NOTE TO USERS

THE ROLE OF BONE MARROW INFLAMMATORY CELL PROGENITORS IN ALLERGEN-INDUCED ASTHMA

By

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ABSTRACT

Asthma is a chronic airways disease characterised by recurrent episodes of wheezing and variable airflow obstruction, which is usually reversible spontaneously or with treatment, and with airway hyperresponsiveness, and airway inflammation. Allergen inhalation by sensitized, atopic asthmatics enhances airway hyperresponsiveness and inflammation, providing a useful model to study allergic asthma.

The aim of this thesis was to characterise the allergen-induced changes in bone marrow eosinophil/basophil (Eo/B) progenitors, their ability to migrate out of the bone marrow and to determine whether these cells accumulate in the airways to participate in the allergen-induced airway inflammatory response. In addition, this thesis attempted to investigate the importance of pro- and anti-inflammatory cytokines and chemokines which contribute to the migration and differentiation of these cells. Bone marrow aspirates, blood and sputum samples were obtained from subjects at various time points both before and following allergen inhalation challenge.

In comparison to isolated early responders, who develop no allergen-induced late asthmatic responses or airway hyperresponsiveness, bone marrow Eo/B progenitor numbers were increased in dual responders, who develop marked allergen-induced airway eosinophilia and airway hyperresponsiveness. Increases in IL-3-responsive progenitors were detected as early as 5 hours post-allergen, and IL-5-responsive progenitors at 12 and 24 hours post-allergen in dual responders only. Bone marrow, blood and sputum IL-5 protein levels increased at 12 and 24 hours in dual responders only and these increases correlated with

increases in IL-5-responsive progenitors. Additionally, bone marrow IFN-γ levels increased in dual responders at 48 hours coinciding with decreases in bone marrow Eo/B progenitors.

Expression of the receptor for eotaxin (CCR3) was detected on primitive (CD34 immunopositive cells) and eosinophil-lineage committed progenitors ($CD34^+IL\text{-}5R\alpha^+$ cells) by flow cytometry and confirmed by co-localization of CCR3 messenger RNA to $CD34^+$ cells using *in-situ* hybridization. When pre-allergen was compared to 24hours post-allergen levels, significant increases in bone marrow $CD34^+CCR3^+$ cells were detected in dual responders, who also developed a significant sputum and blood eosinophilia and increased methacholine airway responsiveness. In contrast, a significant attenuation of bone marrow $CD34^+CCR3^+$ cells was observed in isolated early responders. In a dose dependent fashion eotaxin, but not IL-5, stimulated $CD34^+$ progenitor cell migration *in vitro*.

Allergen-induced dual responders also developed a significant decrease in bone marrow SDF-1α 24 hours post-allergen while developing a significant increase in circulating eotaxin and $CXCR4^+$ cells. These findings indicate that the bone marrow actively participates in the development and persistence of allergen-induced airway inflammation and the participation is regulated by IL-3, IL-5 and eotaxin and down-regulation of SDF-1α for differentiation and migration.

Eo/B progenitors also migrate into the airway as part of the allergen-induced inflammatory response. In comparison to diluent control, sputum $CD34^+$ cells increased at 7 hours following allergen inhalation, an effect which was sustained at 24 hours in the dual responder group only, associated with sustained increases in sputum $CD34^+IL\text{-}5R\alpha^+$ cells,

eosinophils and IL-5 protein levels. These findings indicate that $CD34^+$ cells increase in the airways post-allergen inhalation and have the potential to respond to IL-5, contributing to allergen-induced asthma.

This thesis has demonstrated time-dependent changes in cytokine levels in airway, blood and bone marrow coordinating the activation, recruitment and differentiation of eosinophil/basophil progenitors from the bone marrow to the airways. Furthermore, the activation and trafficking of eosinophil progenitors is more pronounced in asthmatics developing a late inflammatory airway response with associated airway hyperresponsiveness and pronounced eosinophilia. Eosinophil/basophil progenitors, potentially regulated by inflammatory cytokines appear to play an important role in the inflammatory process associated with allergic asthma.

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First and foremost, I would like to thank Dr. Paul O'Byrne for accepting me as a graduate student in his laboratory. From the beginning I knew that Dr. O'Byrne would not lead me astray in the hectic world of research and he has not. Paul is a truly inspirational mentor, especially when you begin to feel uncertain or discouraged. He has a remarkable ability to create enthusiasm for a project and I know this fuels much of the creativity within his lab. For these reasons and many more, thank you Paul.

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such as 'calf.' She also taught me that things outside of the lab are just as, if not more important than things inside; Thanks to Rick Watson for not only being the best respiratory technician on the planet (I am convinced) but for also being a grayt friend; Thanks to George Obminski for his upbeat outlook and all his help with both the subjects and with my computer woes and; I also thank Joceline Otis for her expertise and her undying patience in 'doing things right.'

Last but not least, I thank my husband Tim for being so understanding and so giving of his love and support. I thank my parents and my sister for always standing by me, for encouraging me, for listening and for keeping my spirits high. For these reasons and many more, I would like to dedicate this thesis to all of you.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
APAAP	Alkaline phosphatase anti-alkaline phosphatase
BAL	Bronchoalveolar lavage fluid
BSA	Bovine serum albumin
BFU-E	Burst forming unit-erythroid
CFU	Colony forming unit
DR	Dual responder
EAR	Early asthmatic response
Eo/B CFU	Eosinophil/basophil colony forming unit
ECP	Eosinophil cationic protein
EDN	Eosinophil derived neurotoxin
EPO	Eosinophil peroxidase
FBS	Fetal Bovine Serum
FEV_1	Forced expiratory volume in 1 second
FITC	fluorescein-5-isothicyanate
FSC	linear forward light scatter
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte/macrophage colony stimulating factor
GM-CFU	Granulocyte/macrophage colony forming unit
h	Hour

HDM	House dust mite - D. Farinae
HPSC	Hematopoietic stem cells
Ig	Immunoglobulin
IER	Isolated early responder
IL	Interleukin
IL-R	Interleukin receptor
LAR	Late asthmatic response
MBP	Major basic protein
Meg-CFU	Megakaryocytic colony forming unit
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein - alpha
mRNA	Messenger ribonucleic acid
NAMC	Non-adherent mononuclear cells
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PC_{20}	Provocative concentration required to cause a 20% fall in FEV_1
PE	Phycoerythrin
PFA	Paraformaldehyde
PLP	Periodate-lysine PFA
SDF-1α	Stromal cell-derived factor – 1 alpha
SEM	Standard error of the mean

SSC	Linear side-angle light scatter
RANTES	Regulated on activation normal T expressed and secreted
RW	Ragweed
Th2	T helper 2
TGF	Transforming growth factor
WBC	White blood cell count

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PREFACE

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

THE KINETICS OF BONE MARROW EOSINOPHILOPOIESIS AND ASSOCIATED CYTOKINES AFTER ALLERGEN INHALATION. Sandra C. Dorman, Roma Sehmi, Gail M. Gauvreau, Rick M. Watson, Ronan Foley, Graham L. Jones, Judah A. Denburg, Mark D. Inman and Paul M. O'Byrne.
Published: American Journal of Respiratory and Critical Care Medicine. 2004; 169(5):565-572.

ALLERGEN-INDUCED FLUCTUATION IN CC CHEMOKINE RECEPTOR EXPRESSION ON BONE MARROW CD34$^+$ CELLS FROM ASTHMATIC SUBJECTS: SIGNIFICANCE FOR MOBILIZAION OF HAEMOPOIETIC PROGENITOR CELLS IN ALLERGIC INFLAMMATION. Roma Sehmi, Sandra C. Dorman, Adrian Baatjes, Rick M. Watson, Ronan Foley, Sun Ying, Douglas S. Robinson, A. Barry Kay, Paul M. O'Byrne, and Judah A. Denburg.
Published: Immunology. 2003; 109(4):536-46.

TRANSMIGRATIONAL RESPONSES OF PROGENITOR CELLS TO EOTAXIN AND SDF-1α IN HUMANS. Sandra C. Dorman, Irene Babirad, Julia Post, Rick M. Watson, Ronan Foley, Graham L. Jones, Paul M. O'Byrne and Roma Sehmi
Submitted: Journal of Allergy and Clinical Immunology

SPUTUM CD34$^+$IL-5Rα^+ CELLS INCREASE AFTER ALLERGEN: EVIDENCE FOR IN SITU EOSINOPHILOPOEISIS. Sandra C. Dorman, Ann Efthimiadis, Irene Babirad, Rick M. Watson, Judah A. Denburg, Frederick E. Hargreave, Paul M. O'Byrne, and Roma Sehmi
Published: American Journal of Respiratory and Critical Care Medicine. 2004; 169 (5):573-577.

These studies are clinical studies, completed by the Asthma Research Group in Hamilton, Canada involving the collection of bone marrow aspirates, blood and sputum from human volunteers to assess the activation and migrational responses of progenitor cells following allergen-induced airway responses. These allergen challenge studies required input from many people. Dr. Paul O'Byrne provided and organized the financial support, laboratory space, equipment and technical support. Dr. Kieran Killian supervised the clinical procedures and provided expertise during screening and testing of research subjects. Rick Watson and George Obminski carried out the clinical procedures including allergen and methacholine challenges. Dr. Ronan Foley and Dr. Graham Jones performed the bone marrow aspiration procedures. Julia Post, Irene Babirad and Shauna Denis provided technical support to collect and analyse sputum samples. Dr. Roma Sehmi offered considerable expertise and laboratory space to analyse collected samples. Dr. Judah Denburg, Dr. Gail Gauvreau, Dr. Mark Inman and Dr. Gerald Cox provided additional knowledge and support.

I am the 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 outlining the purpose, methods and analysis of the experiments. I was involved with the clinical procedures and performed methacholine and allergen inhalation challenges, sputum induction, blood sampling and I assisted with the bone marrow aspirations. I was responsible for all the subsequent assays on the samples collected including sputum processing, methylcellulose tissue culture, migration assays and cytochemical and immunochemical

staining of the cells. Pilot studies were required to modify the cytokine levels for the samples collected in these experiments. I collected the data and completed the required statistical analyses for each study. The figures and tables included in each paper are my own work and although feedback was provided by the other authors of the papers, it was my responsibility to write each paper.

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

CHAPTER 1

INTRODUCTION

Asthma

The National Heart, Lung and Blood Institute's Second Expert Panel on the Management of Asthma has defined bronchial asthma as:

> "a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular, mast cells, eosinophils, T lymphocytes and epithelial cells," (Expert Panel Report 2 1997).

This inflammatory process causes recurrent episodes of airway obstruction, characterized by wheezing, breathlessness, chest tightness and a cough that often is worse at night and in the early morning (Szefler 2004). These episodes, are usually reversible either spontaneously or with treatment. The inflammatory process, or its consequences in airway remodelling, is also associated with the other physiological hallmark of asthma which is an increase in airway hyperresponsiveness to a variety of stimuli (Hargreave *et al,* 1981). Asthma prevalence rates and incidence has been shown to steadily increase in the world making it a global health concern (American Lung Association 2000).

Environmental allergens are one important cause of asthma and allergen-induced asthma is characterized by an inappropriate immune response to airborne substances. This is associated with a preferential Th2 type immune dominance (Corrigan *et al,* 1990) and an increased infiltration of eosinophils (Holgate *et al,* 1992), which are bone marrow-derived inflammatory cells, into the lungs. The effecter functions of T cells are determined by the array of cytokines that they produce (Janeway *et al,* 2001). Th2 type cytokines include interleukin (IL)-4 and IL-13 which, along with B cell ligation via

CD40, initiates the humoral immune response by activating naïve antigen-specific B cells to produce IgE antibodies (Anderson *et al,* 1995). Other Th2 type inflammatory chemokines and cytokines include, GM-CSF, eotaxin, IL-3, IL-5, and IL-10, all of which are capable of increasing either eosinophil differentiation, activation or recruitment (Wardlaw *et al,* 1995). Blood and airway eosinophil numbers and severity of asthma are positively correlated (Wardlaw *et al,* 1988), suggesting a causal role for eosinophils, especially since eosinophil-derived products can contribute to the tissue damage seen in asthma (Houston *et al,*1953). Many of the current approaches being studied today to treat allergy and asthma are examining mechanisms to block the differentiation, recruitment and activation of this cell type.

Allergen Inhalation Challenge

Allergen inhalation challenge by sensitized subjects can result in two distinct physiological responses. These are the allergen-induced early response resulting in bronchoconstriction developing within minutes after inhalation of the allergen. This bronchoconstriction is caused by the release of chemical mediators from IgE-coated mast cells, including histamine, cysteinyl leukotrienes and prostaglandins (Gordon *et al,* 1990), which collectively, cause a rapid increase in airway vascular permeability and contraction of airway smooth muscle (Beasley *et al,* 1989; Busse 1998; Mulder *et al,* 1999). This response is transient and usually resolves between 1 to 3 hours (Bentley *et al,* 1997).

In some individuals, the early response is followed by the development of a late phase response (Booij-Noord *et al,* 1972; Herxheimer 1952). The late-phase response

can begin as early as 2 hours after allergen inhalation and is characterized by bronchoconstriction, airway inflammation and increased airway responsiveness. The inflammatory component is due to the release of inflammatory mediators including cysteinyl leukotrienes, chemokines and cytokines from the activated mast cells and other cells, possibly including the airway epithelial cells, and airway smooth muscle cells, inducing smooth muscle contraction, airway edema and the recruitment of inflammatory leukocytes in particular, eosinophils, basophils and Th2 lymphocytes (Bentley *et al,* 1997). These infiltrating cells then provide the potential for an additional source of mediators enhancing the inflammatory response (Weersink *et al,* 1994). Increased airway responsiveness can be demonstrated using inhaled bronchoconstrictor agonists such as methacholine or histamine. This increase is usually measured 24 hours after allergen inhalation and can persist for several days or even weeks (Cockcroft *et al,* 1977).

Early and late-phase asthmatic responses can be elicited by experimental challenge in the laboratory (O'Byrne *et al,* 1987). The development of bronchoconstriction is measured using forced expiratory volume in one second (FEV_1) (O'Byrne *et al,* 1987), which is a reliable and reproducible, albeit indirect, measure of airway caliber. During this test, patients are asked to inspire to total lung capacity and then to exhale as rapidly as possible into a spirometer that records expired air over time. Experimental allergen inhalation challenge, classically involves inhalation of doubling concentrations of allergen until the FEV_1 has fallen by 20% of its baseline value. The FEV_1 is then monitored every 10 minutes until 30 minutes, every 15 until 90 minutes, again at 120 minutes and then every hour for 7 to 12 hours. (O'Byrne *et al,* 1987)

Early studies using this clinical model, demonstrated that only 50% of atopic adult asthmatics challenged with inhaled allergens develop both an early and a late asthmatic reaction (dual responders) (Booij-Noord *et al,* 1972). Those that do not go on to develop late airway responses (isolated early responders) do not develop marked or persisting airway hyperresponsiveness post-allergen inhalation (Cockcroft *et al,* 1977).

Since the development of dual asthmatic responses after allergen inhalation is known to be associated with increases in inflammatory cells in the airway and this development can be quantified in a laboratory setting (Gauvreau *et al,* 2000), it allows for a useful clinical model to study increased inflammatory airway responses and associated airway functional changes, by separating subjects into isolated early responders and dual responders.

Eosinophils in Airway Inflammation

Eosinophils are granule-containing cells that can secrete major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN), all of which can directly cause contraction of smooth muscle (Coyle *et al,* 1994). In addition, they contain lipid bodies which store arachidonic acid and enzymes, including cyclo-oxygenase and 5-lipoxygenase which are capable of producing prostaglandins and leukotrienes respectively. Eosinophils are also able to synthesize cytokines and chemokines which can increase the overall inflammatory response (Giembycz *et al,* 1999).

The association between bronchial asthma and eosinophilia in the blood, sputum and tissue is strong and has been noted since the early 1900's (Ellis *et al,* 1908). However, the debate about a central role of the eosinophil in asthma continues. A causative role has been difficult to prove. While it is generally agreed that the symptoms and alterations in airway physiology are the result of inflammation, in the past, no direct assessment of inflammation of the airways has been used to establish a diagnosis.

Autopsy findings from patients that died during an acute severe asthmatic episode have provided considerable information about the pathological changes seen in the airways. The general pathologic changes include sub-epithelial fibrosis, goblet cell hyperplasia, mucous gland hypertrophy, smooth muscle hyperplasia, mucous plugging, airway edema, epithelial sloughing, increased vascular permeability and inflammatory cellular infiltrate (Laitinen *et al,* 1994). It has been hypothesized that this 'airway remodeling' is the consequence of long standing airway inflammation. Biologically, eosinophils have several features which would allow them to contribute directly to the inducement of these pathological findings. MBP, ECP and EPO have all been shown to cause extensive epithelial damage with epithelial sloughing as seen in asthma (Ayars *et al,* 1989; Gundel *et al,* 1991; Motojima *et al,* 1989). Positive correlations were observed between the concentrations of MBP, the numbers of desquamated epithelial cells in BAL fluid and the degree of bronchial hyperresponsiveness (Wardlaw *et al,* 1988). In addition, decreases in MBP levels are measurable in successful asthma therapy (Gleich *et al,* 1979). Further evidence for a role of MBP in asthma comes from Gundel *et al.*

demonstrating that instillation of MBP into the trachea of primates causes a dose-dependent increase in airway hyperresponsiveness (Gundel *et al,* 1991).

Other products released from eosinophils include platelet activating factor (PAF) and cysteinyl leukotrienes (Cromwell *et al,* 1990; Weller *et al,* 1983). PAF has a number of proinflammatory activities including leukocyte chemoattractant and activating agent (Wardlaw *et al,* 1986). Inhalation of PAF causes bronchoconstriction in humans and increases in bronchial hyperresponsiveness (Cuss *et al,* 1986). Eosinophils can produce substantial quantities of PAF (Cromwell *et al,* 1990). Cysteinyl leukotrienes also exhibit a number of biological properties that may be relevant to asthma including smooth muscle contraction, mucous hypersecretion and increased vascular permeability. Cysteinyl leukotrienes are currently targeted directly in asthma therapy with varying degrees of success (Reviewed in (O'Byrne, 1997)).

Lastly, eosinophils are known to be able to produce proinflammatory cytokines and chemokines including interleukin(IL)-5, Granulocyte/macrophage-colony stimulating factor (GM-CSF), IL-3, IL-8, macrophage inflammatory protein (MIP)-1alpha and regulated on activation normal T expressed and secreted (RANTES)(Braun *et al,* 1993; Broide *et al,* 1992; Costa *et al,* 1993; Kita *et al,* 1991; Nakajima *et al,* 1996). Production of these cytokines as well as an ability to present allergen, via major histocompatatibily complex (MHC) II, to T cells gives eosinophils the ability to not only be terminal effectors in asthma, but to also elicit immunomodulatory effects; that being the ability to drive naïve T cells towards a Th2 phenotype (Lucey *et al,* 1989; Weller *et al,* 1993).

Eosinophilic infiltration into the airways has in the past, been shown to be significantly correlated with allergen-induced late asthmatic responses (Wardlaw *et al,* 1988) and subjects developing an early and late asthmatic response also develop significantly greater airway and circulating eosinophilia (Takanashi *et al,* 1995). In addition, sputum eosinophilia has been shown to correlate with asthma severity (Bousquet *et al,* 1990) and with airway hyperresponsiveness in asthmatics (Louis *et al,* 2002; Walker *et al,* 1991); however, eosinophil numbers in biopsies and sputum have not consistently correlated with bronchial hyperresponsiveness in subjects with mild asthma (Djukanovic *et al,* 1990; Rosi *et al,* 1999).

Recently, a study reported by Leckie *et al.* has intensified the scrutiny of the role of eosinophils in asthma. This group infused a humanized monoclonal antibody against IL-5 into atopic asthmatic subjects and showed significantly decreased circulating eosinophil counts. They also described a lack of effect on the development of allergen-induced late asthmatic response, or airway hyperresponsiveness and concluded that eosinophils were not essential in the development of these features of allergic asthma (Leckie *et al,* 2000). Unfortunately, this study was not adequately designed to properly evaluate the role of airway eosinophils in these responses (O'Byrne *et al,* 2001). Animal studies examining the eosinophil's role in asthma have been equally contradictory and inconclusive (Corry *et al,* 1998; Hogan *et al,* 1997; Hogan *et al,* 1998; Karras *et al,* 2000; Lee *et al,* 1997; Mathur *et al,* 1999; Shardonofsky *et al,* 1999).

Inhalation of IL-5 by asthmatic subjects has been shown to cause increased airway eosinophilia and airway hyperresponsiveness (Shi *et al,* 1997). Furthermore,

human studies continuing the examination of anti-IL-5's ability to deplete eosinophils, found that eosinophils in the tissues of the airways and in the bone marrow are only reduced by 50% and treatment had no appreciable effect on eosinophil MBP levels (Flood-Page *et al,* 2003; Menzies-Gow *et al,* 2003). No changes in clinical measures of asthma were noted in this study, however since eosinophils were not depleted in the airways, conclusive statements could not be made. Similarly, Kips *et al.* using a different anti-IL-5 monoclonal antibody found significant reductions in blood eosinophils, without significant differences in sputum eosinophils in subjects with severe persistent asthma treated with oral or high doses of steroids (Kips *et al,* 2003). As yet, eosinophils cannot be excluded as a target for asthma therapy and in fact, current results provide support for the increased examination of this cell type.

Hematopoiesis

Hematopoeitic Stem Cells (HPSC) are self-renewing progenitors that give rise to all lineages of blood cells and are found in all hematopoietic organs, including the bone marrow, blood and spleen of adults (Chertkov 1986). The generation of mature cells from these pluripotent stem cells involves the highly regulated progression through successive stages including commitment to a specific cell lineage, terminal differentiation of lineage restricted progenitors and growth arrest (Bedi *et al,* 1995). The majority of HPSC are thought to be dormant (G_0) or in the steady state of the cell cycle, with only a few cells supplying all of the hematopoietic cells at a given time. HPSC are capable of extensive replication from which daughter cells may either replace the HPSC or

differentiate along a hematopoietic lineage. The mechanisms controlling the decision to differentiate or renew are unknown, but two theories currently predominate. The stochastic model (Till *et al,* 1964) proposes that the HPSC randomly divide and commit to either differentiation or self-renewal. Considerable evidence exists to support this concept (Leary *et al,* 1984; Leary *et al,* 1985; Suda *et al,* 1983; Suda *et al,* 1984; Till *et al,* 1964).

Alternatively, the inductive model argues that lineage commitment is dependent on external stimuli, particularly cytokines, which alter gene transcription and regulate the survival of cell colonies (Ogawa 1993; Williams *et al,* 1990). A number of different cytokines are involved in regulating and modulating hematopoiesis, many of which have now been identified and characterized, at least at the cellular level. Cytokine effects include the proliferation/differentiation, survival/programmed cell death and migration/homing of stem cells (Byung *et al,* 2000). Cytokine activity can be categorized as stimulatory, inhibitory or co-stimulatory, although some cytokines can elicit more than one activity, either via different cell types or different receptors.

It is now generally agreed that a combination of the two proposals exist. Stochastic processes may determine the preferred differentiation pattern of HPSC daughter cells, but these may be reinforced or overridden, by external factors along the developmental pathway (Inman *et al,* 1998). Whatever the mechanism, the further differentiation of cells gives rise to committed progenitors which can proliferate and mature along a single pathway.

An important component to the development and functioning of HPSC is the hemopoietic microenvironment. The marrow stroma tissue is a network of cells which physically support and can directly influence HPSC proliferation, differentiation and migration (Naeim *et al,* 1996). Accessory cells to the stroma, including T cells and monocytes can also contribute to the microenvironment by secreting multiple cytokines (Mayani *et al,* 1992). Overall, microenvironmental cells can influence hemopoiesis in either a positive or negative way, by different mechanisms including: direct cell-to-cell contact; secretion of proteins constituting the extracellular matrix and; via the production of cytokines. It is evident that more than one of these mechanisms can operate simultaneously (Mayani *et al,* 1992).

The development of *in vitro* assays for hematopoietic progenitor cells has significantly contributed to our knowledge of the regulation of hematopoiesis. Due to the scarcity of progenitor cells in hematopoietic tissue, these cells could only be quantified by their ability to produce clones and these were grown in semisolid media. These 'clones' are made up of a single cell capable of giving rise to a hematopoietic cell colony, termed a 'colony forming unit (CFU)'. The various CFU that can be identified using this technique are, the 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) (Naeim *et al,* 1996). Identification of progenitor colonies 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. Systematic functional analysis of cell surface antigen expression has led to the identification of rare populations highly enriched for stem cell activity. The cell surface sialomucin-like adhesion molecule CD34 was originally discovered as the result of a strategy to recognize subsets of human marrow cells, but not mature blood and lymphoid cells (Civin *et al,* 1984). It is now used as a convenient marker for HPSC. CD34 is expressed on 1-5% of mononuclear bone marrow cells, on a subpopulation of hematopoietic cells – both HPSC and early committed progenitors (Krause *et al,* 1996).

Eosinophilopoiesis

Eosinophils share a common progenitor with basophils (Denburg *et al,* 1985) and the major cytokines controlling the production of these cell types are IL-3, GM-CSF and IL-5 (Denburg *et al,* 1989). Of these IL-3 and GM-CSF appear to be important for expansion of the stem cell populations and IL-5 appears to be the growth factor involved in commitment and maturation of the myeloid cells along the eosinophil (Clutterbuck *et al,* 1989) and basophil pathway (Denburg *et al,* 1989). Significantly, IL-5, the cytokine responsible for eosinophil survival, activation, migrational responses and adhesion to endothelium for extravasation is an eosinophilopoietin (Clutterbuck *et al,* 1988). Furthermore, the mRNA for the IL-5 receptor alpha (IL-5Rα) has been co-localized to $CD34^+$ cells, indicating that this may be the phenotype of the earliest Eo/B lineage-committed progenitor cell (Sehmi *et al,* 1997).

