification of colonies
Analysis of data
Preparation of manuscript

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# Changes in Bone Marrow Inflammatory Cell Progenitors after Inhaled Allergen in Asthmatic Subjects

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**Increases in inflammatory cell progenitors, particularly eosinophil/basophil colony-forming cells (Eo/B-CFU), occur in peripheral blood after allergen provocation. The role of bone marrow (BM) in these reactions is unclear. We examined the effect of allergen challenge on human bone marrow progenitor cell growth. Fifteen asthmatic subjects, eight dual responders (DR) and seven isolated early responders (IER), were challenged with inhaled allergen. BM aspirates were taken before and 24 h after challenge and progenitors were enumerated by a colony-forming assay. Eo/B-CFU numbers increased in both groups after allergen challenge ($p < 0.0001$). For DR, the increases were significant for BM incubated with optimal GMCSF and IL-5, but not with IL-3. For IER, the increases were significant for all three cytokines tested. At a suboptimal concentration of IL-5, there was a significant increase in the number of Eo/B-CFU after allergen in the DR, from $5.25 \pm 1.2$ to $9.68 \pm 2.1$ per $2.5 \times 10^5$ cells plated ($p < 0.01$), which was not demonstrated in the IER ($p = 0.94$). The responses at this concentration of IL-5 were different between groups ($p < 0.05$). These results demonstrate that inhaled allergen increases BM Eo/B-CFU, and that the bone marrow of dual responders is more responsive to IL-5 after allergen. Wood LJ, Inman MD, Watson RM, Foley R, Denburg JA, O'Byrne PM. Changes in bone marrow inflammatory cell progenitors after inhaled allergen in asthmatic subjects.**

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Asthma is a disease characterized by bronchoconstriction, airway hyperresponsiveness, and airway inflammation. Inhalation of allergen by sensitized subjects is an important cause of asthma, and is characterized by biphasic responses known as the early- and late-phase asthmatic responses. Late asthmatic responses (LAR) are associated with transient increases in airway hyperresponsiveness (1), usually lasting several days, and increases in the numbers of activated eosinophils and metachromatic cells in the airways (2); however, subjects developing isolated early asthmatic responses also develop airway hyperresponsiveness, but to a much lesser extent than those patients developing late responses (3).

The predominant cell infiltrating the airways during the late response is the eosinophil. These cells are selectively increased in sputum (4) and bronchoalveolar lavage fluid (BAL) (5), in association with the late response. The eosinophils are also in an activated state in asthma, as indicated by elevated levels of eosinophil cationic protein (ECP) in BAL fluid (6), and enhanced immunostaining with the EG2 monoclonal antibody, which recognizes the cleaved and activated form of ECP.

We have previously provided evidence that an important aspect of allergic inflammatory responses is the induction of increases in inflammatory cell progenitors, which contribute to disease through the continued production of inflammatory effector cells (7, 8). Higher numbers of both circulating eosinophil/basophil colony-forming units (Eo/B-CFU) and $CD34^+$ hemopoietic progenitor cells are demonstrable in the blood of atopic subjects when compared with normal subjects (7, 9). In addition, the numbers of Eo/B-CFU in the bloodstream of asthmatic subjects at the time of an acute exacerbation is significantly higher than those measured after resolution of the exacerbation (10). Also, increased Eo/B-CFU can be stimulated from the peripheral blood of atopic subjects in the presence of conditioned media from atopic nasal, mucosal, and epithelial cells (11), suggesting the production of colony-stimulating activity from these cells. *In vivo* studies in atopic subjects have shown that there are fluctuating numbers of circulating Eo/B-CFU during seasonal exposure to allergen (12) and significantly higher numbers 24 h after allergen inhalation (13). Finally, in dogs with allergen-induced airway hyperresponsiveness and airway inflammation, bone marrow granulocyte-macrophage colony-forming units (GM-CFU) are significantly increased after allergen challenge (14).

Although such studies suggest that inflammatory cell progenitors in the blood arise from the bone marrow, this has not been shown in humans. The purposes of this study were fourfold: (*1*) to determine whether allergen inhalation increases

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the production of bone marrow inflammatory cell progenitors in sensitized subjects with mild asthma; (*2*) to evaluate whether, in subjects who develop both an early and a late asthmatic response (dual responders) and in subjects who develop isolated early responses, the differential physiologic and inflammatory responses to allergen are reflected in different bone marrow responses; (*3*) to establish whether the changes in bone marrow progenitors are selective for Eo/B colonies; (*4*) to determine whether there were differences in the bone marrow's responsiveness after allergen to optimal and suboptimal concentration of hemopoietic cytokines.

## METHODS

### Subjects

Fifteen patients with mild asthma (eight dual responders and seven isolated early responders) were studied (Table 1). Subjects were classified as dual responders if they developed both early- and late-phase asthmatic responses as defined by a greater than 15% drop in $FEV_1$ from baseline, or as isolated early responders if they developed only an early fall in $FEV_1$ greater than 15% from baseline. The study was approved by the Research Advisory Group at McMaster University Medical Centre, and all subjects provided their written informed consent prior to entering the study. Subjects were atopic, as indicated by one or more positive wheal-and-flare responses to skin prick tests. All subjects were nonsmokers and none had experienced a respiratory infection during the 4 wk prior to the study. Asthmatic subjects were stable at the time of study, requiring only intermittent use of inhaled $\beta_2$-agonists, with baseline $FEV_1$ values > 70% predicted.

### Study Design

Subjects attended the laboratory on three occasions. At the initial visit a full medical history was taken and skin prick tests were performed. In addition, spirometry and methacholine inhalation challenge were performed followed by induction of sputum. Within 1 wk subjects returned to the laboratory to undergo an allergen-challenge procedure. Prior to allergen challenge, a bone marrow aspirate and blood sample were taken to determine baseline measurements. At 5 h after allergen inhalation a second blood sample was withdrawn. The third visit to the laboratory occurred 24 h after allergen inhalation. At this visit, postallergen bone marrow aspirate and blood samples were obtained and spirometry and a methacholine inhalation challenge were performed, followed by a sputum induction procedure to evaluate the airway cellular responses.

### Methacholine Inhalation Challenge

Methacholine inhalation was performed as described by Cockcroft (15). Subjects inhaled normal saline and then doubling concentrations of methacholine phosphate from a Wright nebulizer for 2 min. Increasing concentrations of methacholine were administered until the $FEV_1$ decreased by > 20% of the baseline value. Results were expressed as the geometric mean provocative concentration causing a 20% decrease in $FEV_1$ ($PC_{20}$). For the skin prick titration test, the allergen to be used in the allergen inhalation challenge was administered in doubling dilutions in duplicate.

### Allergen Inhalation Challenge

Allergen inhalation challenge was performed as described by O'Byrne (16). The allergen producing the largest skin wheal diameter was diluted in normal saline. The concentration of allergen extract for inhalation was determined from a formula described by Cockcroft and colleagues (17) using results from the skin test and the methacholine $PC_{20}$. The starting concentration of allergen was chosen to be three doubling doses below that predicted to cause a 20% fall in $FEV_1$. Doubling concentrations of allergen were inhaled at 10-min intervals until a decrease of 15% or more occurred in the $FEV_1$ from baseline. Measurements of $FEV_1$ were performed at 10, 20, 30, 45, 60, 90, and 180 min, and then every hour for 7 h after the final inhalation. The allergen-induced early response was determined as the maximal decrease in $FEV_1$ between 0 and 2 h after allergen, and the late response was determined as the maximal decrease between 3 and 7 h after allergen inhalation.

### Blood Samples

Venous blood samples were obtained from each subject before and at 5 and 24 h after allergen inhalation. Samples were collected in tubes treated with ethylenediaminetetraacetic acid (EDTA) for total and differential WBC counts. Total cell counts were performed using a neubauer hemocytometer, and differential cell counts were made from blood smears stained by Diff-Quik (American Scientific Products, McGaw Park, IL). Differential cell counts were performed by one investigator in a blinded fashion and the mean of two slides was obtained (300 cells counted per slide). Cells were classified using standard morphologic criteria. Results were expressed as absolute counts ($10^9$ cells/L).

TABLE 1

SUBJECT CHARACTERISTICS

| | Age (yr) | Sex | Allergen (*inhaled dilution*) | EAR (*Max % fall in* $FEV_1$) | LAR (*Max % fall in* $FEV_1$) |
|---|---|---|---|---|---|
| Isolated early responders | | | | | |
| 1 | 23 | F | HDM(1:32) | 25.0 | 5.4 |
| 2 | 22 | M | RW(1:8) | 30.5 | −8.5 |
| 3 | 19 | F | HDM(1:64) | 31.5 | 11.0 |
| 4 | 23 | M | HDM(1:4) | 17.9 | 10.3 |
| 5 | 29 | M | HDM(1:64) | 18.8 | 8.3 |
| 6 | 22 | F | HDM(1:128) | 25.0 | 11.8 |
| 7 | 30 | M | HDM(1:8) | 20.5 | 4.5 |
| Mean ± SEM | 24 ± 1.5 | | | 24.2 ± 2.1 | 6.1 ± 2.7 |
| Dual responders | | | | | |
| 1 | 24 | M | HDM(1:512) | 25.0 | 40.8 |
| 2 | 22 | F | HDM(1:4,096) | 21.2 | 21.2 |
| 3 | 26 | F | HDM(1:64) | 23.5 | 16.9 |
| 4 | 22 | M | GRASS(1:2,048) | 21.6 | 22.4 |
| 5 | 22 | M | CAT(1:128) | 21.1 | 15.5 |
| 6 | 21 | F | HDM(1:512) | 24.4 | 22.2 |
| 7 | 31 | M | HDM(1:256) | 40.7 | 18.6 |
| 8 | 22 | M | HDM(1:1,024) | 29.4 | 23.5 |
| Mean ± SEM | 23.8 ± 1.2 | | | 25.9 ± 2.3 | 22.6 ± 2.8 |

*Definition of abbreviations*: EAR = early asthmatic response; LAR = late asthmatic response; HDM = house dust mite *D. farinae*; RW = ragweed.

### Bone Marrow Aspirate and Culture

Bone marrow aspirates were obtained from the iliac crest using a bone marrow aspiration needle (16 × 2"; Sherwood Medical, St. Louis, MO). Three milliliters of bone marrow were aspirated into a 10-ml syringe containing 1 ml sterile heparin (1,000 U/ml) (Leo Laboratories, ON, Canada) and semisolid methylcellulose cultures of low density nonadherent mononuclear cells were performed. Briefly, heparinized bone marrow was diluted to 50 ml with McCoy's 5A medium (GIBCO, Grand Island, NY) and separated over 65% percoll (Pharmacia, Uppsala, Sweden). The interface mononuclear-rich cell fraction was washed in McCoy's 5A medium and then incubated in McCoy's 5A medium supplemented with 15% fetal calf serum (FCS) (GIBCO), 1% penicillin/streptomycin (GIBCO), and $5 \times 10^{-5}$ M 2-mercaptoethanol (final concentration) (Sigma Chemical, St. Louis, MO) for 2 h in plastic flasks at 37° C and 5% $CO_2$. Nonadherent mononuclear cells (NAMC) containing progenitor cells and lymphocytes were then cultured ($2.5 \times 10^5$ cells per 35 × 10 mm tissue culture dish; Falcon Plastics, Oxnard, CA), in duplicate, in supplemented Iscove's modified Dulbecco's medium (GIBCO) with 1% penicillin/streptomycin and $5 \times 10^{-5}$ M 2-mercaptoethanol (final concentration), 0.9% methylcellulose (Sigma Chemical), and 20% FCS, either alone or in the presence of one of the following growth factors: recombinant human IL-3 (1 or 0.1 ng/ml; Pharmingen, San Diego, CA), recombinant human GM-CSF (10 or 1 ng/ml; Pharmingen), or recombinant human IL-5 (1 or 0.1 ng/ml; Pharmingen). Cultures were incubated for 14 d at 37° C and 5% $CO_2$ after which colonies were identified as either Eo/B-CFU or GM-CFU according to previously described criteria (18) and expressed as colony-forming units (CFU) per $2.5 \times 10^5$ NAMC plated.

### Sputum Analysis

Sputum was induced and processed according to the method of Popov and colleagues (19). Subjects inhaled 3, 4, and then 5% saline for 10 min each until an adequate sample was obtained or if the $FEV_1$ dropped 20% from baseline. Cell plugs were selected from the sample and processed using 0.1% dithiothreitol (Sputolysin; Calbiochem, San Diego, CA) and Dulbecco's phosphate-buffered saline (GIBCO). Cytospins were prepared on glass slides, and differential counts were performed in a blinded fashion on Diff-Quik-stained slides. Mean counts from duplicate slides were obtained (500 cells counted per slide) and expressed as percentages. When possible, cytospins were also prepared on APTEX-coated slides and fixed in periodate-lysine-paraformaldehyde (PLP) for immunocytochemical staining for eosinophil cationic protein (ECP) using a monoclonal antibody against cleaved ECP (EG2) (Kabi Pharmacia) (20). Results are expressed as percentages of 500 cells counted under light microscopy.

### Statistical Analysis

*Airway hyperresponsiveness.* Methacholine $PC_{20}$ values were $\log_{10}$ transformed prior to analysis. Preallergen versus postallergen comparisons were performed for each group using Student's paired *t* tests. The preallergen versus postallergen change in logged $PC_{20}$ was compared between groups using Student's independent *t* test.

*Sputum.* Preallergen versus postallergen changes in induced sputum cell percentages were assessed for each group using Student's paired *t* tests. Differences in preallergen versus postallergen changes in cell percentages between each group were assessed using Student's nonpaired *t* tests. Regression analysis was used to detect significant relationships between induced sputum and changes in airway function (21).

*Blood.* Differences in blood differentials before and 5 and 24 h after allergen were assessed for each group using Student's paired *t* test.

*Bone marrow colonies.* Differences in bone marrow progenitor colonies were investigated using a mixed model analysis of variance (nonrepeated factor: early versus dual; repeated factors: preallergen versus postallergen, GM-CSF versus IL-3 versus IL-5, low versus high concentration) (22). Preallergen versus postallergen colony growth with each cytokine at each concentration comparisons were also performed as planned comparisons, using Student's paired *t* tests. Differences in the preallergen versus postallergen change in colony numbers between the two groups were analyzed using Student's nonpaired *t* tests. Statistical significance was assumed at $p < 0.05$.

## RESULTS

### Bronchoconstrictor Responses

The mean maximal percent fall in $FEV_1$ during the early asthmatic response was 25.9 ± 2.3% in the dual responders and 24.2 ± 2.1% in the isolated early responders (Table 1). The maximal percent fall in $FEV_1$ during the late asthmatic response was 22.6 ± 2.8% in the dual responders and 6.1 ± 2.7% in the isolated early responders (Table 1).

### Airway Hyperresponsiveness

Methacholine airway hyperresponsiveness developed in the dual responders but not in the isolated early responders 24 h after inhaled allergen. The geometric mean methacholine $PC_{20}$ values in the dual responders fell from 1.66 mg/ml (%SEM, 1.28) before to 0.52 mg/ml (%SEM, 1.32) after allergen ($p < 0.001$), whereas in the isolated early responders the values were 0.98 mg/ml (%SEM, 1.61) before and 1.14 mg/ml (%SEM, 1.77) after allergen ($p = 0.72$) (Figure 1). In addition, there was a significant difference between the two groups in the allergen-induced shift in logged $PC_{20}$ values ($p < 0.001$) (Figure 1).

### Airway Inflammation

The proportion of eosinophils in sputum after allergen challenge increased significantly in the dual responder group, from 3.4 ± 0.8% before to 32.7 ± 9.9% after allergen ($p < 0.05$) (Table 2). A smaller, not significant increase was seen in the isolated early responder group, where the values were 6.9 ± 2.1% before and 18.9 ± 9.7% after allergen ($p = 0.28$) (Table 2). There were no significant differences in the increases in eosinophil numbers between the two groups ($p = 0.23$). Also, there were nonsignificant increases in the proportion of EG2-

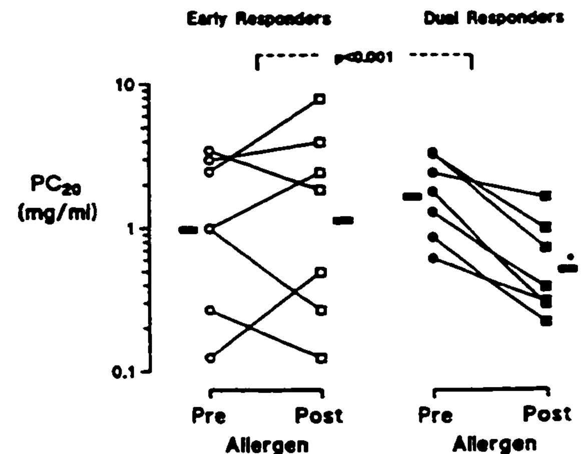


*Figure 1.* Allergen-induced methacholine hyperresponsiveness. Methacholine $PC_{20}$ values before (*circles*) and after (*squares*) allergen inhalation in isolated early (*open symbols*) and dual (*closed symbols*) responder subjects. Geometric means are indicated by *solid bars*. There was a significant drop in $PC_{20}$ values after allergen in the dual responder group (*$p < 0.001$), but not in the isolated early responder group. In addition, there was a significant difference between the two groups in the allergen-induced differences in $PC_{20}$ values ($p < 0.001$).

TABLE 2

SPUTUM AND BLOOD INFLAMMATORY CELL NUMBERS AFTER ALLERGEN INHALATION*

| | Isolated Early Responders | | | Dual Responders | | |
|---|---|---|---|---|---|---|
| | Preallergen | 5-Hour Postallergen | 24-Hour Postallergen | Preallergen | 5-Hour Postallergen | 24-Hour Postallergen |
| Sputum | | | | | | |
| Eosinophils, % | 6.9 ± 2.1 | ND | 18.9 ± 9.7 | 3.4 ± 0.8 | ND | 32.7 ± 9.9[†] |
| Neutrophils, % | 16.7 ± 4.5 | ND | 28.9 ± 13.0 | 23.2 ± 9.3 | ND | 27.3 ± 9.9 |
| Macrophages, % | 73.2 ± 5.1 | ND | 49.6 ± 13.2 | 69.3 ± 9.1 | ND | 35.6 ± 7.3[‡] |
| EG2+ve cells, % | 2.1 ± 1.0 | ND | 11.2 ± 7.3 | 2.5 ± 1.1 | ND | 18.9 ± 10.4 |
| Blood | | | | | | |
| Eosinophils, $\times 10^9$/L | 0.40 ± 0.03 | 0.37 ± 0.08 | 0.49 ± 0.08 | 0.40 ± 0.11 | 0.22 ± 0.05[†] | 0.32 ± 0.05[§] |

* Values are means ± SEM.
[†] $p < 0.05$ compared with baseline.
[‡] $p < 0.01$ compared with baseline.
[§] $p < 0.05$ compared with 5 hours.

positive cells after allergen inhalation in both groups: 2.5 ± 1.1% before and 19.0 ± 10.4% after allergen in the dual responders ($p = 0.15$), and 2.1 ± 1.0% before and 11.2 ± 7.3% after in the isolated early responders ($p = 0.28$) (Table 2). There were no significant correlations between changes in either sputum eosinophils or sputum EG2$^+$ cells, with changes in log $PC_{20}$ values ($p > 0.05$). The only other change in sputum inflammatory cells was a significant reduction in the percent macrophages in the dual responders ($p = 0.007$) (Table 2).

### Blood Eosinophils

Blood eosinophils in the dual responder group fell from 0.40 ± 0.11 × $10^9$/L before allergen to 0.22 ± 0.05 × $10^9$/L 5 h after allergen ($p < 0.05$) (Table 2). Subsequently, blood eosinophils increased from 0.22 ± 0.05 × $10^9$/L to 0.32 ± 0.05 × $10^9$/L between 5 and 24 h after allergen ($p < 0.05$) (Table 2). There were no significant differences in blood eosinophils after allergen inhalation in the isolated early responders.

### Bone Marrow Progenitors

The number of bone marrow Eo/B-CFU increased in both groups after allergen challenge ($p < 0.0001$) (Table 3). In the dual responders, the increases were significant for bone marrow grown in the presence of GM-CSF (10 ng/ml) and IL-5 (1 ng/ml), but not IL-3 (1 ng/ml) (Table 3). In the isolated early responders, the increases were significant for the high concentrations of all three cytokines tested (Table 3). At these concentrations, there were no significant differences in the magnitude of the Eo/B-CFU response between groups ($p > 0.05$). However, at the lower concentrations of IL-5 (0.1 ng/ml), there was a significant increase in the number of Eo/B-CFU after allergen in the dual responders, from 5.3 ± 1.2 before to 9.7 ± 2.1 per 2.5 × $10^5$ cells plated after allergen ($p < 0.01$), but not in the isolated early responders where the Eo/B-CFU were 7.8 ± 3.0 before and 7.8 ± 2.6 per 2.5 × $10^5$ cells plated after allergen ($p = 0.94$) (Figure 2 and Table 3). There was a significant difference in the preallergen to postallergen change in Eo/B-CFU between the two groups at this concentration of IL-5 ($p < 0.05$). There were no significant increases in Eo/B-CFU after allergen inhalation in the presence of lower concentrations of IL-3 (0.1 ng/ml) or GM-CSF (1 ng/ml) in either subject group (Table 3).

There were no changes in the number of GM-CFU grown from the postallergen bone marrow in either group when incubated in the presence of IL-3 or IL-5 (Table 3). There was, however, a significant increase in the number of GM-CFU grown from the postallergen bone marrow compared with the preallergen bone marrow when the cells were incubated with GM-CSF (10 ng/ml) in the dual responders (Table 3). There was a tendency towards an increase in the number of GM-CFU after allergen seen in the isolated early responder group (Table 3). There were no significant differences in the magnitude of the GM-CFU response between the two groups with any of the cytokines tested (Table 3). Finally, there were no signifi-

TABLE 3

BONE MARROW PROGENITOR CELL NUMBERS*

| | Eo/B-CFU | | | | GM-CFU | | | |
|---|---|---|---|---|---|---|---|---|
| | IER | | DR | | IER | | DR | |
| Cytokine Stimulus | Preallergen | Postallergen | Preallergen | Postallergen | Preallergen | Postallergen | Preallergen | Postallergen |
| IL-3, 1 ng/ml | 9.4 ± 2.0 | 14.6 ± 1.2[†] | 5.6 ± 1.8 | 9.2 ± 2.8 | 0.4 ± 0.2 | 1.2 ± 0.5 | 0.4 ± 0.3 | 0.5 ± 0.2 |
| IL-3, 0.1 ng/ml | 0.8 ± 0.4 | 1.1 ± 0.6 | 0.4 ± 0.2 | 0.7 ± 0.4 | 0.3 ± 0.2 | 0.3 ± 0.1 | 0.0 ± 0.0 | 0.2 ± 0.1 |
| GMCSF, 10 ng/ml | 14.0 ± 2.7 | 21.5 ± 3.3[‡] | 9.7 ± 1.2 | 15.2 ± 2.6[†] | 3.1 ± 1.0 | 7.7 ± 2.6 | 2.0 ± 0.8 | 4.4 ± 1.0[†] |
| GMCSF, 1 ng/ml | 15.2 ± 2.6 | 20.1 ± 5.0 | 10.9 ± 1.6 | 12.7 ± 2.4 | 3.6 ± 1.8 | 6.7 ± 2.1 | 2.3 ± 0.6 | 3.4 ± 1.1 |
| IL-5, 1 ng/ml | 11.0 ± 2.3 | 15.2 ± 2.2[†] | 10.3 ± 1.9 | 16.9 ± 3.3[†] | 0.3 ± 0.1 | 0.1 ± 0.1 | 0.4 ± 0.2 | 0.1 ± 0.1 |
| IL-5, 0.1 ng/ml | 7.8 ± 3.0 | 7.8 ± 2.6 | 5.3 ± 1.2 | 9.7 ± 2.1[‡] | 0.3 ± 0.2 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.2 ± 0.1 |

*Definition of abbreviations:* IER = isolated early responder; DR = dual responder.
* Values are means ± SEM. Preallergen and postallergen bone marrow cells were plated in the presence of IL-3 (0.1 or 1 ng/ml), GM-CSF (1 or 10 ng/ml), or IL-5 (0.1 or 1 ng/ml). Colonies are expressed as colony-forming units per 2.5 × $10^5$ NAMC plated.
[†] $p < 0.05$ compared with baseline.
[‡] $p < 0.01$ compared with baseline.

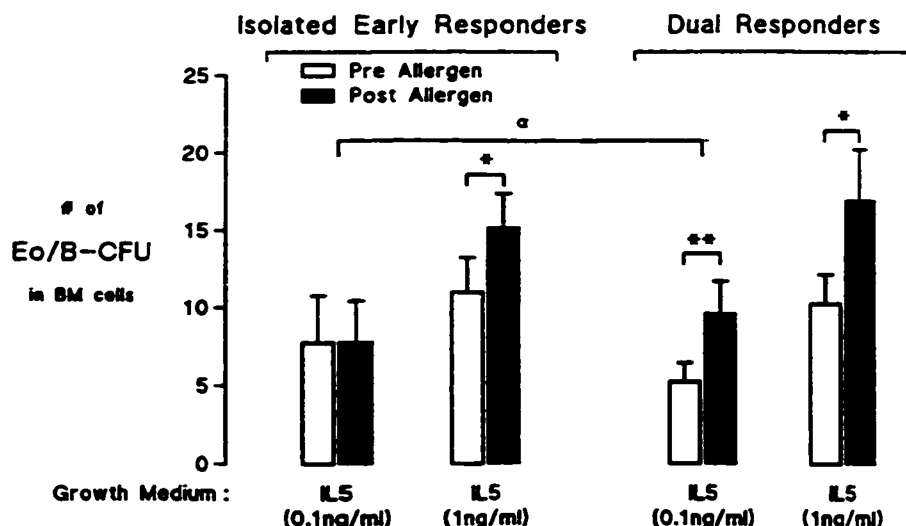


*Figure 2.* Changes in bone marrow Eo/B-CFU. Bone marrow Eo/B-CFU per $2.5 \times 10^5$ NAMC plated before (*open columns*) and after (*closed columns*) allergen inhalation in both isolated early and dual responder groups. Colonies were grown in the presence of IL-5 (0.1 or 1 ng/ml). Preallergen to postallergen differences were significant as indicated. *$p < 0.05$, **$p < 0.01$. Preallergen to postallergen changes were significantly different in the dual responder group compared with the isolated early responder group as indicated $^a p < 0.05$.

cant changes in the number of GM-CFU in the postallergen bone marrow samples from either group, when incubated with IL-3, GM-CSF, or IL-5 at suboptimal cytokine concentrations (Table 3).

## DISCUSSION

This study has demonstrated an increase in the cytokine-induced production of bone marrow inflammatory cell progenitors associated with the development of airway hyperresponsiveness and airway inflammation after allergen inhalation in sensitized asthmatic subjects. In addition, only the dual responders demonstrated measurable methacholine airway hyperresponsiveness after allergen, and increases in sputum eosinophils were larger and significant in the dual responders. This result is consistent with other studies that have contrasted the airway inflammatory response between isolated early and dual responders (23).