Evidence for Bone marrow Progenitor Activation in Asthma

Evidence of a role for bone marrow progenitors, in particular eosinophil/basophil progenitors in asthma, began with studies that demonstrated: *i)* elevated blood levels of Eo/B-CFU in atopic subjects compared with normals (Otsuka *et al,* 1986; Otsuka *et al,* 1987); *ii)* increased circulating Eo/B-CFU during acute asthma exacerbations and following allergen inhalation challenge in atopic asthmatics (Gibson *et al,* 1990; Gibson *et al,* 1991); *iii)* increases in Eo/B-CFU during seasonal exposure to allergen in allergic rhinitis (Linden *et al,* 1999). Together, these results provided indirect evidence that changes in circulating progenitor cell numbers reflected dynamic hemopoietic processes within the bone marrow and supported the hypothesis for progenitor cell transmigration from bone marrow to blood.

The first studies to examine changes in the bone marrow were performed in a dog model and showed significant increases in the number of GM-CFU colonies grown 24 hours after allergen inhalation. In dogs that develop bronchoconstriction with allergen inhalation, the only cell which increases in bronchoalveolar lavage after allergen is the neutrophil (Woolley *et al,* 1994). Therefore, an increase in GM-CFU was an appropriate bone marrow response. Furthermore, in a murine model, it was demonstrated that intranasal or airway challenge with specific antigen, elicited an airway eosinophilic inflammatory response with significantly increased Eo/B CFU production from the bone marrow (Denburg *et al,* 1996; Inman *et al,* 1999).

Wood *et al.* (Wood *et al,* 1998) were the first to examine the number of bone marrow Eo/B-CFU grown from humans both before and 24 hours after allergen challenge

in dual and isolated early responders. They found that bone marrow progenitors from dual responders significantly increased 24 hours post-allergen and were more responsive to IL-5 than isolated early responders, consistent with the induction of a late asthmatic response (Wood *et al,* 1998). This work was supported by work done by Sehmi *et al.* which showed an increase in IL-5Rα/$CD34^+$ cells from dual responders 24 hours after allergen inhalation challenge (Sehmi *et al,* 1997). The coinciding increase in both eosinophils and eosinophil/basophil progenitors, in dual responders only, has led to the hypothesis that the bone marrow hemopoietic processes are an essential component to the persistence of airway eosinophilia. As such, Th2 cytokines, with the ability to promote the terminal differentiation and maturation of eosinophil committed lineages, are likely important in promoting eosinophilopoiesis within the bone marrow. In agreement with this, Wood *et al.* demonstrated significant increases in the number of $CD3^+$ cells expressing IL-5 mRNA in the bone marrow of dual responders compared to isolated early responders 24 hours post-allergen inhalation (Wood *et al,* 2002). Recent findings now suggest that hematopoietic processes outside of the bone marrow also contribute to the persistence of airway eosinophilia.

Progenitor Cell Trafficking

In conjunction with activation of eosinophilopoiesis within the bone marrow, allergen inhalation challenge may also trigger the release of primitive and lineage-committed progenitors into the blood. The majority of HPSC are located in the bone marrow, but over the last 40 years, it has become clear that among circulating peripheral

blood cells are a low number of progenitor cells (Barnes *et al,* 1967). During steady state hematopoiesis, HPSC give rise to mature erythrocytes and leukoctyes that enter the circulation along with a minimal number of stem cells. During inflammatory states or following the administrations of hematopoietic growth factors more HPSC are mobilized. However, circulating progenitor cells still retain the capability to repopulate the hematopoietic compartment of the bone marrow (homing).

Trafficking, mobilizaion and homing of HPSC are multifactorial processes that are regulated partially at the level of the bone marrow endothelium. Recruitment from the bone marrow to the blood is an important but poorly understood early step in egress and likely involves down-regulation of specific adhesive interactions, increased motility, migration through the hematopoietic compartment, and transmigration across the sinus endothelium. There also appears to be a chemokinetic as well as a chemoattractive component to mobilization and egress. Recent studies have indicated in particular the chemokine, stromal cell-derived factor-1 alpha (SDF-1α) and its receptor CXCR4, to be involved in regulation of mobilization and homing of HPSC (Aiuti *et al,* 1997; Mohle *et al,* 1998; Naiye *et al,* 1999; Peled *et al,* 1999).

SDF-1α is a member of the CXC chemokine family and is a potent chemoattractant for hematopoietic stem cells (Murdoch 2000). SDF-1α interacts with its sole G-protein coupled receptor CXCR4 and together are thought to be critical to bone marrow stromal cell homing as mice deficient in either of these molecules have dramatically reduced hematopoiesis (Nagasawa *et al,* 1996)

$CD34^+$ cells migrate towards SDF-1α in a concentration dependent manner, (Aiuti *et al,* 1997; Jo *et al,* 2000; Kim *et al,* 1998) and inhibition of this migration has been reported and correlated with down-regulation of receptor expression, desensitization of receptor signaling or both (Kollet *et al,* 2001; Peled *et al,* 1999; Petit *et al,* 2002).

In addition to migrational responses, studies have also shown a role for SDF-1α /CXCR4 interactions in the retention of progenitor cells within the bone marrow. Petit *et al.* had previously demonstrated that Granulocyte-colony stimulating factor (G-CSF) enhanced the number of circulating $CD34^+$ cells (Petit *et al,* 2002). Liles *et al.* expanded this work, examining circulating $CD34^+$ cells stimulated with a CXCR4 antagonist, AMD-3100, in comparison and in combination with G-CSF. They found that AMD 3100 significantly increased circulating $CD34^+$ cells after administration, although not significantly greater than those induced by G-CSF. However, the combination of AMD 3100 and cytokine further enhanced the release of $CD34^+$ cells (Liles *et al,* 2003).

SDF-1α is a highly basic protein that binds avidly to negatively charged molecules including heparan sulfate-like glycosaminoglycans. These molecules capture SDF-1α on cell surfaces and on the extracellular matrix within the bone marrow and facilitate the establishment of local concentration gradients as well as providing attachment sites for progenitor cells (Sweeney *et al,* 2002). Thus is has been suggested that in homeostatic conditions, many SDF-1α molecules are in an immobilized state. Release and egress of progenitors therefore, is thought to be partially mediated through the inhibition of progenitor attachment to the bone marrow sinus endothelium (Sweeney *et al,* 2002).

HPSC can also be mobilized from the bone marrow following stimulation with various agents, including hematopoietic growth factors, inflammatory cytokines, chemokines and complement components (Lapidot *et al,* 2002). Eotaxin, an important inflammatory chemokine up-regulated in asthma (Lamkhioued *et al,* 1997), has been shown to selectively stimulate the rapid egress of eosinophil progenitor cells from the bone marrow sinuses of guinea pigs into the peripheral circulation (Palframan *et al,* 1998). It is likely that inflammatory cytokines like eotaxin act by both directly stimulating progenitor cells and by down-regulating adhesion molecules upon the bone marrow endothelial cells.

The purpose of HPSC mobilization is unclear. Possibly, the circulating progenitor cells are just on their way to niches at other bone marrow sites (van der Schoot *et al,* 1994), however the fact that progenitor mobilization increases during inflammation, a state associated with the increased production and release of inflammatory cells suggests that mobilization serves another purpose.

Evidence for *In situ* Hematopoiesis

The earliest studies examining *in situ* hematopoiesis of the airways were performed by Otsuka *et al.* using nasal-polyp tissue (Otsuka *et al,* 1987). This group demonstrated that fluctuations in circulating metachromatic cell progenitors were inversely related to nasal metachromatic cell counts and nasal symptoms in allergic rhinitis (Otsuka *et al,* 1986). They continued this area of research and quantified nasal metachromatic cell progenitors from nasal polyp tissue using colony assays. In the same

study, highly potent metachromatic cell colony stimulating activity was detected in the supernantants from cultured nasal epithelial scrapings, from atopic polyp nasal mucosa only (Otsuka *et al,* 1987). Onishi *et al.* in a similar study showed that hemopoietic progenitor cells can be recruited to and subsequently induced to differentiate within nasal allergic mucosa (Ohnishi *et al,* 1988). Together, these results provided indirect evidence that changes in airway inflammation reflects dynamic hemopoietic processes within the airway tissue and support the hypothesis for progenitor cell transmigration from blood to airway where they can mature *in situ* into effecter cells stimulated by locally produced cytokines.

More recently, Robinson *et al.* have demonstrated an increase in $CD34^+$ cells in the airways of atopic asthmatics and atopic non-asthmatics compared to normal control subjects. They suggest that patients with chronic inflammation recruit progenitor cells to the site of inflammation (Robinson *et al,* 1999). Further evidence comes from human studies where $CD34^+$ cells have been extracted from nasal polyp and nasal explant tissue and shown to undergo IL-5-driven proliferation and differentiation into Eo/B-CFU *in-vitro* and *ex-vivo* highlighting the presence of true blast cells (Cameron *et al,* 1999; Kim *et al,* 1999). Together these studies suggest that mobilization of progenitor cells can lead to recruitment and local expansion within tissues contributing to local production of inflammatory cells.

The aim of this thesis is to examine the role of bone marrow inflammatory cell eosinophil/basophil progenitors in allergen-induced airway inflammation and airway hyperresponsiveness with respect to the following hypothesis:

Overall Hypothesis

Allergen-induced airway inflammation and its associated late responses and airway responsiveness is propagated in part by the activation, mobilization and recruitment of bone marrow eosinophil/basophil progenitors, leading to the increased numbers of airway eosinophils after allergen inhalation.

Specific Hypothesis

1) Allergen inhalation will be associated with time-dependent fluctuations in the number of eosinophil/basophil progenitor cells in the bone marrow, peripheral blood and airways of atopic asthmatic subjects.

2) The allergen-induced changes in eosinophil/basophil progenitors correlate with changes in eosinophil numbers in the bone marrow, blood and airways.

3) The quality and quantity of changes in eosinophil/basophil progenitors differ in subjects with different degrees of airway inflammation and differences in the magnitude of the allergen-induced physiological responses.

4) The differences between subjects with different degrees of airway inflammation will correlate with changes in blood and bone marrow pro-inflammatory cytokines and chemokines.

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CHAPTER 2

KINETICS OF BONE MARROW EOSINOPHILOPOIESIS AND ASSOCIATED CYTOKINES AFTER ALLERGEN INHALATION

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Sandra Dorman's contribution:

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

Kinetics of Bone Marrow Eosinophilopoiesis and Associated Cytokines after Allergen Inhalation

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Allergen inhalation is associated with increased eosinophil/basophil progenitors in bone marrow 24 hours after allergen inhalation. This study examined the kinetics of eosinophilopoiesis in dual (n = 14), compared with isolated early, responders (n = 12). Dual responders, in contrast to isolated early responders, develop significant sputum and blood eosinophilia and prolonged airway hyperresponsiveness. Bone marrow aspirates were taken before and 5, 12, 24, and 48 hours after allergen inhalation. In dual responders, increases in interleukin (IL)-3–responsive progenitors were detected as early as 5 hours after allergen inhalation, and IL-5–responsive progenitors were detected at 12 and 24 hours. No changes were detected in isolated early responders. Bone marrow IL-5 protein levels increased at 12 and 24 hours in dual responders only and these increases correlated with increases in IL-5–responsive progenitors. In addition, bone marrow IFN-γ levels increased in dual responders at 48 hours. These data demonstrate that, in dual responders, there is allergen-induced activation of an eosinophilopoietic process that is rapid and sustained, and a relationship between increased bone marrow IL-5 levels and increased eosinophil production. We propose that after allergen inhalation, time-dependent changes in cytokine levels in the bone marrow control differentiation of eosinophil/basophil progenitors.

Keywords: allergy; cellular differentiation; eosinophils; human; lung

Asthma is recognized by the presence of reversible bronchoconstriction, airway hyperresponsiveness (AHR), and airway inflammation. Environmental allergens are an important cause of asthma and can be studied in the laboratory by allergen inhalation challenge. Allergen inhalation challenge in sensitized subjects typically induces an immediate bronchoconstriction (the early asthmatic response), which is maximized within 30 minutes and resolves between 1 and 3 hours. A proportion of subjects will proceed to develop a second, delayed bronchoconstrictor response (the late asthmatic response), which is associated with prolonged AHR and pronounced airway eosinophilia (1–4). Although in a given individual the development of either a single or a dual response is generally consistent, it is believed that the development of the late response occurs as a continuum within and between individuals, largely on the basis of the dose of allergen inhaled (5). The development of the late response and associated increase in airway responsiveness results in a lowering of the threshold for subsequent allergen exposure, setting up a vicious circle for continuing allergic inflammation in the airways (6, 7).

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The size of the late response and the increase in AHR have been shown to correlate with the degree of airway eosinophilia (8). Likewise, studies comparing numbers of circulating eosinophils have shown significant increases 24 hours after allergen inhalation in dual responders only (9–11). Eosinophils, through their ability to release granule-associated proteins, including pro-inflammatory lipid mediators and cytokines, are considered to play a central role in the development of airway inflammation leading to the production of the late asthmatic response. This role, however, is based on circumstantial evidence and identifying mechanism(s) contributing to the development of tissue eosinophilia will provide a better understanding of the importance of this cell in asthma (12–14).

There is now substantial evidence supporting the view that activation of specific hematopoietic pathways within the bone marrow is associated with allergen-induced eosinophilic airway inflammation. Early studies demonstrated higher numbers of circulating eosinophil/basophil colony-forming units (Eo/B-CFU) and $CD34^+IL\text{-}5R\alpha^+$ (interleukin [IL]-5 receptor-positive) cells 24 hours after allergen inhalation in dual responders (15, 16). In addition, bone marrow IL-5–responsive Eo/B-CFU were shown to increase 24 hours after allergen inhalation only in dual responders (17). The coinciding increases in both eosinophils and eosinophil progenitors, in dual responders only, has led to the hypothesis that the bone marrow hematopoietic processes are an essential component to the persistence of airway eosinophilia. As such, helper T cell Type 2 cytokines, with the ability to promote the terminal differentiation and maturation of eosinophil-committed lineages, are likely important in promoting eosinophilopoiesis within the bone marrow. In agreement with this, we have shown significant increases in the number $CD3^+$ cells expressing IL-5 mRNA in the bone marrow of dual responders compared with isolated early responders 24 hours after allergen inhalation (18). In addition, Sehmi and coworkers have demonstrated a significant increase in the number of bone marrow-derived $CD34^+$ cells expressing IL-5Rα 24 hours after allergen inhalation challenge in dual, but not isolated early, responders (16). Together these results suggest that an IL-5–driven phenotypic switch in the bone marrow environment occurs in dual responders only, favoring eosinophil production and contributing to the development of blood and airway eosinophilia. On the basis of this view, it is plausible that, in isolated early responders—who do not develop a significant or sustained eosinophilic response—cytokines such as IFN-γ and IL-10 may be produced that downmodulate the helper T cell Type 2 environment and therefore prevent or ameliorate the development of airway eosinophilia.

The aims of the current study were twofold. The first was to investigate whether the short-lived airway eosinophilic response mounted by early responders is due to a limited activation of bone marrow eosinophilopoietic processes. For this reason, we examined the changes in bone marrow eosinophilopoiesis, before and 5, 12, 24, and 48 hours after allergen inhalation, both in isolated early and dual responders. The second was to investigate whether any observed differences in bone marrow responsiveness

between the two groups is due to the differences in bone marrow cytokines, including the cytokines necessary for eosinophilopoiesis (IL-5 and IL-3) and the downregulatory cytokines (IFN-γ and IL-10). Overall, understanding how these changes relate to AHR and airway inflammation will help to understand the importance of bone marrow eosinophilopoiesis in the persistence of allergen-induced airway responses.

These results have been previously reported in abstract form (19).

METHODS

Subjects/Design

Twenty-six patients with mild, stable, atopic asthma with baseline FEV_1 > 70% predicted were studied (Table 1). Patients were screened and, after allergen inhalation, isolated early responders (n = 12) developed an early fall in FEV_1 > 20% predicted from baseline between 0 and 2 hours, whereas dual responders developed an early and late fall (FEV_1 > 15% predicted) from baseline between 3 and 7 hours (n = 14). Subjects were nonsmokers, using inhaled β_2-agonists intermittently (withheld 8 hours before each visit), and had not experienced respiratory infection or were exposed to altered allergen levels 2 weeks before allergen inhalation. One subject withdrew because of discomfort from aspiration. Six additional subjects were studied to examine the direct effects of IFN-γ on Eo/B-CFU. This study was approved by McMaster University Health Sciences Centre Ethics Committee and subjects gave written, informed consent before enrollment.

Because of difficulty in obtaining five consecutive bone marrow aspirates, the study was randomized into phases. In Phase 1, aspirates and blood were taken at baseline and at 5 and 24 hours, sputum was induced at 7 and 24 hours, and methacholine PC_{20} (provocative concentration inducing a 20% fall in FEV_1) was measured 24 hours after allergen inhalation. In Phase 2, aspirates and blood were taken at baseline and at 12 and 48 hours, sputum was induced at 7, 24, and 48 hours, and methacholine PC_{20} was measured 24 and 48 hours after allergen inhalation. Phases were separated by 1 month.

TABLE 1. SUBJECT CHARACTERISTICS

	Age (*yr*)	Sex	FEV_1 (*% Predicted*)	Allergen (*Inhaled Dilution*)
IER				
2	29	F	101.2	Cat (1:8)
3	24	F	85.3	Cat (1:32)
5	30	F	93.9	HDM (1:16)
6	26	M	117.1	RW (1:16)
8	24	F	92.2	Grass (1:1,024)
10	23	M	98.6	Cat (1:64)
14	51	F	80.2	Cat (1:8)
15	21	F	98.9	Cat (1:64)
18	27	F	98.7	Grass (1:512)
20	19	F	80.7	HDM (1:16)
23	23	M	91.4	HDM (1:256)
25	39	M	78.5	HDM (1:512)
Mean ± SEM	27.8 ± 2.37	8:4	93.1 ± 3.18	
DR				
1	26	F	74.2	RW (1:512)
4	23	F	93.1	HDM (1:64)
7	26	M	85.1	Cat (1:16)
9	24	M	77.8	RW (1:128)
11	23	F	97.3	HDM (1:256)
12	52	M	90	Cat (1:64)
13	33	M	81.9	Grass (1:2,048)
16	40	M	109.4	HDM (1:32)
19	32	M	84.5	RW (1:256)
21	41	M	78.4	CAT (1:64)
22	19	F	84.5	RW (1:2,048)
24	27	M	81.7	HDM (1:512)
26	26	F	90.4	HDM (1:1,024)
Mean ± SEM	30.5 ± 2.77	5:8	86.9 ± 2.83	

Definition of abbreviations: DR = dual responders; HDM = house dust mite (*Dermatophagoides farinae*); IER = isolated early responders; RW = ragweed.

Allergen/Methacholine Inhalation Challenge

Methacholine inhalation was performed as described by Cockcroft (20).

Allergen inhalation challenge was performed as described by O'Byrne and coworkers (21).

Sputum Induction

Sputum was induced and processed according to Popov and coworkers (22). Slides were stained with Congo red and counts from duplicate slides (400 cells per slide) were expressed as a percentage. Immunocytochemical staining was performed with murine monoclonal antibodies to human eosinophil cationic protein (EG2, 1.0 μg/ml; Pharmacia, Uppsala, Sweden).

Bone Marrow Eosinophil/Basophil Progenitors and Blood

Bone marrow aspiration and Eo/B-CFU culture and enumeration were performed according to Wood and coworkers (17). Nonadherent mononuclear cells were cultured with diluent, recombinant human (rh)IL-5 (1 ng/ml), rhIL-3 (1 ng/ml), and rhGM-CSF (granulocyte-macrophage colony-stimulating factor, 10 ng/ml).

Six additional samples were cultured with diluent, recombinant human rhIL-5 (1 ng/ml), and rhIFN-γ (0.1, 1.0, 10, and 100 ng/ml) (BD Biosciences Pharmingen, San Diego, CA).

Blood (400 cells per slide) and bone marrow (1,000 cells per slide) smears were stained with Diff-Quik (VWR International, West Chester, PA) and counts from duplicate slides were expressed as a percentage.

Serum and bone marrow supernatant samples were stored at −70°C. IL-5, IL-3, IL-10, and IFN-γ were quantified using ELISAs (BD Biosciences Pharmingen). The lower limits of detection were 4, 7.4, 4, and 4 pg/ml, respectively. Values below these were assigned a value of 2 pg/ml for statistical analysis.

Statistical Analysis

Results were expressed as means ± SEM, except for methacholine PC_{20}, which was expressed as geometric mean ± % SEM. Methacholine PC_{20}, IL-5, IL-3, IL-10, and IFN-γ were $\log_{10}$ transformed before analysis. Because baseline data from all outcomes showed no differences between phases, analyses were performed and data were presented using the average. The effect of allergen on methacholine PC_{20}, cytokine protein levels; sputum, blood, and bone marrow eosinophils; and Eo/B-CFU was analyzed with a mixed model analysis of variance (between group, isolated early versus dual responders; within group, before versus after allergen inhalation). Correlations were analyzed using Spearman's rho. Significance was accepted as $p < 0.05$.

RESULTS

Allergen-induced Bronchoconstriction and Airway Hyperresponsiveness

The mean maximal fall in FEV_1 during the early asthmatic response was 28.3 ± 1.65% in dual responders and 21.9 ± 1.54% in isolated early responders. The mean maximal fall in FEV_1 during the late asthmatic response was 20.5 ± 2.04% in dual responders compared with 5.4 ± 1.03% in isolated early responders (Table 2).

Dual responders developed methacholine airway hyperresponsiveness 24 and 48 hours after allergen inhalation. The mean methacholine PC_{20} fell from 2.4 mg/ml (% SEM, 1.3) at baseline to 0.7 mg/ml (% SEM, 1.6) at 24 hours ($p < 0.01$) and 1.1 mg/ml (% SEM, 1.3) at 48 hours ($p < 0.05$) (Figure 1). In contrast, no significant changes in mean methacholine airway responsiveness occurred in isolated early responders after allergen inhalation, being 3.3 mg/ml (% SEM, 1.5) at baseline, 2.2 mg/ml (% SEM, 1.5) at 24 hours, and 2.8 mg/ml (% SEM, 1.5) at 48 hours after allergen inhalation.

Eosinophil Counts in Sputum, Blood, and Bone Marrow

Sputum. After allergen inhalation, there was a significant increase in sputum eosinophils in dual responders at 7, 24, and 48 hours. The numbers increased from 2.4 ± 0.6% at baseline to

TABLE 2. AIRWAY RESPONSES

	Arm 1				Arm 2				
	PC_{20}				PC_{20}				
	BL	24 h	EAR	LAR	BL	24 h	48 h	EAR	LAR
IER									
2	2.4	0.7	−15	−9	1.4	1.0	2	−19	−8
3	5.9	13.6	−23	−4	4.6	2.9	10.2	−20	0
5	11.4	4.8	−32	−4	5.7	3.4	3.2	−14	−12
6	30.3	12.2	−24	2	16	7.8	36.8	−20	−4
8	0.7	0.3	−19	−8	0.5	0.9	1.7	−10	−8
10	7.1	6.7	−25	0	5.3	3.2	2.9	−39	−10
14	0.1	0.2	−29	−6	0.3	0.1	0.1	−19	−8
15	4.6	5.3	−24	3	3.2	4	6.2	−12	−7
18	14.0	7.9	−23	−8	14.4	6.2	11.3	−27	−11
20	0.6	0.8	−20	+7	0.9	0.7	0.5	5	0
23	9.3	5.6	−33	−9	5.1	4.4	4	−28	−13
25	2.4	2.6	−26	+2	3.7	1.6	1.1	−21	−10
Mean ± SEM	7.4 ± 2.45	5.1 ± 1.30	−24 ± 1.5	−4 ± 1.4	5.1 ± 1.47	3.0 ± 0.68	6.7 ± 2.93	−20 ± 2.6	−8 ± 1.2
DR									
1	1.2	0.003	−24	−38	4	0.04	0.4	−32	−59
4	1.2	0.9	−22	−18	1.6	1.0	1.3	−19	−17
7	3.0	0.7	−44	−6	7.4	0.7	1.1	−41	−20
9	2.9	1.5	−29	−28	2.6	1.0	1.3	−31	−31
11	7.5	1.2	−23	−18	4.0	1.8	1.4	−18	−10
12	0.9	0.3	−42	−16	0.5	0.1	0.5	−35	−29
13	1.4	0.7	−35	−24	2.3	2.1		−31	−22
16	28.0	11.2	−25	−15	7.7	5.5	3.7	−29	−18
19	7.3	6.1	−19	−17	3.7	1.8	5.6	−19	−7
21	2.6	1.2	−29	−15	2.4	1.5	2.7	−30	−16
22	0.5	0.2	−19	−21	1.0	0.1	0.1	−15	−15
24	3.1	0.3	−24	−28	0.8	0.5	0.6	−36	−25
26	1.3	0.7	−23	−15	1.4	0.7	1.4	−42	−26
Mean ± SEM	4.7 ± 2.04	1.9 ± 0.89	−28 ± 2.2	−20 ± 2.3	3.0 ± 0.64	1.3 ± 0.40	1.7 ± 0.46	−29 ± 2.4	−23 ± 3.6

Definition of abbreviations: BL = baseline; DR = dual responder; EAR = early asthmatic response; IER = isolated early responder; LAR = late asthmatic response; PC_{20} = provocative concentration of methacholine inducing a 20% fall in FEV_1.