Increases in bone marrow Eo/B-CFU were demonstrated in both subject groups after allergen challenge, when optimal concentrations (as determined in preliminary studies) of IL-3, IL-5, and GM-CSF were used. There was, however, a significant difference between the two groups when the cells were incubated with a suboptimal concentration of IL-5. This indicates that after allergen challenge, the bone marrow of the dual responders is more responsive to IL-5, which may reflect either a specific induction of a population of more committed eosinophil/basophil progenitors or an upregulation of the IL-5 receptor on the surface of these cells. Studies performed by Sehmi and colleagues (24) in our laboratory has confirmed an increase in the proportion of $CD34^+$ cells expressing the alpha subunit of the IL-5 receptor after allergen in dual but not in isolated early responders, which supports the latter hypothesis. This increase in responsiveness to IL-5 in dual responders was associated with significant increases in airway eosinophils and methacholine airway responsiveness at 24 h after allergen challenge, which did not occur in the isolated early responders. These results may indicate that the responsiveness of the bone marrow to IL-5 after allergen is a determinant of the magnitude of the eosinophilic responses to inhaled allergen and of the degree of the subsequent physiologic abnormalities. Conversely, the degree of IL-5 sensitivity in the bone marrow may be determined by the events occurring in the airway that ultimately give rise to the LAR. However, these speculations will only be adequately addressed with the use of specific tools that block the activity of IL-5 on the bone marrow such as monoclonal antibodies directed against the cytokine.

Other studies have supported an important role for IL-5 in the development of allergen-induced airway inflammation. In studies of transgenic mice that overexpress IL-5, there is marked circulating and tissue eosinophilia (25), whereas in animal models, antibodies to IL-5 can block allergen-induced local and systemic eosinophilia and airway hyperresponsiveness for periods lasting as long as 6 mo. In murine, guinea-pig, and primate models, treatment of sensitized animals with the anti-IL-5 monoclonal antibody TRFK-5 inhibits the allergen-induced eosinophil infiltration into BAL fluid and lung tissue, and in primates the effect of this antibody could be detected for as long as 3 mo (26–28). In addition, TRFK-5 inhibited the increase in airway responsiveness in both the guinea-pig and primate models. A humanized anti-IL-5 antibody (Sch 55700) has been constructed, which has been shown to inhibit pulmonary eosinophilia in both sensitized guinea pigs and primates; the latter inhibitory effect on allergen-induced BAL eosinophilia also lasts as long as 6 mo (29), suggesting an ongoing effect on eosinophil production in the bone marrow.

The time point of 24 h used to measure eosinophil/basophil progenitors in the bone marrow was chosen based on the increased responsiveness of the bone marrow at 24 h after allergen inhalation in the canine model of allergen-induced AHR (14). Although increases were seen at this time point, this study does not provide any evidence on the timing of the bone marrow response. A more detailed kinetics study may provide further information on whether the bone marrow response after allergen is primary or secondary to events occurring in the airways.

The number of GM-CSF-stimulated GM-CFU colonies were also significantly higher in the dual responders after allergen inhalation. These results show that there is also a neutrophilic/monocytic progenitor response in the bone marrow after allergen inhalation; indeed, previous studies have implicated neutrophils during the late response, albeit transiently, just prior to development of eosinophilic inflammation (30, 31).

Previous studies from our laboratory, in a canine model of allergen-induced airway hyperresponsiveness and airway inflammation, have demonstrated that allergen inhalation increases bone marrow GM-CFU production (14), and that this is due to an as yet unidentified hemopoietic activity released into the bloodstream after allergen inhalation, which stimulates the bone marrow (32). The present study raises the possibility that after allergen inhalation by atopic asthmatics, IL-5 may represent one such hemopoietic signal with a significant effect on IL-5 responsive progenitors induced in the bone marrow; this could then lead to increased production of eosinophils and promote eosinophilic inflammation of the airways. Alternatively, a recent study by Minshall and colleagues (33) showed that after allergen inhalation, T-lymphocytes present in the bone marrow were a significant source of IL-5 in sensitized mice, suggesting that events occurring in the airways may result in local bone marrow production of IL-5, leading to increased production of eosinophils. Our studies in dogs have also shown that pretreatment with the inhaled corticosteroid, budesonide, can blunt the response of GM-CFU in the bone marrow after allergen inhalation (14). Thus, inhaled corticosteroids, which attenuate allergen-induced airway responses, as well as the increases in blood and airway eosinophils (34, 35), may partially exert their effect at the level of the bone marrow to prevent either eosinophil production or the release of maturing eosinophils into the bloodstream. These hypotheses require confirmation.

In conclusion, this study has demonstrated, for the first time, increases in human Eo/B and GM progenitors after allergen inhalation in sensitized, asthmatic subjects. However, an increased responsiveness of the Eo/B progenitors to suboptimal concentrations of IL-5 appears to distinguish between subjects who develop the most marked increases in airway eosinophils after allergen inhalation, which is associated with the development of allergen-induced late responses and airway hyperresponsiveness.

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# CHAPTER 3

# ALLERGEN-INDUCED INCREASES IN IL-5 RECEPTOR α-SUBUNIT EXPRESSION ON BONE MARROW-DERIVED $CD34^{-}$ CELLS FROM ASTHMATIC SUBJECTS

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Lorna Wood's contributions

Experimental design
Collection of clinical data
Processing of laboratory samples
Analysis of data

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# Allergen-induced Increases in IL-5 Receptor α-subunit Expression on Bone Marrow–derived CD34+ Cells from Asthmatic Subjects

## A Novel Marker of Progenitor Cell Commitment towards Eosinophilic Differentiation

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## Abstract

We have proposed previously that hemopoietic myeloid progenitors contribute to the ongoing recruitment of proinflammatory cells, namely eosinophils, to sites of allergen challenge in allergic diseases such as asthma. In this study, we investigated the involvement of bone marrow-derived progenitors in the development of allergen-induced pulmonary inflammation in mild asthmatic subjects. By flow cytometry, we enumerated the level of expression of CD34, a hemopoietic progenitor cell marker, on bone marrow aspirates taken before and 24 h after allergen challenge. In addition, the coexpression of the α-subunits of IL-3 receptor (IL-3R) and IL-5 receptor (IL-5R) on CD34+ cells was investigated. After allergen-challenge, although no significant change in total BM CD34+ cell numbers was observed, a significant increase in the proportion of CD34+ cells expressing IL-5Rα, but not IL-3Rα, was detected in the 24-h post-allergen, compared with the pre-allergen bone marrow. This was associated with a significant blood and sputum eosinophilia and increased methacholine airway responsiveness, 24 h post-allergen. Using simultaneous in situ hybridization and immunocytochemistry, we colocalized the expression of messenger RNA for membrane-bound IL-5Rα to CD34+ cells. In summary, our data suggest that increased expression of IL-5Rα on CD34+ cells favors eosinophilopoiesis and may thus contribute to the subsequent development of blood and tissue eosinophilia, a hallmark of allergic inflammation. (*J. Clin. Invest.* 1997. 100:2466–2475.) Key words: CD34 • IL-5 receptor • hemopoiesis • asthma

## Introduction

Asthma is a complex disorder characterized by reversible airflow obstruction, airway hyperresponsiveness, and infiltration of the airways by activated inflammatory cells. Increasing evidence suggests that the clinical severity of asthma parallels the degree of eosinophilic inflammation and, as such, highlights a role for eosinophils in the pathology of asthma (1–4). However, the mechanism(s) underlying the development and persistence of tissue eosinophilia in allergic inflammation remains unresolved.

We have investigated the hypothesis that activation of specific hemopoietic pathways in the bone marrow may contribute to the allergic diathesis through increased production and traffic of lineage-committed inflammatory progenitor cells such as those for eosinophils. This is supported by findings (in semi-solid liquid cultures) that circulating progenitors (colony-forming units, CFU)[1] for eosinophils and basophils (Eo/Baso-CFU) are constitutively increased in atopic individuals (5) and that these cell numbers change in parallel with acute exacerbations and steroid-controlled resolution of clinical asthma (6). Selective and relevant fluctuations in the numbers of blood Eo/Baso-CFU in allergic rhinitics during seasonal exposure to allergen (7, 8) and in atopic asthmatics after allergen inhalation (9) highlight a link between disease severity and progenitor cell numbers. More direct evidence for the involvement of the bone marrow in allergen-driven airway responses is demonstrated in a canine model of airway hyperresponsiveness, where numbers of bone marrow-derived granulocyte-macrophage CFU are increased significantly 24 h after allergen inhalation challenge and are abolished by pretreatment with inhaled corticosteroids (10). These studies suggest that a feedback mechanism exists between the lungs and bone marrow that triggers increased production of bone marrow–derived inflammatory cell progenitors during allergic inflammatory reactions. However, the potential involvement of the bone marrow in the genesis of allergic asthma in humans has not been directly investigated to date.

CD34 is an *O*-sialylated glycoprotein (105–120 kD), whose expression within the hemopoietic system is restricted to primitive progenitor cells of all lineages (11, 12). By flow cytometry, we have shown recently that increased numbers of CD34+ cells are present in the blood and bone marrow of atopics compared with nonatopic control subjects (13). In addition we showed that in atopics, blood progenitors are skewed towards an increased responsiveness to IL-5 as demonstrated by the increased numbers of Eo/Baso-CFU detected in methylcellulose cultures with IL-5 (13). Therefore, we hypothesized that IL-5R+ progenitors would be detectable in the blood and bone marrow of allergic asthmatic subjects and that the level of ex-

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1. *Abbreviations used in this paper:* CFU, colony forming units; DR, dual responders; Eo/Baso-CFU, eosinophil and basophil CFU; FSC, forward light scatter; IER, isolated early responders; IL-3Rα, interleukin-3 receptor alpha-subunit; IL-5Rα, interleukin-5 receptor alpha-subunit; NAMNC, nonadherent mononuclear cells; PE, phycoerythrin; PerCp, peridinin chlorophyll protein; SSC, orthogonal or side light scatter; WBC, white blood cells.

pression of IL-5R would be increased preferentially in situations where eosinophilic inflammation is induced. In order to investigate this hypothesis, we enumerated the level of coexpression of CD34 and receptors for eosinophilopoietic cytokines such as IL-5 and IL-3, on bone marrow–derived progenitors from a group of stable atopic asthmatics, taken both before and 24 h after allergen inhalation challenge (a time point associated with increases in circulating and airway eosinophils).

## Methods

*Materials.* Materials were obtained as follows: Percoll from Pharmacia Biotech AB (Uppsala, Sweden); McCoys 5A, Iscove's modified Dulbecco's medium, and FCS from GIBCO BRL (Gaithersburg, MD); methylcellulose, BSA grade V, heparin, sodium azide, and paraformaldehyde from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada); May Grunwald Giemsa (MGG) stain from BDH (Mississauga, Canada).

*Antibodies.* Phycoerythrin (PE)-conjugated $IgG_1$ CD34 antibody (HPCA-2), FITC-conjugated $IgG_1$ CD45 antibody (anti-HLE1), PE-conjugated isotype control antibody (i.e., anti-$IgG_1$-PE specific for keyhole limpet hemocyanin), and streptavidin-conjugated peridinin chlorophyll protein (PerCp) were purchased from Becton Dickinson, Canada (Mississauga, Ontario, Canada). Nonneutralizing monoclonal antibodies directed against the α-subunit of IL-3R (IL3Rα; 7G2) and IL-5R (IL5Rα; A16) were kind gifts from Dr. A. Lopez (Institute of Medical and Veterinary Science, Adelaide, Australia) and Roche Laboratories (Ghent, Belgium), respectively. Cytokine receptor antibodies and the isotype-matched controls were biotinylated using a long-arm biotin procedure (14) in which biotin was coupled to azide free protein via a hydroxysuccinimide ester after incubation with *N*-hydroxysuccinobiotin (Sigma-Aldrich Canada Ltd.); excess biotin was then removed by dialyses against borate buffered solution, pH 8.6.

*Subjects.* In an attempt to establish if the differential airway responses to allergen are reflected by different progenitor cell responses in the bone marrow, two groups of asthmatic subjects were examined based on their airway responses to allergen: (*a*) subjects ($n = 6$) who only developed an isolated early asthmatic response with no definite late asthmatic response and no change in airway responsiveness to methacholine after allergen challenge and, (*b*) subjects ($n = 7$) who developed both an early- and a late-asthmatic response after allergen inhalation together with increased airway responsiveness to methacholine (Table I). All subjects were atopic as determined by skin prick test positivity and were studied at a time when their asthma was mild, stable and treated by inhaled $\beta_2$-agonist only. All subjects were nonsmokers and had a baseline forced expiratory volume in one second ($FEV_1$) < 70% of the predicted normal on all study days (15) and none had had a respiratory tract infection for at least 4 wk before entering the study. The study was approved by the Ethics Committee of the McMaster University Health Sciences Centre, and each subject gave written informed consent.

*Study design.* Subjects attended the laboratory on three separate occasions. Visit 1: 1 wk before allergen challenge when documentation included a full medical history, skin prick test sensitivity to allergen extracts, spirometry, methacholine inhalation test, and induction of sputum to assess baseline airway inflammation. Visit 2: subjects underwent the allergen challenge procedure. Before allergen challenge, a bone marrow aspirate was collected and spirometry measurements were taken for 7 h after allergen inhalation in order to follow the allergen-induced bronchoconstrictor response. Blood samples were taken before and 5 h after allergen challenge to enumerate allergen-induced changes in the white blood cell count. Visit 3: blood, sputum, and bone marrow aspirates were collected 24 h after allergen challenge. Spirometry measurements and methacholine inhalation

*Table I. Subject Characteristics and Allergen-induced Airway Responses*

| | Age | Gender | Allergen | EAR | LAR | $PC_{20}$ Methacholine | |
|---|---|---|---|---|---|---|---|
| | | | | | | Pre-allergen | Post-allergen |
| | *yr* | | *Inhaled dilution* | *Max percent fall in $FEV_1$* | | *mg/ml* | |
| Isolated Early Responders | | | | | | | |
| 1 | 23 | F | HDM (1:32) | 25.0 | 5.4 | 2.48 | 8.00 |
| 2 | 22 | M | Ragweed (1:8) | 30.5 | −8.5 | 1.00 | 2.45 |
| 3 | 19 | F | HDM (1:64) | 31.5 | 11.1 | 0.27 | 0.13 |
| 4 | 23 | M | HDM (1:4) | 17.9 | 7.7 | 2.99 | 4.00 |
| 5 | 22 | F | HDM (1:128) | 25.0 | 11.8 | 0.13 | 0.50 |
| 6 | 30 | M | HDM (1:8) | 20.5 | 4.5 | 3.48 | 1.86 |
| Mean | | | | 25.1 | 8.2 | 1.73 | 2.82 |
| SEM | | | | 2.2 | 1.2 | 0.59 | 1.18 |
| Dual-Responders and Delayed Hypperresponsiveness | | | | | | | |
| 1 | 24 | M | HDM (1:512) | 25.0 | 40.8 | 0.17 | — |
| 2 | 22 | F | HDM (1:4096) | 21.2 | 21.2 | 1.80 | 0.29 |
| 3 | 26 | F | HDM (1:64) | 23.5 | 16.9 | 2.44 | 1.65 |
| 4 | 22 | M | Grass (1:2048) | 21.6 | 22.4 | 3.38 | 0.73 |
| 5 | 22 | M | Cat (1:128) | 21.1 | 15.5 | 3.31 | 1.00 |
| 6 | 31 | M | HDM (1:256) | 40.7 | 18.6 | 0.62 | 0.31 |
| 7 | 22 | M | HDM (1:256) | 29.4 | 23.5 | 1.30 | 0.39 |
| Mean | | | | 26.1 | 22.7* | 1.86 | 0.73‡ |
| SEM | | | | 2.7 | 3.2 | 0.47 | 0.20 |

EAR, early asthmatic response; LAR, late asthmatic response; $PC_{20}$, provocation concentration of methacholine causing a 20% fall in $FEV_1$; HDM, house dust mite. *$P < 0.001$ for between group comparisons of maximal % fall in $FEV_1$ during the LAR; ‡$P < 0.001$ for within group comparison of pre-allergen versus post-allergen log $PC_{20}$ values.

challenge were also performed to assess the development of airways hyperresponsiveness.

*Methacholine and allergen inhalation challenge.* Methacholine inhalation was performed as described by Cockcroft et al. (16). Spirometry was measured with a Collins water sealed spirometer and kymograph. The test was terminated when a fall in $FEV_1$ of 20% of the baseline value occurred, and the provocative concentration of methacholine causing a 20% fall in $FEV_1$ ($PC_{20}$) was calculated. Allergen challenge was performed as described by O'Byrne et al. (17). The early bronchoconstrictor response was taken to be the maximal percent fall in $FEV_1$ within 2 h after allergen inhalation and the late asthmatic response was taken to be the maximal percent fall in $FEV_1$ between 3 and 7 h after allergen inhalation.

*Sputum and blood differential counts.* Sputum was induced by saline inhalation and processed according to the method of Popov et al. (18). Cytospins of cell plugs collected from the sputum sample and processed using 0.1% dithiothreitol (Sputolysin; Calbiochem Corp., San Diego, CA) and Dulbecco's PBS (GIBCO BRL) were stained with Diff-Quik (American Scientific Products, McGaw Park, IL). Differential counts are expressed as the mean of duplicate slides (500 cells counted per slide). Venous blood was collected into ethylenediaminetetraacetic acid (EDTA)-treated tubes. Total cell counts were performed using a Neubauer hemocytometer and differential cell counts were made from blood smears stained by Diff-Quik. Differential cell counts were obtained from the mean of two slides (300 cells counted per slide) and cell populations were expressed as the absolute counts ($10^9$ cells per liter).

*Cells and myeloid cell lines: controls for cytokine receptor staining.* Both HL60 clone 15 cells and KG1 myeloid leukemic cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were cultured in RPMI 1640 medium (GIBCO BRL). The B9 cell line, an IL-6–dependent mouse B cell hybridoma, was obtained from Dr. Lucien Aarden (Red Cross Transfusion Services, Amsterdam, The Netherlands) and cultured in RPMI 10 medium plus recombinant human IL-6 (19). Peripheral blood-derived neutrophils and eosinophils were isolated by positive and negative selection, respectively, using a CD16 dependent magnetic cell separation technique (20) and monocytes were isolated by collecting the adherent cell population after adherence of low density mononuclear cells to plastic, as described below.

*Preparation of bone marrow cells and immunofluorescence staining.* Heparinized (1,000 U/ml) samples of bone marrow (2–3 ml) were aspirated from the iliac crest and low density mononuclear cells (MNC) were isolated by sedimentation on Percoll density gradients (specific gravity 1.077) as described previously (9, 21). Monocytes were depleted from the MNC fraction by incubation in plastic flasks for 2 h at 37°C. Samples of $1 \times 10^6$ nonadherent mononuclear cells (NAMNC) in a final volume of 100 µl of ice-cold PBS containing 0.1% $NaN_3$ and 0.5% BSA (PAB) were stained with saturating amounts of biotin-conjugated anti–IL-3Rα, anti–IL-5Rα, or $IgG_1$ isotype control antibody (determined in preliminary studies) for 30 min at 4°C. The cells were then washed and stained with streptavidin-conjugated PerCp, together with saturating concentrations of anti-CD45 FITC and anti-CD34 PE or $IgG_1$ isotype control in a final volume of 100 µl of PAB for 30 min at 4°C. The cells were then washed with 3 ml of PBS plus 0.1% azide, fixed in 500 µl of PBS plus 1% paraformaldehyde, and refrigerated until ready for analysis.

*Flow cytometry and gating strategy.* Cells were analyzed using a FACScan flow cytometer equipped with an argon ion laser (Becton Dickinson Instrument Systems, BDIS, Mississauga, Canada). Five data parameters were acquired and stored in listmode files: linear forward light scatter (FSC), linear side-angle light scatter (SSC), log FITC, log PE, and log PerCp fluorescence; each measurement contained 50,000 events. Compensation settings were established using CalBrite beads (BDIS) and confirmed using NAMNC stained with anti–CD34-PE, anti–CD45-FITC, or anti–IL-5Rα–PerCp. Off-line analysis was performed using the PC lysis software as supplied by BDIS.

We used a multi-parameter sequential gating strategy that, we have previously shown, accurately enumerates $CD34^+$ progenitor cell numbers in various biological samples (22, 23). The rationale for sequential gating was to gradually eliminate contaminating cells that nonspecifically take up anti-CD34 (24). Briefly, a primary gate using CD45 staining versus SSC (region R1) was set up to quantitate total leukocytes and distinguish contaminating events such as platelet aggregates and other debris which can nonspecifically take up anti-CD34 (Fig. 1 *A*, *PLOT 1*). Primitive cells characteristically express CD45 at low to intermediate levels (25) and therefore $CD45^+$ events generate a stable denominator in the calculation of the absolute $CD34^+$ value. Sequential gates were then set up: CD34 staining in region R1 versus SSC (region R2) (Fig. 1 *A*, *PLOT 2*), CD45 versus SSC of the $CD34^+$-gated events in R2 (region R3: to identify blast cells) (Fig. 1 *A*, *PLOT 3*), and FSC versus SSC to confirm the lymphoblastoid characteristics of the gated $CD34^+$ cells in region R3 (i.e., low to medium SSC and FSC: region R4) (Fig. 1 *A*, *PLOT 4*). Without changing any of the gates, analyses of the same cell sample stained with CD45-FITC and PE linked isotype control antibody were performed (Fig. 1 *B*, *PLOT 3* and *PLOT 4*). Enumeration data were derived from the gate statistics: events in gate G4 (= events in R1 to R4) after staining with CD45-FITC/CD34-PE minus events in G4 stained with CD45-FITC/PE–linked control antibody were used to calculate the absolute number of true $CD34^+$ blast cells in the test sample.

In three-color analysis, events in region R4 were back scattered onto a dot plot of CD34-PE versus staining by PerCp linked cytokine receptor mAbs or control antibody (Fig. 2), and data were collected as percent positive cells at the 99% confidence limit (i.e., relative to a marker set to include only 1% of cells stained with control antibody). The data presented are the mean of duplicate assessments. The intra-assay variability was always less than 5%.

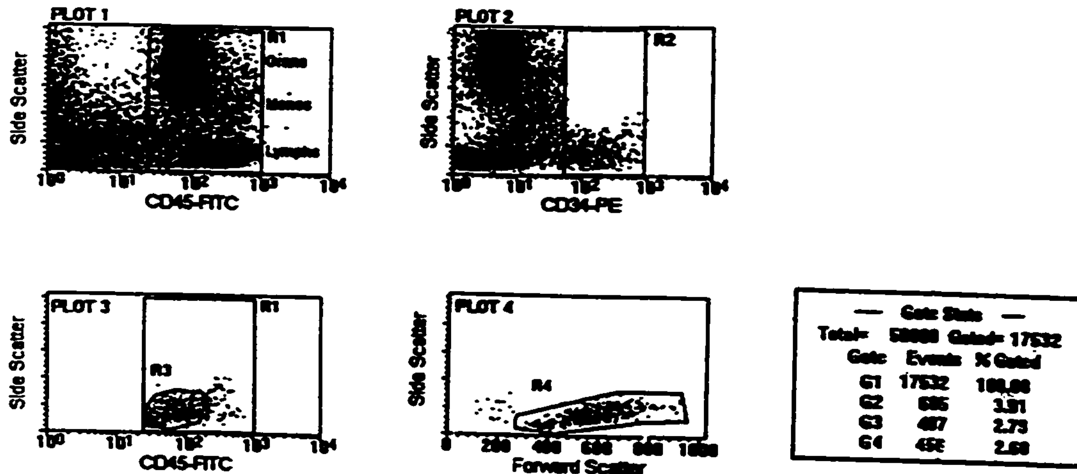
*Simultaneous in situ hybridization and immunohistochemistry.* To confirm the association of the membrane bound form of IL-5Rα messenger RNA to $CD34^+$ progenitor cells, simultaneous in situ hybridization and immunocytochemistry was performed (26). Messenger RNA for membrane bound IL-5Rα was detected by autoradiography and CD34 immunoreactivity was detected by an alkaline phosphatase antialkaline phosphatase technique (APAAP). A population of $CD34^+$ cells was enriched from cord blood by positive selection using a magnetic cell separation technique, as described previously (13). These cells were cytospun on poly-L-lysine–coated slides, fixed in 4% paraformaldehyde in PBS for 30 min, and washed in 15% sucrose in PBS. Preparations were hybridized with $^{35}S$- labeled membrane-bound IL-5Rα antisense riboprobe and simultaneously immunostained with a mouse anti–human mAb against CD34 (QBEND 10: Becton Dickinson, San Jose, CA) (26).

*Statistical analysis.* The data are presented as absolute numbers of $CD34^+$ progenitor cells (Fig. 3) and as arithmetic mean±SEM (Fig. 4, Tables I and II), except $PC_{20}$ values (Table II) that were logarithmically transformed and expressed as geometric means and standard error of geometric means (percent SEM). For statistical analyses of within group comparisons between pre- and post-allergen challenge time points, a paired Student's *t* test (two-tailed) was performed (Table I, Figs. 3 and 4). Student's non-paired *t* tests (two-tailed) were performed for all between group comparisons (Table I) and changes in blood differentials after allergen were assessed for each group using repeated measures analysis of variance (rmANOVA) (Table II). Significance was accepted at the 95% confidence level.

## Results

*Allergen-induced bronchoconstrictor responses and airway hyperresponsiveness.* Subjects ($n = 6$) in whom the maximal percent fall in $FEV_1$ during the late asthmatic response was < 15% were labeled isolated early responders (IER; mean percent fall in $FEV_1$, 8.2±1.2%). Subjects in whom that late maximal percent fall in FEV1 was > 15% were labeled as dual responders

A Staining with CD45-FITC/ CD34-PE




B Staining with CD45-FITC/ $IgG_1$-PE

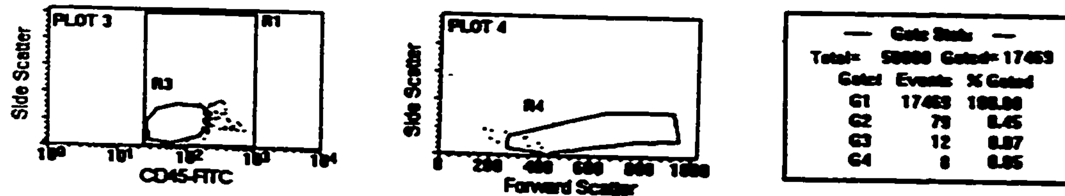



*Figure 1.* Enumeration of CD34⁺ progenitor cells from bone marrow samples taken from an atopic asthmatic subject. (*A*) Plots 1–4 represent staining of bone marrow–derived low density nonadherent mononuclear cells with CD45 FITC/CD34 PE. (*B*) Represents staining of low density NAMNC with CD45 FITC/isotype $IgG_1$ PE. Precise details of the gating strategy are described in Methods. Identical gating regions to those shown in (*A*) were used to analyze the staining of the same bone marrow sample with the control reagents (*B*).

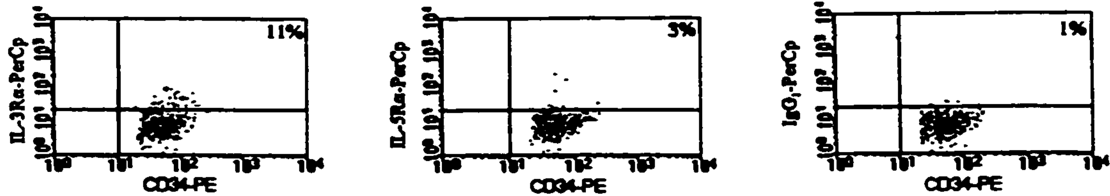
(DR; mean percent fall in $FEV_1$, 22.7±3.2%). The dual responders, but not the isolated early responders, developed a significant increase in methacholine airway responsiveness 24 h after allergen challenge ($P < 0.001$, Table I).

*Airway and blood eosinophilia.* A significant increase in sputum eosinophilia was detected in DR asthmatics but not in the IER group, when pre-allergen levels were compared with 24 h post-allergen values (Table II). Similarly, a significant increase in blood eosinophil levels was observed between 5 and 24 h post-allergen in DR asthmatics ($P < 0.05$), although this was preceded by a significant reduction in eosinophil numbers 5 h post-allergen compared with baseline values ($P < 0.05$, Table II). In contrast, in IER, no significant change in the numbers of blood eosinophils was observed at either 5 or 24 h post-allergen compared with pre-allergen values.