12.2 ± 2.2% at 7 hours ($p < 0.001$), 11.5 ± 1.6% at 24 hours ($p < 0.001$), and 10.8 ± 2.6% at 48 hours ($p < 0.001$) (Figure 2). Isolated early responders also had an allergen-induced increase in sputum eosinophils from 2.6 ± 0.4% at baseline to 6.9 ± 2.0% at 7 hours, 7.2 ± 1.8% at 24 hours ($p < 0.05$), and 7.6 ± 1.8% at 48 hours ($p < 0.05$) (Figure 2). The magnitude of the allergen-induced change in sputum eosinophils was significantly greater in dual responders than in isolated early responders at all time points ($p < 0.05$). Baseline values were not significantly different between the two groups of subjects. After allergen inhalation, there was a significant increase in the percentage of activated sputum eosinophils (EG2-positive cells) in dual responders, from 1.65 ± 0.6% at baseline to 7.39 ± 1.7% at 7 hours ($p = 0.05$), 8.68 ± 2.1% at 24 hours ($p < 0.005$), and 8.7 ± 2.4% at 48 hours ($p < 0.005$). In contrast, no significant change in percentage of activated eosinophils was detected in isolated early responders at any time point. In addition, the magnitude of the allergen-induced change in sputum eosinophils was significantly greater in dual responders than in isolated early responders at all time points ($p < 0.05$).

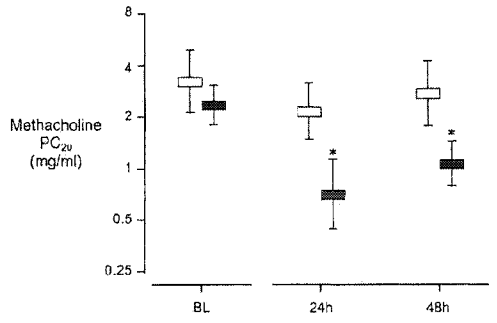

Figure 1. Airway responsiveness to methacholine PC_{20} at baseline (BL) and after allergen inhalation in isolated early responders (*open rectangles*) and dual responders (*solid rectangles*). In dual responders only, airway hyperresponsiveness (AHR) increased significantly at 24 and 48 hours compared with baseline. No significant changes occurred in the isolated early responders over time. Difference compared with baseline, *$p < 0.05$.

Blood. Allergen inhalation significantly increased the percentage of blood eosinophils in dual responders from 3.1 ± 0.6% at 5 hours and 2.7 ± 0.5% at 12 hours to 5.2 ± 0.8% at 24 hours ($p < 0.05$) and 5.7 ± 0.8% at 48 hours ($p < 0.005$) and had a nearly significant increase from baseline, being 4.0 ± 0.5% ($p = 0.055$) (Figure 2). There was no allergen-induced change in the percentage of blood eosinophils detected in isolated early responders.

Bone marrow. The baseline numbers of eosinophils were not significantly different between the two arms of the study; the mean difference being 1.4 ± 0.3 (Table 3). Allergen inhalation significantly increased bone marrow eosinophils in dual responders at 48 hours when compared with 5 hours ($p < 0.005$) but not when compared with baseline (Figure 2). In contrast, there was no significant allergen-induced change in the percentage of bone marrow eosinophils in isolated early responders at any time point (Figure 2). The percent change in eosinophils was significantly lower at 5 hours ($p < 0.05$) in dual responders when compared with isolated early responders.

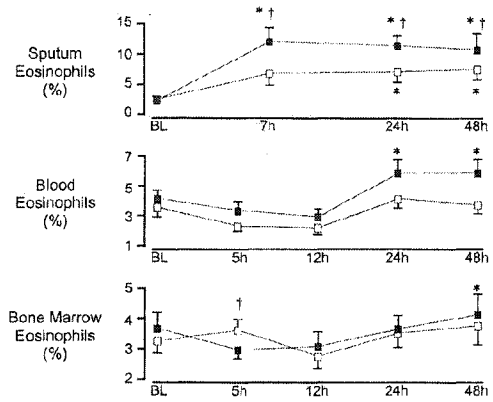

Figure 2. Changes in sputum, blood, and bone marrow eosinophils measured at baseline (BL) and after allergen inhalation in isolated early responders (*open squares*) and dual responders (*solid squares*). Sputum eosinophils significantly increased compared with baseline; at 24 and 48 hours in isolated early responders; and at 7, 24, and 48 hours in dual responders after allergen inhalation. The magnitude of the allergen-induced increase in sputum eosinophils was significantly greater in dual responders at 7, 24, and 48 hours when compared with isolated early responders. Blood eosinophils increased significantly in dual responders only at 24 and 48 hours when compared with 5 and 12 hours. Bone marrow eosinophils increased significantly in dual responders only, at 48 hours when compared with 5 hours. This decrease at 5 hours was significantly lower in dual responders compared with bone marrow eosinophils in the isolated early responders at the same time point. Difference over time, $^*p < 0.05$; difference between groups, $^\dagger p < 0.05$.

Bone Marrow Progenitors

Interleukin-3. The baseline numbers of IL-3–responsive Eo/B-CFU were not significantly different between the two arms of the study; the mean difference being 4 ± 4.5 (Table 3). After allergen inhalation, there was a significantly increased number of IL-3–responsive bone marrow Eo/B-CFU in dual responders at 5 hours, but not at 12, 24, or 48 hours. The numbers increased from 5.1 ± 1.6 at baseline to 12.2 ± 2.7 at 5 hours ($p = 0.05$) (Figure 3). There was no allergen-induced change in the number of IL-3–stimulated Eo/B-CFU from isolated early responders. The number of Eo/B-CFU was significantly greater at 5 hours ($p < 0.05$) in dual responders when compared with isolated early responders (Figure 3).

Interleukin-5. The baseline numbers of IL-5–responsive Eo/B-CFU were not significantly different between the two arms of the study; the mean difference being 8 ± 7.0 (Table 3). After allergen inhalation, there was a significantly increased number of IL-5–responsive Eo/B-CFU in dual responders at 12 and 24 hours after allergen inhalation, increasing from 33.2 ± 4.2 at baseline, to 43.2 ± 5.3 at 5 hours, 51.0 ± 8.1 at 12 hours ($p < 0.01$), 52.7 ± 5.7 at 24 hours ($p < 0.005$), and 46.8 ± 7.3 at 48 hours (Figure 3). In contrast, there was no change in the number of IL-5–responsive Eo/B-CFU in the isolated early responders at any of the time points tested after allergen inhalation, being 29.7 ± 4.0 at baseline, 38.0 ± 4.6 at 5 hours, 36.0 ± 5.5 at 12 hours, 34.9 ± 4.3 at 24 hours, and 30.9 ± 5.6 at 48 hours after allergen inhalation. The magnitude of the change in IL-5–responsive Eo/B-CFU at 24 hours was significantly greater in dual responders when compared with isolated early responders ($p < 0.05$) (Figure 3).

GM-CSF. There was no significant allergen-induced difference over time in the number of GM-CSF–responsive Eo/B-CFU in either isolated early or dual responders (data not shown).

IL-5 Protein

Serum. After allergen inhalation, there was a significant increase in serum IL-5 protein in dual responders at 12 and 24 hours, increasing from 20.1 ± 8.7 pg/ml at baseline to 21.8 ± 8.3 pg/ml at 5 hours, 32.9 ± 10.2 pg/ml at 12 hours ($p < 0.05$), 33.5 ± 10.5 pg/ml at 24 hours ($p < 0.01$) and 28.6 ± 8.9 pg/ml at 48 hours (Figure 4). There was no changes in the level of serum IL-5 protein in isolated early responders at any time points (Figure 4). The level of serum IL-5 protein was significantly greater in dual responders when compared with isolated early responders at all time points ($p < 0.01$).

Bone marrow. The baseline levels of IL-5 were not significantly different between the two arms of the study; the mean difference being 4 ± 6.3 (Table 3). After allergen inhalation, there was a significant increase in bone marrow IL-5 protein in dual responders at 12 and 24 hours increasing from 19.2 + 8.4 pg/ml at baseline to 25.5 ± 10.3 pg/ml at 5 h, 32.6 ± 10.8 pg/ml at 12 hours ($p < 0.01$), 29.6 ± 9.8 pg/ml at 24 hours ($p < 0.01$) and 22.9 ± 7.2 pg/ml at 48 hours (Figure 4). In contrast, there was no change in the level of bone marrow IL-5 protein in isolated early responders at any time point (Figure 4).

There was a significant positive relationship between levels of IL-5 protein detected in the bone marrow and in the serum as measured by the area under the curve from baseline to 48 hours ($r = 0.973$, $p < 0.0001$). Similarly, there was a significant positive correlation between levels of bone marrow IL-5 protein and the number of IL-5-responsive Eo/B-CFU as measured by the area under the curve from baseline to 48 hours after allergen inhalation ($r = 0.448$, $p < 0.05$).

IFN-γ Protein

Serum. After allergen inhalation there was no change in serum IFN-γ protein levels at any time point in either group of subjects studied.

Bone marrow. The baseline levels of IFN-γ were not significantly different between the two arms of the study; the mean difference being 34 ± 91.7 (Table 3). After allergen inhalation there was a significant increase in the level of bone marrow IFN-γ protein in dual responders at 48 hours compared with baseline increasing from 126.6 ± 27.9 pg/ml at baseline to 146.5 ± 34.4 pg/ml at 5 hours, 148.4 ± 41.5 pg/ml at 12 hours, 165.5 ± 46.5 pg/ml at 24 hours, and 168.9 ± 30.6 pg/ml at 48 hours ($p < 0.01$) (Figure 4). In contrast, there was no change in the level of IFN-γ protein detected in the bone marrow of isolated early responders at any time point tested (Figure 4).

There was a significant positive correlation between level of bone marrow IFN-γ and level of bone marrow IL-5 ($r = 0.54$, $p < 0.05$), but not between bone marrow IFN-γ and bone marrow, blood, or sputum eosinophils or Eo/B-CFU.

IL-3 and IL-10 Protein

Serum and bone marrow. There was no significant allergen-induced difference over time in the amount of IL-3 or IL-10 in either isolated early or dual responders (data not shown).

Cell Culture with IFN-γ

There were no significant changes in the number of IL-5–stimulated eosinophil/basophil colonies grown when incubated with any concentration of IFN-γ. The value with IL-5 alone was 31.8 ± 2.8; and values with IFN-γ were as follows: IFN-γ (100 ng/ml), 33.3 ± 8.1; IFN-γ (10 ng/ml), 40.8 ± 6.8; IFN-γ (1 ng/ml), 40.9 ± 5.5; IFN-γ (0.1 ng/ml), 40.4 ± 6.8.

DISCUSSION

This study has demonstrated, for the first time, that allergen inhalation by dual responders is associated with a rapid onset of

TABLE 3. BONE MARROW RESPONSES

	Arm 1					Arm 2				
		Eo/B-CFU		BM			Eo/B-CFU		BM	
	% BM Eo	IL-3	IL-5	IL-5	IFN-γ	% BM Eo	IL-3	IL-5	IL-5	IFN-γ
IER										
2	NA	3.0	29.5	18	285	NA	10.5	36.5	16	246
3	NA	2.5	54.0	5	138	NA	5.5	69.0	6	128
5	3.4	1.0	21.5	NA	NA	2.7	2.0	30.0	3	122
6	3.1	3.0	4.5	3	80	1.4	0.5	30.5	3	59
8	2.9	2.0	34.0	5	80	5.5	1.0	15.0	5	92
10	2.6	6.5	20.0	4	70	7.4	6.5	24.5	4	79
14	4.1	0.5	42.5	13	81	3.4	11.5	32.5	11	66
15	NA	10.5	21.5	4	63	5.8	4.0	23.0	5	71
18	1.9	1.5	29.5	6	70	NA	0.5	24.5	4	93
20	1.7	4.0	16.5	136	NA	2.9	4.0	19.0	119	NA
23	2.9	8.5	44.5	12	59	2.8	2.0	42.0	4	75
25	3.9	2.0	26.0	4	71	2.6	1.0	23.0	4	73
Mean ± SEM	4.0 ± 0.6	3.8 ± 0.9	29 ± 3.9	19 ± 2	100 ± 2	3.3 ± 0.5	4.1 ± 1.1	31 ± 4.1	15 ± 9	101 ± 16
DR										
1	NA	5.0	32.5	4	74	NA	1.0	19.0	3	80
4	NA	4.5	27.0	8	77	NA	10.0	22.0	8	93
7	3.0	0.0	29.5	3	60	2.4	0.0	43.0	10	94
9	3.2	1.5	77.5	2	50	4.4	5.0	61.5	2	61
11	5.2	0.0	34.5	4	61	2.6	2.5	36.0	4	71
12	7.2	3.0	47.0	57	282	4.6	19.0	36.0	52	313
13	NA	14.0	23.5	4	109	3.5	1.0	33.0	30	558
16	5.3	8.5	38.0	31	205	6.7	8.0	45.5	28	206
19	2.9	16.5	19.5	4	66	2.2	4.0	25.0	4	70
21	0.9	9.5	28.5	114	NA	1.0	9.0	24.0	99	NA
22	3.1	0.5	26.5	4	82	3.6	0.5	27.0	4	69
24	5.5	0.0	31.0	6	91	2.0	0.0	9.5	9	126
26	4.1	0.0	10.0	3	67	4.5	0.0	13.0	3	74
Mean ± SEM	2.9 ± 0.2	5.3 ± 1.6	35 ± 4.4	19 ± 9	102 ± 20	3.7 ± 0.6	5.0 ± 1.6	30 ± 4.0	20 ± 8	151 ± 43

Definition of abbreviations: BM = bone marrow; DR = dual responder; Eo = eosinophils; Eo/B-CFU = eosinophil/basophil colony-forming unit; IER = isolated early responder; IL = interleukin; NA = not available.

IL-3–dependent eosinophilopoiesis in the bone marrow, detectable as early as 5 hours. In addition, there is sustained IL-5–dependent eosinophilopoiesis at 12 and 24 hours after allergen inhalation in dual responders. IL-5 protein levels increased significantly in serum and bone marrow at 12 and 24 hours after allergen inhalation in dual responders only. Furthermore, there was a significant correlation between IL-5–responsive Eo/B-CFU and IL-5 protein levels in the bone marrow, suggesting that both airway and blood eosinophilia in dual responders are sustained by an IL-5–responsive, eosinophil-differentiative process within the bone marrow. Finally, bone marrow IFN-γ protein levels increased 48 hours after allergen inhalation, in association with the reduction of bone marrow IL-5 protein levels and eosinophilopoiesis. Together these results are consistent with the hypothesis that upregulation of bone marrow IL-5 and IL-5–responsive Eo/B-CFU plays an important role in the persistence of allergen-induced airway eosinophilia and airway hyperresponsiveness over 48 hours and that increases in bone marrow IFN-γ downregulate these responses.

The development of both early and late asthmatic responses after allergen inhalation is known to be associated with increases in circulating eosinophils (11), greater increases of activated eosinophils in the airway (23), and the development of airway hyperresponsiveness (24) when compared with isolated early responders. All of these changes were confirmed in this study, although markers for eosinophil activation may not be ideal (25). Contrasting these two groups allows for a useful clinical model of increased eosinophilic airway responses and associated airway functional changes. The groups are, however, not dichotomous and the levels of bronchoconstriction used to discriminate between them are arbitrary. Also, increases in airway eosinophils were seen in the isolated early responders in the present study and in other studies from our laboratory (17, 23). Despite this, it was only the significantly greater increases in circulating and airway eosinophils in dual responders that were associated with increases in bone marrow eosinophilopoiesis.

An increase in peripheral blood Eo/B-CFU was first shown in atopic, compared with nonatopic, subjects (26), and in dual responders 24 hours after allergen inhalation (15). Subsequently, Wood and coworkers (17) described a significant increase in IL-5–responsive Eo/B-CFU 24 hours after allergen inhalation in dual responders, but not in isolated early responders. An increase in the expression of IL-5Rα on bone marrow $CD34^+$ cells in dual responders 24 hours after allergen inhalation helped explain these initial findings (16). The data from the present study extend these observations, demonstrating a rapid activation of eosinophilopoiesis, detectable as early as 5 hours after allergen inhalation, which is at first IL-3 dependent followed by an IL-5–dependent phase for at least 24 hours after allergen inhalation.

The development of tissue eosinophilia in allergic inflammatory responses is known to be orchestrated by a number of mediators, of which IL-5 appears to be central. Studies have shown that IL-5 protein levels are increased in induced sputum after allergen inhalation challenge (4, 27). In addition, we have also shown that IL-5 mRNA-positive cells colocalized with $CD3^-$ cells are increased significantly in the bone marrow of dual responders when compared with isolated early responders (18). This suggests that the local production of IL-5 by T cells in the bone marrow is involved in inducing bone marrow hematopoietic processes. However, it is likely that other mediators are also

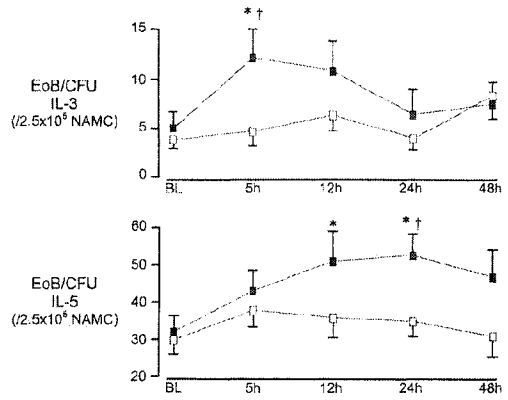

Figure 3. Changes in eosinophil/basophil colony-forming units (Eo/B-CFU) measured at baseline (BL) and after allergen inhalation in isolated early responders (*open squares*) and dual responders (*solid squares*). IL-3–responsive Eo/B-CFU significantly increased after allergen inhalation at 5 hours, compared with baseline in dual responders, and the magnitude of this change was significantly greater when compared with isolated early responders. IL-5–responsive Eo/B-CFU significantly increased after allergen inhalation at 12 and 24 hours compared with baseline in dual responders, and the magnitude of this change at 24 hours was significantly greater when compared with isolated early responders. Difference compared with baseline, *$p < 0.05$; difference between groups, †$p < 0.05$. NAMC = nonadherent mononuclear cells.

involved in the generation of mature eosinophils from pluripotent hematopoietic stem cells, including IL-3. Indeed, IL-3, GM-CSF, and IL-5 all regulate the commitment of progenitor cells along a basophil/eosinophil lineage. IL-3 can induce eosinophil/basophil differentiation, but has other hematopoietic activities,

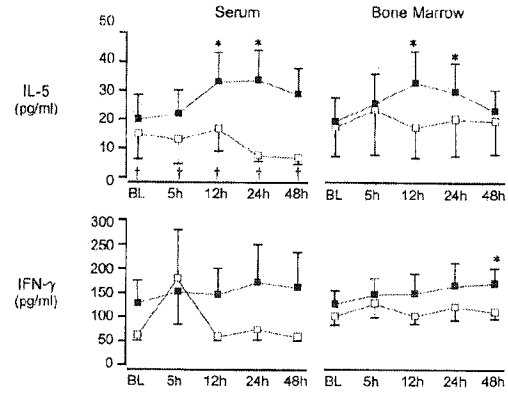

Figure 4. Changes in protein levels of IL-5 and IFN-γ in serum and bone marrow supernatant measured at baseline (BL) and after allergen inhalation in isolated early responders (*open squares*) and dual responders (*solid squares*). In dual responders only, IL-5 protein levels significantly increased after allergen inhalation at 12 and 24 hours, compared with baseline, in both serum and bone marrow supernatant. Serum IL-5 protein levels were significantly higher at all time points in dual responders compared with isolated early responders. In dual responders only, IFN-γ levels significantly increased after allergen inhalation at 48 hours compared with baseline, in bone marrow supernatant. Difference over time, *$p < 0.05$; difference between groups, †$p < 0.01$.

whereas IL-5 is a specific eosinophilopoieten. Early stem cells express receptors for IL-3 and Clutterbuck and coworkers have shown that IL-3 has the ability to enhance the number of eosinophil clusters (28). Tavernier and coworkers, on the other hand, have shown that IL-5 is able to upregulate its own receptor on $CD34^+$ cells (29). Therefore, with early stimulation via IL-3 the number of eosinophil progenitor clusters increases and in the presence of IL-5 the number of these cells differentiating into mature eosinophils also increases.

IL-3 has been shown in murine models to regulate commitment of early myeloid progenitors to the eosinophil lineage (30). Indeed, this role for IL-3 is supported for the first time in humans by our finding of a rapid increase in *ex vivo* IL-3–responsive eosinophil/basophil progenitors 5 hours after allergen inhalation. Although IL-3 was not detectable in bone marrow supernatant in this study, it may be that the sensitivity of the assay was inadequate. It is likely that the increased colony numbers seen 12 and 24 hours after allergen inhalation are due to both the early regulation by IL-3 and the terminal differentiation by IL-5. This is supported by observations in an IL-5–deficient murine model in which symptoms and signs of allergic rhinitis were delayed, but not abolished, after nasal allergen inhalation challenge (31, 32). Therefore, after allergen inhalation, time-dependent changes in bone marrow cytokines control the expansion and differentiation of eosinophil/basophil progenitors.

The turnover of eosinophils is likely slower in normal hematopoiesis compared with that seen during inflammatory situations. Terashima and coworkers have used bromodeoxyuridine (BrdU) in rabbits to examine progenitor cell expansion and transit times. They have shown a transit time for circulating $BrdU^+$ cells beginning at 24 hours and peaking between 66 and 78 hours. However, when *Streptococcus pneumoniae* was instilled in the lung the transit time was significantly shortened, with $BrdU^+$ cells increasing at 12 hours and peaking between 24 and 48 hours. They also found significantly shorter time periods for bone marrow cells in both the mitotic and postmitotic pools (33). This suggests that either there is a shorter time for each division or that cells skip a number of divisions. Similarly, we have examined $BrdU^+$ cells in dogs exposed to allergen or saline inhalation and found significant increases in $BrdU^+$ cells in both the blood and airways of allergen-challenged dogs at 24 hours compared with diluent challenge (34). Together these data show that under stressful circumstances such as during an allergic response, the bone marrow is able to rapidly produce inflammatory cells.

IL-5 is also recognized as a potent eosinophil-activating cytokine (35). Studies have demonstrated the importance of IL-5 in facilitating the release of eosinophils from bone marrow (36). The present study found that in dual responders, IL-5 protein levels peak significantly in the serum before increases in blood eosinophils occur. It is therefore probable that IL-5 regulates eosinophilic responses at several levels, including enhanced bone marrow eosinophil differentiative processes, release of eosinophils from the bone marrow to the blood, and trafficking from the blood into the airways.

Durham and Kay demonstrated an initial fall in circulating eosinophil numbers coinciding with the development of the late airway inflammatory response, followed by a gradual increase peaking at 24 hours (11). Our study corroborates these findings and has shown, for the first time, a similar trend in allergen-induced changes in bone marrow eosinophils, with a drop in eosinophils at 5 and 12 hours, followed by an increase at 24 and 48 hours in dual responders only.

At any given time, the number of eosinophils present in the blood after allergen inhalation depends on three factors: the recruitment of cells into the airways or other tissues, the "margination" of eosinophils in the pulmonary vasculature, and the

recruitment of cells from the bone marrow or other tissues. Likewise, the number of eosinophils present in the bone marrow depends on the release of mature cells into the blood and the ongoing maturation of progenitors. Both margination and bone marrow responses likely contribute to the increases in the number of circulating eosinophils seen at 24 hours, whereas decreased bone marrow eosinophil numbers at 5 hours may be due to egress of mature eosinophils from the bone marrow into the blood. We suggest that movement of eosinophils between compartments is induced, in part, by increases in circulating IL-5, whereas maturation of progenitors at 12 and 24 hours is dependent on bone marrow IL-5 protein levels. As circulating IL-5 decreased, so too did the egress of eosinophils into the blood. Likewise, as bone marrow IL-5 levels diminished, so too did the production of eosinophils by maturing Eo/B-CFU. Therefore, by 48 hours, the number of bone marrow eosinophils was not significantly higher than baseline numbers.

Similarly, in the airways the number of eosinophils present is dependent on three factors: the number of eosinophils available to recruit from the circulation, the quantity of chemokines produced, and the ability of the tissue to express adhesion molecules for the uptake of eosinophils into the airways. The number of eosinophils available to recruit is dependent on the baseline level of circulating eosinophils, the number of cosinophils in reserve in the tissues, and the number of eosinophils produced by the bone marrow. Expression of chemokines and adhesion molecules will depend on the degree of activation and on the baseline levels of inflammatory cells in the airways. The *in situ* production of IL-5 in the bone marrow suggests that the bone marrow is not only responding to the airway's inflammation, but is also driving the ongoing inflammatory response.

In contrast to the stimulatory effects of IL-3 and IL-5, IFN-γ, through the attenuation of helper T cell Type 2-driven processes, may play an important role in the resolution of allergen-induced eosinophilic responses (37). One primary antiinflammatory effect of IFN-γ is the reduction of IL-5 production (38). Rais and coworkers have also shown that IL-12 suppressed eosinophilopoiesis via an IFN-γ–dependent mechanism in a murine model (39). On the basis of these results, the present study looked at the production of IFN-γ as a potential downmodulatory cytokine. We found that in the bone marrow supernatant, there is a delayed increase in IFN-γ protein levels in dual responders only. However, when eosinophil/basophil progenitors were cultured in the presence of IFN-γ and IL-5, we were unable to show a direct suppression of Eo/B-CFU. This suggests that, because the increase in bone marrow IFN-γ occurred at 48 hours, a time point that was coincident with a reduction in bone marrow IL-5 protein levels, IFN-γ may be suppressing eosinophil/basophil progenitors indirectly, via suppression of IL-5 production in the bone marrow. However, this must remain speculative, because we were unable to detect a difference in IFN-γ levels between isolated early and dual responders.