*IL-3Rα– and IL-5Rα-subunit expression in various cell types.* To verify the specific binding capacity of the cytokine receptor antibodies used in this study, we tested the staining of anti–IL-3Rα (7G2) and anti–IL-5Rα (A16) on various leukemic cell lines and mature peripheral blood leukocytes. Compared with the isotype matched control antibody, the significant expression of the IL-3Rα–subunit was detected on KG1 cells [Fig. 3 *A (i)*] and peripheral blood monocytes [Fig. 3 *A (iii)*] (27). As expected, no expression of IL-3Rα was detected on B9 cells, an IL-6–dependent mouse B cell hybridoma cell line [Fig. 3 *A (ii)*] or on mature neutrophils [Fig. 3 *A (iv)*] (27). The expression of IL-5Rα was detected on HL60 clone 15 cells, known to overexpress receptors for IL-5 (8), and on peripheral blood eosinophils (Fig. 3 *B*). No specific expression of IL-5Rα was detected on either neutrophils [Fig. 3 *B (iv)*] or monocytes (data not shown) (28).

*Allergen-induced changes in the phenotype of bone marrow progenitors.* By multiparameter flow cytometric analyses, allergen-induced changes in the absolute numbers of bone marrow–derived CD34⁺ progenitor cells and the absolute numbers of CD34⁺ cells expressing the α-subunit of IL-3R and IL-5R were investigated. In DR asthmatics, the total number of bone marrow–derived CD34⁺ cells increased from 3,715 cells/0.25 × $10^6$ white blood cells (WBC) (percent SEM 830) before allergen to 5,623 cells/0.25 × $10^6$ WBC (percent SEM 2,627) 24 h

**A Pre-Allergen**




**B 24h post-Allergen**

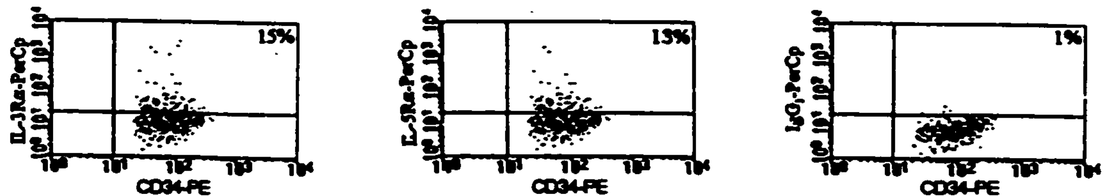



*Figure 2.* Enumeration of bone marrow–derived CD34$^+$ cells that express the α-subunit of IL-3R and IL-5R. Samples of bone marrow–derived NAMNC collected pre- and 24 h post-allergen from an atopic asthmatic subject were stained with CD45 FITC/CD34 PE and either PerCp linked IL-3Rα, IL-5Rα, or isotype control antibody. Events in R4 (see Fig. 1 *A, Plot 4*) were backscattered onto a dot-plot of CD34 PE versus PerCp staining. Quadrant statistics (i.e., data in upper right hand corner) are presented as the percent of total CD34$^+$ cells that demonstrated positive staining with anti–IL-3Rα, anti–IL-5Rα, or mouse $IgG_1$ isotype matched control antibody. Comparisons between pre- and post-allergen bone marrow samples demonstrate that increased numbers of CD34$^+$ cells express IL-3Rα and IL-5Rα.

after allergen challenge, although these changes were not significant ($P = 0.06$) (Fig. 4). In IER, no differences in CD34$^+$ cell numbers was detected when pre-allergen levels (2,291 cells/ 0.25 × $10^6$ WBC, percent SEM 1,009) were compared with 24 h post-allergen levels (2,570 cells/0.25 × $10^6$ WBC, percent SEM 782, $P = 0.70$) (Fig. 4). No significant differences in either the baseline values of bone marrow CD34$^+$ cells or the magnitude of increase of CD34$^+$ cells after allergen challenge were detected between the two groups of asthmatic subjects.

In DR asthmatics, the absolute number of bone marrow–derived CD34$^+$ cells expressing IL-5Rα increased 30-fold from 26 cells/0.25 × $10^6$ WBC (percent SEM 3.3) before allergen to 724 cells/ 0.25 × $10^6$ WBC (percent SEM 1.6) 24 h after allergen ($P = 0.04$) (Fig. 4). In contrast, there was a negligible increase in the numbers of CD34$^+$IL-5Rα$^+$ cells in IER when pre-allergen levels (6 cells/0.25 × $10^6$ WBC, percent SEM 2.7) were compared with post-allergen levels (11 cells/0.25 × $10^6$ WBC, percent SEM 2.7, $P = 0.55$) (Fig. 4). Although there was a ninefold increase in the absolute number of bone marrow CD34$^+$IL-3Rα$^+$ cells in DR asthmatics when pre-allergen levels were compared with 24 h post-allergen levels, these changes were not significant (37 cells/0.25 × $10^6$ WBC versus 324 cells/ 0.25 × $10^6$ WBC, $P = 0.051$) (Fig. 4). In addition, no significant increase in the absolute numbers of bone marrow CD34$^+$IL-3Rα$^+$ cells was detected in IER 24 h post-allergen (Fig. 4).

It is conceivable that the observed increases in absolute numbers of cytokine receptor positive progenitor cells 24 h post-allergen may be entirely due to increases in total CD34$^+$ cell numbers (Fig. 4). In order to exclude this effect and to determine whether a distinct phenotypic change of a fixed progenitor cell pool had occurred after allergen challenge, we expressed the data as a percent of total CD34$^+$ cell numbers (Fig. 5). In DR, but not IER asthmatics, a significant increase in the percent of CD34$^+$ cells expressing IL-5Rα was detected when pre-allergen bone marrow samples were compared with 24 h post-allergen aspirates (DR: 6.6±3.1 versus 15±2.7% of CD34$^+$ cells, $P = 0.045$, and IER: 1.9±1.08 versus 2.2± 1.2% of CD34$^+$ cells, $P = 0.84$) (Fig. 5). In contrast, in both groups of asthmatic subjects no significant increase in the proportion of CD34$^+$ cells expressing IL-3Rα, after allergen challenge, was observed (DR: 8.5±3.4 versus 15±4.5% of CD34$^+$ cells, $P = 0.22$, and IER: 2.7±1.2 versus 9.2±3.5% of CD34$^+$ cells, $P = 0.19$) (Fig. 5). In comparisons between the two groups of asthmatics, no significant differences were observed in the baseline values of percent CD34$^+$ cells expressing either cytokine receptors (Fig. 5). However, allergen-induced increases in the proportion of CD34$^+$ cells expressing IL-5Rα$^+$ cells were significantly greater in dual responders compared with isolated early responder asthmatics ($P = 0.038$).

*In situ hybridization and immunohistochemistry.* Colocalization experiments using in situ hybridization and immunocytochemistry were performed in order to confirm that CD34$^+$ progenitor cells can express IL-5Rα mRNA. Because of the small sample sizes of bone marrow aspirates, these experiments were performed on an enriched population of unstimulated CD34$^+$ cells isolated from cord blood (purity determined by flow cytometry, > 65%). Of all the CD34$^+$ cells, 50% were IL-5Rα mRNA positive. A representative example is shown in Fig. 6.

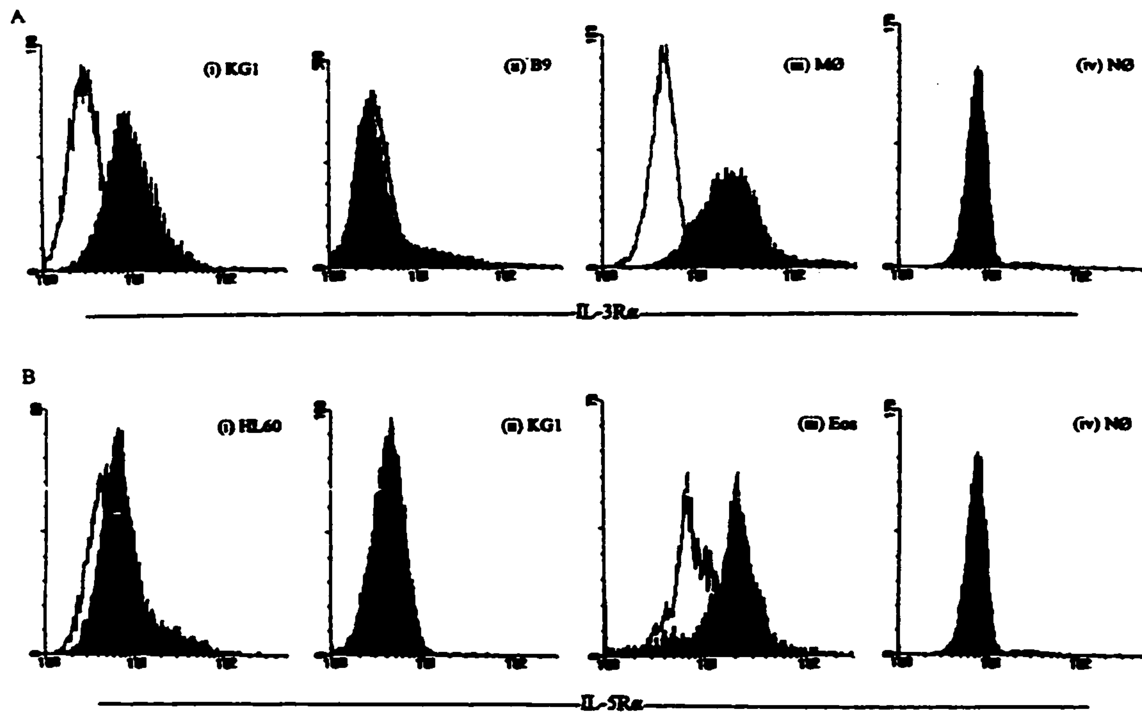



*Figure 3.* Expression of IL-3Rα and IL-5Rα on various cell types. Cells were incubated with saturating amounts of the biotinylated antireceptor antibodies, washed and incubated with streptavidin-linked PerCp (*shaded area*). For negative control (*blank area*), cells were stained with the equivalent amount of the isotype matched biotinylated antibody (mouse $IgG_1$) in place of the antireceptor antibody.

## Discussion

The novel observation from this study is that the proportion of bone marrow–derived $CD34^+$ cells expressing IL-5Rα is increased preferentially in DR asthmatics who characteristically develop allergen-induced late-bronchoconstrictor responses, methacholine airways hyperresponsiveness, and a significant sputum eosinophilia 24 h after allergen inhalation (Fig. 5, Tables I and 2) (2, 29). In contrast, this distinct change in cytokine receptor expression on bone marrow progenitor cells was not seen in asthmatics who did not develop airway hyperresponsiveness or a marked eosinophil infiltration after allergen inhalation (Fig. 5). Although the data presented herein do not prove a direct association between activation of the bone marrow and development of airway pathology as a result of increased inflammatory cell production, they are consistent with the view that a feedback mechanism exists between tissues involved in allergic inflammation and distal sites such as the

*Table II. Inflammatory Cell Count in Sputum and Blood Samples from Asthmatic Subjects*

| | Early single responders | | | Dual responders | | |
|---|---|---|---|---|---|---|
| | Pre-allergen | 5 h Post-allergen | 24 h Post-allergen | Pre-allergen | 5 h Post-allergen | 24 h Post-allergen |
| Sputum | | | | | | |
| Eosinophils (percent) | 6.7±2.0 | ND | 16.4±9.9 | 3.3±0.9 | ND | 32.3±11.7* |
| Neutrophils (percent) | 15.7±4.3 | ND | 28.4±10.2 | 26.0±10.5 | ND | 26.9±11.8 |
| Macrophages (percent) | 72.2±4.1 | ND | 48.0±13.5 | 66.3±10.1 | ND | 36.1±8.6‡ |
| Blood | | | | | | |
| Eosinophils ($\times 10^9$/liter) | 0.35±0.04 | 0.36±0.20 | 0.46±0.50 | 0.44±0.12 | 0.23±0.05* | 0.32±0.06§ |

Data for sputum cell counts are presented as the percentage of 500 white cells counted per slide. Cells were stained with Diff-Quik. Data represent the mean±SEM of $n = 6$ early single responders and $n = 7$ dual responders. *$P < 0.05$; ‡$P < 0.001$ for comparisons of pre-allergen versus post-allergen challenge values; §$P < 0.05$ for 5 h post-allergen versus 24 h post-allergen comparisons.

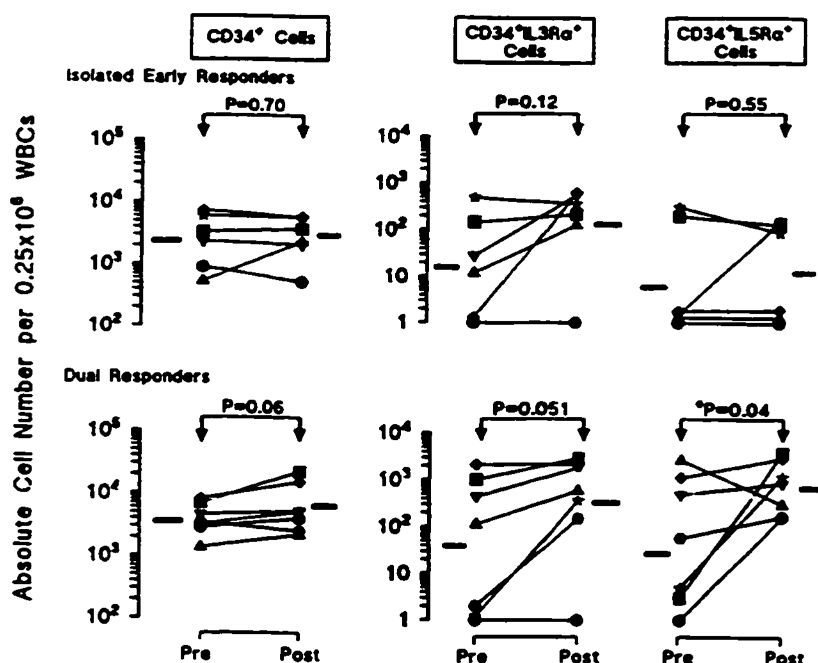



*Figure 4.* Flow cytometric enumeration of the absolute number of bone marrow derived CD34$^+$ progenitor cells expressing the α-subunit of receptors for IL-3 and IL-5. Samples from ($n$ = 6) isolated early- and ($n$ = 7) dual-responder asthmatics were taken pre- and 24 h post-allergen challenge. After allergen challenge, a significant increase in the numbers of CD34$^+$ IL-5Rα$^+$ cells were detected in bone marrow samples taken from DR asthmatics. Horizontal bars represent the geometric mean of each data set.

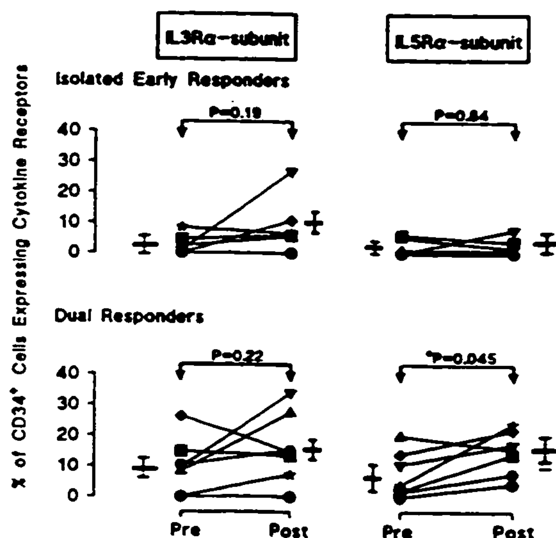



*Figure 5.* Percent of bone marrow derived CD34$^+$ cells that express the α-subunit of receptors for IL-3 and IL-5. Samples from isolated early- ($n$ = 6) and dual-responder ($n$ = 7) asthmatics were taken pre- and 24 h post-allergen challenge. After allergen challenge, a significant increase in the percent of CD34$^+$ cells expressing IL-5Rα, but not IL-3Rα, was observed in bone marrow samples taken from dual responder asthmatics but not isolated early responders. Horizontal bars represent the arithmetic mean of each data set.

bone marrow. We have shown that a distinct phenotypic switch occurs within the bone marrow progenitor cell population and we suggest that in the presence of eosinophil growth factors such as IL-5, increased expression of IL-5Rα on CD34$^+$ cells may favor eosinophilopoiesis and thus contribute to the subsequent development of blood and tissue eosinophilia, a hallmark of allergic inflammatory diseases such as asthma.

Of the cytokines which can support eosinophilopoiesis (IL-5, IL-3, and GM-CSF), IL-5 is unique in its ability to specifically promote the terminal differentiation and maturation of eosinophil/basophil lineage-committed progenitors in liquid and semi-solid cultures (30, 31). In mice that overexpress the IL-5 transgene, IL-5 has been shown to be the predominant regulator of eosinophilia (32). Furthermore, a pivotal role for IL-5 in chronic allergic inflammation has been confirmed by the capacity of neutralizing anti–IL-5 mAb to inhibit antigen- or virus-induced airway hyperresponsiveness and eosinophil infiltration in the airways of mice, guinea pigs, and primates (33–39). In contrast, IL-3 is a pluripotential hemopoietic factor; mice that overexpress either IL-3 or GM-CSF, show only modest eosinophilia, but succumb early owing to massive tissue infiltration and destruction by myeloid cells, especially neutrophils and macrophages (32). Our evidence suggests that both GM-CSF and IL-3 commit pluripotential CD34$^+$ CD33$^-$ progenitors to an eosinophil lineage and that IL-5 brings about the terminal differentiation of the less primitive, myeloid lineage-committed progenitor cells (i.e., CD34$^-$CD33$^+$ cells) derived from CD34$^+$ CD33$^-$ precursors (40). In this study, however, we have demonstrated for the first time the expression of IL-5Rα on CD34$^+$ cells indicating the existence of specific binding sites for IL-5 on more primitive progenitor cells. This

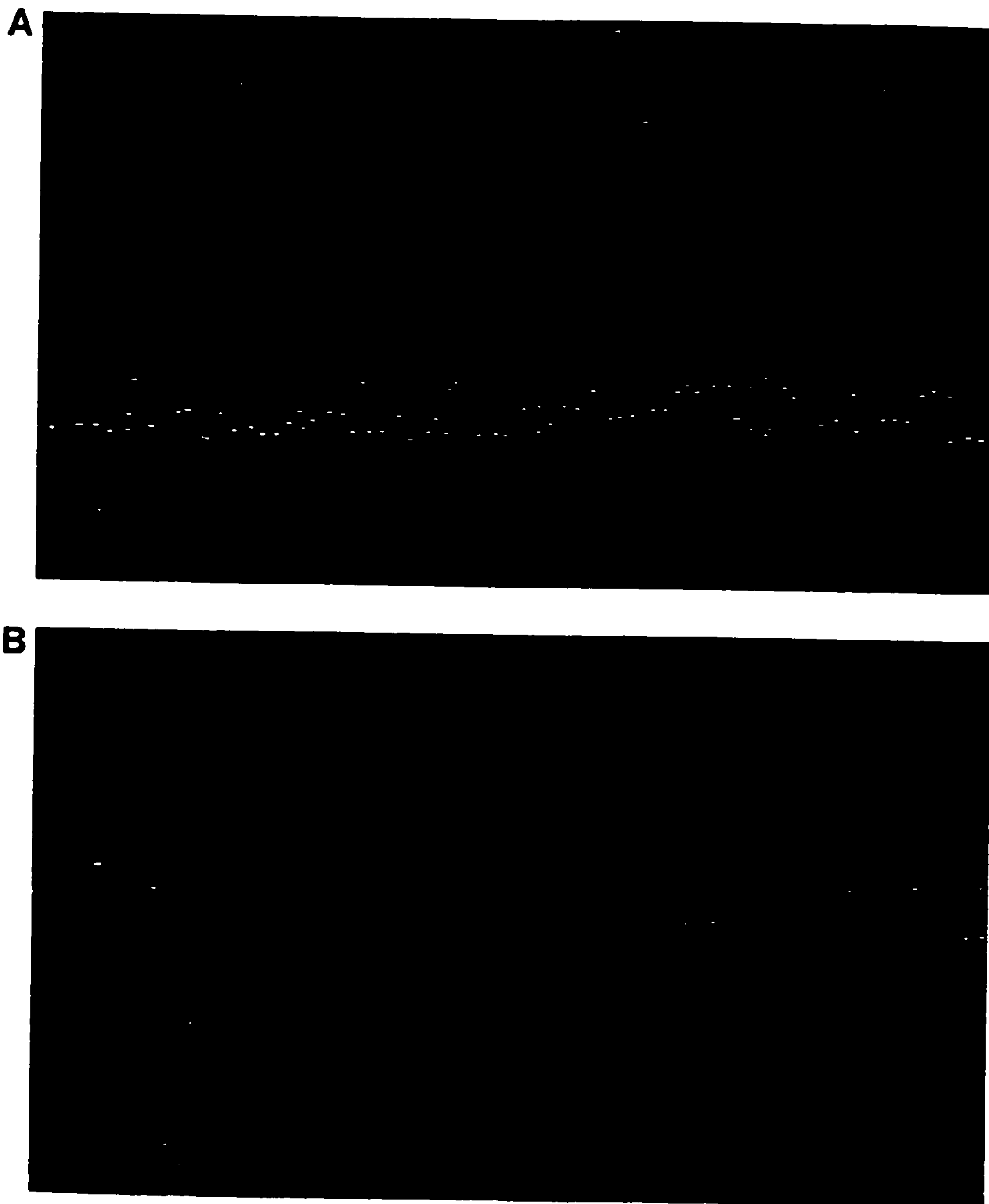



*Figure 6.* **Detection of mRNA for membrane bound form of IL-5Rα in human cord blood–derived CD34⁺ cells. (*A* and *B*) CD34 enriched cell preparations were hybridized with ³⁵S-labeled membrane bound IL-5Rα antisense riboprobe and simultaneously immunostained with a mouse anti-human CD34 (QBEND10). (*A*) Messenger RNA for membrane bound IL-5Rα was detected by autoradiography and visualized by dark field illumination (*arrow*). (*B*) Subcellular localization of membrane bound IL-5Rα mRNA in CD34⁺ cells immunostained simultaneously with mouse anti-human CD34 and APAAP and viewed by bright field illumination (*arrows*).**

is supported by results from in situ hybridization which demonstrate the colocalization of mRNA for membrane bound IL-5Rα to cells immunostained with anti-CD34. Therefore, we propose that the CD34⁺ IL-5Rα⁺ phenotype may be representative of the earliest eosinophil/basophil lineage-committed progenitor. However, until additional cloning experiments have been performed to assess the progeny of progenitors of this specific phenotype, this proposal cannot be confirmed.

Molecular cloning of cytokine receptors have revealed that IL-3R, IL-5R, and GM-CSFR are uniquely composed of heterodimeric structures consisting of a distinct α-subunit that binds the cognate cytokine with low affinity and a common, shared, β-subunit which, although failing to bind the ligand itself, forms high affinity cytokine binding sites in association with the α-subunit (41, 42). Deletion mutation experiments of IL-5R have now revealed that, like the β-subunit, the cytoplasmic domain of the α-subunit is also essential for signal transduction, in particular mediating growth signals through IL-5R (43, 44). Since the α-subunit functions as a cytokine-specific binding site, it has been proposed that this subunit may transduce cytokine-specific growth signals while the common β-chain provides the molecular basis for functional redundancy of IL-3, IL-5, and GM-CSF. Therefore, the preferential increase in the proportion of bone marrow-derived CD34⁺ cells expressing membrane bound IL-5Rα-subunit on bone marrow progenitor cells may increase the ability of the cells to respond more readily to IL-5, and thus differentiate terminally into mature eosinophils and basophils. In support of our findings, Wood et al. have shown that bone marrow aspirates taken from DR 24 h post-allergen are more responsive to IL-5 in vitro, as determined by the significantly greater numbers of Eo/Baso-CFU detected in methylcellulose cultures with suboptimal doses of IL-5 compared with bone marrow cells from IER (45).

Since increases in expression of cytokine receptors on CD34⁺ cells were detected within 24 h after allergen challenge in DR asthmatics, these changes may have occurred as a consequence of cell division and proliferation (Fig. 4). However, when expressed as a percentage of total CD34⁺ cells, the increase in IL-5Rα, but not in IL-3Rα, expression on CD34⁺ cells was independent of changes in the number of progenitors (Fig. 5). This indicates that after allergen challenge in DR asthmatics, a fixed pool of bone marrow CD34⁺ progenitors undergo a distinct phenotypic change resulting in increases in IL-5Rα surface expression. In contrast, the increased production of small amounts of primitive progenitor cells may account for the near significant increases in absolute numbers of CD34⁺IL-3Rα cells observed in DR 24 h post-allergen (Fig. 4) (27).

Evidence of the generation of a serum hemopoietic factor during airway allergen challenge that can prime the bone marrow for increased production of granulocyte progenitor cells has been recently demonstrated in a canine model of airways hyperresponsiveness (46). Similarly, increased numbers of Eo/Baso-CFU were grown from the peripheral blood of atopic individuals when antigen-stimulated lymphomononuclear cell conditioned medium was included in colony assays, suggesting the generation of a hemopoietic signal after in vitro allergen challenge (5). Furthermore, in studies of nematode infection in IL-5 transgenic mice, Strath et al. have provided evidence that the level of blood eosinophilia may not only be controlled by the amount of IL-5 produced but, in addition, by the frequency of eosinophil progenitors in the bone marrow during a chronic inflammatory response (47). Thus, investigation of the nature of the signal(s) that modulate the expression of IL-5R on CD34⁺ progenitor cells may provide insight into the control of eosinophil differentiation from pluripotential stem cells and, potentially provide a novel therapeutic target for controlling the development of the eosinophilic component of the allergic inflammatory response in asthmatic airways. From in vitro studies, little is currently known regarding the modulation of IL-5R expression on normal progenitor cells. Preincubation of peripheral blood CD34⁺ cells with GM-CSF and IL-3 enhances their subsequent ability to differentiate into eosinophils in response to IL-5 (48). While this finding implies upregulation of IL-5R expression, this has not been formally demonstrated, at least not for progenitor cells. On mature eosinophils, preincubation with GM-CSF, but not IL-3, will enhance expression of IL-5R (49). Downregulation of the IL-5Rα-subunit at the mRNA level in myeloid leukemic cell lines has been shown to be due to factors that either promote eosinophil apoptosis such as $TGF\beta_1$ (50), or by pharmacological agents, such as all-trans retinoic acid (RA), which inhibit eosinophil/basophil differentiation from pluripotential progenitor cells, while favoring neutrophil maturation (51, 52). This is further support for the view that the level of expression of IL-5Rα on CD34⁺ cells may be directly related to commitment to the eosinophilopoietic pathway.

In conclusion, the results from this study demonstrate that relevant fluctuations occur in the expression of a specific cytokine receptor, IL-5Rα, on bone marrow progenitors in response to allergen challenge in atopic asthmatics. We propose that this selective increase in expression of IL-5Rα on CD34⁺ progenitors may favor eosinophilopoiesis that may play a role in the generation of increased numbers of eosinophils during an allergic inflammatory response in the airways of asthmatic subjects.