The role of the eosinophil in allergen-induced airway responsiveness is currently a subject of much debate. Leckie and coworkers (40) have suggested that IL-5 and eosinophils do not play an important role in allergen-induced airway hyperresponsiveness, although this study was not adequately designed to address this question (12). Murine models have also provided conflicting results (41–45). Foster and coworkers have suggested that these differences may be due to the presence of low levels of airway eosinophils in mouse models, which demonstrate persisting AHR (46). This suggestion is supported by Flood-Page and coworkers, who have shown that although an anti–IL-5 monoclonal antibody was able to reduce blood and bronchoalveolar lavage eosinophils, airway tissue and bone marrow eosinophils were reduced by only 50%. In addition, major basic protein (an inflammatory mediator prominently produced by eosinophils) showed no significant reduction with treatment (47). In the current study, only dual responders, with significantly greater bone marrow and airway eosinophilic responses, developed airway hyperresponsiveness. These findings are therefore consistent with a role for eosinophils in the development of allergen-induced airway hyperresponsiveness.

In conclusion, this study has demonstrated that allergen-induced eosinophilopoiesis begins within 5 hours of allergen inhalation by upregulation of progenitor responsiveness to IL-3 and then increases in terminal differentiative responses to IL-5 over 48 hours. We have previously shown this is due to increases in progenitors expressing the IL-5 receptor and, in this study, increased levels of bone marrow IL-5. These changes occur only in dual responders who develop both a peripheral blood and airway eosinophilia and an associated prolonged airway hyperresponsiveness. The resolution of bone marrow eosinophilopoiesis may result from IFN-γ–mediated downregulation of IL-5 production. These results support a role for bone marrow eosinophilopoiesis and airway eosinophilia in allergen-induced airway hyperresponsiveness.

Conflict of Interest Statement: S.C.D. has no declared conflict of interest; R.S. has no declared conflict of interest; G.M.G. has no declared conflict of interest; R.M.W. has no declared conflict of interest; R.F. has no declared conflict of interest; G.L.J. has received honoraria from Actelion for presentations and from AstraZeneca for marketing research; J.A.D. has no declared conflict of interest; M.D.I. has no declared conflict of interest; P.M.O. has no declared conflict of interest.

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CHAPTER 3

ALLERGEN-INDUCED FLUCTUATION IN CC CHEMOKINE RECEPTOR 3 EXPRESSION ON BONE MARROW $CD34^+$ CELLS FROM ASTHMATIC SUBJECTS: SIGNIFICANCE FOR MOBILIZATION OF HAEMOPOIETIC PROGENITOR CELLS IN ALLERGIC INFLAMMATION

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Allergen-induced fluctuation in CC chemokine receptor 3 expression on bone marrow $CD34^+$ cells from asthmatic subjects: significance for mobilization of haemopoietic progenitor cells in allergic inflammation

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SUMMARY

There is increasing evidence that primitive progenitors migrate from the bone marrow (BM) via the peripheral circulation to tissue sites where they undergo *in situ* differentiation to provide a continued source of effector cells, such as eosinophils, during an allergic inflammatory response. To study mechanisms of progenitor cell mobilization in allergic reactions, we investigated fluctuations in the expression of the eotaxin receptor, CC chemokine receptor 3 (CCR3), on $CD34^+$ cells from stable asthmatics following allergen (i.e. antigen) challenge. BM aspirates were taken from seven early responder (ER) and 10 dual responder (DR) asthmatics who, following antigen challenge developed only an early bronchoconstrictor response and an early and late-bronchoconstrictor response, respectively. Expression of CCR3 was detected on primitive ($CD34^+$ cells) and eosinophil-lineage committed progenitors ($CD34^+$ interleukin-5 receptor alpha-subunit$^+$ cells) by flow cytometry and confirmed by co-localization of CCR3 messenger RNA to CD34 immunopositive cells using *in situ* hybridization. When preantigen levels were compared to 24-hr postantigen levels, significant increases in BM $CD34^+$ $CCR3^+$ cells were detected in DR, who also developed a significant sputum and blood eosinophilia and increased methacholine airway responsiveness. In contrast, a significant attenuation of BM $CD34^+$ $CCR3^+$ cells was observed in ER. In a dose-dependent manner eotaxin, but not interleukin (IL)-5, stimulated $CD34^+$ progenitor cell migration *in vitro*. This migrational response to eotaxin was abrogated by anti-CCR3 monoclonal antibody and primed by preincubation with IL-5. We propose that fluctuations in CCR3 expression on human BM $CD34^+$ cells may facilitate chemokine-mediated progenitor cell mobilization to the peripheral circulation and the resultant development of pulmonary eosinophilia, a cardinal feature of asthma.

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Abbreviations: APAAP, alkaline phosphatase–anti-alkaline phosphastase; BrdU, bromodeoxy uridine; CCR3, CC chemokine receptor 3; CFU, colony-forming units; DR, dual responders; ER, early responders; FACS, fluorescence activated cell sorter; FCS, fetal calf serum; FEV_1, forced expiratory volume in 1 second; FITC, fluorescein isothiocyanate; ICC, immunocytochemistry; IL-5Rα, interleukin-5 receptor alpha-subunit; ISH, *in situ* hybridization; mAb, monoclonal antibody; MACS, magnetic antibody cell sorting; MNC, mononuclear cells; NAMNC, non-adherent mononuclear cells; PE, phycoerythrin; PerCp, peridinin chlorophyll protein; SDF-1, stromal cell derived factor-1; SSC, orthogonal or side light scatter.

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INTRODUCTION

There is a considerable body of evidence that increased production of eosinophil-lineage committed progenitors within the bone marrow is associated with the onset and maintenance of upper and lower airway eosinophilic inflammation in response to allergen exposure in atopic subjects.[1,2] We have shown that increased numbers of haemopoietic progenitors (CD34$^+$ cells) and CD34$^+$ cells expressing the membrane-bound isoform of interleukin-5 receptor α-subunit (IL-5Rα) are found in the bone marrow of atopic asthmatics, compared with chronic bronchitic and normal subjects.[3,4] To establish that 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: early responders (ER), who only develop an early bronchoconstrictor response; and dual responders (DR), who, following allergen inhalation, develop both an early and a late bronchoconstrictor response. Significant increases in bone marrow CD34$^+$ IL-5Rα$^+$ cells and IL-5-responsive eosinophil/basophil colony-forming units (Eo/Baso-CFU) were only detected in DR who also developed delayed airway hyperresponsiveness and airway eosinophilia 24 hr postallergen.[5,6] Similar antigen-driven increases in CD34$^+$ IL-5Rα$^+$ cell numbers, and resultant enhancement of IL-5-dependent eosinophilopoiesis, have become evident in the bone marrow following nasal allergen challenge in various mouse models of eosinophilic airway inflammation.[7,8]

Although bone marrow haemopoietic events may trigger the increased production and ultimate egress of mature eosinophils from the bone marrow, there is increasing evidence that primitive and lineage-committed progenitors migrate to mucosal tissue sites, where local haemopoietic events may contribute to the increased recruitment of effector cells. In animal studies, using bromodeoxy uridine (BrdU) to label actively proliferating cells undergoing DNA synthesis within the bone marrow, increases in the numbers of BrdU$^+$ cells in the bronchoalveolar lavage fluid 24-hr postallergen are interpreted to reflect the ingress of newly divided myeloid cells in the airways in response to antigen challenge.[9,10] In human studies, CD34 immunopositive cells were extracted from human nasal polyp and nasal explant tissue and shown to undergo IL-5-driven proliferation and differentiation into Eo/Baso-CFU *in vitro* and *ex vivo*,[11,12] confirming the presence of true blast cells in mucosal tissue. Increased numbers of CD34$^+$ IL-5Rα$^+$ cells have also been detected in induced sputum[13] and bronchial biopsy tissue[14] from atopic asthmatics compared with normals. In the latter study, CD34$^+$ IL-5Rα$^+$ cell numbers correlated with asthma severity, as judged by airway calibre, suggesting that control of progenitor cell trafficking may be a prerequisite for reduction of eosinophilic inflammation in the airway in asthma. Little, however, is known about the factors that orchestrate the mobilization of progenitors during an allergic inflammatory response.

Intravenous infusion of eotaxin has been shown to stimulate the rapid egress of eosinophil progenitor cells from the bone marrow sinuses of guinea-pigs into the peripheral circulation.[15] In human studies, intravenous infusion of IL-5 stimulated an increase in the numbers of circulating progenitor cells and of eosinophils expressing CCR3, proposing an essential interplay between IL-5 and eotaxin in mobilization of pro-inflammatory cells from the bone marrow.[16] In the present study we have investigated the hypothesis that ligation of the CC chemokine receptor 3 (CCR3)[17] may stimulate the migration of progenitor cells during an allergic inflammatory response. We have studied fluctuations in CCR3 expression on CD34$^+$ progenitors in asthmatic subjects in response to allergen challenge and investigated the function of this receptor on bone marrow progenitors in the context of allergen-induced development of an airway eosinophilic response.

MATERIALS AND METHODS

Materials

Materials were obtained as follows: Percoll from Pharmacia (Uppsala, Sweden); McCoys 5A, Iscove's modified Dulbecco's phosphate-buffered saline (PBS) and fetal calf serum (FCS) from Gibco (Burlington, Ont., Canada); methylcellulose, bovine serum albumin (BSA) grade V, heparin, sodium azide and paraformaldehyde from Sigma Chemical Co. (Mississauga, Ont., Canada); May–Grunwald–Giemsa stain from BDH (Mississauga, Ont., Canada), Diff-Quik stain from American Scientific Products (McGaw Park, IL) and dithiothreitol (Sputolysin) from Calbiochem (San Diego, CA). Stromal cell derived factor-1 (SDF-1), eotaxin and IL-5 were purchased from R & D Systems (Minneapolis, MN).

Antibodies

Phycoerythrin (PE)-conjugated immunoglobulin G1 (IgG1) CD34 (HPCA-2), fluorescein isothiocyanate (FITC)-conjugated IgG1 CD45 (anti-HLE1), PE-conjugated isotype-control antibody (i.e. anti-IgG1–PE) and streptavidin-conjugated peridinin chlorophyll protein (PerCp) were purchased from Becton-Dickinson (Mississauga, Ont., Canada). Non-neutralizing monoclonal antibodies (mAbs) directed against the α-subunits of IL-5R (IL-5Rα; α16) were a kind gift from Dr Jan Tavernier (University of Ghent, Belgium). Anti-CCR3 mAb (MAB155) and IgG2a isotype control were purchased from R & D Systems. Cytokine receptor antibodies were biotinylated as previously described.[5]

Subjects

Seventeen non-smokers with mild, stable asthma (seven ER and 10 DR) were studied (Table 1). The asthmatic subjects were classified into two groups based on their airway responses to allergen:

(1) ER, who only developed an early fall (of >15% from baseline) in the forced expiratory volume in 1 second (FEV_1); and
(2) DR, who, following allergen inhalation, developed both an early and a late bronchoconstrictor response (defined as a fall in FEV_1 of >15% and >12% from baseline, respectively).

The early and late bronchoconstrictor responses were taken to be the maximal percentage fall in FEV_1 within 2 hr after allergen inhalation and between 3 and 7 hr after allergen inhalation, respectively. The definitions of early and late asthmatic response were established before the study, and the subjects were characterized as ER and DR by their airway

Table 1. Subject characteristics and allergen-induced airway responses

	Early responder asthmatics	Dual responder asthmatics
Age	24 (20–30)	23 (21–50)
Sex	3 M : 7 F	2 M : 5 F
% fall in FEV_1		
EAR	31 (43.9–22.1)	27.4 (51.3–17.9)
LAR	6.1 (9.7–2.9)	16.6 (37.1–12.5)
Methacholine PC_{20}		
Pre-allergen	5.2 (11.3–1.0)	4.2 (11.4–0.1)
Post-allergen	5.8 (11.7–0.3)	2.5†,** (17.3–0.1)

Values are presented as medians with ranges except for PC_{20} values which are geometric means with ranges.**$P < 0.005$ for within group comparison of pre-allergen versus 24 hr post-allergen log PC_{20} values and †$P < 0.05$ for between group comparisons of pre-allergen versus 24 hr post-allergen log PC_{20} values. Dual responder, but not the early responder, asthmatics developed a significant increase in methacholine airway responsiveness 24 hr post-allergen $P < 0.005$). In addition, there was a significant difference between the two groups in the allergen-induced shift in log PC_{20} values ($P < 0.05$).

M, male; F, female.

responses to a screening allergen inhalation challenge. All subjects had a baseline FEV_1 of >70% of the predicted normal on all study days, none had had a respiratory tract infection for at least 4 weeks prior to entering the study and required only infrequent use of inhaled α_2-agonist.[18] This study was approved by the Research Advisory Board of McMaster University Health Sciences Corporation, and each subject gave written, informed consent.

Study design

Subjects attended the laboratory on three occasions, as follows:

(1) Visit 1. One week prior to allergen challenge to document full medical history, and to undergo skin-prick test sensitivity testing to allergen extracts, spirometry, methacholine-inhalation test and induction of sputum to assess baseline airway inflammation.
(2) Visit 2. Allergen-challenge procedure. Prior to allergen challenge, a bone marrow aspirate was collected and spirometry measurements were taken hourly for 7 hr post-allergen inhalation to follow the allergen-induced bronchoconstrictor response. Blood samples were taken pre- and 5 hr postallergen challenge.
(3) Visit 3. Blood, sputum and bone marrow aspirates were collected 24 hr postallergen challenge. Spirometry measurements and methacholine inhalation challenge were also performed to assess the changes in airway calibre and hyperresponsiveness.

Allergen-inhalation challenge and methacholine-inhalation challenge

Allergen challenge was performed as described by O'Byrne *et al.*,[19] and the concentration of the allergen extract for inhalation was determined from a formula using skin-prick test and methacholine PC_{20} results (PC_{20} = provocative concentration of methacholine causing a 20% fall in FEV_1).[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 incremental concentrations of allergen were inhaled at 10-min intervals until a decrease of ≥15% occurred in the FEV_1 from baseline.

Methacholine inhalation was performed by the tidal breathing method, as described by Cockcroft *et al.*[21] Subjects inhaled normal saline and then incremental doubling concentrations of methacholine phosphate from a Wright nebulizer for 2 min. The test was terminated when a fall in FEV_1 of 20% of the postsaline value occurred, and the PC_{20} was calculated.

Indices of inflammation: sputum, blood and bone marrow differential cell counts

Sputum was induced by inhalation of hypertonic saline and processed according to the method of Pizzichini *et al.*[22] Sputum cell plugs were processed using 0·1% dithiothreitol and Dulbecco's PBS. Cytospins were prepared from the pelleted cells and differential counts were performed on Diff-Quik-stained slides. Means of duplicate slides were obtained (500 cells counted/slide) and expressed as absolute counts (10^4 cells/ml). Venous blood was collected into EDTA-treated tubes. Total cell counts were performed using a Neubauer haemocytometer and differential cell counts were made from blood smears stained by Diff-Quik. Similarly, differential cell counts were made from smears of whole bone marrow stained by Diff-Quik. Differential cell counts were obtained from the mean of two slides (1000 cells counted/slide) and cell populations were expressed as the absolute counts (10^4 cells/ml) for sputum and blood and as percentage values for bone marrow samples.

Isolation and immunofluorescence staining of bone marrow and blood progenitors

For progenitor cell enumeration, heparinized (1000 U/ml) samples of bone marrow (5 ml) were aspirated from the iliac crest, and venous blood (20 ml) was collected. From each sample, low-density mononuclear cells (MNC) were isolated by sedimentation on Percoll density gradients (specific gravity 1·077), as previously described.[23] Monocytes were depleted from the MNC fraction by incubation in plastic flasks for 2 hr at 37°. Non-adherent MNC (NAMNC; containing progenitor cells and lymphocytes) were first stained with saturating amounts of biotin-conjugated anti-IL-5Rα, anti-CCR3 or the isotype-control antibody (determined in preliminary studies) in a final volume of 100 µl of ice-cold fluorescence-activated cell sorter (FACS) staining buffer (PBS plus 0·1% NaN_3, 2·5% each of mouse serum and human serum) for 30 min at 4°. The cells were then washed with 3 ml of PBS + 0·1% sodium azide, resuspended in FACS staining buffer and stained with streptavidin-conjugated PerCp together with saturating concentrations of FITC–CD45 IgG1 (anti-HLE1) and PE–CD34 IgG1 (HPCA-2) for 30 min at 4°. Lysis buffer (Becton Dickinson Canada) was then added and the cells incubated for 5 min, after which they were washed twice with 3 ml of PBS + 0·1% sodium azide and finally fixed in 500 µl of PBS + 1% paraformaldehyde. Cells were refrigerated until acquired by flow cytometry.

Flow cytometry and gating strategy

The stained NAMNC were analysed using a FACScan flow cytometer equipped with an argon ion laser [Becton Dickinson Instrument Systems (BDIS)] using the CELLQUEST programme

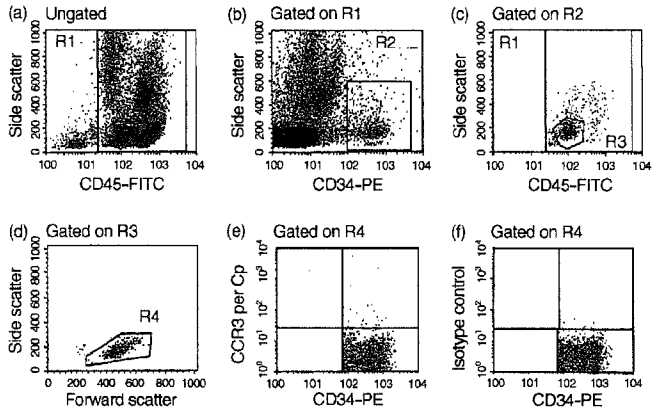


Figure 1. Detection of CC chemokine receptor 3 (CCR3) expression on bone marrow CD34$^+$ cells from an atopic asthmatics by multigating flow cytometry. Plots (a)–(d) represent staining of bone marrow low-density non-adherent mononuclear cells with CD45–fluorescein isothiocyanate (FITC)/CD34–phycoerythrin (PE). Details of the gating strategy are described in the Materials and methods, and have been published.[5] Events in region R4 (true progenitor cells) were further analysed for staining with peridinin chlorophyll protein (PerCp)-linked CCR3 monoclonal antibody (mAb) (e) or isotype control (f). Data were collected as the number of positive cells at the 99% confidence limit (i.e. relative to a marker set to include only 1% of cells stained with control antibody).

(BDIS). Progenitor cells were identified based on their unique cell size, granularity and immunofluorescence characteristics.[5] Off-line analysis was performed using the PC LYSIS software supplied by BDIS. True CD34$^+$ blast cells were identified as cells with CD34^{high}/CD45^{dull} staining and low side scatter (Fig. 1a, A–D). Within the true CD34$^+$ population, specific staining of PerCp-linked cytokine/chemokine receptor mAbs (Fig. 1a, E) or control antibody (Fig. 1a, F) was detected, and data were collected as numbers of cells at the 99% confidence limit (i.e. relative to a marker set to include only 1% of cells stained with control antibody).

In situ *hybridization and immunohistochemistry*

To confirm the cell-surface expression of CCR3 on CD34$^+$ progenitor cells detected by flow cytometry, we co-localized CCR3 messenger RNA to CD34 immunopositive cells by sequential non-radioactive *in situ* hybridization (ISH) and immunocytochemistry (ICC), as previously described.[24] Briefly, a population of unstimulated CD34$^+$ cells was enriched from a bone marrow aspirate by positive selection using magnetic antibody cell sorting (MACS) (purity determined by flow cytometry to be >85%), and cytospin preparations were prepared as previously described.[5] Peripheral blood eosinophils, purified by negative selection using CD16-coated MACS beads as previously described,[25] were used as a positive control for detection of CCR3 mRNA. The cell phenotypes were first identified by ICC using an alkaline phosphatase–anti-alkaline phosphatase (APAAP) technique and phenotype-specific murine anti-human mAb (CD34; Dako, High Wycombe, UK) (EG2$^+$ Pharmacia). After developing with Fast Red for CD34$^+$ immunostaining, slides were hybridized with 200 ng of digoxigenin-labelled riboprobes for CCR3 (antisense and sense) in hybridization buffer at 50° overnight, then washed in 2 × saline sodium citrate (SSC), 1 × SSC and 0·5 SSC, respectively; unhybridized probe was removed by digestion with RNAse A.[24] After blocking with 2% normal sheep serum, the slides were incubated with sheep anti-digoxigenin Fab fragment-conjugated alkaline phosphatase (1 : 200 dilution; Boehringer Mannheim, Mannheim, Germany) at room temperature for 3 hr. The signals were developed with BCIP/NBT (X-phosphate-5-bromo-4-chloro-3-indoly phosphate/nitro-blue tetrazolium). Positive cells expressing phenotypic markers (red colour), mRNA for CCR3 (dark blue colour), or both (mixed colours), were counted (Fig. 2b). A minimum of 200 cells were counted per slide.

Transmigration assay

The migrational response of progenitors *in vitro* was assessed using transwell chambers (24-well cell clusters with 5-μm pore polycarbonate filters (Costar, Boston, MA), as previously described with minor modifications.[26] An enriched population of bone marrow CD34$^+$ cells (purity >85%), isolated from atopic asthmatic subjects by MACS,[3] were loaded into each filtered transwell insert (1×10^5/100 μl). These transwell inserts were then placed in a larger well containing 600-μl dilutions of the chemoattractant in serum-free medium, previously incubated for 15 min at 37° in 5% CO_2. After 4 hr, the filtered transwell insert was carefully removed and the cells in the bottom chamber (representing migrated cells) were aspirated. These cells were resuspended in FACS staining buffer and stained with PE-conjugated CD34 mAb and FITC-conjugated CD45 mAb, as described above. To obtain absolute values of migratory cells, flow cytometric counts for each sample were obtained during a constant predetermined time period. Data for the enumeration of CD34$^+$ cells are expressed as the percentage of total numbers of CD45$^+$ cells counted in the bottom chamber.

Colony-forming assay

Bone marrow-derived NAMNC were isolated, as described above, and cultured in duplicate in 0·9% methylcellulose at $0{\cdot}25 \times 10^6$ cells per 35 × 10-mm tissue culture dish (Falcon Plastics, Oxnard, CA) in Iscove's modified Dulbecco's medium

containing 20% FCS, 1% penicillin-streptomycin and 5×10^{-5} M 2-mercaptoethanol in the presence of various cytokine growth factors, including IL-5 and eotaxin. Cultures were incubated for 14 days at 37° and 5% CO_2 and then enumerated by light microscopy. Eo/Baso-CFUs were identified by their distinct morphology, previously described as tight granulated, compact round refractile cell aggregates.[23]

Statistical analysis

The data are presented as arithmetic mean ± standard error of the mean (SEM), except PC_{20} values and absolute numbers of $CD34^+$ progenitor cells, which were logarithmically transformed and are expressed as geometric mean and standard error of geometric mean (%SEM). For statistical analyses of within-group comparisons between pre- and postallergen challenge time-points, a paired Student's *t*-test (two-tailed) was performed. Student's non-paired *t*-tests (two-tailed) were performed for all between-group comparisons. Changes in blood and sputum differentials at several time-points after allergen, and data from the transmigration and priming assays, were assessed using repeated measures analysis of variance (rm ANOVA; Statistica version 5·1, Tulsa, OK). Significance was accepted at the 95% confidence level.

Table 2. Allergen-induced changes in sputum, peripheral blood and bone marrow eosinophils

	Early responder asthmatics	Dual responder asthmatics
Sputum	($n = 7$)	($n = 10$)
Pre-allergen	8 ± 1	21 ± 4
7 hr post-allergen	54 ± 21**	147 ± 19**,†
24 hr post-allergen	32 ± 12**	80 ± 8**,†
Peripheral blood	($n = 7$)	($n = 10$)
Pre-allergen	33 ± 7	25 ± 3
24 hr post-allergen	34 ± 8	42 ± 4**
Bone marrow	($n = 6$)	($n = 5$)
Pre-allergen	2.9 ± 0.8%	3.8 ± 1.6%
24 hr post-allergen	2.5 ± 0.6%	3.5 ± 1.4%

Data in the sputum and blood are presented as cells/104 per ml and as a percentage of total WBC in the bone marrow. In sputum, significant increases from baseline in eosinophils were detected at 7 hr and 24 hr post-allergen in both early- and dual- responder asthmatics. However, the allergen-induced shift at 7 hr and 24 hr was greater in dual- compared with early- responder asthmatics. A significant increase in blood eosinophil numbers was detected 24 hr post-allergen inhalation challenge in dual responder asthmatics only. There were no significant allergen-induced changes in bone marrow eosinophils in either group of asthmatic subjects when pre-allergen were compared with 24 hr post-allergen levels (**$P < 0.005$ for within group analysis; †$P < 0.05$ for between group analysis).

RESULTS

Allergen-induced changes in airway physiology

Allergen-induced changes in airway responses (bronchoconstrictor and methacholine PC_{20}) for all asthmatic subjects in this study are summarized in Table 1. Compared with preallergen levels, a significant increase in methacholine airway responsiveness 24 hr after inhaled allergen was detected in DR only ($P < 0·005$). In addition, there was a significant difference between ER and DR in the allergen-induced shift in log PC_{20} values ($P < 0·05$) (Table 1).

Allergen-induced changes in sputum, blood and bone marrow eosinophils

Allergen-induced changes in sputum, blood and bone marrow eosinophil numbers are summarized in Table 2. A significant increase in the absolute number of sputum eosinophils was found in both ER and DR when preallergen levels were compared with those at 7- and 24-hr postallergen inhalation challenge. However, the magnitude of the allergen-induced increase in sputum eosinophil numbers at 7 and at 24 hr was significantly greater in DR compared with ER ($P < 0·05$) (Table 2). In the peripheral blood there was a significant increase in eosinophil numbers at 24 hr postallergen challenge compared to preallergen levels in DR only ($P < 0·005$). In contrast, no significant change in bone marrow eosinophil numbers was detected in either ER or DR when 24-hr postallergen levels were compared with preallergen levels (Table 2).