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# CHAPTER 4

# ALLERGEN CHALLENGE INCREASES CELL TRAFFIC BETWEEN BONE MARROW AND LUNG

Published in the American Journal of Respiratory Cell and Molecular Biology in 1998

Lorna Wood's contribution:

Experimental design
Collection of clinical data
Processing of laboratory samples
Identification and quantification of colonies
Analysis of data
Preparation of manuscript

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# Allergen Challenge Increases Cell Traffic between Bone Marrow and Lung

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Increases in inflammatory-cell progenitors have been demonstrated in the bone marrow (BM) after inhalation of *Ascaris suum* in dogs at the time of allergen-induced airway hyperresponsiveness (AHR). The aim of this study was to evaluate the effect of allergen challenge on trafficking of inflammatory cells and their progenitors from the BM to the lung, using a marker of proliferating cells, bromodeoxyuridine (BrdU). BrdU is a thymidine analogue taken up by the DNA of dividing cells, and can be detected with immunohistochemistry (IHC). The development of AHR was assessed through acetylcholine (ACh) airway responsiveness before and after allergen inhalation. Two groups of dogs were matched for the degree of AHR after a screening allergen challenge. On the study day, one group inhaled allergen ($n = 8$) and one group inhaled diluent ($n = 8$). All dogs received equal bolus injections of BrdU before and at 5 h after challenge. Blood samples were taken before challenge and at 5 h and 24 h after challenge, and BM aspirate and bronchoalveolar lavage (BAL) samples were taken 24 h after challenge. BrdU-positive cells were detected in cytospin preparations of these samples, using IHC. Allergen inhalation caused AHR ($P < 0.05$) at 24 h after allergen challenge, and also an increase in BrdU-positive cells in blood, which was 5.7 ± 0.6% (mean ± SEM) after allergen challenge and 2.5 ± 0.7% after diluent ($P < 0.005$); in BM the increase in BrdU-positive cells was 27.0 ± 3.4% after allergen challenge and 18.9 ± 3.2% after diluent ($P = 0.1$); and in BAL the increase was 3.2 ± 0.4% after allergen challenge and 0.8 ± 0.3% after diluent ($P < 0.005$). There was a significant correlation between the number of BAL neutrophils and the percentage of BrdU-positive BAL cells ($r^2 = 0.54$, $P < 0.05$). These results demonstrate an allergen-induced increase in proliferating cells, probably in the BM, and indicate that such cells traffic through the circulation into the lungs in response to allergen inhalation. Wood, L. J., M. D. Inman, J. A. Denburg, and P. M. O'Byrne. 1998. Allergen challenge increases cell traffic between bone marrow and lung. Am. J. Respir. Cell Mol. Biol. 18:759–767.

An important feature of asthma is the development of airway inflammation (1, 2), which is associated with the presence of several cell types such as eosinophils, neutrophils, metachromatic cells, and lymphocytes (3–6). In addition, airway inflammation is related to the presence of airway hyperresponsiveness (AHR), another important characteristic of asthma (7, 8).

We have previously provided evidence that a potentially important aspect of allergic inflammatory responses is the induction of increases in inflammatory-cell progenitors, which may then contribute to disease through the ongoing, enhanced production of inflammatory effector cells (9, 10). Larger numbers of both circulating eosinophil/basophil colony-forming units (Eo/B-CFU) and CD34+ hemopoietic progenitor cells are demonstrable in the blood of atopic subjects than in that of normals (9, 11). In addition, the numbers of Eo/B-CFU in the circulation of asthmatic subjects at the time of an acute exacerbation are significantly greater than those measured after resolution of the exacerbation (12). Studies with atopic subjects have shown that there are fluctuating numbers of Eo/B-CFU during seasonal exposure to allergen (13, 14) and significantly larger numbers 24 h after allergen inhalation (15). Additionally, bone-marrow (BM) inflammatory progenitors are significantly increased after allergen challenge both in humans and dogs (16, 17). In humans, Eo/B-CFU are significantly increased in subjects with mild asthma after allergen inhalation (16), whereas in dogs with allergen-induced AHR and airway inflammation, granulocyte-macrophage colony forming units (GM-CFU) are increased (17), which is reflected by the predominant neutrophilia seen in bronchoalveolar lavage fluid (BALF) after allergen challenge (18). In addition, the increase in BM GM-CFU has been shown to be associated with the production of a serum hemopoietic factor generated after allergen challenge (18). Although these studies demonstrate increased production of inflammatory progenitor cells in association with allergen inhalation, little is known about the release and trafficking of these cells and their progeny in response to allergen challenge.

The development of a monoclonal antibody to the thymidine analogue 5'-bromo-2'-deoxyuridine (BrdU) (19)

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*Abbreviations:* airway hyperresponsiveness, AHR; alkaline phosphatase-anti-alkaline phosphatase, APAAP; bone marrow, BM; bromodeoxyuridine, BrdU; immunohistochemistry, IHC; Tris-buffered saline, TBS.

has been a major development in studies of cell kinetics. BrdU is incorporated into the nuclei of S-phase cells (20), and can be identified by immunohistochemical staining with anti-BrdU antibody (21, 22). In the present study, we used BrdU to label proliferating myeloid and other hemopoietic cells *in vivo* in a canine model of allergen-induced AHR, to evaluate the effect of allergen challenge on trafficking of these, and by implication inflammatory cells and their progenitors, from the BM through the circulation and into the lungs.

## Materials and Methods

### Study Design

Two groups of dogs (random-source mongrels of 21 to 36 kg) were paired for study, based on changes in airway responsiveness after a screening inhalation of *Ascaris suum* extract. After a 4-wk period, one group inhaled *A. suum* ($n = 8$) and the other inhaled *A. suum* diluent ($n = 8$). Prior to challenge, baseline measurements of airway responsiveness to acetylcholine (ACh) were made. All dogs received a bolus injection of BrdU before and at 5 h after challenge. Blood samples were taken before challenge and at 5 h and 24 h after challenge, and BM aspirate and BALF samples were taken 24 h after challenge. Development of AHR was assessed by changes in airway responsiveness to ACh at 24 h after challenge.

### Procedures

Dogs were anesthetized with intravenous pentobarbitol sodium (30 mg/kg; Somnotol; MTC Pharmaceuticals, Mississauga, Ontario, Canada). Additional anesthetic was administered as required during the experiment. An endotracheal tube (10 mm inner diameter) was inserted and connected to a constant-volume ventilator (Model 551; Harvard Apparatus, South Natick, MA) set at a $V_T$ of 10 ml/kg and a rate of 30 breaths/min. An esophageal balloon catheter was inflated as previously described (23), and was placed in the esophagus at the point of most negative end-expiratory pressure. The esophageal catheter and a port at the proximal end of the endotracheal tube were connected to a differential pressure transducer (Model 267; Hewlett Packard, Inc., Palo Alto, CA).

### Measurement of Total Pulmonary Resistance

Total pulmonary resistance ($R_L$) was measured as described by Woolley and colleagues (17). Briefly, transpulmonary pressure was measured as the differential pressure between the endotracheal tube and the esophageal pressure (Pes). Flow was measured with a pneumotachometer (Fleisch No. 1; Instrumentation Associates, New York, NY), a differential pressure transducer (Hewlett Packard 270), and a pressure amplifier (Hewlett Packard 8805C). A continuous measurement of total $R_L$ was computed from the flow and transpulmonary pressure, using a respiratory analyzer (Hewlett Packard 8816A) that utilizes the method described by Mead and Whittenberger (24).

### Measurement of Airway Responsiveness

Airway responsiveness was determined as described by Woolley and associates (17). Briefly, a dose–response relationship of $R_L$ against doubling concentrations of ACh (0.7 to 80.0 mg/ml; Sigma Chemicals, St. Louis, MO) was established. After baseline $R_L$ was measured, the dogs inhaled normal saline and then increasing concentrations of ACh at 5 min intervals until an increase was obtained of at least 5 cm $H_2O$/liter/s above the postsaline value. The response was expressed as the concentration of ACh causing an increase in $R_L$ of 5 cm $H_2O$/liter/s above the baseline measurement, which was termed the ACh provocative concentration. A decrease in this value represents an increase in airway responsiveness.

### Allergen/Diluent Challenge

Allergen challenges involved inhalation of *A. suum* (stock extract, $10^{-1}$, wt/vol; Greer Laboratories, Lenoir, NC) as previously described (18). During the initial screening challenge, increasing concentrations of *A. suum* ($10^{-5}$, $10^{-4}$, $10^{-3}$, $10^{-2}$, and $10^{-1}$, wt/vol) were inhaled until $R_L$ increased by 10 cm $H_2O \cdot liter^{-1} \cdot s^{-1}$ above preallergen levels. The concentration of *A. suum* producing this change in resistance was used for the allergen challenge during the study (for some dogs, resistance did not increase by 10 cm $H_2O \cdot liter^{-1} \cdot s^{-1}$, in which case a concentration of $10^{-1}$ [wt/vol] was used). *A. suum* was delivered in 50 inhalations of 3-s duration each, using the same nebulizer as used for ACh challenges. A total of 10 min was allowed between doses during screening, and the postchallenge resistance was taken as the peak value in the 10 min after the inhalation. The diluent used in the *A. suum* preparations (0.4% phenol) was inhaled in the same concentration and manner as allergen.

### Group-matching Protocol

To ensure that the study groups contained dogs with similar allergen-induced changes in airway responsiveness, dogs were matched for this attribute. Changes in airway responsiveness to ACh during a screening allergen challenge were expressed as shifts (prechallenge provocative concentration/postchallenge provocative concentration). Dogs with shifts that differed by less than 15% were paired, and each dog was randomly assigned to either the allergen or diluent group. However, dogs were not treated as pairs for statistical analysis.

### Administration of BrdU

BrdU (Sigma Chemicals) was administered as equal intravenous bolus injections at 30 min before and 5 h after allergen challenge (total BrdU = 25 mg/kg). The total amount of BrdU per dog was calculated and dissolved in 60 ml endotoxin-free 0.9% sodium chloride solution (Baxter Corporation, Toronto, Canada). The solution was then filter-sterilized, and 30 ml was given per injection. The dose of BrdU was similar to that used by Bicknell and colleagues in a rabbit model of *Streptococcus pneumoniae*-induced inflammation (21).

### Blood Samples

Venous blood samples were obtained from each dog before and at 5 h and 24 h after inhalation. The sample taken before challenge was also taken before the first injection of BrdU, and the 5-h sample was taken before the second

BrdU injection. Samples were collected into heparin sodium-containing Vacutainer tubes (Hausser Scientific, Blue Bell, PA) for total and differential white blood cell (WBC) counts. WBC counts were made with a Neubauer hemocytometer, and differential cell counts were made from blood smears stained with the Diff-Quik method (American Scientific Products, McGaw Park, IL). Differential cell counts were made by one investigator in a blinded fashion, and the mean of two slides (300 cells counted per slide) was obtained. Cells were classified according to standard morphologic criteria. Results were expressed as absolute counts ($\times 10^6$ cells/ml). One milliliter of blood was used to make cytospin preparations for immunohistochemistry (IHC), as subsequently discussed.

### BM Aspiration

BM aspirates were obtained from the iliac crest of anesthetized dogs with a 16-gauge Rosenthal needle. Three milliliters of BM were aspirated into a 10-ml syringe containing 1 ml of sterile heparin (1,000 U/ml; Leo Laboratories, Canada), and the syringe contents were then immediately resuspended in 50 ml of 1% bovine serum albumin (BSA) (Sigma Chemicals) in phosphate-buffered saline (PBS). Prior to cytospin preparation, the sample was spun for 10 min at $1{,}500 \times g$, and cytospins were then prepared for IHC from the cell pellet, as subsequently discussed.

### Preparation of Cytospins from Blood and BM for IHC

One milliliter of each blood sample, and the BM pellet, were lysed for 30 s with 10 ml of cold 0.2% PBS to remove erythrocytes, and were then resuspended in 40 ml of BSA/PBS to restore the normal concentration of PBS. The samples were allowed to stand for at least 30 min to allow the WBCs to recover from the lysing process, and were then resuspended in BSA/PBS. The cell concentration was adjusted to $2 \times 10^6$/ml, and cytospins were prepared on APTEX-coated (Sigma Chemicals) slides.

### Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) was performed as previously described (18). Briefly, a fiberoptic bronchoscope (BF-Be; Olympus, Tokyo, Japan) (optical density = 6 mm) was passed into a third-generation airway of the right middle lobe. Five 20-ml aliquots of PBS warmed to 37°C were injected into the airway via the bronchoscope, and immediately after injection of each aliquot, BALF was aspirated through the bronchoscope into collection traps. The BALF was then pooled and spun at $1{,}500 \times g$ for 10 min, and the cell pellet was resuspended in BSA/PBS. A total cell count (TCC) was made, the cell count was adjusted to $2 \times 10^6$/ml, and cytospin samples were prepared on APTEX-coated slides for IHC. Cytospin preparations were also made on glass slides, and differential counts were performed in a blinded fashion on Diff-Quik-stained slides. Mean counts from duplicate slides were obtained (500 cells counted per slide) and expressed as the number of cells per milliliter of BALF recovered ($\times 10^6$/ml BALF).

### Immunohistochemical Staining for BrdU-labeled Cells

Immunohistochemical staining for BrdU-labeled cells was performed according to a method described by Bicknell and coworkers (21), with some modifications. All blood, BM, and BAL cytospin preparations were fixed for 10 min in 1% paraformaldehyde (BDH Inc., Toronto, Canada) in PBS, and then digested at 37°C for 5 min in 0.001% pepsin (Sigma Chemicals) solution acidified to pH 2.5. DNA in the cytospin samples was denatured at 37°C for 1 h in 2 N HCl, followed by neutralization in three washes of 0.1 M borate buffer, pH 8.5, each lasting 10 min. A final 10-min wash in 50 mM Tris(hydroxymethyl) aminomethane hydrochloride (Sigma Chemicals) and 150 mM NaCl, pH 7.6, containing 0.1% Tween 20 (TBS–Tween) (Sigma Chemicals), was used to restore neutrality. The alkaline phosphatase–anti-alkaline phosphatase (APAAP) technique (25) was used to detect BrdU-labeled DNA in cells. Briefly, cytospin preparations were incubated consecutively in 5% rabbit serum (GIBCO, Grand Island, NY) for 15 min, and then in 2 µg/ml mouse anti-BrdU antibody (DAKO Laboratories, Copenhagen, Denmark) prepared with 1% BSA in TBS–Tween at room temperature in a humidified chamber for 1 h. Nonimmune mouse $IgG_1$ (Sigma Chemicals) at 2 µg/ml was used as a negative control for each specimen. Incubation in a 1:20 dilution of rabbit antimouse IgG (DAKO Laboratories) for 30 min was followed by 30 min in a 1:50 dilution of a mouse monoclonal APAAP complex (DAKO Laboratories). Slides were washed three times (3 min each) in TBS–Tween following each antibody incubation. The alkaline phosphatase reaction was developed for 20 min, using the Fast Red Substrate System (DAKO Laboratories), and the resulting preparation was counterstained with Mayer's hematoxylin for 60 s and mounted in an aqueous medium. The nuclei of positive cells stained bright red, and duplicate slides from each sample were analyzed with light microscopy. An average number of cells per high power field (hpf) was calculated by counting 5 hpfs. The number of hpfs required to count 10,000 cells was then calculated, and the number of BrdU-positive cells in 10,000 cells was recorded and expressed as the percentage of BrdU-positive cells. For some slides, on which insufficient cells were present, the number of cells counted was always between 5,000 and 10,000.

### Statistical Analysis

*AHR.* ACh provocative concentrations were $\log_{10}$-transformed prior to analysis. Pre- versus postchallenge comparisons were made for each group of dogs, using paired Student's *t* tests. Values are expressed as geometric means (antilog of the mean logged data) and % SEM (antilog of the SEM of the logged data). The pre- versus postchallenge change in logged provocative concentrations was compared between groups with an independent Student's *t* test.

*Blood.* Differences in blood neutrophil counts and the percentage of BrdU-positive cells before and 5 h and 24 h after challenge were assessed for each group of dogs, using a mixed-model analysis of variance (ANOVA) (nonrepeated factor: allergen versus diluent; repeated factors: pre- versus 5 h post- versus 24 h postchallenge values) (26). *Post hoc* comparisons were made where indicated, using the Newman-Keuls test.

*BM.* The percentage of BrdU-positive cells in the BM in different groups was compared through an independent Student's *t* test.

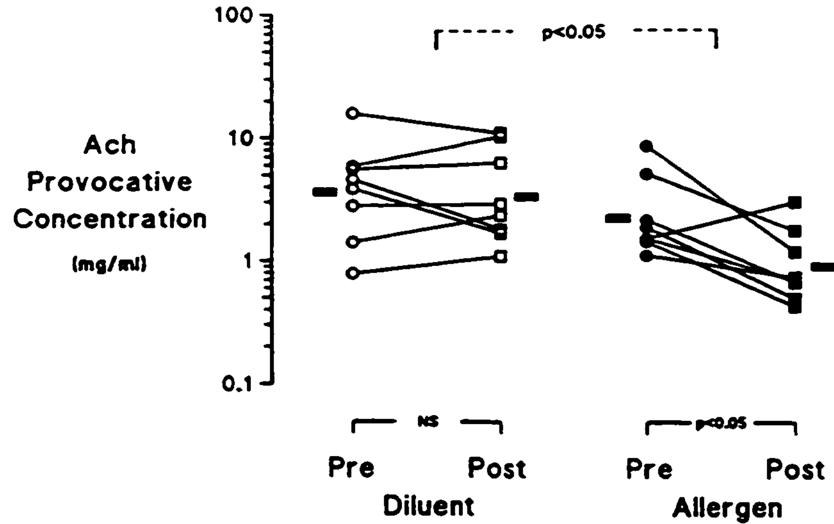


*Figure 1.* Acetylcholine airway responsiveness. ACh provocative concentration values before (*circles*) and after (*squares*) diluent (*open symbols*) and allergen (*closed symbols*) inhalation. Geometric means are indicated by *solid bars*. There was a significant reduction in ACh provocative concentration values following allergen ($P < 0.05$) but not diluent inhalation. In addition, there was a significant difference between the pre- and postinhalation values of ACh provocative concentrations between the two groups ($P < 0.05$).

*BALF.* Differences between groups in the TCC, differential count, and percentage of BrdU-positive cells in the BALF were compared through an independent Student's *t* test. Regression analysis was used to detect significant relationships between BALF neutrophils and the percentage of BrdU-positive cells in the BALF in the allergen-challenge group only (27). Statistical significance was assumed at $P < 0.05$.

## Results

### AHR

AHR to ACh developed in the allergen-challenged group but not in the diluent group at 24 h after challenge. The geometric mean provocative concentration values for ACh in the allergen-challenge group fell from 2.31 mg/ml (%SEM = 1.29) before to 0.94 mg/ml (%SEM = 1.26) after allergen challenge ($P < 0.05$), whereas in the diluent group the values were 3.68 mg/ml (%SEM = 1.38) before and 3.49 mg/ml (%SEM = 1.36) after diluent ($P = 0.81$) (Figure 1). In addition, there was a significant difference between the two groups in the challenge-induced shift in logged ACh provocative concentration values ($P < 0.05$) (Figure 1).

### Blood and BALF Neutrophils

Blood neutrophils increased at 24 h after allergen challenge to 11.19 ± 1.18 × $10^6$/ml from 6.18 ± 0.62 × $10^6$/ml

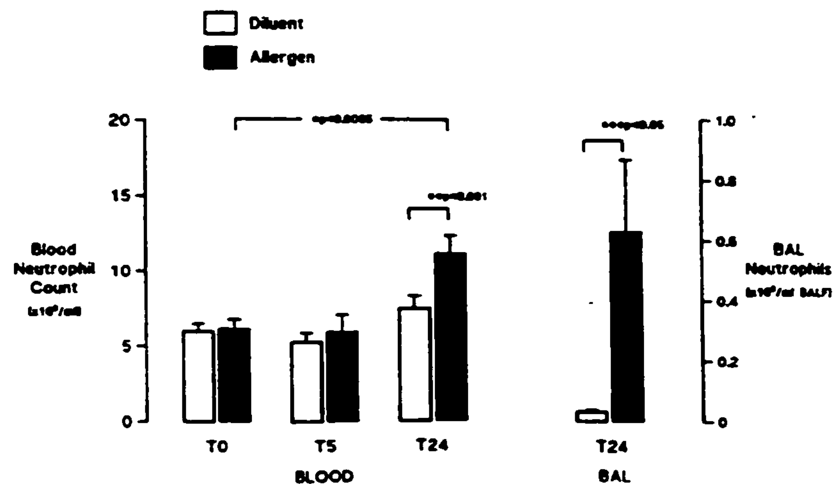


*Figure 2.* Blood and BALF inflammatory cells following challenge. Changes in neutrophil numbers in blood (× $10^6$/ml) and BALF (× $10^6$/ml BALF) following allergen (*solid bars*) and diluent (*open bars*) inhalation. Significant increases in blood neutrophils from baseline were apparent at 24 h after allergen (*$P < 0.0005$) but not diluent inhalation. In addition, blood neutrophils at 24 h after allergen inhalation were significantly different from the neutrophil count at the same time point following diluent (**$P < 0.001$). There were significantly more BALF neutrophils at 24 h after allergen challenge than at the same time point after diluent (***$P < 0.05$).

TABLE 1

*BAL and blood inflammatory cells following challenge*

| | Diluent Challenge | | | Allergen Challenge | | |
|---|---|---|---|---|---|---|
| | Prediluent | 5 h Postdiluent | 24 h Postdiluent | Preallergen | 5 h Postallergen | 24 h Postallergen |
| BAL cells, $\times 10^6$/ml BALF | | | | | | |
| TCC | ND | ND | $0.69 \pm 0.11$ | ND | ND | $1.24 \pm 0.26$ |
| Neutrophils | ND | ND | $0.03 \pm 0.005$ | ND | ND | $0.63 \pm 0.24$* |
| Macrophages | ND | ND | $0.42 \pm 0.04$ | ND | ND | $0.44 \pm 0.09$ |
| Lymphocytes | ND | ND | $0.07 \pm 0.02$ | ND | ND | $0.03 \pm 0.005$ |
| Eosinophils | ND | ND | $0.18 \pm 0.06$ | ND | ND | $0.15 \pm 0.07$ |
| Blood | | | | | | |
| Neutrophils, $\times 10^6$/ml | $5.99 \pm 0.51$ | $5.28 \pm 0.61$ | $7.51 \pm 0.84$ | $6.18 \pm 0.62$ | $5.98 \pm 1.12$ | $11.19 \pm 1.18$†‡ |

*Definition of abbreviations*: BAL = bronchoalveolar lavage; BALF = bronchoalveolar lavage fluid; TCC = total cell count; ND = not done.

* $P < 0.05$ compared with diluent.

† $P < 0.001$ compared with postdiluent.

‡ $P < 0.0005$ compared with preallergen.

before challenge ($P < 0.0005$), but did not increase at 5 h after allergen challenge, being $5.98 \pm 1.12 \times 10^6$/ml ($P = 0.97$) (Figure 2, Table 1). In addition, blood neutrophils at 24 h after allergen challenge were significantly increased as compared with their values at the same time point after diluent ($P < 0.001$) (Figure 2, Table 1). There were no significant differences in blood neutrophils at 5 h or 24 h after diluent inhalation (Figure 2, Table 1).

BALF neutrophils increased at 24 h after allergen challenge as compared with their values at 24 h after diluent, being $0.63 \pm 0.24 \times 10^6$/ml BALF after allergen versus $0.03 \pm 0.005 \times 10^6$/ml BALF after diluent ($P < 0.05$) (Figure 2, Table 1). There were no significant differences between the two groups in numbers of other cell types (Table 1).

### Trafficking of BrdU-positive Cells

BrdU-positive nuclear staining could be demonstrated with IHC in BM, blood, and BALF samples (Figures 3 and 4) after both allergen and diluent challenge. Positive-staining cells in the blood were of the band and metamyelocyte forms typical of granulocytes (Figures 3B and 3C). By contrast, cells staining positive for BrdU in the BM consisted of various nucleated cell types, including both mononuclear and polymorphonuclear cells (Figures 4A and 4B). The cellular morphology of BrdU-positive cells in the BALF following diluent was mainly mononuclear (Figure 4C), whereas after allergen inhalation, polymorphonuclear cells were also apparent (Figure 4D).

There was a statistically insignificant increase in the percentage of BrdU-positive cells in the postallergen BM as compared with the postdiluent BM, at: $27.0 \pm 3.4\%$ versus $18.9 \pm 3.2\%$, respectively ($P = 0.1$) (Figure 5). BrdU-positive cells in the blood increased at 24 h after challenge in both groups, from $0 \pm 0\%$ to $5.7 \pm 0.6\%$ after allergen challenge ($P < 0.0005$) and from $0 \pm 0$ to $2.5 \pm 0.7\%$ after diluent ($P < 0.0005$) (Figure 5). There was a significant difference between the two groups in the percentage of BrdU-positive cells in the blood at 24 h after challenge ($P < 0.0005$) (Figure 5). There were no significant increases in the percentage of BrdU-positive cells in the blood at 5 h after challenge. Additionally, there was a greater percent-

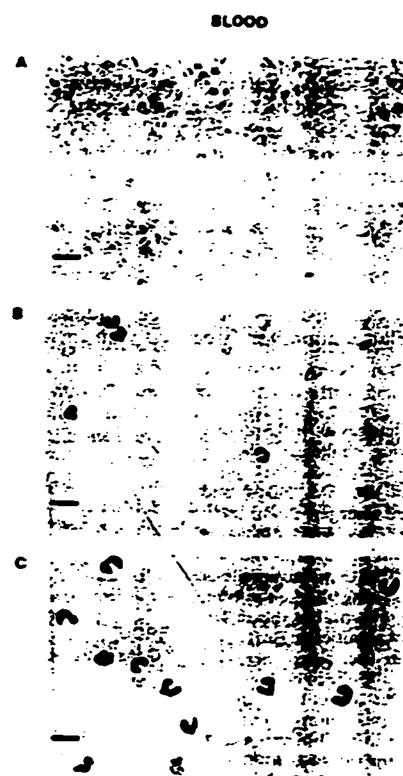


*Figure 3.* BrdU-positive blood cells. BrdU-positive nuclear staining in the blood before challenge (*A*), 24 h after diluent (*B*), and 24 h after allergen challenge (*C*). Cells staining positive for BrdU are of the metamyelocyte type and band forms typical of granulocytes. *Bar* = 25 μm.

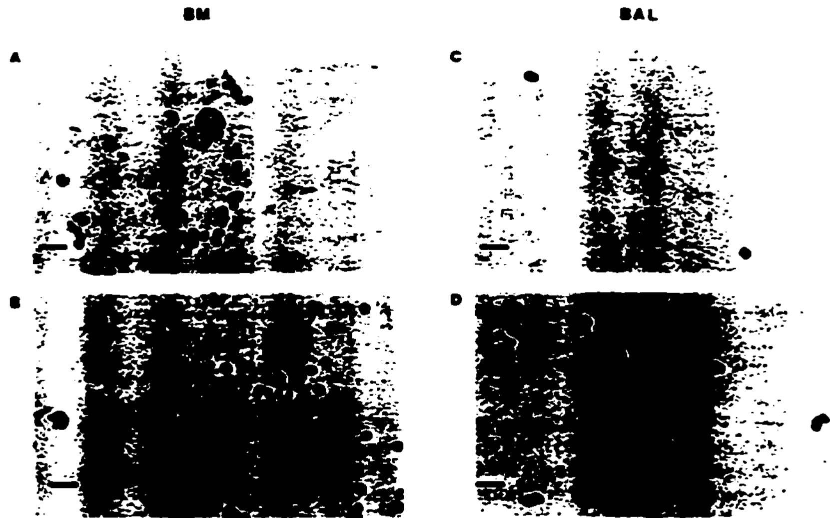


*Figure 4.* BrdU-positive BM and BALF cells. BrdU-positive nuclear staining in BM and BALF. Cells staining positive for BrdU in the BM following diluent (*A*) and allergen challenge (*B*) consist of various nucleated cell types, including both mononuclear and polymorphonuclear cells. Cellular staining in the BALF following diluent is mainly mononuclear (*C*), whereas that following allergen inhalation is mainly polymorphonuclear (*D*). *Bar* = 25 μm.

age of BrdU-positive cells in the postallergen BALF sample than in the postdiluent BALF sample, at 3.25 ± 0.39% versus 0.84 ± 0.25%, respectively ($P < 0.0005$) (Figure 5). There was a significant correlation between the number of BALF neutrophils and the percentage of BrdU-positive BALF cells in the allergen group ($r^2 = 0.54$, $P < 0.05$) (Figure 6).

## Discussion

This study demonstrates that allergen-induced AHR and airway inflammation are associated with increased numbers of newly formed cells in the blood and airways, suggesting trafficking into the airways of inflammatory cells from the blood or BM. The results are consistent with those in our previous studies, which have shown an increased production of BM neutrophil progenitors (GM-CFU) after allergen inhalation in sensitized dogs (17, 18). Taken together, studies in this canine model suggest that allergen inhalation may initiate a cascade of events, with a stimulatory effect on the BM to produce and release newly formed inflammatory cells, followed by trafficking of these cells, through the circulation and into the airways.

The BM is the site of proliferation and terminal differ-

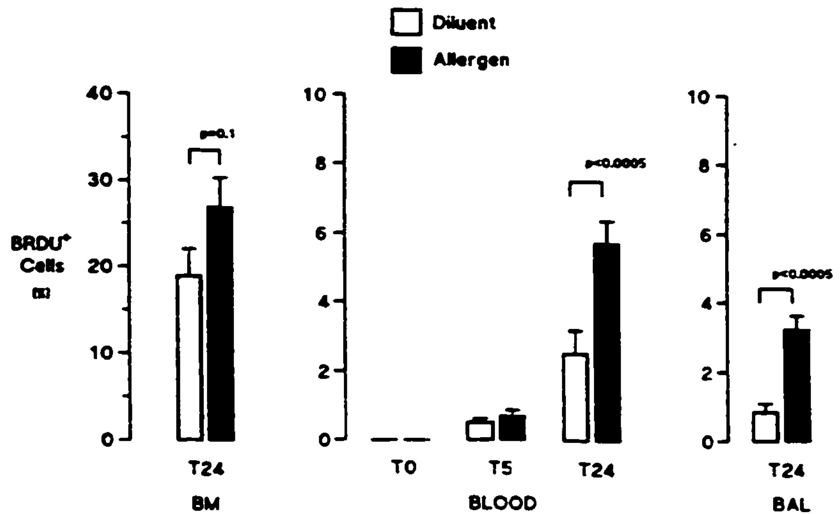


*Figure 5.* Changes in BrdU-positive cells following challenge. Changes in the percentage of BrdU-positive cells in the BM, blood, and BALF after allergen (*solid bars*) and diluent (*open bars*) inhalation. There was an increase in the percentage of BrdU-positive cells in the BM following allergen as compared with diluent, but this difference was not significant. BrdU-positive cells in the blood increased 24 h after challenge in both groups, and there was a significant difference in the percentage of BrdU-positive cells in the blood between the two groups at 24 h after challenge ($P < 0.0005$). There was also an increase in the percentage of BrdU-positive cells in the BALF following allergen as compared with diluent ($P < 0.0005$).

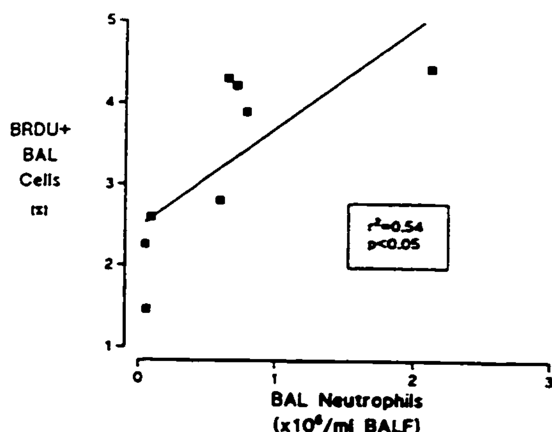


*Figure 6.* There was a significant correlation between the number of BALF neutrophils and the percentage of BrdU-positive BALF cells ($r^2$ = 0.54, $P < 0.05$).

entiation of neutrophilic granulocytes (28). In humans, neutrophil proliferation and differentiation consist of approximately five divisions, which take place during the first three stages of the neutrophil maturation process. There is one mitosis each at the myeloblast and promyelocyte stages, two successive mitoses in the myelocyte stage, and a minimum of 3 h from terminal myelocyte mitosis to the appearance of labeled cells in the metamyelocyte compartment (29). An additional mitosis may occur at the myelocyte level during times of granulocytic inflammation (30). After the myelocyte stage, the cells become "end-stage" cells (no longer capable of mitosis), a compartment that includes metamyelocytes, band forms, and mature neutrophils.

Studies with dogs have found a mean transit time of tritiated-thymidine-labeled cells from the myelocyte stage to their appearance in the circulation of 102 h in the steady state (31). The appearance of labeled granulocytes in our study began at 5 h and was significantly increased at 24 h after allergen inhalation. The kinetics appear much faster than in the steady state, but are in accordance with the findings in other studies in which neutrophilic inflammation was initiated. For example, Cronkite and colleagues (30) induced a sterile inflammation in the uterus of dogs, and showed that the myeloid-to-erythroid ratio of cells was 3-fold greater than in control dogs, peaking at 24 h. The time to appearance of tritiated thymidine-labeled cells in the blood was reduced from 40 h in the control dogs to 16 h in the dogs with inflammation. Also, infusion of starch into the peritoneal cavity of dogs caused a rapid rise in tritiated-thymidine-labeled cells in the blood within 10 h after infusion (32). In addition, the induction of pneumococcal pneumonia in dogs has been shown to increase the rate of production of neutrophils from their precursors, shortening their maturation time and decreasing their storage time in the marrow, with release of mature and immature neutrophils into the circulation (33). BrdU has also been used in rabbits, in which, after the introduction of *Streptococcus pneumoniae* into the lung, the transit times of labeled neutrophils through the proliferating and nonproliferating pools in the BM were shortened considerably (22).

In the current study, we demonstrated the appearance of labeled cells in the circulation 24 h after diluent challenge, which represents the steady-state turnover of cells for this model and is in contrast with the finding in studies using tritiated thymidine, in which the first appearance of labeled cells in the circulation was at 40 h (30). This suggests that BrdU labeling of dividing cells may be a more sensitive indicator of proliferating cells than tritiated thymidine labeling. Our findings are supported by studies with rabbits, in which BrdU-labeled cells began to appear in the circulation at between 24 h and 48 h in the steady state (21).

In asthmatic subjects, it is known that the influx of inflammatory cells into the airways begins at 3 to 4 h after allergen challenge. Since BrdU is cleared rapidly from the circulation, it was administered in two doses, to ensure availability of this thymidine analogue over the period in which inflammatory-cell production and trafficking may occur in response to allergen in the dog model. However, because of this study design, no inference can be made from the study data with regard to the true time course of the appearance in the circulation of labeled cells from the BM. This could only be addressed by administration of one dose of BrdU, at or shortly after the challenge procedure.

We have demonstrated a significantly higher number of BrdU-positive cells in the BALF at 24 h after allergen as compared with diluent inhalation. Morphologically, these cells appear to be of the granulocyte series, and there was a significant correlation between the number of BALF neutrophils and the percentage of BrdU-positive cells in the BALF. This response is in accordance with the study by Bicknell and colleagues, in which there were significantly larger numbers of labeled neutrophils in the inflammatory foci and alveolar spaces at 4 h after instillation of *S. pneumoniae* than in the control lung (21). Unfortunately, we did not examine the BALF at earlier time points to follow the time course of this response.

Previous results from our laboratory (18) have shown that increases in BALF neutrophils after allergen inhalation in dogs are associated with the production of a serum factor that has a hemopoietic effect on the marrow, increasing the number of myeloid progenitors. In the current study there was an increase in BrdU-labeled BM cells after allergen inhalation, but this was not significant. Since the marrow is a site of ongoing cell proliferation, it is probable that the cells staining positive for BrdU in this compartment include precursor cells for other hemopoietic lineages in addition to the myeloid series. The study did, however, demonstrate an allergen-induced increase in the number of proliferating cells appearing in the circulation and BALF. It is likely that BrdU-positive cells in the circulation and BALF are derived from progenitor cells in the BM. However, we cannot exclude the possibility that some of these cells were derived from progenitor cells that were already present and divided in those compartments, although this has never been previously shown to occur. Fu-

ture studies, done with markers of hemopoietic progenitor cells and/or lineage-specific markers, are required to confirm the BM involvement in this process.

The present study does not provide information on the specificity of the trafficking of cells in response to allergen. It is feasible that allergen deposited in another site, such as the skin or nose, would induce similar trafficking of inflammatory cells from the BM in association with the inflammatory reaction at that site.

The study does not make clear the extent to which the inflammatory response contributes to the development of allergen-induced AHR in dogs. However, our previous studies, in which we have pretreated dogs with the inhaled corticosteroid budesonide before allergen inhalation, have shown that this corticosteroid attenuates allergen-induced increases in BM progenitors, as well as airway inflammation and AHR (17). However, it is not yet known whether the trafficking of these inflammatory cells contributes to the AHR. There was no significant correlation between the percentage of BrdU-positive cells in the lung and the development of AHR. An estimated 27 dogs would have to be studied in order to achieve significance for these parameters.

On the basis of our findings, we can hypothesize that there is both a more rapid production of neutrophils in the BM and a movement of newly divided neutrophils through the circulation into the lung in states of neutrophilic inflammation during the development of AHR. Similar events may occur in allergen-induced asthma, in which we have observed increased eosinophil progenitors in the BM concurrent with the appearance of eosinophilic inflammation and hyperresponsiveness in the airways (16). In addition, eosinophils have been shown to be increased in the BM, circulation, and BALF in a murine model of allergen-induced eosinophilic inflammation of the airways (34). The precise mechanisms responsible for these types of inflammatory-cell trafficking remain to be elucidated, but probably involve mediators released as a result of the immune response to allergen, or secondarily, in response to the ensuing inflammation in the airway. In either of these scenarios, it is possible that the trafficking of cells is important in initiating and/or supporting airway inflammation and the ongoing physiologic disturbance. Uncovering such possible mechanisms for inflammatory-cell trafficking may reveal new avenues for therapeutic intervention in diseases with chronic allergic inflammation.

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# CHAPTER 5

# AN INHALED CORTICOSTEROID, BUDESONIDE, REDUCES BASELINE BUT NOT ALLERGEN-INDUCED INCREASES IN BONE MARROW INFLAMMATORY CELL PROGENITORS IN ASTHMATIC SUBJECTS

Accepted (pending review) by the American Journal of Respiratory and Critical Care Medicine in 1998

Lorna Wood's contribution:

Experimental design
Collection of clinical data
Processing of laboratory samples
Identification and quantification of colonies
Analysis of data
Preparation of manuscript

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November 20, 1998

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RE: **9808123, An Inhaled Corticosteroid, Budesonide, Reduces Baseline but not Allergen-Induced Increases in Bone Marrow Inflammatory Cell Progenitors in Asthmatic Subjects**

Dear Dr. O'Byrne:

I am pleased to inform you that your manuscript referenced above has been recommended for acceptance in the *American Journal of Respiratory and Critical Care Medicine* pending your response to reviewer comments.

Both reviewers find your paper of interest. Reviewer #1 has a few questions which you should be able to address. Reviewer #2 (Point 1) suggests an additional set of experiments to define whether the budesonide action is on the bone marrow vs. lung signal; if you have such data, it would be of interest to include it. The issues raised in points 2 and 3 need your attention. Finally, are the doses given to humans and dogs equivalent? If you can address these issues in a revised manuscript, I will be able to act on the modified manuscript.

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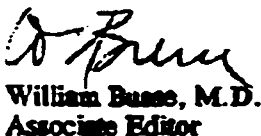

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**Title:** An inhaled corticosteroid, budesonide, reduces baseline but not allergen-induced increases in bone marrow inflammatory cell progenitors in asthmatic subjects

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Running Head: Effect of inhaled budesonide on bone marrow inflammatory cell progenitors

## ABSTRACT

We have previously shown that allergen inhalation by asthmatics is associated with increases in bone marrow eosinophil/basophil colony forming cells (Eo/B-CFU), and increases in CD34$^+$ hemopoietic progenitors expressing the $\alpha$-subunit of the IL-5 receptor (IL-5R$\alpha$). This study investigated the effect of inhaled corticosteroid on baseline numbers and allergen-induced increases in these parameters. Nine mild, stable asthmatics inhaled budesonide (400$\mu$g/day) for 8 days in a placebo-controlled, double blind, randomized crossover study. On day 7, subjects inhaled allergen, with bone marrow sampling before, and 24h after challenge. Budesonide inhalation significantly attenuated the allergen-induced early and late asthmatic responses, degree of increase in sputum and blood eosinophils, as well as the baseline numbers of total bone marrow CD34$^+$ cells ($p<0.05$), CD34$^+$IL-3R$\alpha^+$ cells ($p<0.01$) and IL-5-responsive Eo/B-CFU ($p<0.05$). Allergen inhalation significantly increased Eo/B-CFU grown in the presence of IL-3, GM-CSF or IL-5 alone ($p<0.05$) and in combination ($p<0.01$), as well as the number of CD34$^+$IL-5R$\alpha^+$ cells ($p<0.01$). However, these increases in Eo/B-CFU and CD34$^+$IL-5R$\alpha^+$ cells were not affected by budesonide treatment. These data demonstrate that short term-inhaled budesonide treatment has a systemic effect in inhibiting the turnover of a sub-population of bone marrow-derived progenitors, but that inhalation of allergen overcomes this inhibitory effect.

**Key Words:** Eosinophils, progenitors, cytokine receptors, inhaled budesonide

•

## INTRODUCTION

Asthma is a disease characterised by bronchoconstriction, airway hyperresponsiveness and airway inflammation. Inhalation of allergen by sensitized subjects is an important cause of asthma, and is characterised by biphasic changes in airway physiology known as the early and late phase asthmatic responses. Late asthmatic responses (LAR) are associated with transient increases in airway hyperresponsiveness (1), usually lasting several days, and increases in the numbers of activated eosinophils and metachromatic cells in the airways (2).

The predominant cell infiltrating the airways during the late response is the eosinophil, which is selectively increased in sputum (2,3), and bronchoalveolar lavage fluid (BAL) (4), in association with the late response. Eosinophils are also in an activated state in asthma, as indicated by elevated levels of eosinophil cationic protein (ECP) in BAL fluid (5), and enhanced immunostaining with the EG2 monoclonal antibody, which recognises the cleaved and activated form of ECP (2).

We have previously provided evidence that increases in inflammatory cell progenitors, which contribute to disease through the continued production of inflammatory effector cells, is an important aspect of allergic inflammatory responses (6,7). Higher numbers of both circulating eosinophil/basophil colony forming units (Eo/B-CFU) and $CD34^+$ hemopoietic progenitor cells are demonstrable in the blood of atopic subjects compared with normals (6,8), and there are significantly higher numbers of progenitors in the circulation 24 hours following allergen inhalation in atopic asthmatic subjects (9). In

addition, the numbers of both Eo/B-CFU and $CD34^+$ progenitors expressing the α-subunit of the IL-5-receptor (IL-5Rα$^+$) in the bone marrow of asthmatic subjects are preferentially increased 24 hours following allergen inhalation (10,11).

Inhaled glucocorticosteroids are known to improve airway hyperresponsiveness in asthmatics (12,13), to inhibit both allergen-induced late responses and airway hyperresponsiveness (14-16), and to attenuate allergen-induced increases in blood eosinophils and sputum total and activated eosinophils (2). Inhaled budesonide has been shown to attenuate allergen-induced increases in bone marrow granulocyte progenitors (GM-CFU) in dogs with allergen-induced airway hyperresponsiveness and airway inflammation (17). We therefore postulated that inhaled budesonide would, as part of its activity in attenuating allergen-induced airway inflammation in allergic asthmatic subjects, also inhibit allergen-induced increases in bone marrow progenitors. The purpose of this study was to determine whether treatment with inhaled budesonide, administered for 8 days, could alter baseline or attenuate allergen-induced increases in Eo/B-CFU, total $CD34^+$ hemopoietic progenitor cells, and $CD34^+$IL-5Rα$^+$ cells, as well as attenuating physiologic parameters and airway and blood eosinophilia.

## METHODS

*Subjects*

Nine subjects with mild asthma and a previously documented early and late asthmatic response following allergen inhalation were studied (Table 1). The study was approved by the Research Advisory Group at McMaster University Medical Centre and all subjects provided their written informed consent prior to entering the study. Subjects were atopic, as indicated by one or more positive wheal-and-flare responses to skin prick tests. All subjects were non-smokers and none had experienced a respiratory infection during the four weeks prior to the study. Asthmatic subjects were stable at the time of study, requiring only intermittent use of inhaled $\beta_2$-agonists with baseline $FEV_1$ values >70% predicted.

*Study Design*

The study was carried out using a double-blind, placebo-controlled, randomized, crossover design. Subjects completed two treatment periods with either inhaled budesonide (400µg/day) or identical placebo for 8 days. Each treatment period consisted of four visits to the laboratory. A baseline measurement of the provocative concentration of methacholine causing a 20% fall in $FEV_1$ ($PC_{20}$) was determined prior to the study. Baseline measurements of $FEV_1$, blood and induced sputum differential and total cell counts were determined before treatment, and on day 6 of treatment with budesonide or placebo. Allergen challenges were performed on the morning of day 7 and blood and sputum samples were taken at 24h post challenge, on day 8. An additional sputum was performed at 7h after allergen challenge.

**Table 1 - Subject characteristics on screening**

| SUBJECT | AGE (Yrs) | GENDER | $FEV_1$ (% predicted) | $PC_{20}$ (mg/ml) | ALLERGEN (Inhaled Dilution) |
|---|---|---|---|---|---|
| 1 | 23 | M | 85.5 | 2 | HDM (1:512) |
| 2 | 23 | M | 83.1 | 1.35 | Cat (1:16) |
| 3 | 22 | F | 77.9 | 0.5 | HDM (1:1024) |
| 4 | 21 | F | 91.4 | 6.3 | HDM (1:256) |
| 5 | 28 | F | 81.7 | 0.25 | HDM (1:2048) |
| 6 | 37 | M | 94.7 | 2.17 | HDM (1:1024) |
| 7 | 22 | M | 97.8 | 1.5 | RW (1:4) |
| 8 | 26 | F | 78 | 1.25 | HDM (1:256) |
| 9 | 21 | F | 89 | 0.35 | RW (1:32) |
| **Mean±SEM** | **24.8±1.7** | | **86.6±2.4** | **1.74±0.61** | |

*Definition of abbreviations:* $PC_{20}$ = provocative concentration of methacholine causing a 20% fall in $FEV_1$; HDM=House dust mite - D.farinae; RW=Ragweed

Bone marrow aspirates were performed immediately prior to allergen inhalation, and at 24h post challenge. Each treatment period was separated by a washout period of at least 4 weeks.

*Methacholine Inhalation Challenge*

Methacholine inhalation was performed as described by Cockcroft *et al* (18). Subjects inhaled normal saline and then doubling concentrations of methacholine phosphate from a Wright nebulizer for 2 min. Increasing concentrations of methacholine were administered until the $FEV_1$ decreased by >20% of the baseline value. Results were expressed as the provocative concentration (mg/ml) causing a 20% decrease in $FEV_1$ ($PC_{20}$).

*Allergen Inhalation Challenge*

Allergen inhalation challenge was performed as described by O'Byrne *et al* (19). The concentration of allergen extract for inhalation was determined from a formula described by Cockcroft *et al* (15), using results from the skin test and the methacholine $PC_{20}$. The starting concentration of allergen was chosen to be three doubling doses below that predicted to cause a 20% fall in $FEV_1$. Doubling concentrations of allergen were inhaled at 10-min intervals until a decrease of 15% or more occurred in the $FEV_1$ from baseline. Measurements of $FEV_1$ were performed at 10, 20, 30, 45, 60, 90 and 180 min, and then every hour for 7h after the final inhalation. The allergen-induced early response was determined as the maximal decrease in $FEV_1$ between 0 and 2 hours after allergen and the late response as the maximal decrease between 3 and 7h after allergen inhalation.

*Sputum Analysis*

Sputum was induced and processed according to the method of Popov *et al* (20).

Subjects inhaled 3%, 4% then 5% saline for 10 minutes each until an adequate sample was obtained or if the $FEV_1$ dropped 20% from baseline. Cell plugs were selected from the sample and processed using 0.1% dithiothreitol (Sputolysin, Calbiochem-Behring, San Diego, CA) and Dulbecco's phosphate buffered saline (GIBCO, Grand Island, NY). The total number of cells obtained was recorded and expressed as absolute counts ($10^6$ cells/ml). Cytospins were prepared on glass slides and differential counts were performed in a blinded fashion on Diff-Quik stained slides. Mean eosinophil counts from duplicate slides were obtained (400 cells counted per slide) and expressed as absolute counts ($10^4$ cells/ml).

*Blood Samples*

Venous blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-treated tubes for total and differential WBC. Total cell counts were performed using a Neubauer hemocytometer and differential cell counts were made from blood smears stained by Diff-Quik (American Scientific Products, McGaw Park, IL). Differential cell counts were performed by one investigator in a blinded fashion and the mean of two slides obtained (300 cells counted per slide). Eosinophils were classified using standard morphologic criteria. Results were expressed as absolute eosinophil counts ($10^4$ cells/ml).

*Bone Marrow Aspirate and Culture*

Bone marrow aspirates were obtained from the iliac crest using a bone marrow aspiration needle (16x2";Sherwood Medical, St Louis, MO). Three millilitres of bone marrow were aspirated into a 10 ml syringe containing 1 ml sterile heparin (1000 U/ml) (Leo Laboratories, Canada) and semi-solid methylcellulose cultures of low density non-adherent

mononuclear cells (NAMC) were performed. Briefly; heparinized bone marrow was diluted to 50ml with McCoy's 5A medium (GIBCO, Grand Island, NY) and separated over 65% percoll (Pharmacia, Uppsala, Sweden). The interface mononuclear-rich cell fraction was washed in McCoy's 5A medium and then incubated in McCoy's 5A medium supplemented with 15% fetal calf serum (FCS) (GIBCO, Grand Island, NY), 1% penicillin/streptomycin (GIBCO, Grand Island, NY) and 5 x $10^{-5}$M 2-mercaptoethanol (final concentration)(SIGMA Chemicals, St Louis, MO) for 2 hours in plastic flasks at 37°C and 5% $CO_2$. Non-adherent mononuclear cell populations (NAMC; containing progenitor cells and lymphocytes) were then cultured (2.5 x$10^5$ cells per 35 x 10mm tissue culture dish; Falcon plastics, Oxnard, CA), in duplicate, in supplemented Iscove's Modified Dulbecco's medium (GIBCO, Grand Island, NY) (with 1% penicillin/streptomycin and 5 x $10^{-5}$M 2-mercaptoethanol (final concentration)), 0.9% methylcellulose (SIGMA Chemicals, St Louis, MO) and 20% FCS, either alone or in the presence of one of the following growth factors: recombinant human IL-3 (10 or 1ng/ml; Pharmingen, San Diego, Ca), recombinant human GM-CSF (10 or 1ng/ml; Pharmingen, San Diego, Ca) recombinant human IL-5 (1 or 0.1ng/ml; Pharmingen, San Diego, Ca), or a combination of all three. Cultures were incubated for 14 days at 37°C and 5% $CO_2$ after which colonies were identified as Eo/B-CFU according to previously described criteria (21) and expressed as Eo/B-CFU per 2.5 x$10^5$ NAMC plated.

*Immunofluorescence Staining of Bone Marrow Cells*

Bone marrow-derived low density NAMC (1x$10^6$ /tube), isolated as described above, were resuspended in phosphate buffered saline (PBS) plus 0.1% sodium azide (BDH Inc.,

Toronto, Ont.) and incubated for 45 min at 4°C with saturating amounts (determined in preliminary studies) of biotin-conjugated monoclonal antibodies directed against the α-subunit of either IL-3R (IL-3Rα; 7G2), IL-5R (IL-5Rα; SP491), GM-CSF (GM-CSFR; 6E10), the β-common subunit (βc; 8E4) or $IgG_1$ isotype control antibody. These non-neutralizing monoclonal antibodies were all kind gifts from Dr. A. Lopez, Institute of Medical and Veterinary Science, Adelaide, South Australia except for SP491 which was supplied by Schering Plough Research Institute, Kenilworth, NJ, USA. The cells were then washed and stained with streptavidin-conjugated peridinin chlorophyll protein (PerCp) (Becton Dickinson Canada, Mississauga, Ont.), together with saturating concentrations of fluorescein isothiocyanate (FITC) conjugated $IgG_1$ CD45 antibody (anti-HLE1) and phycoerythrin (PE)-conjugated $IgG_1$ CD34 antibody (HPCA-2), or PE-linked $IgG_1$ isotype control (Becton Dickinson Canada, Mississauga, Ont.) for 30 min at 4°C. Lysis buffer (Becton Dickinson Canada, Mississauga, Ont.) was then added to the cells and incubated for 5 min after which the cells were washed twice with PBS plus 0.1% sodium azide and finally fixed in 500μl of PBS plus 1% paraformaldehyde (BDH Inc., Toronto, Ont.). The cells were refrigerated until ready for analysis.

*Flow Cytometry and Gating Strategy*

Cells were analysed using a FACScan flow cytometer equipped with an argon ion laser (Becton Dickinson Instrument Systems, San Diego, Ca.). Five data parameters were acquired and stored in list mode files: linear forward light scatter (FSC), linear side -angle light scatter (SSC), log FITC, log PE and log PerCp fluorescence; each measurement

contained 50,000 events. Compensation settings were established using CalBrite beads (BDIS) and confirmed using NAMC stained with anti-CD34-PE, anti-CD45-FITC or anti-IL-5Rα-PerCp. Off-line analysis was performed using the PC lysis software as supplied by BDIS. As previously described in detail, we used a multi-parameter five sequential-gating strategy to accurately enumerate true bone marrow-derived $CD34^+$ progenitor cells that co-expressed the above-mentioned cytokine receptors (11).

*Statistical Analysis*

The area under the curve of both the early and late asthmatic responses was compared between treatment groups using a Student's t-test for paired comparisons. The numbers of $CD34^+$ cells and cytokine receptor-positive progenitors were $\log_{10}$ transformed prior to analysis and the summary statistics are expressed as the geometric mean and %SEM. A two-way, repeated measures analysis of variance (ANOVA) was used to assess the interaction between allergen-induced increases and the effect of treatment on blood eosinophils; on sputum total cell counts and eosinophils; on bone marrow Eo/B-CFU progenitor colonies for each cytokine at each concentration; on absolute $CD34^+$ cell numbers and co-expression of each cytokine receptor on $CD34^+$ cells: and the effect of treatment with budesonide on baseline measurements of sputum total cell and absolute eosinophil counts, and blood eosinophils (repeated factors: pre- vs post-allergen, placebo vs budesonide)(22). Statistical significance was assumed at $p<0.05$.

## RESULTS

*Airway Responses*

Inhaled budesonide attenuated both the mean maximal allergen-induced early and late asthmatic responses. The maximal fall in $FEV_1$ during the early response was 27.9±2.8% after placebo and 14.7±4.5% after budesonide treatment, and the maximal fall in $FEV_1$ during the late response was 23.4±4.5% after placebo and 4.6±1.6% after budesonide treatment (Figure 1). Budesonide treatment also significantly reduced the area under the curve of both the early (placebo 26.9±4.2 vs budesonide 12.5±3.6 $p<0.05$) and late (placebo 47.8±10.6 vs budesonide 2.8±2.9 $p<0.005$) responses (Figure 1).