CCR3 expression on progenitor cells

CCR3 expression was detected on bone marrow $CD34^+$ progenitor cells from atopic asthmatic subjects, as determined by flow cytometry (Fig. 1). In addition, to investigate the expression of CCR3 on eosinophil-lineage committed progenitors, we performed triple staining on an enriched population of bone marrow $CD34^+$ cells (purity >85%). As shown in Fig. 2, expression of CCR3 was detected on $CD34^+$ IL-$5R\alpha^+$ cells. In this experiment, the number of $CD34^+$ $CCR3^+$ IL-$5R\alpha^+$ cells represented 11% of the $CD34^+$ $CCR3^+$ cells and 50% of the $CD34^+$ IL-$5R\alpha^+$ cells. Similar findings were obtained in three separate experiments, indicating that CCR3 is expressed by both primitive and lineage-committed progenitor cells.

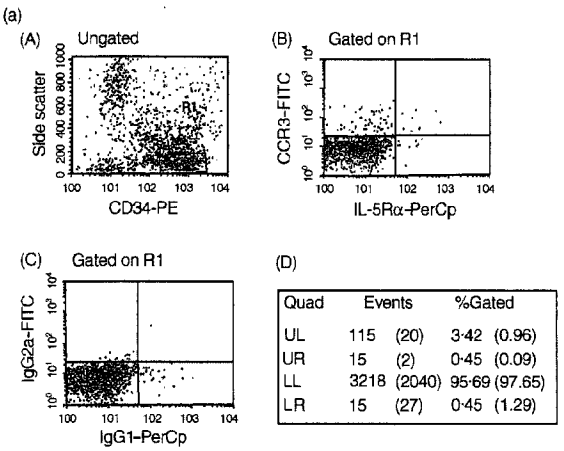
Allergen-induced changes in the phenotype of progenitor cells from bone marrow and peripheral blood

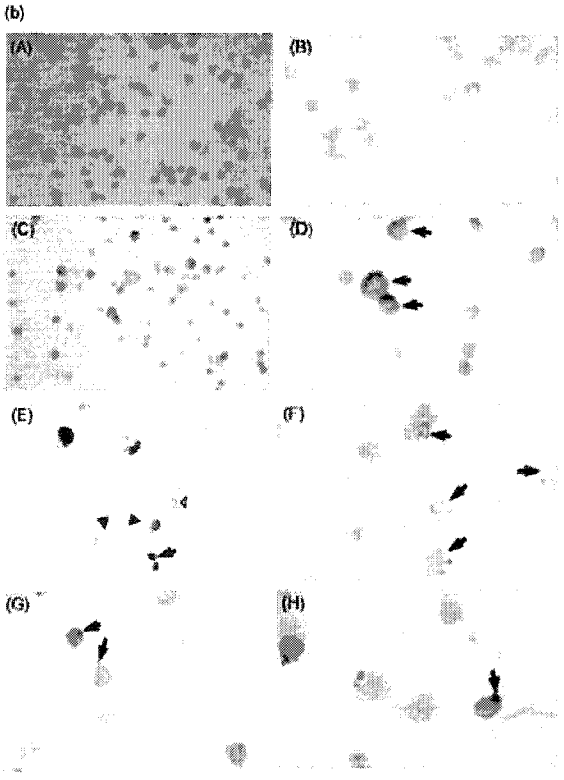
In accordance with previously published findings, no significant change in the total number of bone marrow $CD34^+$ cells was found when preallergen levels were compared with 24-hr postallergen levels in either group of asthmatics [DR: 4898 (%SEM 720) to 5623 (%SEM 910); ER: 5129 (%SEM 628) to 4169 (%SEM 510) cells/$0·25 \times 10^6$ white blood cells (WBC)] (Fig. 3).[5] A significant increase in the absolute number of $CD34^+$ IL-$5R\alpha^+$ cells when preallergen levels were compared with 24-hr postallergen levels in bone marrow samples from DR [46 (%SEM 9) to 83 (%SEM 18) cells/$0·25 \times 10^6$ WBC; $P < 0·05$], but not in ER [55 (%SEM 9) to 48 (%SEM 26) cells/$0·25 \times 10^6$ WBC], was also detected (Fig. 3).

In enumerating the expression of the eotaxin receptor, CCR3, on bone marrow $CD34^+$ cells, we found similar levels of expression at baseline for both groups of asthmatic subjects (DR: 47 (%SEM 12) versus ER: 79 (%SEM 18) cells/$0·25 \times 10^6$ WBC). When preallergen levels were compared with 24-hr postallergen levels, a significant increase in the absolute numbers of bone marrow $CD34^+$ $CCR3^+$ cells was

detected in DR [47 (%SEM 12) to 100 (%SEM 19) cells/0·25 × 10^6 WBC, $P < 0·05$] (Fig. 3). In contrast, a significant decrease in the absolute number of CD34$^+$ CCR3$^+$ cells was detected in bone marrow samples from ER, 24 hr postallergen [79 (%SEM 18) to 38 (%SEM 8) cells/0·25 × 10^6 WBC, $P < 0·01$].

In blood samples, when preallergen levels were compared with 24-hr postallergen levels, there were no significant changes in the total numbers of progenitor cells or the phenotype of CD34$^+$ cells in both groups of asthmatics (Table 3). However,





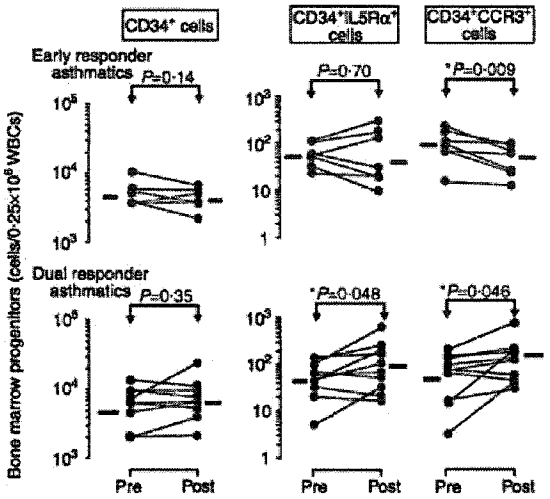


Figure 3. Allergen-induced changes in CD34$^+$ cells and CD34$^+$ cells co-expressing interleukin-5 receptor alpha-subunit (IL-5Rα) and CC chemokine receptor 3 (CCR3) in bone marrow aspirates taken from early responder ($n = 7$) and dual-responder ($n = 10$) asthmatics. Significant increases in both CD34$^+$ IL-5Rα$^+$ and CD34$^+$ CCR3$^+$ cell numbers were detected in dual responders 24 hr postallergen. In contrast, a significant attenuation in CD34$^+$ CCR3$^+$ cell number was detected in early responders. Horizontal bars represent the geometric mean of each data set. (For statistical significance, a Student's paired *t*-test was performed on log-transformed data.) WBCs, white blood cells.

Figure 2. (a) Detection of CC chemokine receptor 3 (CCR3) and interleukin-5 receptor alpha-subunit (IL-5Rα) co-expression on CD34$^+$ cells by flow cytometry. An enriched population of CD34$^+$ cells (purity >85%) from bone marrow non-adherent mononuclear cells (NAMNC) was collected by a magnetic cell separation technique. Cells were stained with CD34–phycoerythrin (PE), anti-CCR3–fluorescein isothiocyanate (FITC) and anti-IL-5Rα–peridinin chlorophyll protein (PerCp). (A) CD34-positive staining events were gated (gate R1); (B) cells within R1 were assessed for co-expression of both CCR3 and IL-5Rα; (C) a 99·9% confidence limit was set for this rare cell type (>0·1% staining with isotype-control antibodies); (D) region statistics show that of the total number of CD34$^+$ cells counted, 3·42% were CD34$^+$ CCR3$^+$, 0·45% were CD34$^+$ IL-5Rα$^+$ and 0·45% were CD34$^+$ CCR3$^+$ IL-5Rα$^+$ (isotype-control values are expressed in parenthesis). The proportion of CD34$^+$ CCR3$^+$ IL-5Rα$^+$ cells represented 11% of the CD34$^+$ CCR3$^+$ cells and 50% of the CD34$^+$ IL-5Rα$^+$ cells in this sample. (b) Co-localization of mRNA for CCR3 to CD34$^+$ cells using sequential immunocytochemistry (ICC) and *in situ* hybridization (ISH) on cytospins of bone marrow-derived CD34$^+$ cells from an atopic asthmatic subject. (A)–(D) Staining with Dig-labelled CCR3 sense (A, B) and antisense (C, D) riboprobes, at low (magnification × 400; a and c) and high (magnification × 1000; b and d) power. Some of the CCR3 mRNA$^+$ cells (dark blue) are indicated (→). (E) A combination of ICC and ISH in eosinophils (positive control cells) was performed. Single EG2$^+$ stained red is indicated (△) and single ISH$^+$ (CCR3 mRNA) are dark blue (▲). Double positive cells are dark red (→). (F) Cytospins of purified bone marrow CD34$^+$ cell ICC showing CD34$^+$ cells (→). (G) Single ISH of CCR3 mRNA$^+$ cells (→). (H) Double ICC/ISH of CD34$^+$/CCR3$^+$ cells (→).

Table 3. Summary of FACS data from peripheral blood

	Early responder asthmatics ($n = 7$)	Dual responder asthmatics ($n = 10$)
$CD34^+$		
Pre-allergen	741 (%77)	832 (%109)
24 hr post-allergen	776 (%108)	1068 (%89)
$CD34^+IL5R\alpha^+$		
Pre-allergen	7 (%3)	25 (%8)*
24 hr post-allergen	15 (%6)	18 (%6)
$CD34^+CCR3^+$		
Pre-allergen	6 (%3)	19 (%2)*
24 hr post-allergen	5 (%2)	17 (%6)

Data are presented as cells/106 WBC and expressed as geometric mean and standard error of geometric mean (%SEM). Significantly higher baseline (pre-allergen) levels of $CD34^+$ $IL5R\alpha^+$ and $CD34^+CCR3^+$ cells were detected in the blood of dual responder compared with early responder asthmatics. However, when pre-allergen were compared with 24 hr post-allergen levels, no significant change in numbers of blood $CD34^+IL5R\alpha^+$ and $CD34^+CCR3^+$ cells were detected in either group of asthmatics. $*P < 0.05$ for between group comparisons of baseline levels of progenitor cells.

in comparing pre-allergen blood levels of both $CD34^+$ IL-$5R\alpha^+$ and $CD34^+$ $CCR3^+$ cells, significantly greater numbers of both cell types were detected in DR, compared with ER, asthmatics (Table 3).

In situ hybridization and immunohistochemistry

Co-localization experiments using simultaneous *in situ* hybridization and immunocytochemistry were performed to confirm that $CD34^+$ progenitor cells express mRNA for CCR3. Non-radioactive *in situ* hybridization showed that 56·9% of bone marrow CD34-immunopositive cells expressed CCR3 mRNA. A representative example from an atopic subject is shown in Fig. 2(b).

Transmigration assay

In order to assess the functional role of CCR3 expression on $CD34^+$ cells from atopic asthmatic subjects ($n = 4$), an *in vitro* migrational assay was used (Fig. 4). The CXC chemokine, SDF-1 (optimal concentration 100 ng/ml), previously described as a potent progenitor cell chemoattractant, was included in this assay as a positive control.[26] Compared with diluent, a significant (but weak) dose-dependent migratory response was detected for bone marrow $CD34^+$ cells stimulated by eotaxin, which was optimal at 500 ng/ml (diluent: 18 ± 2% versus eotaxin (500 ng/ml): 24 ± 1% of $CD45^+$ cells in the bottom well, $n = 6$, $P < 0·05$) (Fig. 4). Eotaxin (500 ng/ml) stimulated a migratory response of 4 ± 2·5% $CD34^+$ cells and this was comparable with the level of CCR3 expression detected on an enriched population of bone marrow $CD34^+$ cells by flow cytometry (CCR3 expression on 3·42% of $CD34^+$ cells, Fig. 2).

In the presence of a neutralizing CCR3 mAb (25 μg/ml), eotaxin-stimulated, but not SDF-1-stimulated, $CD34^+$ cell migration was completely abrogated (Fig. 4). In three separate

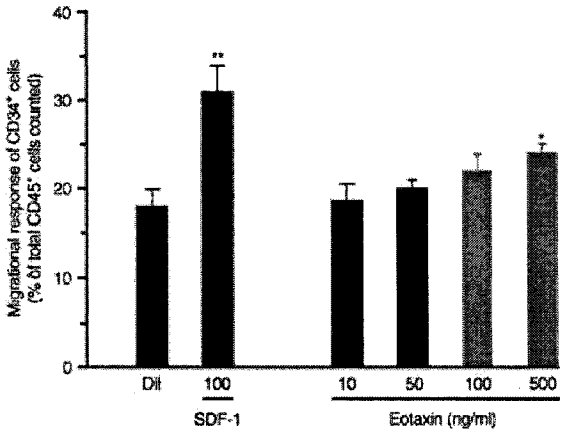


Figure 4. Transwell migrational assay of bone marrow $CD34^+$ cells, *in vitro*. Compared with diluent control, stromal cell derived factor-1 (SDF-1) and eotaxin stimulated a significant dose-dependent migrational response of $CD34^+$ cells, which was optimal at 100 ng/ml and 500 ng/ml, respectively ($*P < 0·05$; $**P < 0·01$). The eotaxin-, but not SDF-1-stimulated progenitor cell migratory response was completely attenuated in the presence of anti-CC chemokine receptor 3 (CCR3). Data are expressed as mean ± standard error of the mean (SEM) of six separate experiments performed in duplicate.

experiments, there was no appreciable migratory response of progenitor cells stimulated by IL-5 over a wide concentration range (5–500 ng/ml) compared with diluent control.

In priming experiments where an enriched population of bone marrow $CD34^+$ cells were preincubated with IL-5 (50 ng/ml) for 1 hr at 37°, we found a significant increase in the subsequent migratory responses of $CD34^+$ cells to sub-optimal concentration of eotaxin (50 ng/ml, $P < 0·05$) (Fig. 5).

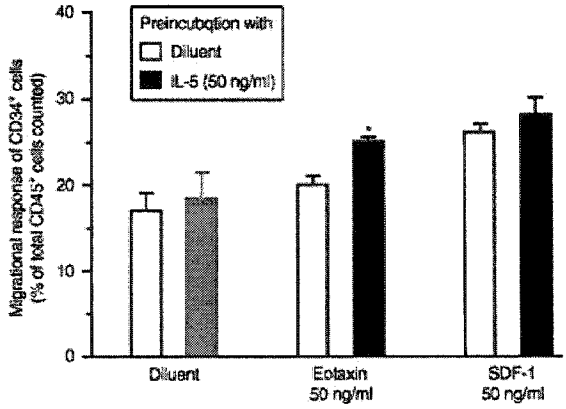


Figure 5. Interleukin-5 (IL-5) priming of bone marrow $CD34^+$ cell migratory responses *in vitro*. Preincubation with IL-5 (50 ng/ml) for 1 hr at 37° significantly increased the migratory response of $CD34^+$ to a suboptimal dose of eotaxin (50 ng/ml, $*P < 0·05$) but not suboptimal doses of SDF-1 (50 ng/ml) or diluent. Data are expressed as mean ± standard error of the mean (SEM) of six separate experiments performed in duplicate.

Table 4. Hemopoietic activity of eotaxin

	Eo/Baso-CFU	GM-CFU
Diluent	0	0
Eotaxin (1–500 ng/ml)	0	0
IL-5 (1 ng/ml)	12.5 ± 2**	0
IL-5 (1 ng/ml) + Eotaxin (1 ng/ml)	12.0 ± 1**	0
IL-5 (1 ng/ml) + Eotaxin (10 ng/ml)	11.5 ± 3**	0
IL-5 (1 ng/ml) + Eotaxin (100 ng/ml)	12.0 ± 2**	0

Data are presented as Eo/Baso-CFU or GM-CFU detected in 14 day methycellulose colonies for 0.25x106 BM-derived NAMNC plated as previously described 6. Eotaxin (1–500 ng/ml) did not stimulate the growth of either Eo/Baso-CFU or GM-CFU. In addition, Eotaxin did not enhance the numbers of Eo/Baso-CFU grown in the presence of previously determined optimal concentrations of either IL-5 (1 ng/ml). (**$P < 0.01$ for comparisons between diluent and cytokine growth factor-stimulated colonies).

In contrast, preincubation with IL-5 did not stimulate the migrational response of progenitor cells to suboptimal doses of SDF-1 (50 ng/ml). The priming effect of IL-5 on the migrational responses of eotaxin-mediated progenitor cells was not enhanced further by longer incubation (either 4 or 24 hr) with CD34$^+$ cells.

Colony-forming assay

In 14-day methycellulose cultures of bone marrow NAMNC, eotaxin, over a wide concentration range (1–500 ng/ml) did not stimulate the significant growth of either Eo/Baso or granulocyte–macrophage (GM) colonies compared with diluent control (Table 4). In addition, in cultures of NAMNC with optimal concentrations of IL-5 (1 ng/ml), the addition of eotaxin did not further increase the number of Eo/B-CFU detected.

DISCUSSION

The findings from this study confirm that:

(1) the CC chemokine receptor, CCR3, is expressed on human primitive (CD34$^+$) and eosinophil lineage-committed (CD34$^+$ IL-5Rα^{+}) progenitors;
(2) the number of bone marrow CD34$^+$ CCR3$^+$ cells is significantly increased in DR asthmatics (who characteristically develop a late-bronchoconstrictor response, sputum and blood eosinophilia and increased methacholine airway responsiveness 24 hr postallergen inhalation);
(3) a significant attenuation in the number of CD34$^+$ CCR3$^+$ cells was observed in ER asthmatics who do not develop a blood eosinophilia and airway hyperresponsiveness following allergen inhalation;
(4) using an *in vitro* transwell migration assay to assess the function of CCR3 on progenitor cells, eotaxin stimulates a migratory response by CD34$^+$ cells; and
(5) preincubation with IL-5, which itself does not stimulate CD34$^+$ cell migration, significantly primed the migratory response of CD34$^+$ cells to eotaxin.

Together, these findings suggest that following allergen-inhalation challenge in asthmatics, upregulation of CCR3 expression on CD34$^+$ cells may facilitate chemokine-mediated egress of eosinophil-lineage committed progenitors from the bone marrow to the blood, thereby promoting the development of an eosinophilic response, a distinctive feature of allergic inflammation.

Eotaxin, first described as a potent eosinophil-selective chemotactic activity, was identified by microsequencing as a CC chemokine[27–29] and shown to mediate its effect through ligation of a specific receptor, CCR3.[30–32] Although several ligands for CCR3 have been described, including regulated on activation, normal, T-cell expressed, and secreted (RANTES), monocyte chemotactic peptide (MCP)- 2, -3 and -4, it is clear that only eotaxin and other, less potent, eosinophil-selective chemoattractants (eotaxin-2 and eotaxin-3) mediate their agonist effects mainly through ligation of CCR3.[33] To date, the expression of CCR3 has been shown to be restricted to mature eosinophils and, to a lesser extent, to basophils, some T helper 2 (Th2) cells, mast cells[34] and dendritic cells.[35] Although the expression of CCR3 mRNA has been co-localized to mature and immature eosinophils in bone marrow samples, the expression of CCR3 on CD34$^+$ progenitor cells in asthmatic subjects has not been reported to date.[36] Studies on the sequence of expression of CCR3 on eosinophils have described this receptor as a late differentiation marker.[17,37] However, these studies were performed on transformed leukaemic cell lines, including HL-60 clone 15 cells and AML14 cells, which may sometimes provide different results from findings with freshly isolated cells. Using flow cytometry and limiting-dilution assays to assess eotaxin-stimulated cell proliferation, the expression of CCR3 has been reported on primitive progenitor cells from murine bone marrow.[38] We confirm the expression of CCR3 on human bone marrow and blood CD34$^+$ progenitor cells, at the protein level by flow cytometry and at the mRNA level by sequential immunocytochemistry and *in situ* hybridization (Fig. 2b). We have previously shown that CD34$^+$ cells can express membrane-bound IL-5Rα, and suggested that this may be the phenotype of the earliest eosinophil lineage-committed progenitor cell.[5] As we have, in the present study, shown for the first time that CCR3 is co-expressed on a subset of CD34$^+$ IL-5Rα^+ cells (Fig. 2), we propose that this may be the phenotype of an eosinophil lineage-committed progenitor cell that has the potential to respond to a chemokine signal and egress from the bone marrow sinus into the peripheral circulation during an allergic inflammatory response. This may account for our findings that increased numbers of both CD34$^+$ CCR3$^+$ and CD34$^+$ IL-5Rα^+ cells were detected in the blood of DR, compared with ER, asthmatics (Table 3).

Eosinophils arise from CD34$^+$ pluripotent stem cells that differentiate and mature under the influence of lineage-specific growth factors such as IL-5.[39] An important step in the selective recruitment of eosinophils to sites of allergic inflammation is the mobilization of mature and immature eosinophil pools from the bone marrow sinuses into the peripheral circulation. This traffic has been shown to be mediated by IL-5, acting systemically, and eotaxin, acting locally, at the site of inflammation.[40–42] Although in some animal models eotaxin has been shown to have myeloproliferative properties,[38,43] we were unable to detect any colony-forming activity of eotaxin on bone marrow CD34$^+$ cells, either alone or in combination with stem cell factor or IL-5 in 14-day methylcellulose cultures (Table 4). It has been shown that in guinea-pigs eotaxin acts systemically to

activate the bone marrow and stimulate mobilization of both mature eosinophils and eosinophil progenitors: using an *in situ* perfusion system of guinea-pig femoral bone marrow, eotaxin infusion into the arterial supply stimulated not only a rapid selective release of eosinophils into the draining vein but the release of eosinophil colony-forming progenitor cells as well.[15] In contrast, infusion of IL-5 stimulated the delayed and sustained release of mature eosinophils, but not of eosinophil progenitors. In the present study, we used an *in vitro* transwell migration assay to investigate the role of the CCR3 receptor on human $CD34^+$ progenitor cells. We showed that ligation of the CCR3 receptor resulted in a migratory response by bone marrow $CD34^+$ cells to eotaxin and that the proportion of progenitors that migrated to eotaxin was comparable with the level of CCR3 expression detected on enriched $CD34^+$ cells (Fig. 4). The migrational response elicited by eotaxin was modest compared with that elicited by SDF-1, previously described as a potent progenitor cell chemoattractant.[26] Our findings with eotaxin are in contrast to results reported by Auiti *et al.* who showed a negligible chemotactic effect of eotaxin on $CD34^+$ cells isolated from normal donors.[26] This disparity is further supported by our recent findings that $CD34^+$ cells from atopic asthmatics have a significantly greater migrational response to eotaxin than progenitor cells from normal subjects.[44] Based on our findings that IL-5 can prime a $CD34^+$ cell migratory response to eotaxin (Fig. 5), it is probable that elevated levels of IL-5 in atopic asthmatics compared with normal subjects may cause *in vivo* priming, resulting in the observed migratory responsiveness of $CD34^+$ cells from atopic asthmatics to eotaxin.[45]

In the present study, we have shown that although no changes in the total numbers of $CD34^+$ cells were detected in the bone marrow, the number of $CD34^+$ $CCR3^+$ and $CD34^+$ IL-$5R\alpha^+$ cells were significantly increased in DR 24 hr postallergen (Fig. 3). We propose that whereas upregulation of IL-5Rα on $CD34^+$ cells within the bone marrow may favour increased eosinophilopoiesis within that microenvironment, the upregulation of CCR3 on $CD34^+$ cells may favour increased traffic of progenitor cells from the bone marrow to the peripheral circulation in the presence of a positive concentration gradient of chemokines, such as eotaxin. The inability to detect similar significant changes in the blood at 24 hr may reflect the speed of homing of the progenitors or the dilutional effect of the blood (Table 3). Moreover, the kinetics of change in the progenitor cell population may be different within the blood compared to the bone marrow compartment; this is the subject of an ongoing study.

In long-term culture studies, it has been shown that IL-5 upregulates CCR3 expression on differentiating eosinophils within 3–7 days.[37,46] Relevant to this we have recently reported that mRNA levels of IL-5 are significantly increased within the bone marrow of DR compared with ER asthmatics 24 hr postallergen challenge.[47] It is therefore probable that local increases in IL-5 alone or in combination with other pro-inflammatory factors within the bone marrow may upregulate CCR3 expression on $CD34^+$ progenitor cells from DR asthmatics. Conversely, our findings of significantly lower levels of blood-derived $CD34^+$ $CCR3^+$ and $CD34^+$ IL-$5R\alpha^+$ cells in ER compared with DR asthmatics (Table 3), and downregulation of CCR3 expression on $CD34^+$ cells from ER asthmatics in response to allergen challenge (Fig. 3), may reflect an active means of preventing progenitor-cell mobilization from the bone marrow sinuses. Studies have shown that eotaxin treatment of differentiated acute myeloid leukemia (AML) cells results in marked downregulation of CCR3 expression for at least 18 hr,[17] and that IL-3 causes a significant downregulation of CCR3 expression on eosinophils.[48] Whether increased local generation of eotaxin, or possibly IL-3, within the bone marrow of ER compared with DR asthmatics causes the ligand-mediated internalization of CCR3 and thus the observed significant attenuation of $CD34^+$ $CCR3^+$ cell numbers postallergen, still remains to be investigated.

Under normal conditions of haemopoiesis, the bone marrow acts as a site for the turnover and traffic of mature leucocytes to the peripheral circulation. However, in inflammatory conditions, such as atopic asthma, in addition to the increased release of mature eosinophils, increased egress of primitive and eosinophil-lineage committed progenitor cells occurs. Although the precise mechanisms which trigger progenitor cell traffic remain to be fully elucidated, this study provides evidence to support the hypothesis that, through upregulation of CCR3 *in vivo*, increased responsiveness to an eotaxin gradient may result in the mobilization of eosinophil progenitors from the bone marrow to the peripheral circulation during an allergic inflammatory response. Our results also point out the likelihood that the effects of both IL-5 and eotaxin may have to be abrogated if eosinophil inflammation is to be fully controlled.