*Sputum Eosinophils*

Inhaled budesonide significantly reduced the baseline number of sputum eosinophils measured before allergen inhalation, from 11.4±2.4 $x10^4$/ml before budesonide treatment to 2.9±1.1 $x10^4$/ml after treatment ($p<0.005$) (Figure 2). Baseline measurements of sputum eosinophils did not change significantly during placebo treatment, being 5.5±1.9 $x10^4$/ml before placebo, and 8.7±2.5 $x10^4$/ml after placebo (Figure 2).

Allergen inhalation caused a significant increase in sputum eosinophils during treatment with placebo both at 7h ($p<0.01$) and 24h ($p<0.005$) post allergen, being 64.1±17.4 $x10^4$/ml at 7h, and 97.6±28.7 $x10^4$/ml at 24h (Figure 2). There was a trend for sputum eosinophils to increase 24h following allergen inhalation during budesonide treatment, being 41.0±13.0 $x10^4$/ml at 24h, but this increase did not achieve significance ($p=0.076$). Inhaled budesonide significantly attenuated the allergen-induced increases in sputum eosinophils to

$10.4\pm3.1\times10^4$/ml at 7h and $41.0\pm13.0\times10^4$/ml at 24h ($p<0.05$) (Figure 2).

*Blood Eosinophils*

The baseline number of blood eosinophils was unaffected by treatment with either placebo or budesonide treatment (Figure 2). However, allergen inhalation caused a significant increase in blood eosinophils during treatment with placebo, from $40.1\pm4.5\times10^4$/ml before allergen to $71.9\pm9.8\times10^4$/ml 24h after allergen ($p<0.005$). Inhaled budesonide significantly attenuated this increase ($p<0.05$) from $36.2\pm6.1\times10^4$/ml before and $43.1\pm6.4\times10^4$/ml after allergen (Figure 2).

*Bone Marrow-derived Eo/B Progenitors*

Treatment with inhaled budesonide significantly reduced the baseline numbers of bone marrow Eo/B-CFU when grown in the presence of IL-5 at both 1 ng/ml ($p<0.05$) and 0.1 ng/ml ($p<0.01$) (Figure 3), but not when grown in the presence of IL-3 or GM-CSF (Table 2). Likewise, budesonide treatment suppressed the total baseline numbers of bone marrow $CD34^+$ progenitor cells ($p<0.05$) (Figure 4) and the numbers of $CD34^+IL\text{-}3R\alpha^+$ progenitors ($p<0.01$) (Figure 5).

Inhaled allergen significantly increased the number of bone marrow Eo/B-CFU grown in the presence of either IL-3 alone (10 ng/ml, $p<0.05$), GM-CSF alone (10 ng/ml, $p<0.05$), IL-5 alone (1 ng/ml, $p<0.05$) (Figure 3) or a combination of all three cytokines ($p<0.01$), following both placebo and budesonide treatment (Table 2). In contrast to the effects on the baseline IL-5-stimulated Eo/B-CFU, treatment with inhaled budesonide did

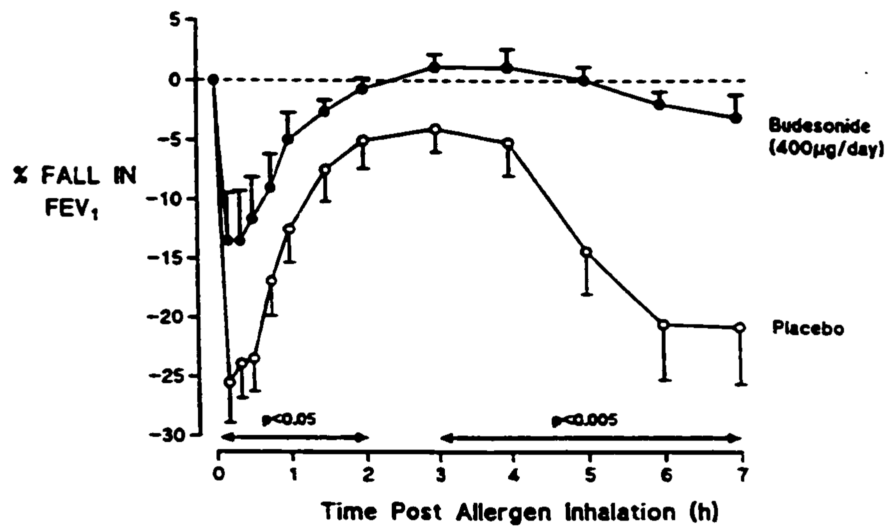


**Figure 1: Allergen-induced bronchoconstrictor responses**

Percent change in $FEV_1$ following inhalation of allergen with placebo (*open circles*) or budesonide (400μg/day) treatment (*closed circles*). Both the early (0-2 h)($p<0.05$) and the late (3-7 h) ($p<0.005$) asthmatic responses were significantly reduced with budesonide treatment.

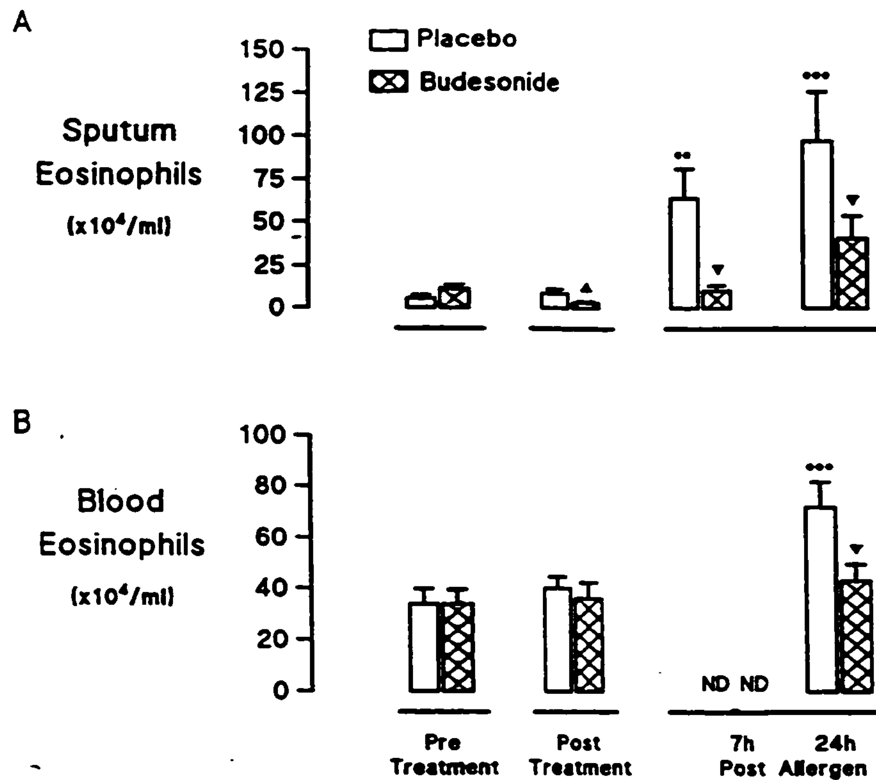


**Figure 2: Allergen-induced airway inflammation and blood eosinophilia**

Sputum (top panel) and blood (bottom panel) eosinophil values before and after allergen inhalation, following treatment with placebo (*open bars*) or budesonide (400μg/day) (*hatched bars*). Baseline measurements of sputum (▲$p<0.005$) but not blood eosinophils were affected by treatment with budesonide. Compared with the post-treatment, pre-allergen baseline levels, a significant increase in both sputum eosinophils at 7h (** $p<0.01$) and 24h post-allergen (***$p<0.005$) and blood eosinophils at 24 h post-allergen (***$p<0.005$) was observed. These allergen-induced increases were significantly reduced by treatment with inhaled budesonide (400μg/day)(▼$p<0.05$). ND = not done.

not significantly attenuate the allergen-induced increases in Eo/B-CFU in response to any cytokine (Table 2).

Inhaled allergen significantly increased the number of CD34$^+$IL-5R$\alpha^+$ progenitors, and again this effect was not attenuated by treatment with inhaled budesonide (Figure 5). After inhaled allergen during placebo treatment, the number of CD34$^+$IL-5R$\alpha^+$ progenitors increased from 45 cells/ 0.25x10$^6$ WBC (%SEM 24) to 112 cells/ 0.25x10$^6$ WBC (%SEM 33)($p<0.01$); during budesonide treatment these numbers increased from 33 cells/ 0.25x10$^6$ WBC (%SEM 11) to 71 cells/ 0.25x10$^6$ WBC (%SEM 18)($p<0.01$) (Figure 5). In contrast, no significant allergen-induced increase in the numbers of bone marrow-derived CD34$^+$IL-3R$\alpha^+$ progenitors was detected during either placebo or budesonide treatment (Figure 5).

There was a trend for increases in the number of both CD34$^+$GM-R$\alpha^+$ and CD34$^+\beta c^+$ progenitors following allergen inhalation in the placebo group only, but these changes were not significant (Figure 5, Table 3).

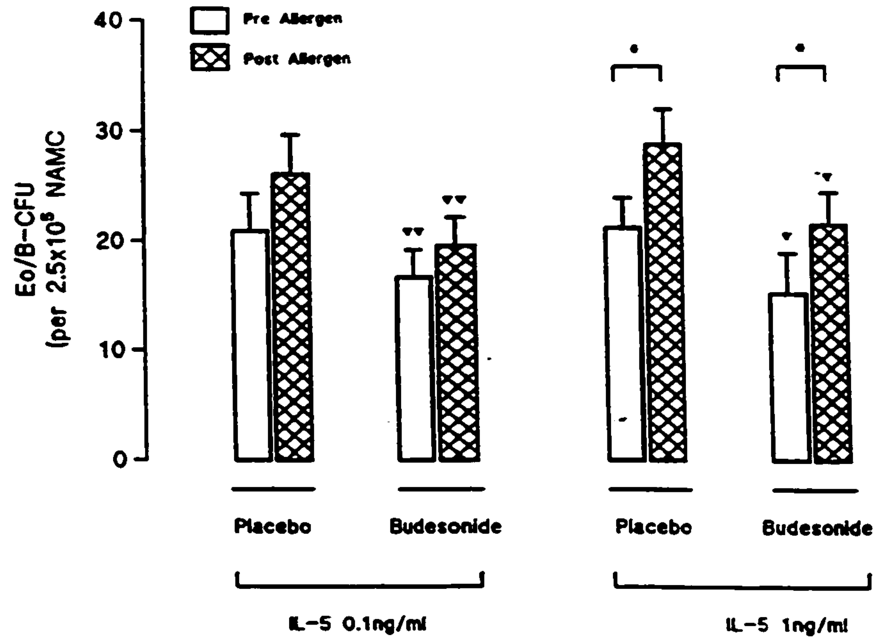


**Figure 3:** IL-5-induced changes in bone marrow Eo/B-CFU

Bone marrow Eo/B-CFU per 2.5 $x10^5$ NAMC were incubated with IL-5 at 1ng/ml or 0.1ng/ml, before (*open bars*) and 24h after (*hatched bars*) allergen inhalation, following treatment with placebo or budesonide (400µg/day). Significant allergen-induced increases in Eo/B-CFU were seen following allergen inhalation when bone marrow cells were incubated with IL-5 at 1ng/ml (*$p<0.05$) but not at 0.1ng/ml. These allergen-induced changes were not affected by treatment with budesonide. However, budesonide treatment caused a significant overall suppression of IL-5 responsive Eo/B-CFU numbers compared with placebo numbers at both 1ng/ml (▾$p<0.05$) and 0.1 ng/ml (▾▾$p<0.01$).

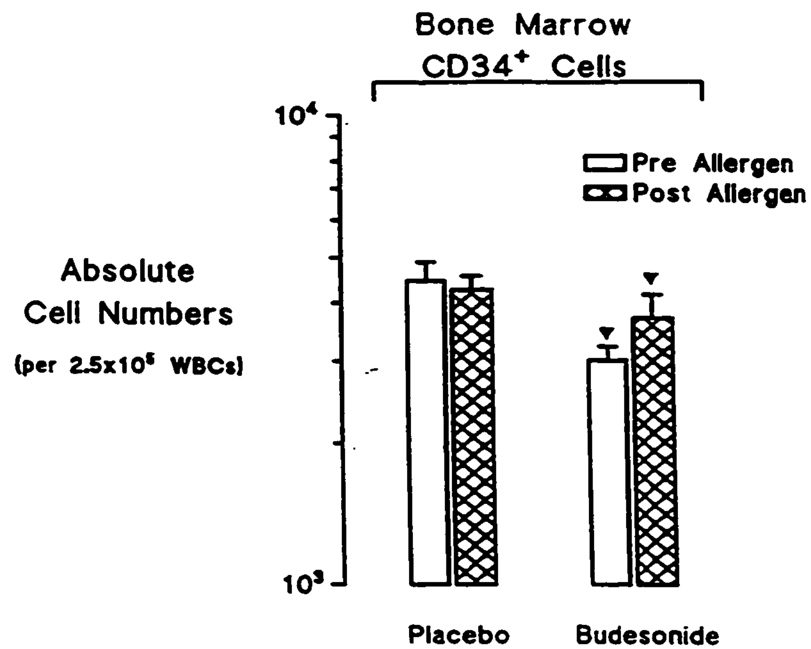


**Figure 4: Measurement by FACS analysis of total bone marrow CD34⁺ progenitors.** Following either placebo or budesonide (400µg/day) pretreatment bone marrow aspirates from (n=9) dual-responder asthmatics were taken before (*open bars*) and 24h after (*hatched bars*) allergen challenge. Due to constraints in bone marrow sample sizes, estimation of total numbers of CD34⁺ progenitors was performed in only n=8 subjects. No significant increase in CD34⁺ cell numbers was detected following allergen challenge in both treatment groups. However, treatment with budesonide caused a significant overall attenuation in bone marrow CD34⁺ cell numbers compared to the placebo pretreatment group (▾$p<0.05$).

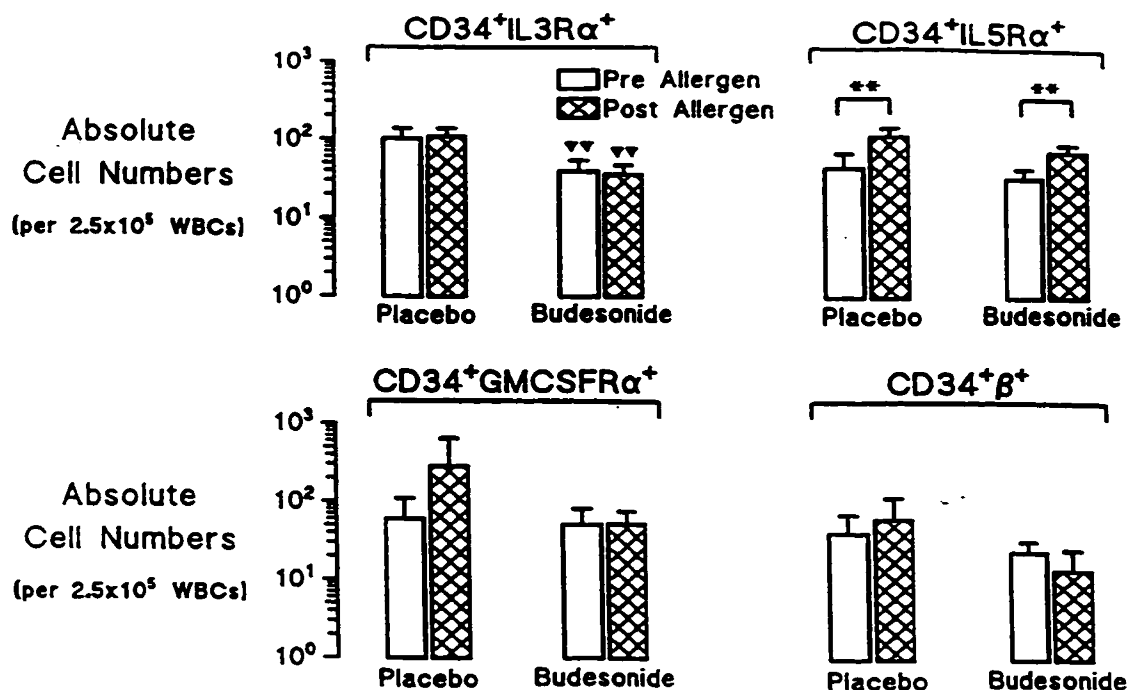


**Figure 5: Total bone marrow CD34⁺ progenitors co-expressing cytokine receptor subunits.** Bone marrow aspirates from asthmatics were taken before (*open bars*) and 24h after (*hatched bars*) allergen challenge and IL-3Rα or IL-5Rα expression on CD34⁺ progenitor cell numbers was enumerated. Following allergen challenge there was a significant increase in CD34⁺IL-5Rα⁺ (**$p<0.01$) but not CD34⁺IL-3Rα⁺ cell numbers in both treatment groups. Budesonide treatment however caused a significant overall attenuation in CD34⁺IL-3Rα⁺ cell numbers (▼▼$p<0.01$).

**Table 2 - Bone marrow progenitor (Eo/B-CFU) numbers**

Pre- and post-allergen bone marrow cells were plated in the presence of IL-3 (1 or 10ng/ml), GM-CSF (1 or 10ng/ml) or IL-5 (0.1 or 1ng/ml) or a combination of all three. Colonies are expressed as Eosinophil/basophil colony forming units per 2.5 x$10^5$ NAMC plated.

| Cytokine Stimulus | PLACEBO | | BUDESONIDE | |
|---|---|---|---|---|
| | Pre Allergen | Post Allergen | Pre Allergen | Post Allergen |
| IL-3 (10ng/ml) | 19.9±3.5 | 29.9±6.8† | 17.8±4.2 | 22.7±6.0† |
| IL-3 (1ng/ml) | 10.7±3.7 | 12.2±3.8 | 11.2±3.5 | 11.4±3.7 |
| GM-CSF (10ng/ml) | 23.2±3.5 | 30.7±6.5† | 19.8±4.0 | 24.2±5.0† |
| GM-CSF (1ng/ml) | 25.2±5.3 | 24.3±4.6 | 21.1±3.0 | 20.8±2.9 |
| IL-5 (1ng/ml) | 21.2±2.8 | 28.8±3.2† | 15.2±3.7▼ | 21.5±2.9†▼ |
| IL-5 (0.1ng/ml) | 20.9±3.4 | 26.0±3.6 | 16.7±2.5▼▼ | 19.6±2.6▼▼ |
| Combination (high) | 38.3±4.0 | 52.1±7.1†† | 33.6±3.8 | 41.9±5.6†† |
| Combination (low) | 41.2±10.8 | 43.9±7.2 | 29.61±3.5 | 36.1±3.3 |

† P<0.05 compared to baseline, †† P<0.01 compared to baseline; ▼(p<0.05) Budesonide numbers were significantly reduced compared with placebo numbers; ▼▼(p<0.01) Budesonide numbers were significantly reduced compared with placebo numbers; Combination (high) = IL-3 (10ng/ml) + GM-CSF (10ng/ml) + IL-5 (1ng/ml); Combination (low) = IL-3 (1ng/ml) + GM-CSF (1ng/ml) + IL-5 (0.1ng/ml)

**Table 3 - Cytokine receptor expression by bone marrow progenitors**

Pre- and post-allergen bone marrow cells were collected prior to and 24h post allergen inhalation, following placebo and budesonide treatment. Data from n=8 subjects are presented as geometric mean (%SEM)and are expressed as numbers of $CD34^+$ cells co-expressing the specific cytokine receptor subunit per 2.5 x$10^5$ NAMC.

| Cytokine Receptor Subunit | PLACEBO | | BUDESONIDE | |
|---|---|---|---|---|
| | Pre Allergen | Post Allergen | Pre Allergen | Post Allergen |
| GM-Rα | 60 (12) | 282 (8) | 51 (2) | 52 (2) |
| β common subunit | 41 (1) | 62 (2) | 24 (2) | 14 (1) |

No significant increase in $CD34^+$ cells co-expressing either GM-Rα or βc were detected post allergen challenge in either treatment group although a trend for an increase was detected following placebo treatment only.

## DISCUSSION

This study has demonstrated that treatment with a low daily dose of the inhaled corticosteroid budesonide (400μg/day) for 1 week, caused a selective suppression of baseline numbers of IL-5-responsive eosinophil/basophil progenitors and in the absolute numbers of $CD34^+$ and $CD34^+IL\text{-}3R\alpha^+$ hemopoietic progenitors in the bone marrow of allergic asthmatic subjects. Treatment with the inhaled corticosteroid also attenuated allergen-induced early and late asthmatic responses, as well as allergen-induced increases in blood and airway eosinophils. In addition, this study has confirmed our previous findings of allergen-induced increases in bone marrow Eo/B-CFU (10) and $IL\text{-}5R\alpha^+$ expression on $CD34^+$ progenitor cells (11). Inhaled budesonide, however, had no effect on the allergen-induced increase in this progenitor subpopulation in the bone marrow, despite the fact that the treatment regimen did attenuate both allergen-induced blood and airway eosinophilia.

The ability of inhaled budesonide treatment prior to allergen inhalation, to attenuate the magnitude of allergen-induced early and late asthmatic responses and increases in both sputum and blood eosinophils at 24 hours has been previously described (2), and was an expected result. However, the lack of effect of inhaled budesonide on the allergen-induced increases in IL-5-responsive Eo/B CFU and on $CD34^+IL\text{-}5R\alpha^+$ progenitors was unexpected. This is because of our previous observations of the ability of inhaled budesonide to abolish inhaled allergen-induced increases in GM-CFU in allergic dogs (17), and to attenuate the number of circulating Eo/B-CFU in asthmatic subjects with an acute exacerbation (23). However, the dose of budesonide used in the canine study was approximately 15 times

greater than that used in this study, which may explain, in part, the lack of effect of budesonide on allergen-induced increases in bone marrow eosinophil progenitors. In this study, the dose of inhaled budesonide that is effective in preventing the allergen-induced increases in both blood and airway eosinophils is not sufficient to prevent the bone marrow from increasing its production of eosinophil progenitors. However, pretreatment with inhaled budesonide did have a significant suppressive effect on the baseline number of IL-5-responsive Eo/B-CFU, the absolute number of $CD34^+$ progenitors, and the absolute number of $CD34^+IL\text{-}3R\alpha^+$ progenitors demonstrating that these doses of inhaled budesonide have a systemic effect in inhibiting the turnover of specific sub-populations of bone marrow progenitor cells. It is not possible from this study to decide whether this effect on bone marrow eosinophil progenitors occurs because of inhibition of signals from the airways or a direct effect on the bone marrow, or a combination of both. It would be of interest to look at the effect of inhaled budesonide on bone marrow eosinophil progenitors in normal subjects, since these subjects would likely not release a signal from the airways and any demonstrated suppression in baseline progenitor numbers would be as a result of a direct systemic effect on the bone marrow.

It is possible that IL-3, GM-CSF, IL-5 or a combination of all three, may be involved in the upregulation of the IL-5 receptor on progenitor cells, and that the level of budesonide present in the bone marrow may be sufficient to inhibit the production of these cytokines in the steady state, resulting in a lower level of IL-5-responsive cells. However, following a

stimulus such as inhaled allergen, the production of these cytokines may increase to an extent that cannot be over-ridden by the presence of budesonide, resulting in the allergen-induced increases observed in this study. IL-3 responsiveness usually appears earlier during the differentiation process, and on more primitive hemopoietic progenitor cells than IL-5 responsiveness (24); since budesonide had a suppressive effect on the baseline levels of $CD34^{+}IL\text{-}3R\alpha^{+}$, it is possible that higher inhaled doses of budesonide or a longer treatment period may result in the inhibition of allergen-induced increases of more lineage-committed progenitors such as $CD34^{+}IL\text{-}5R\alpha^{+}$ cells. The differences in the effect of inhaled budesonide on baseline $CD34^{+}IL\text{-}5R\alpha^{+}$ cells compared to IL-5-responsive Eo/B-CFU may potentially be explained by differences in the cell populations assayed by these two methods: the group of progenitor cells responding to IL-5 in the colony assay is very heterogenous, while the $CD34^{+}IL\text{-}5R\alpha^{+}$ cell is a relatively homogenous, primitive eosinophil progenitor bearing CD34, a surface receptor which is progressively lost as the progenitor matures, and not present on the colony-forming cell. It is the latter which is likely more sensitive to budesonide.

Another surprising result obtained in this study was the complete attenuation by inhaled budesonide, of the allergen-induced blood eosinophilia, measured 24h after allergen, at the time when the bone marrow eosinophil progenitors were significantly increased. This suggests that inhaled budesonide may prevent maturation of the eosinophil progenitors in the bone marrow, or may prevent release of the mature cells from the bone marrow. However, there was a trend for sputum eosinophils to increase at 24 hours following allergen inhalation

in the budesonide treatment arm, suggesting that trafficking of some eosinophils is occurring into the airways from the blood. If this is the case, it could account for the lack of increase in blood eosinophils at 24 hours following challenge, and it may be that eosinophils are being released from the bone marrow into the blood, even though the results suggest that they are being inhibited by budesonide.

Increases in bone marrow Eo/B-CFU were demonstrated after allergen challenge, when optimal concentrations of IL-3, IL-5 and GM-CSF either alone or in combination were used, *in vitro (ex vivo)*. These findings suggest that there is an increase in the numbers of lineage-committed progenitor cells that are able to respond to these "eosinopoietic" cytokines following allergen challenge in asthmatic subjects. Molecular cloning of cytokine receptors has revealed that IL-3R, IL-5R and GM-R are uniquely composed of heterodimeric structures consisting of a distinct α-subunit that binds the cognate cytokine with low affinity, and a common, shared, β-subunit which, although failing to bind the ligand itself, forms high affinity cytokine binding sites in association with the α-subunit (25,26). Therefore, the current findings showing a preferential increase in the proportion of bone marrow $CD34^+$ cells expressing IL-5Rα-subunit in response to allergen inhalation may suggest an increase in the ability of these progenitor cells to respond more readily to IL-5, and thus differentiate terminally into mature eosinophils and basophils.

Previous studies from our laboratory in a canine model of allergen-induced airway hyperresponsiveness and airway inflammation, have demonstrated that allergen inhalation increases bone marrow GM-CFU production (17), and that this is due to an as yet

unidentified hemopoietic activity released into the circulation following allergen inhalation, which stimulates the bone marrow (27). The present study raises the possibility that, following allergen inhalation by atopic asthmatics, IL-5 may represent one such hemopoietic signal, with a significant effect on IL-5-responsive-progenitors induced in the bone marrow; this could then lead to increased production of eosinophils, and promote eosinophilic inflammation of the airways. Alternatively, a recent study by Minshall *et al* showed that following allergen inhalation, T-lymphocytes in the bone marrow were a significant source of IL-5 in sensitized mice (28) suggesting that events occurring in the airways may result in local bone marrow production of IL-5, leading to increased production of eosinophils. In support of a bone marrow source of these signals, differentiating eosinophils themselves are a potent source of GM-CSF and IL-5, especially after allergen challenge (29).

The allergen-induced increase in sputum eosinophils was inhibited, but not completely attenuated by treatment with inhaled budesonide. A considerable body of evidence now exists suggesting that IL-5 and eotaxin co-operate in mediating a rapid transfer of eosinophils from the bone marrow to the lung in response to allergen challenge (30,31). Recent findings show that the generation of eotaxin, unlike IL-5, may not be inhibited by budesonide (31), thus preventing the complete ablation of sputum eosinophilia in response to allergen challenge. Studies in both mice and guinea-pigs have shown that while eotaxin may play a predominant role in eosinophil recruitment into the lungs, IL-5 is pivotal in triggering the traffic of mature eosinophils from the bone marrow into the peripheral circulation (30-33). In addition, it is possible that budesonide treatment may inhibit the

allergen-induced eosinophilia in the present study through attenuating allergen-induced increases in IL-5 generation.

In summary, this study has demonstrated that inhaled budesonide reduces baseline numbers of bone marrow $CD34^+$ progenitors, $CD34^+IL\text{-}3R\alpha^+$ progenitors and IL-5-responsive Eo/B-CFU in allergic asthmatic subjects, as well as allergen-induced increases in blood and airway eosinophils. However, inhaled budesonide did not significantly attenuate allergen-induced increases in bone marrow IL-5-stimulated Eo/B CFU or $CD34^+IL\text{-}5R\alpha^+$ progenitors. This indicates a systemic anti-inflammatory property of even low doses of inhaled budesonide on bone marrow responsiveness and that, during inhaled budesonide treatment, allergen-induced increases in subpopulations of early eosinophil/basophil progenitors may be blocked from fully differentiating into mature cells, or that the mature cells are not released from the bone marrow.

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# CHAPTER 6

# SUMMARY OF FINDINGS AND GENERAL DISCUSSION

## SUMMARY OF FINDINGS

The results of studies described in this thesis indicate that the bone marrow may play a pivotal role in allergic airway disease, through the increased production, and release of inflammatory cell progenitors in response to an airway stimulus. The studies suggest that these cells are contributing to the development of both airway hyperresponsiveness and airway inflammation in allergic asthma.

In the first two studies, the effect of allergen inhalation on bone marrow progenitor cell production in mild asthmatic subjects was examined. We demonstrated, for the first time, that bone marrow progenitors were increased following allergen inhalation in asthmatic subjects. Eosinophil progenitors (Eo/B-CFU) were significantly increased 24 hours after an allergen challenge in isolated early responders and both early and late responders. These increases were seen when bone marrow progenitors were incubated in the presence of optimal concentrations of IL-3, GM-CSF or IL-5. However, when these cells were incubated in the presence of a sub-optimal concentration of IL-5, increases in eosinophil progenitors were seen only in the dual responder group, suggesting that the bone marrow of subjects that develop late asthmatic responses contains a population of progenitor cells that are more responsive to IL-5. This could be due to an increase in numbers of this specific sub-population of cells or due to an up-regulation of the IL-5 receptor on the surface of these

cells. In support of the latter, the second study examined cell surface marker expression using flow cytometry and demonstrated that preferential increases in the proportion of $CD34^+$ cells expressing the α-subunit of the IL-5 receptor (IL-5Rα) occurred in dual responders, but not isolated early responders following allergen inhalation. These data suggest that a distinct phenotypic switch occurs within the bone marrow progenitor cell population and that, in the presence of IL-5, increased expression of IL-5Rα on $CD34^+$ cells may favor eosinophil production, thus contributing to the development of blood and tissue eosinophilia seen in allergic responses.

The third study examined the effect of allergen inhalation on trafficking of newly formed cells from the bone marrow in a canine model of allergen-induced AHR. In this study, BrdU was used to label proliferating cells *in vivo,* and allowed us to follow their movement through different compartments following allergen inhalation. We demonstrated increased numbers of BrdU positive cells in the blood and BAL fluid 24 hours following allergen inhalation compared with a group of dogs that inhaled diluent, and these cells were of the granulocytic series as assessed by morphological examination. There was a significant positive correlation between the number of BAL neutrophils and the number of BrdU positive cells in the BAL. These findings suggest that the increased progenitor cell production seen in the bone marrow following allergen inhalation can contribute towards the inflammatory response occurring in the airways by trafficking of these cells and their progeny to the inflamed airway site.

In the fourth study, we examined the effect of inhaled budesonide on the production

of bone marrow eosinophil progenitors in mild asthmatic subjects. Budesonide inhalation significantly attenuated the airway responses compared with placebo and suppressed the baseline numbers of $CD34^{+}$ cells, $CD34^{+}IL\text{-}3R\alpha^{+}$ cells and IL-5 responsive Eo/B-CFU. Allergen inhalation significantly increased the number of Eo/B-CFU and $CD34^{+}IL\text{-}5R\alpha^{+}$ cells confirming the results in the first and second study. However, inhalation of budesonide had no suppressive effect on these increases. These findings suggest that short term inhalation of budesonide has a systemic effect in inhibiting the turnover of a subpopulation of bone marrow progenitor cells, but that this effect can be overcome by inhalation of allergen.

In summary, the findings in this thesis suggest that inflammatory cell progenitors in the bone marrow are affected by inhalation of allergen, with increased production and trafficking to the airways. These data are consistent with our overall hypothesis and our specific hypotheses 1, 2 and 3. These data also suggest that while budesonide may have an inhibitory effect on the steady state turnover of eosinophil progenitors, it has no effect on the allergen-driven events occurring in the bone marrow, suggesting that the inhibitory effects of short term budesonide inhalation on inflammation and airway responses may not be a consequence of interference with allergen-induced IL-5 receptor upregulation. This result rejects our specific hypothesis 4, which proposed that the allergen-induced increases in eosinophil progenitors would be reduced by treatment with inhaled budesonide. The significance of these findings is discussed in the following section.

GENERAL DISCUSSION

The aim of this thesis was to examine the role of bone marrow inflammatory cell progenitors in models of allergen-induced AHR and inflammation. The results obtained suggest that inflammatory progenitor cells contribute to the ongoing airway pathology through increased marrow production, and/or subsequent release and trafficking of inflammatory cell progenitors and their progeny to the inflamed airway site. The data also suggests that IL-5 plays an important role in this process through increased responsiveness of these progenitor cells to IL-5 and by up-regulation of specific IL-5 receptors on progenitor cells.

IL-5 is produced by T-lymphocytes, eosinophils and mast cells as a 40-45KD glycoprotein with a unique disulphide-linked, anti-parallel homodimeric structure (Milburn *et al*, 1993). As previously discussed, IL-5 is unique in its ability to promote the terminal differentiation and maturation of eosinophil-committed lineages (Clutterbuck *et al* 1989) and is a predominant regulator of eosinophilia *in vivo* (Dent *et al*, 1990). An important role for IL-5 in allergic inflammation has been demonstrated by the ability of a neutralizing anti-IL-5 monoclonal antibody to inhibit both antigen- or virus-induced AHR and eosinophil infiltration into the airways of mice (Nagai *et al*, 1993; Kung *et al*, 1995), guinea pigs (Chand *et al*, 1992; Gulbenkian *et al*, 1992; Van Oosterhout *et al*, 1995) and primates (Mauser *et al*, 1995; Egan *et al*,1995). Similar observations have also been made in IL-5 deficient mice (Foster *et al*, 1996). The human IL-5 receptor (IL-5R) has been identified on mature eosinophils and basophils but not neutrophils or monocytes (Lopez *et al*, 1991), and

the IL-5Rα subunit has been shown to exist as either a membrane or soluble isoform (Tavernier *et al*, 1991; Devos *et al* 1993; Koike & Takatsu, 1994). The soluble isoform binds to IL-5 with the same affinity as the membrane isoform and can antagonize the action of IL-5 on its target cells (Koike & Takatsu, 1994; Tavernier *et al*, 1992). As much as 90% of the expressed IL-5Rα may be in the soluble (inactivating) isoform while the remainder is in the membrane anchored (activating) isoform, thereby allowing the soluble isoform to potentially act as a natural antagonist for IL-5. In a recent study of asthmatic subjects, the level of messenger RNA (mRNA) expression for the membrane bound isoform of IL-5Rα in endobronchial biopsies was shown to be inversely correlated with $FEV_1$ values, while the expression of mRNA for the soluble isoform of IL-5Rα was directly correlated with the $FEV_1$ (Yasruel *et al*, 1997). These findings indicate that the generation of the two isoforms of IL-5Rα may be inversely related to each other and that up-regulation of mIL-5Rα transcripts may influence eosinophil responses and accompanying airway responses.

It is possible that following allergen inhalation, there is a systemic release of tissue-derived signal(s) from the airways that provide a communicative link with the bone marrow. This factor(s) could function by acting directly on the bone marrow or by stimulating the local production of factor(s) within the bone marrow micro-environment. One of these factors to consider is IL-5. Support for this comes from the observed upregulation of IL-5 receptors on a subpopulation of progenitor cells, leading to an increase in IL-5 responsiveness and subsequent increase in production of eosinophils, and also from recent evidence that IL-5 can itself up-regulate IL-5R (Bachert *et al* 1998). There is *in vivo* evidence

for the generation of a serum hemopoietic factor during allergen challenge, where serum taken from dogs at the time of allergen-induced AHR is capable of stimulating the production of GM-CFU from naive (pre-allergen), autologous bone marrow (Inman *et al*, 1996[a]) and from the bone marrow of dogs who do not develop either AHR or bone marrow responses following allergen inhalation (Inman *et al*, 1996[b]). The identity of this serum factor, however, remains to be elucidated.

Evidence for the generation of an antigen-specific T-cell-derived hemopoietic activity in humans has been shown *in vitro*, where increased numbers of eosinophil progenitors were grown from the peripheral blood of atopic subjects in the presence of antigen-stimulated lymphomononuclear cell conditioned medium (Denburg *et al*, 1985). While the factor(s) generated were not classified, IL-5 is certainly a candidate molecule.

Finally, studies in balb/c mice have demonstrated increased local generation of IL-5 within the bone marrow, following allergen challenge, which was shown to be co-localized to T-cells (Minshall *et al*, 1998). This supports the view that IL-5 may play an important role in expanding eosinophil production within the bone marrow during an allergic inflammatory response.

Another important finding in this thesis is that differential bone marrow responses exist between dual and isolated responders. Eosinophil progenitors from dual responder subjects demonstrated both an increased responsiveness to IL-5 and increased up-regulation of IL-5 receptors compared with isolated early responders. In addition to bone marrow progenitors, increases in peripheral blood Eo/B-CFU (Gibson *et al*, 1991) and $CD34^+$ cell

numbers (Sehmi *et al*, 1996[a]) have been demonstrated in dual but not isolated early responders. The data shows that the degree of responsiveness of eosinophil progenitors in the marrow and trafficking of these progenitors through the circulation, are associated with the magnitude of the eosinophilic response in the airways. The nature of this association remains to be elucidated, but it is possible that the increase in bone marrow eosinophil production seen following allergen inhalation is contributing to the inflammatory response in the airways, and that the degree of bone marrow responsiveness may be a determinant of the magnitude of the subsequent airway eosinophilia.

We have demonstrated an increased trafficking of progenitor cells from the bone marrow through the circulation, to the site of inflammation in the lungs. This suggests that progenitor cells are being recruited to the site of inflammation where presumably they undergo differentiation and maturation within the tissue. A number of studies have shown that a wide variety of hemopoietic cytokines are expressed or produced in increased amounts by the epithelial cells and fibroblasts of inflamed airways in patients with nasal polyposis, allergic rhinitis and asthma (Denburg *et al*, 1990; Ohnishi *et al*, 1989; Ohtoshi *et al*, 1991; Kim *et al*, 1996; Nakagawa *et al*, 1995). More specifically, IL-5 has been detected both at the mRNA (Hamid *et al*, 1991; Robinson *et al*, 1992 & 1993) and protein levels (Broide *et al*, 1992) at sites of allergic inflammatory reactions. This supports the view that local tissue generation of cytokines may provide the appropriate micro-environment required to induce *in situ* differentiation of eosinophil-committed progenitors.

In both murine (Kung *et al*, 1995; Mould *et al*, 1997) and guinea pig (Collins *et al*,

1995) models, intravenously administrated IL-5 has been shown to play a role in the egress of eosinophils from the bone marrow to blood. Thus, IL-5 may play a role in the increased trafficking of mature eosinophils and possibly their progenitors, from the bone marrow to the airways following allergen challenge, in addition to its role in the increased production of bone marrow eosinophil progenitors.

While the data in this thesis is supportive of a major role of IL-5 in eosinophil production and trafficking to inflamed areas, it is unlikely that it is the only cytokine involved. The study in chapter 5 demonstrated that the allergen-induced increase in sputum eosinophils was inhibited, but not completely attenuated by treatment with inhaled budesonide. This is in agreement with a previous study, where inhalation of budesonide caused an approximately 50% inhibition of allergen-induced sputum eosinophilia (Gauvreau *et al*, 1996). Recent evidence suggests that IL-5 and eotaxin co-operate in mediating a rapid transfer of eosinophils from the bone marrow to the lungs (Collins *et al*, 1995) and that, unlike IL-5, the generation of eotaxin may not be inhibited by corticosteroid action (Humbles *et al*, 1997); this would prevent the complete ablation of sputum eosinophilia in response to allergen challenge. More recently, eotaxin has been shown to induce the release of both mature eosinophils and their progenitors from the bone marrow when administered intravenously (Palframan *et al*, 1998). Thus eotaxin, like IL-5, may be also be involved in the mobilization and trafficking of eosinophil progenitors, in addition to the recruitment of eosinophils to sites of allergic inflammation.

A surprising result of our work was the inability of inhaled budesonide to inhibit the

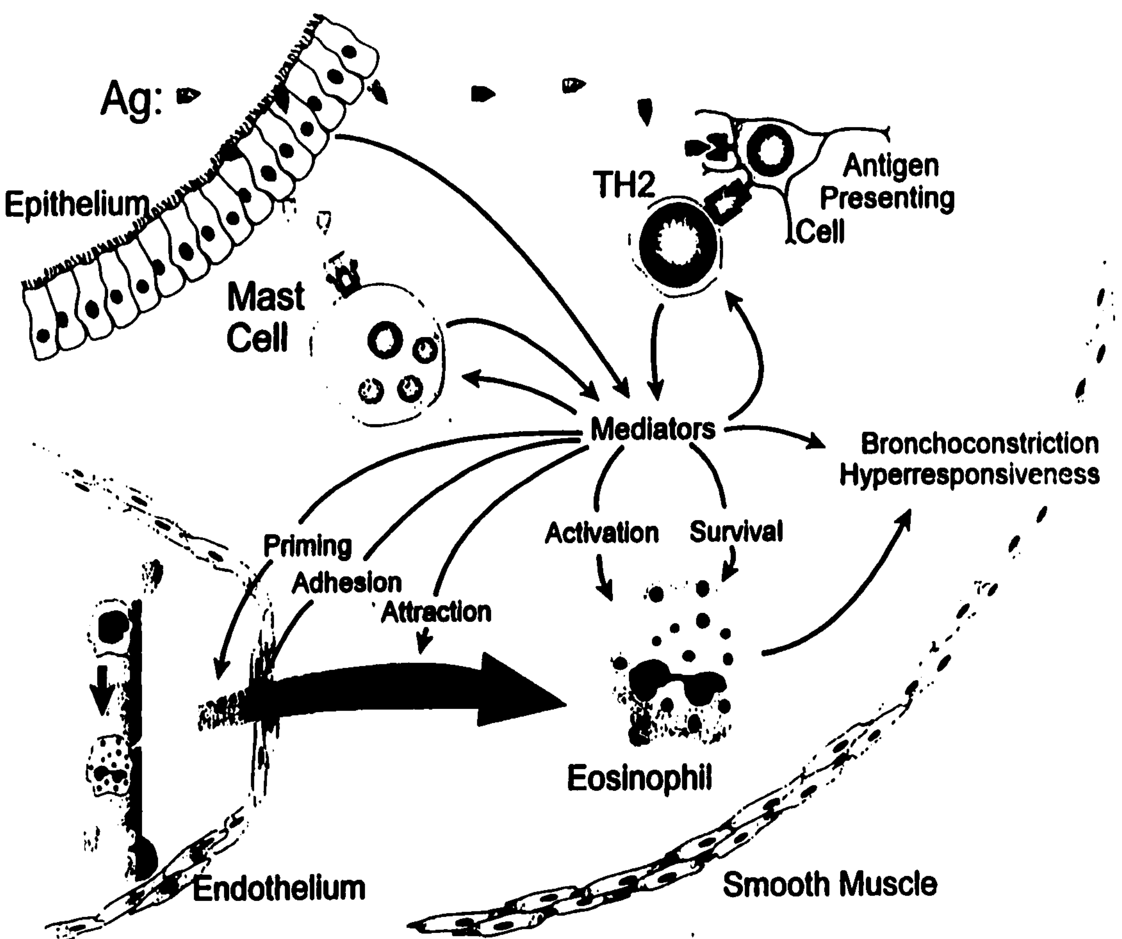
allergen-induced increase in eosinophil progenitors, despite suppressing baseline numbers. Corticosteroids exert their effects by binding to glucocorticoid receptors (GR), which are localized to the cytoplasm of target cells. (reviewed by Barnes *et al*, 1998). GR are widely distributed within the human lung, with the greatest expression on airway epithelial cells and bronchial vascular endothelial cells (Adcock *et al* 1996). Once bound, the steroid/GR complex locates to the nucleus where it binds to DNA and exerts its effects by altering gene transcription. Alternatively they may act indirectly by binding to transcription factors such as Activator Protein-1 (AP-1) (Jonat *et al*, 1990) and nuclear factor-kappa B (NF-κB) (Adcock *et al*, 1994), preventing the interaction of these molecules with DNA and the resultant gene transcription. Thus corticosteroids may control airway inflammation in asthma through increased transcription of anti-inflammatory genes and/or by decreasing the transcription of inflammatory genes (Barnes & Adcock,1993; Barnes, 1996). Recent studies have shown that steroids are capable of inhibiting mediator release from alveolar macrophages (John *et al*, 1997), eosinophils (Kita *et al*, 1991; Evans *et al*,1990), T lymphocytes (Krouwels *et al*, 1996) and epithelial cells (Schweibert *et al*, 1996) which may, in part, explain the inhibitory effects of steroids on the airways. It is possible that the systemic levels of budesonide achieved after inhalation, and reaching the bone marrow, were sufficient to suppress the cytokine production that is required for the steady state production of eosinophils, thus lowering baseline numbers of eosinophil progenitors. Following an allergic stimulus, however, the production of these cytokines may increase to a level where they could overcome the effect of budesonide, resulting in the allergen-induced increases

seen. If the signal for increased bone marrow production is coming from the lungs, then it is a signal that is probably unaffected by steroid action. However, if the signal is bone marrow-derived signal, such as IL-5, then an increased dose and/or a longer duration of treatment with budesonide may be required to see an inhibitory response in the bone marrow. In addition, the complete attenuation of blood eosinophils seen in this study suggests that budesonide could be acting by preventing the release of mature eosinophils from the bone marrow.

In summary, allergen challenge in the airways leads to increases in local mediator release which, in addition to their direct effects on the development of bronchoconstriction and airway hyperresponsiveness, are also involved in the recruitment of inflammatory cells to the airways (Figure 6.1). The results from this thesis suggest that inhalation of allergen is also associated with increased eosinophil progenitor activity in the bone marrow, particularly in those subjects that develop more severe airway responses, and with increased trafficking of newly formed cells from the bone marrow to the airways. Finally, inhalation of budesonide at a dose sufficient to inhibit the airway responses, has an inhibitory effect on baseline numbers of bone marrow eosinophil progenitors but is unable to overcome the allergen-induced increases in these cells.

## LABORATORY TECHNIQUES

For the first study, subjects were selected for their airway responses and then allocated to the dual responder or isolated early responder groups. Within this design,




SUMMARY

1. Increased numbers of Eo/B-CFU
2. Upregulation of the IL-5 receptor
3. Increased IL-5 responsiveness and IL-5 R expression in Dual responders
4. Increased trafficking of newly formed cells
5. Inhibition of specific sub-populations of bone marrow progenitor cells

Figure 6.1 - The effect of allergen inhalation on bone marrow progenitor cells

subjects had a bone marrow aspirate before and after allergen challenge, which allowed the pre-allergen bone marrow to serve as the subject's own control. This design allowed the bone marrow samples to be taken very close together, minimizing the time frame over which external, environmental factors could affect the bone marrow response. In addition, bone marrow samples were taken at the same time of the day to control for possible diurnal variation which may also affect the response of the bone marrow. The drawbacks with this design however, were that it did not allow for the investigator to be blinded, since all subjects received only allergen challenge, and the order in which the bone marrow aspirates were performed could not be randomized. In the second human study, this problem was overcome by designing the study as a double-blind, placebo-controlled, randomized, crossover design.

The methyl cellulose colony forming assay used to identify bone marrow Eo/B-CFU has played a crucial role in the characterization of both colony types and evaluation of the hemopoietic properties of new growth factors. Its drawbacks however are in the length of time required to grow colonies (>1 week), poor reproducibility due to lack of standardization between institutions, high variability, and the assumption that colony growth under artificial *in vitro* conditions truly reflects the number of lineage-specific progenitors *in vivo*. The problem of variability was reduced in our studies, firstly by minimizing the time between sampling as discussed and secondly, by preparing all reagents and growth factors beforehand, in sufficient quantities for the entire study, such that all samples were prepared and cultured under identical conditions with the same batch numbers of media and growth factors. This effect is highlighted by looking at the differences in responsiveness of bone marrow samples

to growth factors *between* the two studies, where different cytokine batches were used.

To complement the data obtained from the colony forming assays, in collaboration with Dr Roma Sehmi, we also looked at the surface expression of IL-5Rα on CD34$^+$ cells using antibodies directed against these molecules. Flow cytometry is a rapid and highly reproducible means of surface marker analysis and previous studies of progenitor cell surface markers in allergic disease has shown a close correlation between CD34$^+$ cell numbers and total colony forming units (Sehmi *et al*, 1996[b]). A drawback of flow cytometry is that often only a small number of cells are present that are positive for the relevant marker(s) and it is sometimes difficult to distinguish a true positive stain from non-specific or artifactual staining. This can be overcome by the use of isotype control antibodies, that have no reactivity with the marker being studied, in place of the specific antibody. Isotype control antibodies were used in all flow cytometric analyses performed as part of this thesis. In addition, we used a multi-parameter sequential-gating strategy to gradually eliminate contaminating cells that stain non-specifically with anti-CD34, a technique which has previously been shown to accurately enumerate CD34$^+$ progenitor cells (Sutherland *et al*, 1994). Finally, the expression of mRNA for membrane bound IL-5Rα was colocalized to CD34$^+$ cells, confirming that CD34$^+$ can express this molecule, and that the results obtained from flow cytometry were not due to non-specific staining of the antibody used. In both of the human studies performed, the results from the colony forming assay were corroborated by the flow cytometry data suggesting that our results reflect actual events that are occurring in the bone marrow in response to allergen inhalation.

In the study of the canine model of allergen-induced airway hyperresponsiveness, the design included two groups of dogs; an allergen inhalation group and a diluent control group. In a pilot study, BrdU positive cells were still detectable in dogs at four weeks after allergen challenge, which made it impossible for the dogs to serve as their own controls. Thus, a separate set of dogs served as a control group. However, both groups contained dogs matched for the degree of allergen-induced airway responsiveness measured during a screening allergen challenge.

BrdU positive cells were detected by an immunohistochemical staining technique where a monoclonal antibody against BrdU was used to stain the DNA of cells. The specificity of the antibody for BrdU was checked during staining in two ways. Firstly, an isotype control was used to stain a duplicate slide of each sample, and secondly, the pre-challenge sample acted as a negative control, since it was taken before BrdU was administered, and no positive cells were detected in any of these samples.

Results from this study need to be interpreted with care, especially when comparing the canine to a human model of allergic asthma. The physiological responses after inhaled allergen in dogs differ from humans both in airway responses and cellular infiltration. The dogs do not develop allergen-induced late responses after inhaled A.*suum*, and rarely develop an airway eosinophilia. Neutrophils appear to be a pivotal cell in the development of allergen-induced AHR. In addition, budesonide has been shown to inhibit the allergen-induced increases in bone marrow GM-CFU in dogs, albeit at a high concentration (Woolley *et al,* 1994), while allergen-induced increases in Eo/B-CFU in human bone marrow are

unaffected by budesonide treatment. The study in dogs, however, allowed for the first time, the demonstration of trafficking of newly divided inflammatory cells from the bone marrow to the airways in response to allergen, suggesting that the bone marrow is not only producing more inflammatory cells but that they actively contributing to the ongoing airway inflammation in this model.

## STATISTICS

The statistics used in analyzing the data presented in this thesis were appropriate for the measurements taken. For statistical analysis of within group comparisons between pre- and post-challenge time points, a Student's *t* test for paired observations (two-tailed) was performed, and a Student's *t* test for unpaired observations (two-tailed) were performed for all between group comparisons. Where repeated measures were made on a sample, at repeated time points, and for looking at the effect of budesonide treatment, a repeated measures analysis of variance (rmANOVA) was performed. rmANOVA is a powerful statistical tool in a randomized cross-over study design, since the error is based only on the variability within subjects. In the BrdU study, although dogs were initially paired based on their degree of AHR, they were not treated as pairs for statistical analysis.

## FUTURE DIRECTIONS

We have demonstrated that the bone marrow plays a role in contributing to the ongoing airway inflammation during an allergic response in asthmatic subjects, and that IL-5

is important to this process. However, these studies have not elucidated the exact mechanisms regulating this response.

Future studies will need to determine the kinetics of the allergen-induced increase in eosinophil progenitors following allergen inhalation. This would provide information on how early after allergen challenge the bone marrow becomes stimulated and whether this response plays a role in initiating the airway eosinophilia.

It is also important to establish the exact role that IL-5 plays in the bone marrow response and whether IL-5 is being delivered to the marrow via the circulation or produced locally within the bone marrow. These issues can be addressed by looking at both serum and bone marrow levels of IL-5 at various time points following challenge. In addition, the cellular source of IL-5 in the bone marrow could be determined by immunohistochemistry for IL-5 protein and by *in situ* hybridization for IL-5 message, with a double stain for T cell markers. It would also be interesting to look at eotaxin levels in these studies, to determine the role that this specific eosinophil chemokine may play in the bone marrow response.

It will be important to further investigate the mechanism of steroid action on bone marrow responses. It would be interesting to determine if a longer treatment period or higher dose of steroid inhalation would be effective in blocking the allergen-induced increases in eosinophil progenitors. It would also be of interest to see if inhaled budesonide is effective in attenuating any increase in IL-5 production, either in the sera or bone marrow, following allergen inhalation.

With the recent availability of canine anti-CD34$^{+}$ antibodies (McSweeney *et al,* 1998)

it will be possible to further investigate progenitor levels and trafficking in the canine model of AHR. Initial studies should look at correlations of bone marrow $CD34^+$ numbers with GM-CFU and to look at co-expression of surface markers such as GM-CSFR and G-CSFR. In addition, double staining of BrdU labeled cells with anti-CD34 antibody would further corroborate the bone marrow origin of progenitor cells that have trafficked to the lungs following allergen inhalation. Finally, it would be interesting to examine the role of inhaled budesonide on all of the above parameters, which may provide further information as to the mechanism of steroid action on the inflammatory response.

In conclusion, this thesis has demonstrated a role for bone marrow inflammatory progenitor cells in allergic airway inflammation and has highlighted an important role for IL-5 and up-regulation of its receptor in this process. Further research in this area should be aimed at establishing the mechanisms that regulate eosinophil hemopoiesis, IL-5 production and receptor upregulation. In particular, investigations of agents that can modulate IL-5 receptor expression on $CD34^+$ progenitor cells may provide insight into the control of eosinophil differentiation and IL-5 responsiveness. A more complete understanding of these fundamental concepts may provide a basis for the development of novel therapeutic interventions for allergy and asthma.