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CHAPTER 4

SDF-1α AND EOTAXIN'S ROLE IN PROGENITOR CELL EGRESS FROM THE BONE MARROW AFTER ALLERGEN CHALLENGE

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Sandra Dorman's contribution:

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

Title: **SDF-1α and Eotaxin's role in Progenitor Cell Egress from the Bone Marrow after Allergen Challenge.**

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ABSTRACT

Background: CCR3 expression on progenitors mediates migration to eotaxin, *in vitro*. CXCR4 and SDF-1α are important for stem-cell homing to hemopoietic compartments.

Objective: This study investigated chemokine effects on progenitor egress from bone marrow.

Methods: Marrow aspirates were obtained from normals, atopic non-asthmatics and asthmatics (dual and early). Additionally, aspirates and blood were obtained from dual and early responders before, 5 and 24h after allergen inhalation. Changes in receptor expression and migration were compared between groups and over time.

Results: CXCR4, but not CCR3, expression was greater in normal subjects compared with asthmatics. Likewise, SDF-1α, but not eotaxin, stimulated transmigration of more progenitors from normal subjects. A positive correlation existed between intensity of CXCR4 expression and magnitude of migration to SDF-1α *in vitro*. Allergen inhalation attenuated both intensity of CXCR4 expression and SDF-1α levels in marrow from dual when compared to early responders, 24h post-allergen. In contrast, circulating eotaxin and intensity of CCR3 expression on circulating progenitors increased in dual versus early responders at 5 and 24h post-allergen. Also in dual responders, increases in the proportion of circulating progenitors expressing CXCR4 was detected at 24h post-allergen, while decreases in responsiveness to SDF-1α and an increase in responsiveness to eotaxin were found for marrow progenitors, 24h post-allergen compared to pre-allergen.

Conclusion: Following allergen inhalation, a down-regulation in CXCR4 on progenitors

and subsequent reduced responsiveness to SDF-1α may result in reduced progenitor retention to marrow stroma and subsequent peripheral egress, mediated in part, by CCR3 and its ligand eotaxin.

INTRODUCTION

Activation of hemopoiesis within the bone marrow is associated with the onset and maintenance of airway inflammation in asthma.[1,2] In conjunction with this activation, allergen inhalation may also trigger the release of primitive and lineage-committed progenitors into the blood. Early studies have demonstrated: *i)* elevated blood levels of eosinophil/basophil colony forming units (Eo/B-CFU) in atopic subjects compared with normals;[3] *ii)* increased circulating Eo/B-CFU during acute asthma exacerbations and 24h following allergen inhalation challenge in atopic asthmatics and;[4-6] *iii)* increases in circulating Eo/B-CFU during seasonal exposure to allergen in allergic rhinitis.[7;8] Together, these results provide indirect evidence that in allergic asthma, changes in circulating progenitor cell numbers reflect dynamic hemopoietic processes within the bone marrow and support the hypothesis for progenitor cell transmigration from bone marrow to blood.

After allergen inhalation, allergic asthmatics can be classified into two groups, labelled early responders and dual responders. Dual responders develop prolonged airway hyperresponsiveness, pronounced airway, blood and bone marrow eosinophilia, eosinophil-lineage commitment and increased IL-5-driven eosinophilopoiesis within the bone marrow with an associated increase in circulating Eo/B-CFU, not seen in early responders, following allergen challenge.[4-6;9] The difference in bone marrow responses between these groups suggest that significant progenitor cell migration out of the bone marrow only occurs in the dual responder group.

Homing and retention of hemopoietic cells in the bone marrow occurs via specific

chemokines, in particular, stromal cell-derived factor-1α (SDF-1α).[10-12] Interfering with SDF-1α interactions and its ligand, CXCR4, blocks the *in vivo* homing of HPSC to the bone marrow and mobilizes stem cells for release into the circulation.[11-13] HPSC can also be mobilized from the bone marrow following stimulation with various agents, including hemopoietic growth factors, inflammatory cytokines, chemokines and complement components.[12;14] Eotaxin, an important inflammatory chemokine up-regulated in asthma, has been shown to selectively stimulate the rapid egress of eosinophil progenitor cells from the bone marrow sinuses of guinea pigs into the peripheral circulation.[15] In human studies, we have shown that both $CD34^+$ and $CD34^+$IL-$5R\alpha^+$ cells from bone marrow and blood express CCR3 at a protein and mRNA level and that this receptor is up-regulated on progenitor cells following allergen inhalation challenge.[14] In addition, we found that eotaxin stimulated directional migration of progenitor cells, *in vitro*.[16] More recently, Lamkhioued *et al.* have demonstrated that under conditions that promote Th2 type differentiation, CCR3 surface expression is up-regulated on $CD34^+$ progenitor cells from cord blood.[17] Together, these studies suggest that eotaxin is an important inflammatory chemokine that may promote the egress of HPSC from the bone marrow. The role of various groups of chemokine receptor families including CCR3 and CXCR4 have not been investigated in the context of progenitor cell mobilization during an allergic inflammatory response in asthmatic subjects.

The purpose of this study was twofold: first, to the enumerate the level of chemokine receptor expression on progenitors from asthmatics and normals and to relate this to the magnitude of migrational responsiveness of these cells; and second, to

investigate the effect of allergen challenge on production of the chemokines and the corresponding level of receptor expression on progenitor cells, with a view to understanding how these chemokines modulate the egress of progenitors out of the bone marrow during an allergic inflammatory response.

MATERIALS AND METHODS

Study Design

Subjects were screened and classified as normal, atopic non-asthmatic, early or dual responders. Normal and atopic subjects visited the laboratory once, when medical history, skin prick test sensitivity, spirometry and bone marrow aspirate samples were taken. Early and dual responders attended the laboratory on 4 occasions: visit 1, one week prior to allergen challenge, medical history, skin prick test sensitivity, spirometry, blood and bone marrow aspirate samples were taken; visit 2, one day prior to allergen challenge, methacholine inhalation test was performed; visit 3, allergen challenge was performed and blood and bone marrow aspirate were taken at 5h post-challenge; visit 4, 24h post-challenge, methacholine inhalation challenge, blood and bone marrow aspirate were performed.

Antibodies

Phycoerythrin (PE)-conjugated IgG_1 CD34 antibody (HPCA-2), fluorescein isothiocyanate (FITC) conjugated IgG_1 CD45 antibody (anti-HLE1), PE conjugated isotype control antibody (i.e. anti IgG_1- PE) and streptavidin conjugated peridinin chlorophyll protein (PerCp) were purchased from Becton Dickinson, Canada

(Mississauga, ON). Anti-CXCR4, Anti-CCR3 mAb, IgG_{2a} isotype control and IgG_{2b} isotype control were purchased from R&D systems (Minneapolis, MN, USA).

Subjects

Nine non-atopic, non-asthmatic subjects (determined by negative skin-prick test and PC_{20} greater than 32mg) and five asymptomatic atopic non-asthmatic subjects (determined by clinical history, positive skin prick-test and PC_{20} greater than 32mg) participated. In addition, thirty subjects with mild, stable, atopic asthma and baseline FEV_1>70% predicted were enrolled. For post-allergen comparisons, atopic asthmatics were broken into two groups: early; and dual responders. Dual responders, in contrast to early responders, develop prolonged airway hyperresponsiveness and significant bone marrow activation. Post-allergen, early responders (n=14) developed an early fall in FEV_1>20% from baseline between 0-2h, while dual responders developed an early and late fall (FEV_1>15%) from baseline between 3-7h (n=16). Subjects were non-smokers, using inhaled β_2-agonists intermittently (not used 8h before each visit) and had not experienced respiratory infection nor were exposed to altered levels of allergen two weeks prior to allergen inhalation. This study was approved by the Ethics Committee of McMaster University Health Sciences Centre and subjects gave written, informed consent before study entry.

Methacholine and Allergen Inhalation Tests

Methacholine inhalation challenge was performed as described by Cockcroft.[18] The test was terminated when a fall in FEV_1 of 20% of the post-saline value occurred, and the provocative concentration of methacholine causing a 20% fall in FEV_1 (PC_{20})

was calculated.

Allergen inhalation challenge was performed as described by O'Byrne *et al.* using a Wright nebulizer.[19] The allergen extract was selected and diluted for inhalation at a concentration determined from a formula using both the results from the skin test and the methacholine PC_{20}.[20]

Isolation and Immunofluorescence Staining of Bone Marrow and Blood Progenitors

Bone marrow aspiration and blood progenitor enumeration were performed according to Wood *et al.*[6] Non-adherent mononuclear cells (NAMNC; containing progenitor cells and lymphocytes) were first stained with saturating amounts of biotin-conjugated anti-CXCR4, anti-CCR3 or the relevant isotype control antibody (determined in preliminary studies) in a final volume of 100μl of ice cold FACS staining buffer (PBS plus 0.1% NaN_3, 2.5% each of mouse serum and human serum) for 30 minutes at 4°C. The cells were then washed with 3ml PBS plus 0.1% sodium azide, resuspended in FACS staining buffer and stained with streptavidin-conjugated PerCp together with saturating concentrations of FITC conjugated IgG_1 CD45 antibody (anti-HLE1) and PE-conjugated IgG_1 CD34 antibody (HPCA-2) for 30 minutes at 4°C. Lysis buffer (Becton Dickinson Canada, Mississauga, ON) was then added to the cells and incubated for 5 minutes after which the cells were washed twice with 3ml PBS plus 0.1% sodium azide and finally fixed in 500μl of PBS plus 1% paraformaldehyde. Cells were refrigerated until acquired by flow cytometry.

Cells were analysed using a FACScan flow cytometer equipped with an argon ion laser (BDIS). Progenitor cells were identified based on their unique cell size; granularity

and immunofluorescence characteristics.[9] Off-line analysis was performed using the PC lysis software as supplied by BDIS. True $CD34^+$ blast cells were identified as cells with $CD34^{high}/CD45^{dull}$ staining as previously described.[21] Chemokine receptor expression on progenitors was expressed as either proportion of total progenitor cells (percent of $CD34^+45^+$ cells) or as the intensity of receptor expression (specific mean fluorescence intensity, SMFI) on 4000-5000 $CD34^+45^+$ cells.

Transmigration Assay

The migrational response of progenitors *in vitro* was assessed using transwell chambers (24-well cell clusters with 5-μm pore polycarbonate filters, Costar, Boston, MA, USA) as previously described with minor modifications[21]. An enriched population of bone marrow $CD34^+$ cells (purity >85%) by MACS , were loaded into each filtered transwell insert ($1x10^5/100\mu l$). These transwell inserts were then placed in a larger well containing 600μl dilutions of the chemoattractant (SDF-1α (25, 50, 100 or 200ng/ml), eotaxin (50, 100, 200 or 500ng/ml) or diluent) in serum-free medium, previously incubated for 15 min at 37°C in 5% CO_2. After 4h, the filtered transwell insert was carefully removed and the cells in the bottom chamber (representing migrated cells) were aspirated. These cells were resuspended in FACS staining buffer and stained with PE-conjugated CD34 mAb and FITC-conjugated CD45 mAb, as described above. To obtain absolute values of migratory cells, flow cytometric counts for each sample were obtained during a constant predetermined time period. Since a constant number of $CD34^+45^+$ cells were added to the top well for all migrational assays performed, the migrational data for responding progenitor cells are presented as total $CD34^+45^+$ cell numbers counted in the

lower well.

Serum and Bone Marrow Supernatant

Serum and bone marrow supernatant were stored at -70°C. Eotaxin and SDF-1α were quantified using standard ELISA (R&D systems, MN, USA). The lower limits of detection were 5 and 18pg/ml respectively.

Statistical Analysis

Results were expressed as mean ± SEM, except for methacholine PC_{20} which was expressed as geometric mean and %SEM. Methacholine PC_{20} was $\log_{10}$ transformed prior to analysis. The difference between normals, atopic non-asthmatics and atopic asthmatics at baseline was analysed using a repeated measures ANOVA (between group: normal versus atopic non-asthmatic versus dual responder versus early responder). The effect of allergen on methacholine PC_{20}, $CD34^+45^+$ cell numbers, proportion of $CD34^+45^+$ expressing $CXCR4^+$ and $CCR3^+$ were analysed using a repeated measures ANOVA (between group: early versus dual responders; within group: pre versus 5 and 24 hours post-allergen inhalation). Transmigration data of $CD34^+45^+$ cells were log transformed and analysed using a repeated measures model ANOVA (between treatment: diluent versus various concentrations of chemokines and; between group: normal versus atopic non-asthmatic versus early and dual responder asthmatics). Comparisons of migrational responses of $CD34^+45^+$ cells before versus 24h after allergen were analysed using a student's T test for dependent samples for each concentration of cytokine and diluent control. Cytokine protein levels were analysed using nonparametric testing (Kruskall Wallace between group: early versus dual responders and Wilcoxon for within

group: pre vs 5 and 24 hours post-allergen inhalation) (Statistica version 6.0, Tulsa, OK, USA). Statistical significance was accepted as $p<0.05$.

RESULTS

Chemokine Receptor Expression on Progenitor Cells

In agreement with previous studies, we found that significantly greater numbers of $CD34^+45^+$ cells were detected in the bone marrow of atopics compared with normal subjects[16;21-22] (Figure 1a). More specifically, significantly greater $CD34^+45^+$cell numbers were detected in bone marrow aspirates from atopic non-asthmatics compared with both dual and early responder asthmatics and normal subjects. In addition, significantly greater numbers of $CD34^+45^+$cells from bone marrow were detected in dual responders compared with early responders ($p<0.05$) and normals ($p<0.01$) but not between early responders and normals (atopic non-asthmatics: 7285±1727; dual responders: 6532±435.7; early responders: 4945±389.5; and normal subjects 4206±402 $CD34^+45^+$cells / $0.25x10^6$ WBCs) (Figure1a).

A comparison of various classes of chemokine receptors on bone marrow progenitors showed that the intensity of CXCR4 expression on $CD34^+45^+$ cells was significantly higher in normal subjects (33.9±6.5) when compared to dual responders (5.7±0.6; $p<0.001$) and early responders (6.9±1.0; $p<0.001$) but not atopic non-asthmatics (22.7±4.5) (Figure 1b). By comparison, there was no difference in the intensity of CCR3 expression on $CD34^+45^+$ cells between any of the subject groups (dual responders: 4.8±0.9; early responders: 3.8±0.5; atopic non-asthmatics: and normals:

6.8±1.7). Similar findings were obtained when we analysed the data as the percentage of bone marrow $CD34^+45^+$ cells that expressed either CXCR4 (normals 34±5.0% versus dual responders: 13±3.0%; $p<0.05$; early responders: 7±1.1%; $p<0.05$; atopic non-asthmatic: 27±4.5% $CD34^+45^+$ cells) or CCR3 (normals: 1.4±0.2%; dual responders: 1.8±0.4%; early responders: 1.3±0.1%; atopic non-asthmatic: 1.1±0.3% $CD34^+45^+$ cells).

Baseline Transmigration of Progenitor Cells

To investigate the relationship between levels of chemokine receptor expression and functional responsiveness, we enumerated the migrational responses of bone marrow derived progenitor cells to SDF-1α and eotaxin, *in vitro*. SDF-1α stimulated a significant dose dependent migrational response of $CD34^+45^+$ cells from all subject groups, optimal at 200ng/ml (Figure 2a). For between group comparisons, the number of $CD34^+45^+$ cells transmigrating towards SDF-1α was significantly greater in normals compared to atopic non-asthmatics and asthmatic subjects at optimal concentrations (200ng/ml; 609±173, 279±64 and 111±55 $CD34^+45^+$ cells respectively, $p<0.05$)(Figure 2a). No difference in the magnitude of migrational response to any dose of SDF-1α tested was detected in comparisons between dual and early responder asthmatics (data not shown). For all subjects, a significant positive correlation was observed between the intensity of CXCR4 expression on $CD34^+45^+$ cells and the magnitude of progenitor cell migrational response to SDF-1α at 200ng/ml ($r=0.33$, $p<0.05$) and 100ng/ml ($r=0.43$, $p<0.05$).

Eotaxin stimulated a weak migrational response for bone marrow $CD34^+45^+$ cells which was significantly greater than diluent only at 500ng/ml for all groups of subjects ($p<0.05$) (Figure 2b). There was a trend for a greater migrational response by progenitor

cells from asthmatics compared with normals to eotaxin, but this was not significant (Figure 2b).

Allergen-induced Fluctuations in Bone Marrow and Peripheral Blood Progenitors Cells

To assess the role of the CXCR4/SDF-1α and CCR3/Eotaxin axis on the egress of progenitor cells from the bone marrow in allergic inflammation, allergen challenge was performed on two groups of asthmatic subjects: dual responders and early responders, and progenitor levels in the bone marrow and blood were enumerated. Allergen-induced airway responses are outlined in Table I.

Bone Marrow Changes: In accordance with previous studies, no significant change in the number of bone marrow $CD34^{+}45^{+}$cells was detected over 24h following allergen challenge in either group of asthmatic subjects[6;16;21] (Figure 3). No significant change in the proportion of bone marrow $CD34^{+}45^{+}$ cells expressing CXCR4 or CCR3 was detected overtime following allergen in either group of asthmatic subjects (Figure 4). Similarly, no significant allergen-induced change in the intensity of CXCR4 or CCR3 on bone marrow $CD34^{+}45^{+}$ cells was detected overtime. However, for between group comparisons, significantly lower intensity of CXCR4 expression on progenitor cells was detected in dual responders compared with early responders at 24h post-allergen (dual responders: 2.9 ± 1.4, early responders: 8.9 ± 2.4, $p<0.05$) (Table II). In contrast, significantly higher intensity of CCR3 expression on $CD34^{+}45^{+}$ cells were detected in the bone marrow of dual responders compared to early responders at 24h post-allergen (dual responders: 4.13 ± 0.9, early responders: 2.4 ± 1.4, $p<0.05$) (Table II).

Peripheral Blood Changes: Enumeration of circulating progenitor cells showed that following allergen inhalation, dual responders developed a significant decrease in the number of $CD34^+45^+$cells at 5h compared to baseline and compared to 24h levels (Figure 3). The numbers of hemopoietic progenitors decreased from 931±139.8 cells pre-allergen to 740±103.4 cells at 5h ($p<0.05$) and subsequently increased to 1015±125.8 cells at 24h ($p<0.05$) post allergen. In contrast, in early responders no change in circulating $CD34^+45^+$cell numbers at any time point was found following allergen challenge (Figure 3).

Following allergen inhalation, dual responders developed a significant increase in the proportion of peripheral blood $CD34^+45^+$ cells expressing CXCR4 at 5h and 24h compared to baseline. Cell numbers increased from 12±3.4% pre-allergen to 28±7.3% at 5h ($p<0.05$) and to 28±8.9% of $CD34^+45^+$ cells at 24h post allergen ($p<0.05$) (Figure 4a). By contrast, in early responders there was no significant change in the proportion of circulating $CD34^+45^+$ cells expressing CXCR4 at any time point post-allergen challenge (Figure 4a). For both groups of asthmatics there was no significant allergen-induced change in the proportion of circulating $CD34^+45^+$ cells expressing $CCR3^+$ cells (Figure 4b).

Allergen-Induced Changes in Transmigration of Progenitor Cells

To investigate the effect of allergen challenge on functional responsiveness of progenitor cells, comparisons of the chemokine-stimulated migrational responses pre versus post-allergen were performed in dual responder asthmatics. Following allergen challenge there was a reduction in the stimulated migrational response of bone marrow

$CD34^{+}45^{+}$ cells to a sub-optimal concentration of SDF-1α at 100ng/ml (Figure 5a). In contrast, the stimulated migrational response to a sub-optimal concentration of eotaxin (200ng/ml) was significantly greater when pre-allergen levels were compared with post-allergen levels (Figure 5b).

Allergen-Induced Changes in SDF-1α and Eotaxin

SDF-1α: Following allergen inhalation, there was no significant change in the amount of circulating SDF-1α over time in either group (Figure 6a). In contrast in the bone marrow, following allergen inhalation, dual responders developed a significant decrease in SDF-1α at 24h post-allergen compared to baseline. Protein levels were1666±61.8pg/ml pre-allergen; 1630±69.9 pg/ml at 5h and 1460±79.2pg/ml at 24h post allergen ($p=0.05$). In early responders, there was no change in SDF-1α levels over time following allergen inhalation. Between group comparisons showed that at 24h post-allergen significantly lower levels of SDF-1α were detected in dual responders compared to early responders ($p<0.05$) (Figure 6a).

Eotaxin: Following allergen inhalation, dual responders developed a significant increase in the amount of eotaxin in the blood at 5h post allergen compared to baseline. Protein levels increased from baseline being: 81±11.2pg/ml; to 101±9.9 pg/ml at 5h ($p<0.05$) and; 96.6±12.1pg/ml at 24h post-allergen. By contrast, there was no significant change in eotaxin levels in early responders following allergen inhalation. There was also significantly more eotaxin at 5h post allergen in dual responders compared to early responders ($p<0.05$)(Figure 6b). No change in bone marrow eotaxin levels was detected over time in either any group pf asthmatics (Figure 6b).

DISCUSSION

The distinctive findings of this study are that in the bone marrow *(i)* at baseline, there are significant differences in the level of CXCR4 but not CCR3 expression on progenitor cells from various groups of subjects, *(ii)* a significant correlation exists between the magnitude of the migrational response and the level of CXCR4 expression on progenitor cells, *(iii)* a down-regulation of CXCR4 expression on $CD34^{+}45^{+}$cells from dual compared with early responders at 24h post-allergen, and (iv) a significant reduction in migrational responsiveness to SDF-1α, *in vitro,* when pre-allergen levels are compared with 24h post allergen levels in dual responders. In addition, the level of eotaxin in the peripheral blood and the level of SDF-1α in the bone marrow also fluctuate in response to allergen challenge at times that are consistent with progenitor cell movement. Together, these findings support the hypothesis that following allergen-inhalation challenge in asthmatics, down-regulation of SDF-1α in the bone marrow and increased eotaxin in the peripheral blood together with relevant fluctuations of the receptors on $CD34^{+}45^{+}$ cells may facilitate chemokine-mediated egress of progenitors from the bone marrow to the blood.

CD34 antigen is a monomeric O-sialylated glycophosphoprotein whose expression is restricted to developmentally early lymphohemopoietic stem and progenitor cells. We have previously shown increases in the number of eosinophil/basophil colony forming units 24h after allergen inhalation and during seasonal flares.[6;8] In addition, Robinson *et al.* have also shown the presence of immunopositive CD34 cells in bronchial biopsies and found an increase in the number of these cells in asthmatics compared to

normals.[23] Concurrent with these findings we have recently shown increases in the number of sputum $CD34^{+}45^{+}$cells in the airways of dual responder asthmatics at 7 and 24h post-allergen inhalation.[24] In the present study, for the first time we have shown fluctuations in the number of circulating $CD34^{+}45^{+}$cells, decreasing significantly at 5h in dual responders only, a time-point preceding the 7h increases previously seen in sputum $CD34^{+}45^{+}$cells.[24] Together these results suggest that during an asthmatic exacerbation, bone marrow progenitor cells are both activated and released into the circulation where they may then home to the airway tissue undergoing an allergic inflammatory response.

SDF-1α, first described as a potent hemopoietic stem cell-selective chemokine, was identified by micro-sequencing as a CXC chemokine and shown to mediate its effect through ligation of a specific receptor, CXCR4.[25] SDF-1α is produced by all stromal cells and is bound within the marrow by heparans and proteolycans. Therefore, ligation of CXCR4 by SDF-1α is not only a migrational stimulus but is also retentive for progenitor cells within the bone marrow.[26] Petit *et al.* have proposed that manipulation of SDF-1α-CXCR4 interactions may be an improved way to control the navigation of progenitor cells between the bone marrow and blood. To examine this hypothesis, they investigated the mobilization of hemopoietic progenitor stem cells (HPSC) induced by intravenous injection of granulocyte colony-stimulating factor (GCSF), a method widely used in clinical transplantation. Using ELISA and immunohistologic analysis of human bone marrow plasma they showed sharp transient increases in marrow plasma SDF-1α followed by significant reduction within 24 hours of treatment which was not seen in the blood. Furthermore, it has been demonstrated that the mobilization of HPSC by GCSF

was due to the disruption of the CXCR4-SDF-1α chemotactic pathway resulting in the loss of chemotactic response of the CXCR4 to its ligand[13] and the proteolytic degradation of SDF-1α.[14] As both SDF-1α and CXCR4 are essential for the homing and retention of HPSC in the bone marrow, the interruption of ligation may represent a critical step in the mobilization of HPSC into the peripheral circulation.[13] The current study is consistent with these finding by showing a reduction in bone marrow SDF-1α levels at 24h, a decrease in the intensity of CXCR4 expression on bone marrow $CD34^+45^+$ cells and increases in circulating $CD34^+45^+$ $CXCR4^+$ cells at 5 and 24h following allergen inhalation. The increase in circulating $CD34^+45^+$ $CXCR4^+$ cells prior to the measured decrease in SDF-1α presumably reflects the initial cleavage process where cells become non-responsive and are released into the circulation. This is supported by the decrease in the number of bone marrow $CD34^+45^+$ cells transmigrating towards SDF-1α at 24h compared to baseline numbers (Figure 5a).

Interestingly SDF-1α ligation has also been shown to arrest progenitor cell cycling, preventing apoptosis, but promoting a transition into quiescence.[27] In the present study, the decrease in measurable SDF-1α occurs at a time which has previously been shown to be associated with an increase in eosinophilopoiesis in dual responders only.[6] Therefore SDF-1α, in addition to allowing the egress of progenitor cells from the bone marrow, may also contribute indirectly to the increased production of inflammatory cells in this site. This is in agreement with our findings that a significant increase in IL-5 responsive Eo/B-CFU formation was detected in dual responders.[9]

Eotaxin, first described as a potent eosinophil-selective chemokine, was identified

by micro-sequencing as a CC chemokine[28-30] and shown to mediate its effect through ligation of a specific receptor, CCR3.[31-33] CCR3 expression on progenitor cells has now been shown by several groups.[16-17;34] We have also previously shown the ability of these cells to transmigrate towards eotaxin which was abrogated by monoclonal antibodies against CCR3. In the same study, we found significant increases in CCR3 expression on bone marrow $CD34^+$ cells from dual responders 24h following allergen inhalation.[16] In the current study, we confirm the expression of CCR3 on human bone marrow and blood $CD34^+45^+$ cells and show that following allergen challenge there was a significant increase in the intensity of CCR3 expression on $CD34^+45^+$ cells from bone marrow of dual responder asthmatics (Table II).

In guinea pigs, eotaxin acts systemically to activate the bone marrow and stimulate mobilization of both mature eosinophils and eosinophil progenitors. Using an *in situ* perfusion system with guinea pig femoral bone marrow, eotaxin infusion into the arterial supply stimulated not only a rapid selective release of eosinophils into the draining vein but the release of eosinophil colony-forming progenitor cells as well.[15] In the current study, we have shown for the first time, significant increases in circulating eotaxin levels at 5h in dual responders only. The inability to detect similar significant increases in circulating $CD34^+CCR3^+$ cells at 5h may reflect the speed of homing of the progenitors or the dilution effect of the blood.

Pre-allergen, CCR3 expression on $CD34^+45^+$ cells was not significantly different between groups. Similarly, there were no differences between groups in the ability of the $CD34^+45^+$ cells at pre-allergen to transmigrate towards eotaxin. In contrast, the

significant differences in CXCR4 expression between groups was associated with increases in the number of $CD34^{+}45^{+}$ cells transmigrating towards SDF-1α. Together these results suggest that chemokine receptor expression reflects the ability of these cells to respond to stimulus. This is further supported by our findings of a significant positive correlation between the intensity of CXCR4 expression on progenitor cells and the magnitude of the migrational response to SDF-1α.

Under normal conditions of hemopoiesis, the bone marrow acts as a site for the turnover and traffic of mature leukocytes to the peripheral circulation. However, in inflammatory conditions such as atopic asthma, in addition to the increased release of mature eosinophils, increased turnover of bone marrow and blood progenitors also occurs. Although the precise mechanisms which trigger progenitor cell traffic remain to be fully elucidated, this study demonstrates that fluctuations in CXCR4 and CCR3 receptor expression on bone marrow $CD34^{+}45^{+}$ cells together with changes in chemokines, SDF-1α and eotaxin protein levels play a role in orchestrating the release from bone marrow stromal compartments and subsequent egress of progenitors to the peripheral circulation in allergic inflammatory responses. These results also point out the likelihood that the activation of the bone marrow may have to be abrogated if airway inflammation is to be fully controlled.

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Table I

Allergen-induced Airway Responses

	Early Responder Asthmatics	Dual Responder Asthmatics
% fall in FEV_1		
EAR	21.9±1.54%	28.3±1.65%
LAR	5.4±1.03%	20.5±2.04‡
Methacholine PC_{20}		
Pre-Allergen	3.3mg/ml (%SEM 1.5)	2.4mg/ml (%SEM 1.3)
Post-Allergen	2.2mg/ml (%SEM 1.5)	0.7mg/ml (%SEM 1.6) †‡

FEV_1: Forced expiratory volume in 1 second; PC_{20}: provocative concentration of methacholine inducing a 20% fall in FEV_1; EAR: early airway response; LAR: late airway response; difference within group: † $P<0.05$; and difference between groups: ‡ $P<0.05$

Blood, bone marrow and airway eosinophil numbers for a subset of these patients has been reported in AJRCCM 2004[34]

Table II

Fluctuations in Chemokine Receptor Expression on Bone Marrow Progenitor Cells Following Allergen Challenge

Receptor Expression on BM $CD34^{+}45^{+}$Cells	Pre-Allergen	5h Post-Allergen	24h Post-Allergen
DR (n=16)			
CCR3 (SMFI)	4.8 ± 0.9	4.9 ± 0.87	6.13 ± 0.9*
CXCR4 (SMFI)	5.7 ± 1.9	9.02 ± 5.26	2.9 ± 1.4
ER (n=14)			
CCR3 (SMFI)	3.8 ± 0.5	3.9 ± 2.21	4.4 ± 1.4
CXCR4 (SMFI)	6.9 ± 1.0	10.5 ± 5.02	8.9 ± 2.4*

BM: bone marrow; h: hour; DR: dual responders; ER early responders; SMFI: Specific mean fluorescence intensity; difference between group: * $P<0.05$.

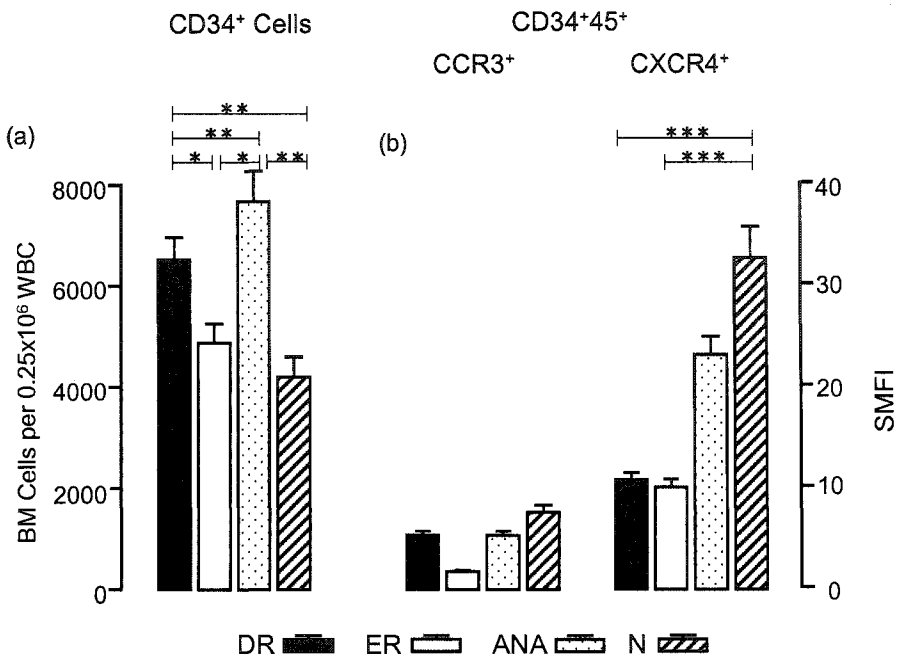

Figure 1 Progenitor cells in bone marrow aspirates from dual responders (DR, n=16), early responders (ER, n=14), atopic non-asthamtics (ANA, n=5) and normals (N, n=9). Data are expressed as mean ± SEM of cells per $0.25x10^6$ WBC for $CD34^+45^+$ cells and as specific mean fluorescence intensity (SMFI) for chemokine receptor enumeration on $CD34^+45^+$ cells. Difference between group: *p<0.05, **p<0.01, ***p<0.001

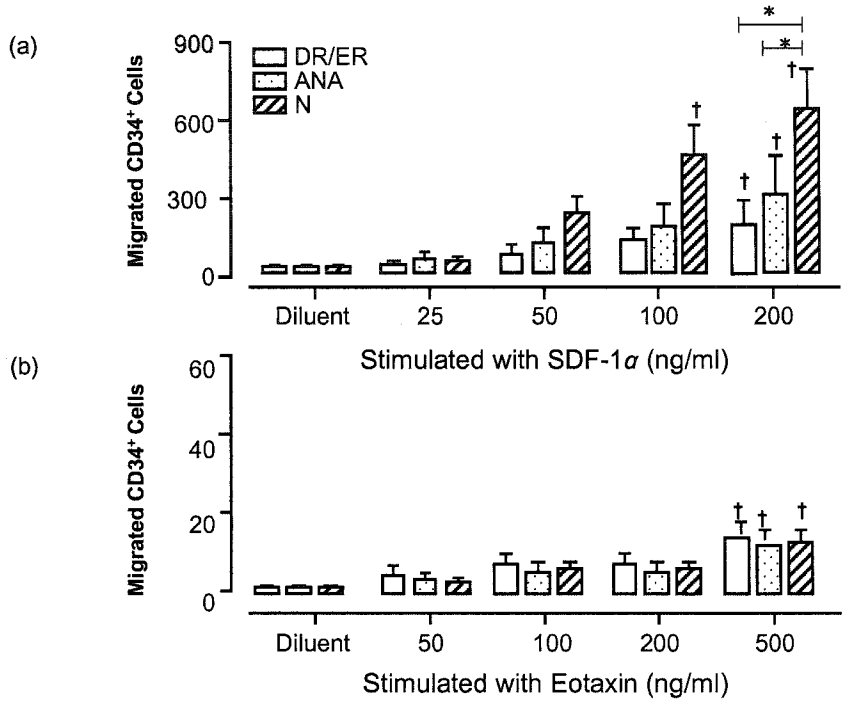

Figure 2 Trans-well migration assay of bone marrow progenitor cells from asthmatics (dual and early responders, DR/ER, n=9), atopic non-asthmatics (ANA, n=5) and normals (N, n=9). SDF-1α stimulated a dose-dependent migrational response of $CD34^+$ cells optimal at 200ng/ml for all groups. The magnitude of transmigration was greatest in normal subjects. Eotaxin stimulated a dose-dependent migrational response of $CD34^+$ cells optimal at 500ng/ml for all groups. Difference within group: † $p<0.05$; difference between group: *$p<0.05$.

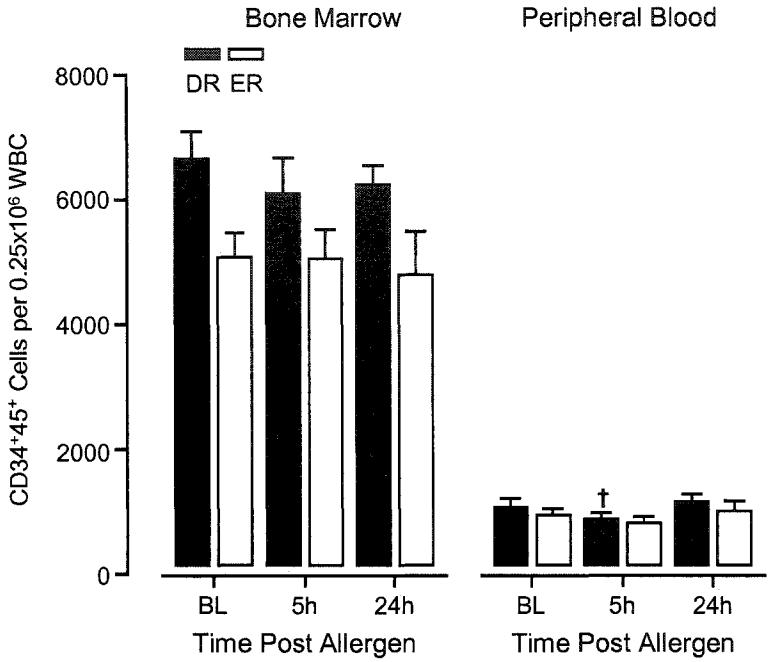

Figure 3 Allergen-induced changes in $CD34^{+}45^{+}$ cells in bone marrow and peripheral blood from dual responders (DR, n=16) and early responders (ER, n=14). Peripheral $CD34^{+}45^{+}$ cells decreased at 5h followed by an increase at 24h post-allergen in DR only. No change in blood progenitors was detected in ER at any time point following allergen challenge. Difference over time: † $p<0.05$.

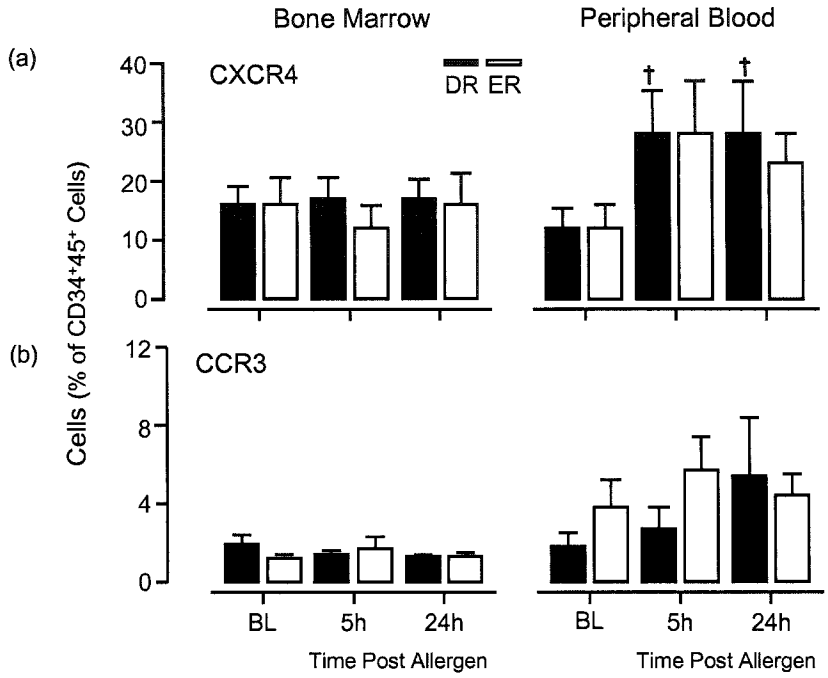

Figure 4 Allergen-induced changes in CXCR4 and CCR3 expression on $CD34^{+}45^{+}$ cells from bone marrow and peripheral blood from dual responders (DR, n=16) and early responders (ER, n=14). Peripheral $CD34^{+}45^{+}$ cells expressing CXCR4 increased at 5h and 24h post-allergen in DR only. Difference within group: † $p<0.05$.

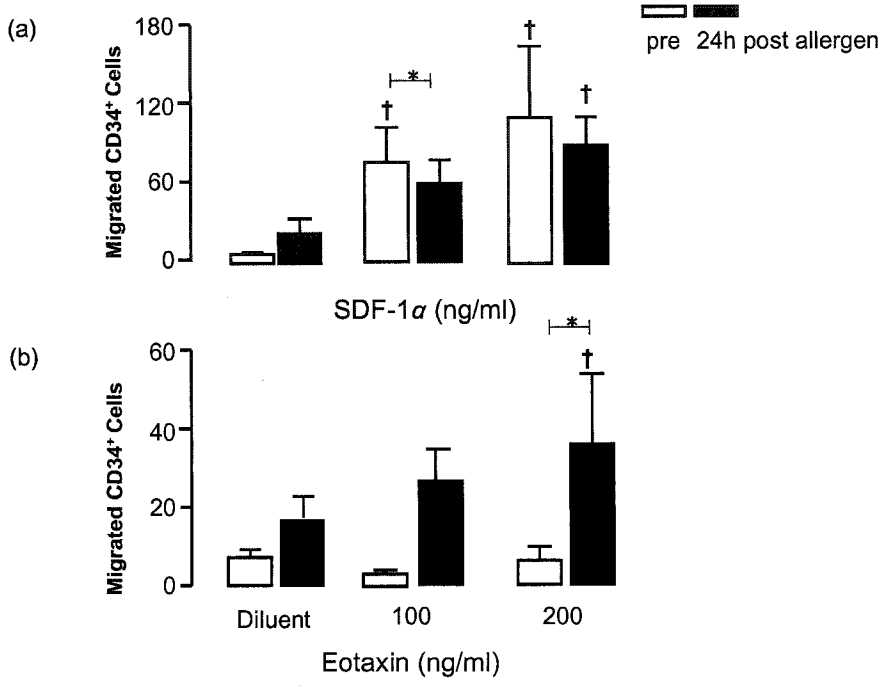

Figure 5 Trans-well migration assay of bone marrow progenitors before and after allergen inhalation in dual responders (n=6). 24h post-allergen, migrational responses to SDF-1α (100 ng/ml) decreased compared to pre-allergen. In contrast, migrational responses to Eotaxin (200ng/ml) increased. SDF-1α and eotaxin stimulated an increased migration compared to diluent. Difference with diluent: † $p<0.05$; difference over time: *$p<0.05$.

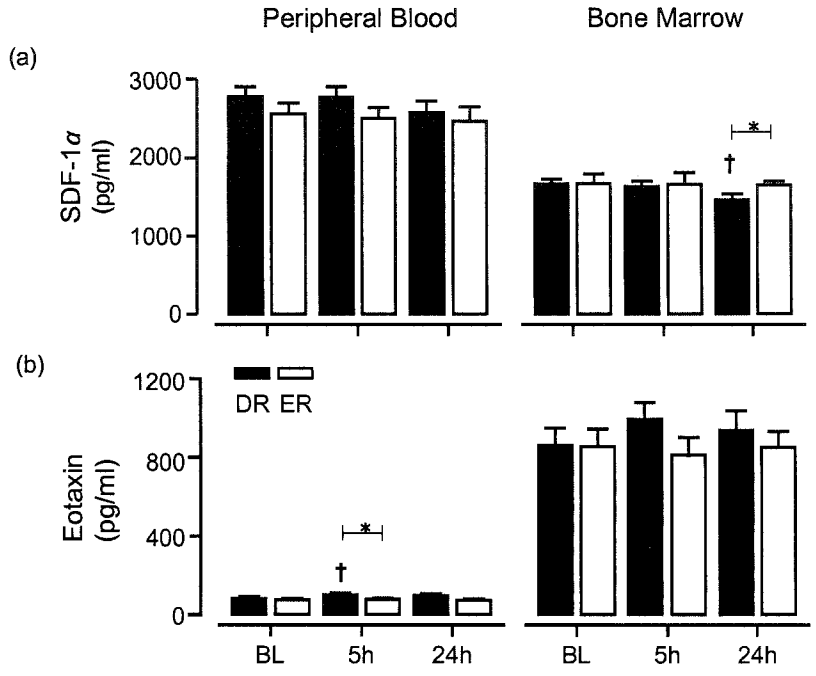

Figure 6 Allergen-induced changes in SDF-1α and eotaxin levels from bone marrow supernatants and plasma taken from dual responders (DR, n=16) and early responders (ER, n=14) at baseline (BL), 5 and 24h post-allergen inhalation. Bone marrow SDF-1α levels decreased at 24h, while circulating eotaxin levels increased at 5h in DR only. Difference within group: † $p<0.05$; difference between groups: *$p<0.05$.

CHAPTER 5

SPUTUM $CD34^+$IL-$5R\alpha^+$ CELLS INCREASE AFTER ALLERGEN

EVIDENCE FOR *IN SITU* EOSINOPHILOPOIESIS

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Sandra Dorman's contribution:

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

Sputum CD34$^+$IL-5Rα^+ Cells Increase after Allergen

Evidence for *In Situ* Eosinophilopoiesis

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Eosinophil lineage-committed progenitors increase in the bone marrow of subjects with asthma developing allergen-induced airway hyperresponsiveness and eosinophilia. Also, higher numbers of circulating eosinophil/basophil cfu have been demonstrated 24 hours after allergen inhalation and in bronchial and nasal biopsies of allergic individuals. These cells may undergo *in situ* eosinophilopoiesis, suggesting that after allergen inhalation, progenitor cells traffic from the bone marrow to the airways, providing an ongoing source of effector cells. To examine this possibility, CD34$^+$ and CD34$^+$IL-5Rα^+ cells were measured in induced sputum from allergic subjects with asthma at baseline and at 7 and 24 hours after allergen and diluent inhalation, using flow cytometry. Isolated early responders (n = 9) were contrasted to dual responders (n = 9), who develop allergen-induced sputum and blood eosinophilia and airway hyperresponsiveness, and to normal control subjects. At baseline, there were significantly fewer sputum eosinophils and CD34$^+$ cells in normal control subjects compared with subjects with asthma. Sputum CD34$^+$ cells increased at 7 hours after allergen inhalation in both groups of subjects with asthma, which was sustained at 24 hours in the dual responder group only, associated with sustained increases in sputum CD34$^+$IL-5Rα^+ cells, eosinophils, and interleukin-5. These results indicate that eosinophil progenitors can migrate to the airways and may differentiate toward an eosinophilic phenotype.

Keywords: stem cell mobilization; asthma; airway

Activation of specific hemopoietic pathways within the bone marrow is associated with allergen-induced eosinophilic airway inflammation (1–3). In conjunction with the activation of eosinophilopoiesis, allergen inhalation challenge may also trigger the release of primitive and lineage-committed progenitors into the blood (4, 5). Evidence to support progenitor cell migration to the site of inflammation comes from recent studies on humans, where CD34 immunopositive cells have been extracted from human nasal polyp and explant tissue and shown to undergo interleukin (IL)-5–driven proliferation and differentiation into eosinophil/basophil colony-forming unit *in vitro* and *ex vivo* (6, 7), highlighting the presence of true blast cells. In addition, increased numbers of CD34$^+$IL-5Rα^+ cells have been detected in asthmatic bronchial biopsy tissue (8).

It is not known whether progenitor cell numbers in the airway increase in response to allergen inhalation in subjects with asthma. Therefore, the aims of the current study were twofold. First, it was investigated whether there are time-dependent changes in the number of CD34$^+$ and CD34$^+$IL-5Rα^+ cells in the airways of subjects with asthma after allergen inhalation. Thus, sputum samples were induced before and 7 and 24 hours after allergen and diluent inhalation. Second, it was determined whether there is an association between progenitor cells and airway eosinophilia. Therefore, sputum samples were induced in dual responders, isolated early responders, and normal control subjects. Some of these results have been reported previously in an abstract (9).

METHODS

Study Design

Nineteen subjects with mild, atopic asthma and baseline FEV_1 greater than 70% predicted participated. Postallergen, isolated early responders (n = 9) developed a fall in FEV_1 greater than 20% from baseline between 0 and 2 hours, whereas dual responders developed an early and late fall (FEV_1 > 15%) from baseline between 3 and 7 hours (n = 9). Subjects were nonsmokers, using inhaled β_2-agonists intermittently (not used 8 hours before each visit), and had neither respiratory infection nor altered allergen levels 2 weeks before study. Also, six subjects without atopy and asthma were enrolled. This study was approved by the Ethics Committee of McMaster University Health Sciences Centre, and subjects gave written, informed consent before study entry.

Allergen/diluent challenges were randomized with a 2-week washout period between. Subjects attended the laboratory on six occasions: at Visits 1 and 4, medical history was documented and skin-prick test sensitivity to allergen extracts (Visit 1 only), spirometry, methacholine inhalation test, and sputum induction were performed; at Visits 2 and 5, allergen/diluent challenge and sputum induction at 7 hours postchallenge were performed; at Visits 3 and 6, methacholine inhalation challenge and sputum induction were performed.

Normal control subjects visited the laboratory once, when medical history was documented and skin prick test sensitivity, spirometry, and sputum induction were performed.

Allergen–Diluent and Methacholine Inhalation Challenge

Allergen challenge was performed as described by O'Byrne and coworkers (10), and the concentration of allergen extract was determined from a formula using skin prick test and PC_{20} (provocative concentration of methacholine causing a 20% drop in FEV_1) (11). Diluent control was physiologic saline.

Methacholine inhalation was performed as described by Cockcroft and coworkers (12).

Sputum Induction

Sputum was induced by hypertonic saline inhalation and processed according to Pizzichini and coworkers (13). Cytospins were prepared and means of duplicate slides (400 cells/slide) were expressed as absolute counts (10^6 cells/ml). Supernatants were separated by centrifugation and stored at −70°C. IL-5 levels were quantified using commercially available ELISAs (Pharmingen, San Diego, CA). The lower limit of detection was 4 pg/ml.

Flow Cytometry

Progenitors were stained and identified according to Sehmi and coworkers (2). True CD34$^+$ blast cells were identified as cells with CD34^{high}/CD45^{dull}

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staining, as described previously, and expressed as absolute cell counts per milliliter of sputum sample. Cytokine receptor antibodies were biotinylated, as described previously (2). Nonneutralizing monoclonal antibodies directed against the α-subunits of IL-5R (IL-5Rα; α16) was a kind gift from Dr. Jan Tavernier (Ghent University, Belgium).

Statistical Analysis

Results were expressed as mean ± SEM, except for PC_{20}, which was expressed as geometric mean ± %SEM. PC_{20}, IL-5, and sputum values were log_{10} transformed to fit a normal distribution before analysis. The allergen-induced changes from baseline in PC_{20}, $CD34^+$ cell numbers, $CD34^+IL\text{-}5R\alpha^+$ cells, cytokine protein levels, and sputum eosinophils were analyzed using a repeated measures analysis of variance (between treatment: allergen vs. diluent; between group: isolated early vs. dual responders vs. normal control subjects; within group: baseline vs. 7 hours vs. 24 hours; Statistica 5.1; Statsoft, Tulsa, OK). Correlations were analyzed using Spearman's ρ. Statistical significance was accepted as p values less than 0.05.

RESULTS

Airway Responses

The baseline FEV_1 values were similar on all study days. Dual responders had a greater fall in FEV_1 between 3 and 7 hours after inhaled allergen and a significant decrease in PC_{20} 24 hours after inhaled allergen ($p < 0.05$), which was not demonstrated in isolated early responders. No changes occurred after diluent challenge in either group (Table 1).

Eosinophils

Both isolated early and dual responders had significantly more eosinophils at baseline compared with normal control subjects, being $1.0 \pm 0.34 \times 10^4$/ml ($p < 0.01$), $1.4 \pm 0.53 \times 10^4$/ml ($p < 0.01$), and $0.02 \pm 0.093 \times 10^4$/ml, respectively (Table 2).