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# APPENDIX 1

# MATERIALS AND METHODS

## 1. HUMAN STUDIES

*Subjects*

In all human studies, mild asthmatic subjects were studied, and were classified as dual responders if they developed both an early and late phase asthmatic response to allergen, as defined by a greater than 15 % drop in $FEV_1$ from baseline, or as isolated early responders if they developed only an early fall in $FEV_1$ of greater than 15% from baseline. All human studies were approved by the Research Advisory Group at McMaster University Medical Centre and all subjects provided their written informed consent prior to entering the studies. On initial visits to the laboratory, a full medical history was taken and skin/prick tests were performed. Subjects were considered atopic if they had one or more positive wheal-and-flare responses to skin/prick tests. All subjects were non-smokers and none had experienced a respiratory infection during the four weeks prior to the studies. Asthmatic subjects were stable at the time of studies, requiring only intermittent use of inhaled $\beta_2$-agonists with baseline $FEV_1$ values >70% predicted.

*Spirometry*

The forced expiratory volume in one second ($FEV_1$) was used as a measurement of lung function and was assessed using a Collins 14L water-filled spirometer.

*Skin/prick test*

The cutaneous skin/prick test was used to assess the atopic status of each subject and to predict the dose of allergen required to cause a 20% fall in $FEV_1$ (predicted allergen $PC_{20}$) (Chai *et al*, 1975). Serially diluted allergen extracts (0.02 ml) were administered cutaneously using a 26-gauge 3/8" needle, to the lateral part of the upper arm. In addition, a diluent control and a histamine based control (0.01 mg/ml) were administered. The reactions were read after 20 min and recorded as the largest diameter of the wheal elevation produced by the diluent control subtracted from that produced by the allergen. The results were expressed in millimetres of wheal.

*Methacholine Inhalation Challenge*

Methacholine inhalation was performed as described by Cockcroft (Cockcroft, 1996) using the 2-min tidal breathing technique. Subjects inhaled normal saline and then increasing, doubling concentrations of methacholine phosphate (0.03 to 16 mg/ml). Aerosols were generated continuously with a Wright nebulizer (output, 0.13 ml/min; airflow, 8 L/min; particle size aerodynamic mass median diameter 1 to 1.5 μm) containing 3 ml of solution for 2 min. The $FEV_1$ was initially measured in triplicate, and then repeated once at 30 and once at 90 (and at 180 if necessary) seconds after each inhalation. Inhalations were continued until the $FEV_1$ decreased by >20% of the baseline value. The percent reduction in $FEV_1$ was calculated for each concentration using the lowest post diluent value to the lowest post methacholine value. The methacholine provocative concentration causing a 20% decrease in $FEV_1$ (Methacholine $PC_{20}$) was calculated using linear interpolation of the last two points

on the noncumulative concentration-response curve.

*Allergen Inhalation Challenge*

Allergen inhalation challenge was performed as described by O'Byrne (O'Byrne *et al* 1987). The concentration of allergen extract for inhalation was determined from a formula described by Cockcroft (Cockcroft *et al,* 1987) using results from the skin test and the methacholine $PC_{20}$. The formula, derived by single linear regression, was: $y = 0.69x + 0.11$ ($r = 0.85$), where $y = \log_{10}$ allergen $PC_{20}$ and $x = \log_{10}$ (methacholine $PC_{20}$ x skin sensitivity). The skin sensitivity value was the smallest allergen dilution that gave a wheal of 2 mm in diameter in the skin prick test. The starting concentration of allergen was chosen to be three doubling doses below that predicted to cause a 20% fall in $FEV_1$. Baseline measurements of $FEV_1$ were followed by inhalation of allergen for 2 min, using the same method as for the methacholine challenge. The $FEV_1$ was repeated 10 min after the inhalation, and inhalations continued until a decrease of 15% or more occurred in the $FEV_1$ from baseline. Measurements of $FEV_1$ were then performed at 10, 20, 30, 45, 60, 90 and 180 min, and then every hour for 7 hours after the final inhalation. The allergen-induced early response was determined as the maximal decrease in $FEV_1$ between 0 and 2 hours after allergen and the late response as the maximal decrease between 3 and 7 hours after allergen inhalation.

*Blood Samples*

Blood samples were obtained by venipuncture into vacucontainers containing ethylenediaminetetraacetic acid (EDTA). Total cell counts were performed using a Neubauer hemocytometer and differential cell counts were made from blood smears stained by Diff-

Quik (American Scientific Products, McGaw Park, IL). Differential cell counts were performed by one investigator in a blinded fashion and the mean of two slides obtained (300 cells counted per slide). Cells were classified using standard morphologic criteria. Results were expressed as absolute counts ($10^9$ cells/L or $10^4$ cells/ml).

*Sputum Analysis*

Sputum was induced and processed according to the method of Popov *et al* (1994). Subjects inhaled 3%, 4% then 5% aerosolized saline for 10 min each until an adequate sample was obtained or if the $FEV_1$ dropped 20% from baseline. Cell plugs were selected from the sample, placed in an eppendorf tube and the weight recorded. Next, dithiothreitol (0.1%) (Sputolysin, Calbiochem Corp., San Diego, CA) was added to the tube in a volume (in µl) equal to double the weight of the sputum portion (in mg) and mixed mechanically with a pasteur pipette. The sample was then further diluted with Dulbecco's phosphate buffered saline (DPBS)(GIBCO, Grand Island, NY) to a volume equal to the sputum plus dithiothreitol. The cell suspension was filtered through 52 µm nylon gauze (BNSH Thompson, Scarborough, Ontario) to remove debris and mucous, and the volume of the filtrate was recorded. The total cell count was measured using a Neubauer hemocytometer and the results expressed as $10^6$ cells/ml. The cell count was adjusted to a concentration of $1x10^6$ cells/ml with DPBS and cytospins were prepared. Cells were cytospun onto glass slides using a Shandon III cytocentrifuge (Shandon Southern Instruments, Sewickley, PA) and differential counts were performed in a blinded fashion on Diff-Quik stained slides. Mean counts from duplicate slides were obtained (500 cells counted per slide) and expressed

as percentages or absolute counts ($10^4$ cells/ml). Where possible, cytospins were also prepared on APTEX (3-aminopropyltriethoxysilane) (SIGMA Chemicals, St Louis, MO) coated slides for immunocytochemical staining for eosinophil cationic protein (ECP) using a monoclonal antibody against cleaved, activated ECP (EG2) (Girgis-Gabardo *et al*, 1994). Slides were fixed in periodate-lysine-paraformaldehyde (PLP) (McLean & Nakane, 1974) for 30 min, washed in 15% sucrose (SIGMA Chemicals, St Louis, MO) in DPBS for 30 min and then stored at -70°C. For staining, the slides were thawed, washed with DPBS, followed by wash buffer (DPBS plus 0.01M HEPES (GIBCO, Grand Island, NY) and 0.01% saponin (SIGMA Chemicals, St Louis, MO)) for a total of three 5 minute washes. Slides were then blocked with 75% human AB serum (SIGMA Chemicals, St Louis, MO) for one hour followed by 25% normal rabbit serum (SIGMA Chemicals, St Louis, MO) for 30 min. Slides were washed for 5 min with wash buffer and then incubated overnight at 4°C with the primary antibody, mouse anti human ECP (EG-2) (1 μg/ml) (Pharmacia, Uppsala, Sweden) or with a non immune mouse $IgG_1$ antibody (1 μg/ml) (SIGMA Chemicals, St Louis, MO) as a negative control. Slides were then washed three times with wash buffer and positive cells were visualized using the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique (Cordell *et al*, 1984). Slides were incubated for 45 min with a 1:50 dilution of rabbit anti-mouse IgG (DAKO Laboratories, Copenhagen, Denmark) followed by 45 min in a 1:50 dilution of a mouse monoclonal APAAP (DAKO Laboratories, Copenhagen, Denmark). Slides were washed 3 times (5 minutes each) in wash buffer following each antibody incubation. The alkaline phosphatase was developed for 3 min using the New

Fuchsin Substrate System (DAKO Laboratories, Copenhagen, Denmark) with added Levamisole (1.25mM) (SIGMA Chemicals, St Louis, MO) to block endogenous alkaline phosphatase activity. Slides were counterstained with Mayer's hematoxylin (SIGMA Chemicals, St Louis, MO) for 3 min and mounted in an aqueous medium (GVA mount, Zymed Labs, San Francisco, CA). Results are expressed as percentages of 500 cells counted under light microscopy.

*Bone Marrow Aspirate and Culture*

Bone marrow aspirates were obtained from the iliac crest using a bone marrow aspiration needle (16x2";Sherwood Medical, St Louis, MO) by a qualified hematologist. Three ml of bone marrow were aspirated into a 10 ml syringe containing 1ml sterile heparin (1000U/ml)(Leo Laboratories, Mississauga, Canada) and semi-solid methylcellulose cultures of low density non-adherent mononuclear cells were performed. Heparinized bone marrow was diluted to 50 ml with McCoy's 5A medium (GIBCO, Grand Island, NY) and separated over 65% percoll (Pharmacia, Uppsala, Sweden). The interface of the mononuclear-rich cell fraction was washed in McCoy's 5A medium and incubated in McCoy's 5A medium supplemented with 15% fetal calf serum (FCS) (GIBCO, Grand Island, NY), 1% penicillin/streptomycin (GIBCO, Grand Island, NY) and 5 x $10^{-5}$M 2-mercaptoethanol (final concentration)(SIGMA Chemicals, St Louis, MO) for 2 hours in plastic flasks at 37°C and 5% $CO_2$. Non-adherent mononuclear cells (NAMC - containing progenitor cells and lymphocytes) were then cultured (2.5 x$10^5$ cells per 35 x 10mm tissue culture dish; Falcon plastics, Oxnard, CA), in duplicate, in supplemented Iscove's Modified Dulbecco's medium

(GIBCO, Grand Island, NY) (with 1% penicillin/streptomycin and 5 x $10^{-5}$M 2-mercaptoethanol (final concentration)), 0.9% methylcellulose (SIGMA Chemicals, St Louis, MO) and 20% FCS, either alone or in the presence of the following growth factors: recombinant human IL-3 (10, 1 or 0.1 ng/ml; Pharmingen, San Diego, CA), recombinant human GM-CSF (10 or 1 ng/ml; Pharmingen, San Diego, CA) or recombinant human IL-5 (1 or 0.1 ng/ml; Pharmingen, San Diego, CA), or with a combination of all three. Cultures were incubated for 14 days at 37°C and 5%$CO_2$ after which colonies were identified as either Eo/B-CFU or GM-CFU according to previously described criteria (Denburg *et al*, 1985) and expressed as colony forming units (CFU) per 2.5 x$10^5$ NAMC plated.

*Immunofluorescence Staining of Bone Marrow Cells*

Bone marrow-derived low density NAMC (1x$10^6$ /tube), isolated as described above, were resuspended in a final volume of 100 µl of ice cold phosphate buffered saline (PBS) supplemented with 0.1% sodium azide (BDH Inc., Toronto, Ont) and 0.5% bovine serum albumin (BSA) (SIGMA chemicals, St Louis, MO) and incubated for 30-45 min at 4°C with saturating amounts (determined in preliminary studies) of biotin-conjugated monoclonal antibodies directed against the α-subunit of either IL-3R (IL-3Rα; 7G2), IL-5R (IL-5Rα; A16 or SP491), GM-CSFR (GM-CSFRα; 6E10), the β-common subunit (βc; 8E4) or $IgG_1$ isotype control antibody. These non-neutralizing monoclonal antibodies were all kind gifts from Dr. A. Lopez, Institute of Medical and Veterinary Science, Adelaide, South Australia except for SP491 which was supplied by Schering Plough Research Institute, Kenilworth, NJ, USA, and A16 which was supplied by Roche Laboratories (Ghent, Belgium). The cells were then

washed and stained with streptavidin-conjugated peridinin chlorophyll protein (PerCp) (Becton Dickinson Canada, Mississauga, Ont.), together with saturating concentrations of fluorescein isothiocyanate (FITC) conjugated $IgG_1$ CD45 antibody (anti-HLE1) and phycoerythrin (PE)-conjugated $IgG_1$ CD34 antibody (HPCA-2), or PE-linked $IgG_1$ isotype control (Becton Dickinson Canada, Mississauga, Ont.) in a final volume of 100 µl PBS (plus sodium azide and BSA) for 30 min at 4°C. Lysis buffer (Becton Dickinson Canada, Mississauga, Ont.) was then added to the cells and incubated for 5 min after which the cells were washed twice with 3 ml PBS plus 0.1% sodium azide and finally fixed in 500 µl of PBS plus 1% paraformaldehyde (BDH Inc., Toronto, Ont.). The cells were refrigerated until ready for analysis.

Cytokine receptor antibodies and the isotype matched controls were biotinylated using a long-arm biotin procedure (Coligan *et al*, 1991[a]) in which biotin was coupled to azide free protein via a hydroxysuccinimide ester after incubation with *N*-hydroxysuccinobiotin (SIGMA Chemicals, St Louis, MO); excess biotin was then removed by dialysis against borate buffered solution, pH 8.6) (BDH, Mississauga, Ontario).

*Flow Cytometry and Gating Strategy*

Cells were analysed using a FACScan flow cytometer equipped with an argon ion laser (Becton Dickinson Instrument Systems, San Diego, Ca.). Five data parameters were acquired and stored in list mode files: linear forward light scatter (FSC), linear side-angle light scatter (SSC), log FITC, log PE and log PerCp fluorescence; each measurement contained 50,000 events. Compensation settings were established using CalBrite beads

(BDIS) and confirmed using NAMNC stained with anti-CD34-PE, anti-CD45-FITC or anti-IL-5Rα-PerCp. Off-line analysis was performed using the PC lysis software as supplied by BDIS.

We used a multi-parameter five sequential-gating strategy that has previously been shown to accurately enumerate CD34$^+$ progenitor cell numbers in various biological samples (Sutherland *et al*, 1994). The rationale for sequential gating was to gradually eliminate contaminating cells that non-specifically take up anti-CD34 (Sutherland *et al*, 1996). Briefly, a primary gate using CD45 staining versus SSC (region R1) was set up to quantitate total leukocytes and distinguish contaminating events such as platelet aggregates and other debris which can non-specifically take up anti-CD34. Primitive cells characteristically express CD45 at low to intermediate levels (Borowitz *et al*, 1993) and therefore CD45$^+$ events generate a stable denominator in the calculation of the absolute CD34$^+$ value. Sequential gates were then set up: CD34 staining in region R1 versus SSC (region R2), CD45 versus SSC of CD34$^+$-gated events in R2 (region R3: to identify blast cells), and FSC versus SSC to confirm the lymphoblastoid characteristics of the gated CD34$^+$ cells in region R3 (i.e., low to medium SSC and FSC; region R4). Without changing any of the gates, analyses of the same cell sample stained with CD45-FITC and PE-linked isotype control antibody were performed. Enumeration data were derived from the gate statistics: events in gate G4 (= events in R1 to R4) after staining with CD45-FITC/CD34-PE minus events in G4 stained with CD45-FITC/PE-linked control antibody were used to calculate the absolute number of true CD34$^+$ blast cells in the test sample.

In three-colour analysis, events in region R4 were back scattered onto a dot plot of CD34-PE versus staining by PerCp-linked cytokine receptor monoclonal antibodies or control antibody, and data were collected as percent positive cells at the 99% confidence limit (i.e. relative to a marker set to include only 1% of cells stained with control antibody). The data presented are the mean of duplicate assessments and the intra-assay variability was always less than 5%.

*Cells and myeloid cell lines: controls for cytokine receptor staining*

To verify the specific binding capacity of the cytokine receptor antibodies used in these studies, we tested the staining of anti-IL-3Rα (7G2) and anti-IL-5Rα (A16) on various leukemic cell lines and mature peripheral blood leukocytes. Both HL60 clone 15 cells and KG1 myeloid leukemic cell lines were obtained from the American Type Culture collection (ATCC, Rockville, MD) and were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY). The B9 cell line, an IL-6-dependent mouse B cell hybridoma, was obtained from Dr Lucien Aarden (Red Cross Transfusion Services, Amsterdam, The Netherlands) and cultured in RPMI 10 medium plus recombinant human IL-6 (Coligan *et al*, 1991[b]). Peripheral blood-derived neutrophils and eosinophils were isolated by positive and negative selection, respectively, using a CD16 dependent magnetic cell separation technique (Sehmi *et al*, 1993) and monocytes were isolated by collecting the adherent cell population after adherence of low density mononuclear cells to plastic as described above.

*Simultaneous in situ hybridization and immunohistochemistry*

To confirm the association of the membrane bound form of IL-5Rα messenger RNA

to $CD34^+$ progenitor cells, simultaneous *in situ* hybridization and immunohistochemistry was performed (Hamid *et al*, 1992). Messenger RNA for membrane bound IL-5R$\alpha$ was detected by autoradiography and CD34 immunoreactivity was detected by the APAAP technique described above. A population of $CD34^+$ cells was enriched from cord blood by positive selection using a magnetic cell separation technique as described previously (Sehmi *et al*, 1996). These cells were cytospun on poly-L-lysine-coated slides, fixed in 4% PFA in PBS for 30 min, and washed in 15% sucrose in PBS. Preparations were hybridized with $^{35}S$-labeled membrane bound IL-5R$\alpha$ antisense riboprobe and simultaneously immunostained with a mouse anti-human monoclonal antibody against CD34 (QBEND 10; Becton Dickenson, San Jose, CA) (Hamid *et al*, 1992).

B. CANINE STUDY

*Study Design*

Two groups of dogs (random-source mongrel 21-36kg) were paired for study based on changes in airway responsiveness following a screening inhalation of *Ascaris suum* extract. After a four week period, one group inhaled *A. suum* (n=8) and the other, *A. suum* diluent (n=8). Prior to challenge, baseline measurements of airway responsiveness to acetylcholine were made. All dogs received a bolus injection of BrdU before, and at 5h following challenge. Blood samples were taken before challenge, and at 5 and 24h, and bone marrow aspirate and bronchoalveolar lavage (BAL) samples were taken 24h following

challenge. Development of AHR was assessed by changes in airways responsiveness to acetylcholine 24h following challenge.

*Group Matching Protocol*

To ensure that the groups contained dogs with similar allergen-induced airway responsiveness changes, dogs were matched for this attribute. Changes in airway responsiveness to acetylcholine during a screening allergen challenge were expressed as shifts (pre-challenge provocative concentration/post-challenge provocative concentration). Dogs with shifts that differed by less than 15% were paired and each dog randomly assigned to either the allergen or diluent group. However, dogs were not treated as pairs for statistical analysis.

*Procedures*

Dogs were anaesthetized with intravenous pentobarbitol sodium (30mg/kg; Somnotol; MTC Pharmaceuticals, Mississauga, Canada). Additional anaesthetic was administered as required during the experiment. An endotracheal tube (10mm internal diameter) was inserted and connected to a constant volume ventilator (Model 551; Harvard apparatus, South Natick, MA) set at a tidal volume of 10 ml/kg and at a rate of 30 breaths/minute. An esophageal balloon catheter was inflated as previously described (Lemens *et al*, 1974) and was placed in the esophagus at the point of most negative end expiratory pressure. The esophageal catheter and a port at the proximal end of the endotracheal tube were connected to a differential pressure transducer (Hewlett Packard 267; Hewlett Packard, Palo Alto, CA).

*Measurement of Total Pulmonary Resistance*

Total pulmonary resistance was measured as previously described (Woolley *et al*, 1994). Transpulmonary pressure was measured as the differential pressure between the endotracheal tube and the esophageal pressure. Flow was measured by use of a pneumotach (Fleisch no. 1; Instrumentation Associates, New York, NY), a differential pressure transducer (Hewlett Packard 270), and a pressure amplifier (Hewlett Packard 8805C). A continuous measurement of total pulmonary resistance was computed from the flow and transpulmonary pressure using a respiratory analyser (Hewlett Packard 8816A), which utilizes the method described by Mead and Whittenberger (1953).

*Measurement of Airway Responsiveness*

Airway responsiveness was determined as previously described (Woolley *et al*, 1994). A dose response relationship of pulmonary resistance against doubling concentrations of acetylcholine (0.7 to 80.0 mg/ml; SIGMA Chemicals, St Louis, MO) was established. After baseline pulmonary resistance was measured, the dogs inhaled normal saline and then increasing concentrations of acetylcholine at 5 min intervals until an increase of at least 5 $cmH_2O$/litre/s above the post-saline value was obtained. The response was expressed as the concentration of acetylcholine causing an increase in pulmonary resistance of 5 $cmH_2O$/litre/s above the baseline measurement and was termed the acetylcholine provocative concentration. A decrease in this value represents an increase in airway responsiveness.

*Allergen - Diluent challenge*

Allergen challenges involved inhalation of *A. suum* (stock extract $10^{-1}$ wt/vol; Greer

Laboratories, Lenoir, NC) as previously described (Inman *et al*, 1996). During the initial screening challenge, increasing concentrations of *A. suum* ($10^{-5}$,$10^{-4}$, $10^{-3}$, $10^{-2}$ and $10^{-1}$ wt/vol) were inhaled until pulmonary resistance increased by 10 cm $H_2O$. $Litre^{-1}.s^{-1}$ above pre-allergen levels. The concentration of *A.suum* producing this resistance change was used for the allergen challenge during the study (for some dogs, resistance did not increase by 10 cm $H_2O$. $Litre^{-1}.s^{-1}$ in which case $10^{-1}$ wt/vol was used). *A.suum* was delivered over 50 inhalations, of 3 second duration each, using the same nebulizer as used for acetylcholine challenges. A total of 10 min was allowed between doses during screening, and the post-challenge resistance was taken as the peak value in the 10 min following the inhalation. For the diluent challenge, the diluent used in *A. suum* preparation (0.4% phenol) was inhaled in the same concentration and manner as allergen.

*Administration of Bromodeoxyuridine (BrdU)*

BrdU (SIGMA Chemicals, St Louis, MO) was administered as equal intravenous bolus injections, 30 min before, and 5h following challenge (total BrdU 25mg/Kg) The total amount of BrdU per dog was calculated and dissolved in 60ml endotoxin free 0.9% sodium chloride solution (Baxter Corporation, Toronto, Canada). The solution was then filter sterilized and 30 ml administered per injection. The dose of BrdU was similar to that used in a rabbit model of *S. pnemoniae*-induced inflammation (Bicknell *et al* 1994 ).

*Blood samples*

Venous blood samples were obtained from each dog before, and at 5 and 24h following inhalation. The sample taken before challenge was also taken before the first

injection of BrdU and the 5h sample was taken before the second BrdU injection. Samples were collected into heparin sodium vacutainer tubes for total and differential white blood cell counts (WBC). WBC counts were performed using a Neubauer hemocytometer and differential cell counts were made from blood smears stained by Diff-Quik (American Scientific Products, McGaw Park, IL). Differential cell counts were performed by one investigator in a blinded fashion and the mean of two slides obtained (300 cells counted per slide). Cells were classified using standard morphologic criteria. Results were expressed as absolute counts ($x10^6$ cells/ml). One ml of blood was used to prepare cytospins for immunohistochemistry as discussed below.

*Bone marrow aspiration*

Bone marrow aspirates were obtained from the iliac crest of anesthetized dogs using a 16-gauge Rosenthal needle. Three ml of bone marrow were aspirated into a 10 ml syringe containing 1 ml sterile heparin (1000U/ml)(Leo Laboratories, Canada) and then immediately resuspended in 50ml of 1% bovine serum albumin (SIGMA Chemicals, St Louis, MO) in phosphate buffered saline (BSA/PBS). Prior to cytospin preparation the sample was spun for 10 min at 1500g and cytospins were then prepared for immunohistochemistry from the cell pellet as discussed below.

*Preparation of cytospins from blood and bone marrow for immunohistochemistry*

One ml of each blood sample and the bone marrow pellet were lysed for 30 seconds with 10 ml of cold 0.2% PBS to remove erythrocytes and then resuspended in 40 ml of

BSA/PBS to restore normal concentration of PBS. The samples were allowed to stand for at least 30 min to allow the white blood cells to recover from the lysing process and then resuspended in BSA/PBS. The cell concentration was adjusted to $2x10^6$/ml and cytospins were prepared on APTEX-coated slides. Slides were stored at room temperature and immunohistochemical staining was performed within 2 days of preparation.

*Bronchoalveolar lavage (BAL)*

Bronchoalveolar lavage (BAL) was performed as previously described (Inman *et al*, 1996). A fibreoptic bronchoscope (BF-Be; Olympus, Tokyo, Japan)(OD 6 mm) was passed into a third generation airway of the right middle lobe. Five 20 ml aliquots of PBS warmed to 37°C were injected into the airway via the bronchoscope, and immediately after injection of each aliquot, BAL fluid was aspirated through the bronchoscope into collection traps. The BAL fluid was then pooled, spun at 1500g for 10 min and the cell pellet resuspended in BSA/PBS. A total cell count (TCC) was performed, the cell count adjusted to $2x10^6$/ml and cytospin samples prepared on APTEX-coated slides for immunohistochemistry. Slides were stored at room temperature and immunohistochemical staining was performed within 2 days of preparation. Cytospins were also prepared on glass slides and differential counts were performed in a blinded fashion on Diff-Quik stained slides. Mean counts from duplicate slides were obtained (500 cells counted per slide) and expressed as the number of cells per ml of BAL fluid (BALF) recovered ($x10^6$/ml BALF).

*Immunohistochemical staining for BrdU-labeled cells*

Immunohistochemical staining for BrdU-labeled cells was performed according to a method previously described (Bicknell *et al,* 1994) with some modifications. All blood, bone marrow and BAL cytospins were fixed for 10 min in 1% paraformaldehyde (BDH Inc, Toronto, Canada) in PBS and then digested at 37°C for 5 min in 0.001% pepsin (SIGMA Chemicals, St Louis, MO) solution acidified to pH 2.5. DNA in the cytospin samples was denatured at 37°C for 1h in 2N HCL, followed by neutralization in three washes of 0.1M borate buffer, pH 8.5 (equal volumes of Boric acid (2mM) (BDH Inc, Toronto, Canada) and Borax (0.5mM) (BDH Inc, Toronto, Canada)), each for 10 min. A final wash in TBS-Tween buffer (equal volumes of 50mM Tris (hydroxymethyl amino-methane hydrochloride) (SIGMA Chemicals, St Louis, MO) and 150mM NaCl (BDH Inc, Toronto, Canada) plus 0.1% Tween 20 (SIGMA Chemicals, St Louis, MO) pH 7.6) was used to restore neutrality. The APAAP technique (Cordell *et al*, 1984) described above was used to detect BrdU-labeled DNA in cells. Slides were incubated consecutively in 5% rabbit serum (GIBCO, Grand Island, NY) for 15 min, then in 2μg/ml mouse anti-BrdU antibody (DAKO Laboratories, Copenhagen, Denmark) prepared with 1%BSA in TBS-Tween at room temperature in a humidified chamber for 1hr. Non-immune mouse $IgG_1$ (SIGMA Chemicals, St Louis, MO) at 2μg/ml was used as a negative control for each specimen. Incubation in a 1:20 dilution of rabbit anti-mouse IgG (DAKO Laboratories, Copenhagen, Denmark) for 30 min was followed by 30 min in a 1:50 dilution of a mouse monoclonal alkaline-phosphatase

anti-alkaline phosphatase complex (DAKO Laboratories, Copenhagen, Denmark). Slides were washed 3 times (3 min each) in TBS-Tween following each antibody incubation. The alkaline phosphatase was developed for 20 min using the Fast Red Substrate System (DAKO Laboratories, Copenhagen, Denmark), counterstained with Mayer's hematoxylin (SIGMA Chemicals, St Louis, MO) for 60 seconds and mounted in an aqueous medium (GVA mount, Zymed Labs, San Francisco, CA). The nucleus of positive cells stains bright red and duplicate slides from each sample were analyzed using light microscopy. An average number of cells per high power field (HPF) was calculated by counting 5 HPF's. The number of HPF's required to count 10000 cells was then calculated, the number of BrdU-positive cells in 10000 cells were recorded and expressed as the percentage of BrdU-positive cells. For some slides, where insufficient cells were present on the slide, the number of cells counted was always between 5000 and 10000 cells.

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