After allergen inhalation, in dual responders, eosinophils increased from $1.6 \pm 0.51 \times 10^4$/ml preallergen to $15.4 \pm 5.72 \times 10^4$/ml at 7 hours ($p < 0.01$) and to $14.7 \pm 4.03 \times 10^4$/ml at 24 hours ($p < 0.001$) postallergen. In addition, cell numbers increased at 7 hours ($p < 0.01$) and at 24 hours ($p < 0.01$), compared with diluent inhalation. Isolated early responder eosinophils increased from $0.78 \pm 0.38 \times 10^4$/ml preallergen to $11.9 \pm 8.68 \times 10^4$/ml at 7 hours ($p < 0.01$) and to $5.8 \pm 2.12 \times 10^4$/ml at 24 hours ($p < 0.005$) postallergen (Figure 1). There was a trend for a difference between the groups in the sputum eosinophil numbers at 24 hours ($p = 0.07$).

TABLE 1. SUBJECT ALLERGEN AND DILUENT-INDUCED AIRWAY RESPONSES

	Early Responder Subject with Asthma	Dual Responder Subject with Asthma
Allergen % decrease in FEV_1		
EAR	35.6 ± 3.11	31.2 ± 3.37
LAR	5.4 ± 1.15	24.2 ± 3.05*†
Diluent % decrease in FEV_1		
EAR	3.1 ± 1.37	2.9 ± 3.27
LAR	1.8 ± 1.72	2.4 ± 1.36
Allergen PC_{20}		
Preallergen	6.1 (1.8)	2.6 (1.5)
Postallergen	4.0 (1.8)	0.9 (1.6)*†
Diluent PC_{20}		
Preallergen	4.7 (1.8)	2.1 (1.5)
Postallergen	9.1 (1.4)	1.4 (1.6)

Definition of abbreviations: EAR = early airway response; LAR = late airway response; PC_{20} = provocative concentration of methacholine causing a 20% drop in FEV_1.

Values are presented as means ± SEM except for PC_{20} values, which are geometric means with percent SEM in parentheses.

Dual but not the isolated early responder subjects with asthma developed a significant increase in methacholine airway responsiveness 24 hours postallergen. In addition, there was a significant difference between the two groups in the allergen-induced shift in log-PC_{20} values ($p < 0.05$). There were no diluent-induced changes.

* $p < 0.01$ for within-group comparison of preallergen versus 24-hour postallergen log-PC_{20} values.

† $p < 0.05$ for between-group comparisons of preallergen versus 24-hour postallergen log-PC_{20} values.

$CD34^+$ Cells

Both isolated early and dual responders had more $CD34^+$ cells at baseline compared with normal control subjects, being 33 ± 17.3/ml, 27 ± 7.4/ml, and 0.9 ± 0.48/ml ($p < 0.05$), respectively (Table 2).

After allergen inhalation, dual responder $CD34^+$ cells increased from 31 ± 11.4/ml preallergen to 79 ± 20.4/ml at 7 hours ($p < 0.05$) and to 114 ± 26.5/ml at 24 hours ($p < 0.05$) postallergen. In addition, cell numbers increased at 7 hours ($p < 0.05$) and at 24 hours ($p < 0.05$) compared with diluent inhalation (Figure 2A). In contrast, isolated early responders showed no change in $CD34^+$ cells postallergen compared with baseline. However, compared with diluent challenge, there was a significant increase in the numbers of $CD34^+$ cells at 7 hours ($p < 0.05$) followed by a significant decrease at 24 hours ($p < 0.001$) (Figure 2A).

There was also a trend for a difference between groups at 24 hours ($p = 0.086$). There was a significant positive relationship between $CD34^+$ cells and eosinophils measured by the area under the curve from baseline to 24 hours ($r = 0.48$; $p < 0.05$).

$CD34^+IL\text{-}5R\alpha^+$ Cells

No significant differences occurred in baseline $CD34^+IL\text{-}5R\alpha^+$ cell numbers between groups (Table 2). After allergen inhalation, dual responder $CD34^+IL\text{-}5R\alpha^+$ cells increased from 2 ± 0.88/ml preallergen to 14 ± 4.24/ml at 7 hours ($p < 0.05$) and to 48 ± 14.7/ml at 24 hours ($p < 0.01$) postallergen. In addition, $CD34^+IL\text{-}5R\alpha^+$ cells increased at 7 hours ($p < 0.05$) and at 24 hours ($p < 0.05$), compared with diluent inhalation (Figure 2B). In contrast, isolated early responders showed no change in $CD34^+IL\text{-}5R\alpha^+$ cells postallergen compared with baseline. However, compared with diluent challenge, there was a significant increase in $CD34^+IL\text{-}5R\alpha^+$ cells at baseline ($p < 0.005$) and at 7 hours ($p < 0.005$). Dual responders also had significantly more $CD34^+IL\text{-}5R\alpha^+$ cells at 24 hours compared with isolated early responders ($p < 0.001$) (Figure 2B). There was a significant positive relationship between $CD34^+IL\text{-}5R\alpha^+$ cells and eosinophils, measured by the area under the curve from baseline to 24 hours ($r = 0.51$; $p < 0.05$).

IL-5 Protein

There were no significant differences in baseline IL-5 levels between groups. Dual responders demonstrated significantly more IL-5 protein at 7 and 24 hours postallergen compared with diluent challenge. Levels increased from 6.3 ± 0.78 pg/ml preallergen to 9.8 ± 1.68 pg/ml at 7 hours ($p < 0.05$) and to 9.2 ± 0.94 pg/ml at 24 hours postallergen ($p = 0.05$) and were 6.2 ± 0.75 pg/ml prediluent to 6.7 ± 0.55 at 7 hours and 6.8 ± 0.67 pg/ml at 24 hours postdiluent. In contrast, isolated early responders showed no significant differences in IL-5 protein levels.

Dual responders also had significantly more IL-5 protein in the sputum supernatant at 24 hours compared with isolated early responders ($p < 0.01$) (Figure 3). The change in IL-5 protein levels at 7 hours correlates positively with the change in IL-5Rα expression at 24 hours in both groups ($r = 0.49$; $p < 0.005$).

TABLE 2. SUMMARY OF BASELINE DATA FROM INDUCED SPUTUM

	Normal Control Subjects	Isolated Early Responders	Dual Responders
Eosinophils, $\times 10^4$ cell/ml	0.2 ± 0.90	1.0 ± 0.34*	1.4 ± 0.53*
CD34$^+$, cells/ml	1 ± 0.48	33 ± 17.3†	27 ± 7.4†
CD34$^+$IL-5Rα$^+$, cells/ml	0 ± 0.01	6 ± 2.10	3 ± 0.78

Values are presented as means ± SEM. Isolated early and dual responder means are calculated from the average of the diluent and allergen baselines.

Isolated early and dual responder subjects with asthma had significantly increased eosinophils and CD34$^+$ cells at baseline compared with normal control subjects. There was no difference in baseline CD34$^+$IL-5Rα$^+$ cells.

* $p < 0.01$ for between-group comparisons.

† $p < 0.05$ for between-group comparisons.

DISCUSSION

The novel findings of this study are: (*1*) CD34$^+$ cells can be detected in human sputum; (*2*) the proportion of airway CD34$^+$ cells is greater in subjects with asthma compared with normal control subjects and fluctuates after allergen inhalation challenge; (*3*) the proportion of CD34$^+$ cells expressing IL-5Rα increases in dual responder subjects with asthma only; and (*4*) this change in IL-5Rα expression occurs in conjunction with increases in IL-5 protein levels. These findings indicate that in subjects with asthma, after allergen inhalation, trafficking of CD34$^+$ progenitors to the airways occurs, with upregulation of IL-5Rα expression, which, in the presence of IL-5, may mediate the *in situ* differentiation of progenitor cells into eosinophils.

Although changes in the levels of sputum progenitor cells would suggest the trafficking of progenitors from the peripheral circulation, one cannot disregard the possibility of *in situ* proliferation of progenitors. Although previous studies would suggest that a systemic source of the progenitors is likely, namely bone marrow via the blood, the current study is limited by the lack of peripheral blood data collected from the same subjects.

The development of both early- and late-asthmatic responses after allergen inhalation is known to be associated with increases in circulating eosinophils (14), greater increases of activated eosinophils in the airway (15), activation of eosinophilopoiesis in the bone marrow (6), and the development of airway hyperresponsiveness (16), when compared with isolated early responders. Contrasting these two groups allows for a useful clinical model of increased eosinophilic airway responses and associated airway functional changes. The current study suggests that the greater increases in eosinophils seen in the dual responders may in part be due to the *in situ* production of eosinophils by CD34$^+$ cells. Although the number of CD34$^+$ cells measured is small when compared with total sputum numbers, this is not surprising considering that progenitors are self-sustaining and differentiate into colonies of cells.

Robinson and coworkers (8) have examined bronchial biopsies and confirmed both the presence of true blast cells and an increase in progenitor cells in subjects with atopy and asthma and subjects with atopy but without asthma compared with normal control subjects, concluding that the increase in CD34$^+$ cells was a feature of atopy. The data from the current study confirm these findings because both groups of subjects with asthma demonstrated significantly more CD34$^+$ cells at baseline compared with normal control subjects and extends them, demonstrating a rapid increase in CD34$^+$ cells, potentially reflecting an influx from the circulation as early as 7 hours and sustained for 24 hours after allergen inhalation challenge in dual responders.

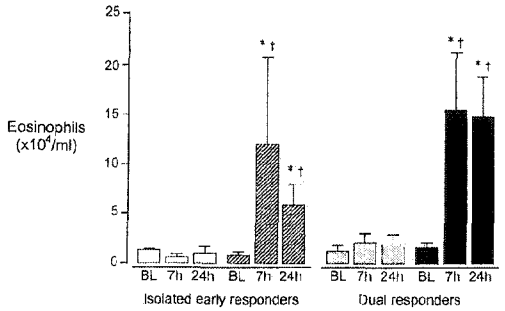

Figure 1. Comparison of sputum eosoinophil numbers at baseline (BL) and at 7 and 24 hours after diluent (*open/stippled*) and allergen (*hatched/solid*) inhalation challenge. Sputum eosinophils significantly increased at 7 and 24 hours postallergen inhalation compared with BL and with diluent challenge in both isolated early and dual responders. *p Values less than 0.01, comparison with BL; †p values less than 0.01, comparison with diluent values.

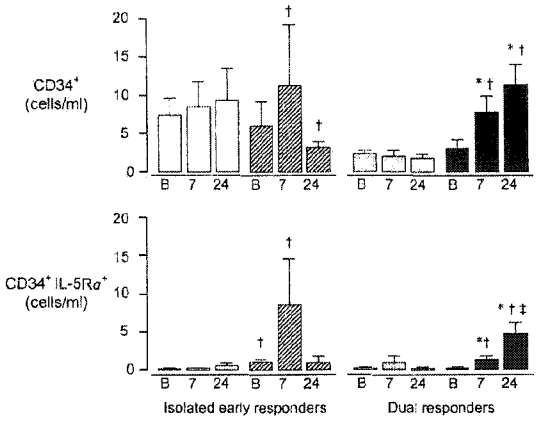

Figure 2. Comparison of sputum CD34$^+$ cells (*upper panel*) and CD34$^+$IL-5Rα$^+$ cells (*lower panel*) at baseline (B) and at 7 and 24 hours after diluent (*open/stippled*) and allergen (*hatched/solid*) inhalation challenge. CD34$^+$ and CD34$^+$IL-5Rα$^+$ cells significantly increased at 7 and at 24 hours postallergen inhalation, compared with B and with diluent challenge in dual responders. In isolated early responders, CD34$^+$ cells increased at 7 hours and decreased at 24 hours compared with diluent challenge, whereas CD34$^+$IL-5Rα$^+$ cells were significantly increased at B and at 7 hours compared with diluent challenge. Dual responders also had significantly more CD34$^+$IL-5Rα$^-$ cells at 24 hours compared with isolated early responders. *p Values less than 0.05, comparison with B; †p values less than 0.05, comparison with diluent values; ‡p values less than 0.001, comparison between groups.

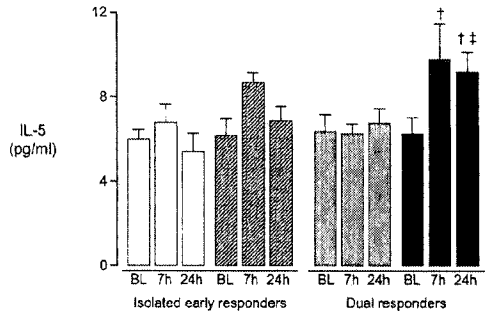

Figure 3. Comparison of sputum interleukin (IL)-5 protein levels at baseline (BL) and at 7 and 24 hours after diluent (*open/stippled*) and allergen (*hatched/solid*) inhalation challenge. IL-5 levels significantly increased at 7 and 24 hours postallergen compared with diluent challenge in dual responders only. Dual responders also had significantly more IL-5 protein at 24 hours compared with isolated early responders. †p Values less than 0.05, comparison with diluent values; ‡p values less than 0.01, comparison between groups.

In the same article, Robinson and coworkers (8) also demonstrated an increase in $CD34^+$ IL-5Rα messenger RNA^+ cell numbers in the airways of subjects with asthma only, compared with subjects with atopy but without asthma and normal control subjects. They concluded that the ability to respond to IL-5 was a feature of asthma. The present study was unable to show differences in the amount of IL-5Rα expression at baseline between groups. However, we did not observe the intracellular messenger RNA levels.

Kim and coworkers (6) have confirmed the presence of $CD34^+$ cells in nasal polyps and, using methylcellulose clonogenic assays, showed that nasal polyp tissue contained myeloid colony-forming cells. In addition, Cameron and coworkers (7), using an *ex vivo* allergen challenge model from nasal mucosa, showed an increase in eosinophils, suggesting that these cells had differentiated locally in an IL-5–dependent process. This study corroborates these findings and demonstrates an increase in $CD34^+$IL-5Rα$^+$ cells in the airways after allergen inhalation. We have as yet not been able to show the presence of true blast cells because sputum culture is hampered by treatment with sputolysin and its lack of sterility. However, these results support the concept that local tissue differentiation and expansion of eosinophils may occur in asthma affecting humans.

IL-5 is likely to be a central cytokine in the development of tissue eosinophilia in asthma. Studies have shown that IL-5 protein levels are increased in induced sputum after allergen inhalation challenge (17, 18), and this study confirms these findings. IL-5 is also a potent eosinophilopoietin (19), and expression of membrane-bound IL-5Rα is believed to be the phenotype of the earliest eosinophil lineage–committed progenitor (2). In the present study, the increase in IL-5 was positively correlated with the delayed upregulation of the IL-5Rα on $CD34^+$ cells in the dual responder group at 24 hours, supporting the hypothesis that IL-5 upregulates its own receptor and suggesting the *in situ* differentiation of these cells in the presence IL-5.

Recently, anti–IL-5 therapy has been developed to block eosinophil production, migration, and activation in allergic asthma. Although this therapy has been shown to be effective in reducing circulating eosinophil numbers (20, 21), it has not been shown to be equally effective in reducing bone marrow and airway tissue eosinophils. Flood-Page and coworkers (21) demonstrated a median percent reduction of only 50% for both airway tissue and bone marrow eosinophils. In a related study, Menzies-Gow and coworkers (22) extended these findings, showing that anti–IL-5 therapy had no effect on blood or bone marrow $CD34^+$ cell numbers but decreased $CD34^+$IL-5Rα messenger RNA^+ cells in the bronchial mucosa. Although eosinophilopoiesis is primarily driven by IL-5, other cytokines, including granulocyte–macrophage colony stimulating factor and IL-3, also induce eosinophil production (19). The presence of $CD34^+$ cells in the airways capable of responding to other inflammatory cytokines may explain why anti–IL-5 could not completely abolish airways eosinophilia.

Under normal conditions of hemopoiesis, the bone marrow acts as a site for the turnover and traffic of mature leukocytes to the peripheral circulation. However, in inflammatory conditions such as atopic asthma, there is an increased release of both mature eosinophils and bone marrow eosinophil lineage–committed progenitor cells. Although the precise mechanisms that trigger progenitor cell traffic remain to be fully elucidated, this study showed for the first time that there are increased numbers of $CD34^+$ cells in the sputum of subjects with asthma and that these cells increase after allergen inhalation. In addition, in the presence of IL-5, phenotypic changes occur in the expression of IL-5Rα, together supporting a potential for local differentiation of progenitors in the airways.

Conflict of Interest Statement: S.C.D. has no declared conflict of interest; A.F. has no declared conflict of interest; I.B. has no declared conflict of interest; R.M.W. has no declared conflict of interest; J.A.D. has no declared conflict of interest; F.E.H. has no declared conflict of interest; P.M.O'B. has no declared conflict of interest; R.S. has no declared conflict of interest.

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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 plays an active role in allergic airways disease, through the increased production and release of eosinophils and eosinophil progenitors in response to an inhaled allergen. These changes are regulated by cytokines produced in the bone marrow, blood and airway tissue. In addition, these studies suggest that these cells contribute to the development of the persistent physiological abnormalities that can occur after an inhaled allergen.

In the first study, the kinetics of the bone marrow's response and bone marrow cytokine production following allergen inhalation were examined in mild asthmatic subjects developing both an early and late airway response, as well as in subjects developing an isolated early airway response. We demonstrated, for the first time, a rapid activation of the bone marrow, via an IL-3 dependent mechanism, followed by a sustained activation, which was IL-5 dependent, in dual responders only. The IL-5 dependent increases correlated with increases in IL-5 protein and IFN-γ in the bone marrow. Peak levels of IFN-γ occurred at a time when eosinophil progenitors were decreasing, although a direct effect of IFN-γ in reducing eosinophil progenitor production *in vitro* could not be shown.

In the second and third study, we examined the biology of progenitor cell recruitment into the blood and the airways by chemokines associated with asthma, namely IL-5 and eotaxin, as well as fluctuations in the production of the chemokines,

eotaxin and SDF-1α, and their receptors, in the blood and bone marrow during an allergic inflammatory response.

In the second study, progenitor cells were stimulated *ex vivo* with cytokines in a concentration dependent manner and migrational responses were measured. We found eotaxin was able to directly stimulate $CD34^+$ cell migration, which was enhanced by the presence of IL-5. We also demonstrated for the first time, that $CD34^+$ cells could express the eotaxin receptor – CCR3 and that there are allergen-induced fluctuations in the expression of this receptor. Specifically, dual responders showed an increase in CCR3 at 24 hours post allergen, whereas isolated early responders had a decrease at 24 hours. These findings suggest that the release of progenitor cells from the bone marrow involves both phenotypic changes on the surface of the cells, which depend on the microenvironment of the bone marrow and that eotaxin and IL-5 synergistically stimulate progenitor mobilization *in vivo.*

This conclusion was supported by the third study which showed increases in eotaxin levels in the peripheral blood and decreases in SDF-1α levels in the bone marrow post-allergen inhalation. Receptor expression was shown to correlate with responsiveness to chemokine-induced migration and there were time-dependent fluctuations in chemokine receptors, specifically increases in bone marrow $CCR3^+/CD34^+$ and decreases in $CXCR4^+/CD34^+$ cells, as well as increases in circulating $CXCR4^+/CD34^+$.

In the fourth study, we examined the changes in airway progenitors in both isolated early and dual responders. We demonstrated, for the first time, that $CD34^+$ cells

can be detected in sputum and these cells are decreased in normal control subjects when compared to mild asthmatic subjects. In addition, following allergen inhalation challenge, increases in $CD34^+$ cells were measured and in dual responders only, these cells had increased expression of IL-5Rα, which is believed to be an indication of eosinophil lineage commitment. The increases in IL-5Rα were correlated with increases in IL-5 protein levels. Following allergen-inhalation in asthmatics, trafficking of progenitors to the airways occurs. These findings suggest that in the presence of IL-5, up-regulation of IL-5Rα expression occurs on $CD34^+$ cells which may mediate the *in situ* differentiation of progenitor cells into eosinophils, contributing to the overall airway eosinophilia and allergic inflammation.

In summary, the studies described in this thesis have demonstrated that eosinophil progenitors are influenced by inhalation of allergen in allergic asthmatic subjects, not only by an increased production of eosinophils in the bone marrow, but also with a controlled trafficking of their progenitors cells into the blood and into the airway tissues. The studies also showed a variation in the degree of bone marrow activation and traffic between asthmatic subjects, with those who develop allergen-induced dual responses having a more pronounced response, when compared to isolated early responders. Since dual responders also develop a more pronounced allergen-induced airway inflammatory response and airways hyperresponsiveness, this suggests a connection between the bone marrow's activation and the development of these airway changes.

The aim of this thesis was to investigate the association between the activation, mobilization and recruitment of bone marrow eosinophil/basophil progenitors and the

allergen-induced airway inflammation and its associated late responses. Specifically, we hypothesized that allergen inhalation would be associated with fluctuations in bone marrow, blood and airway progenitor cells, eosinophils and cytokines and that these changes will vary depending on the magnitude of the allergen-induced physiological response. These data are consistent with our overall hypothesis and our specific hypothesis 1, 2, 3 and 4. These data suggest that while abrogation of IL-5 may reduce eosinophil production, activation and infiltration, it will not completely nullify this cell type, as the allergen-driven events occurring in the bone marrow involve both up-regulatory and down-regulatory cytokines and chemokines, suggesting that the inhibition of this organ will require pleiotropic factors.

GENERAL DISCUSSION

The aim of this thesis was to examine the role of bone marrow inflammatory cell progenitors in clinical models of allergen-induced asthma. The results obtained suggest that inflammatory progenitor cells contribute to the ongoing airway pathology through increased marrow production, trafficking and infiltration into the inflamed airway site. The data also suggest that IL-3, IL-5 and eotaxin play important roles in this process, through increased progenitor cell turnover, increased trafficking to the airways, via the circulation and through up-regulation of specific receptors. In addition, fluctuations in responsiveness to the homeostatic chemokine; SDF-1α and increases in Th1 type cytokines also coordinate and control the progenitor cells' responsiveness.

Eosinophils are derived from the eosinophilic progenitor: Eo/B CFU (Denburg *et al,* 1985). IL-5 is recognized for its unique ability to promote the terminal differentiation and maturation of these progenitors (Clutterbuck *et al,* 1989) and expression of the IL-5Rα on progenitor cells is considered indicative of a commitment towards an eosinophillic phenotype (Sehmi *et al,* 1997). IL-5 has been intensely studied because of this function as well as its ability to activate and induce the migration of mature eosinophils, a cell considered to be important in the development of airway inflammation in asthma (Sanderson *et al,* 1992). Recently, studies examining the role of IL-5 in asthma using a monoclonal antibody against IL-5 have suggested that eosinophils may not be as important in airway inflammation as previously predicted (Leckie *et al,* 2000). Anti-IL-5 has been shown to abolish circulating eosinophils but not airways or bone marrow eosinophils (Flood-Page *et al,* 2003; Menzies-Gow *et al,* 2003). This thesis provides a potential explanation for these results. We demonstrated that in the bone marrow eosinophilopoiesis is occurring at an early stage with IL-3, not IL-5, illustrating how bone marrow eosinophilopoesis could persist in the absence of IL-5. In addition, we found increases in eosinophil progenitors in the airways, suggesting the production of eosinophils can occur *in situ* and does not require infiltration from the circulation. Further work demonstrating the ability of these cells to form colonies is needed to confirm this.

Another important finding of this study was the correlation between IFN-γ and bone marrow activation in dual responders only. We had hypothesized that IFN-γ may be important in preventing the activation of the bone marrow in isolated early responders.

However, increases in bone marrow IFN-γ occurred late after allergen inhalation and appeared to be more important in causing the resolution of eosinophilopoiesis rather than preventing its onset. We have previously shown that T cells producing IL-5 in the bone marrow increase following allergen inhalation (Wood *et al,* 2002), it may now be important to examine changes in T cell types in the bone marrow post-allergen and whether a switch from a Th2 to a Th1 phenotype is beneficial in resolving the inflammatory response. Interestingly, we were unable to show a direct suppressive effect of IFN-γ on Eo/B CFU, suggesting that this cytokine's actions are via an indirect pathway, potentially by suppressing the production of IL-5 or by suppressing the expression of the IL-5Rα (Rais *et al,* 2002).

Another important finding in this thesis is the differences in bone marrow responses between isolated early and dual responders. We had previously shown that dual responders demonstrated an increased responsiveness to IL-5 and increased up-regulation of IL-5Rα, when compared to isolated early responders (Sehmi *et al,* 1997). The current studies extend these findings by showing increased expression of the CCR3 receptor, changes in cytokine and chemokine responses in all three compartments examined, as well as fluctuations in both eosinophilopoiesis and progenitor numbers between these two groups. Overall, we found no change in any of the measurements examined in the bone marrow of the isolated early responders. However, the changes demonstrated in this group reflect the changes seen in the dual responder, without achieving statistical significance. This may be because the identification of isolated early and dual responders is based on an arbitrary cut-off of a decline in FEV_1 of 15% at 7

hours. Many isolated early responders have some airway constriction at 7 hours. This suggests that neither the development of late responses nor the magnitude of the airway inflammatory response are dichotomous, but rather a continuum of responses.

An important, novel finding described in this thesis is the increase in $CD34^+$ cells in the sputum. Induced sputum is a useful technique, which utilizes aerosolized saline to promote the expectoration of cell plugs from the lumen of the airways. This technique has been recognized to be a non-invasive, reliable and responsive technique which can give insight into the inflammatory changes in the airways (Gauvreau *et al,* 1999; Gauvreau *et al,* 1999). In addition, sputum eosinophil numbers have been shown to correlate with airway mucosal biopsy numbers although other cell types have been less representative (Fahy *et al,* 1995). This thesis provides a new method in which to examine airway progenitor cell changes, but it raises questions as to how these changes will correlate with airway tissue and, perhaps more importantly, why progenitor cells would migrate into the lumen of the airways. Airways remodeling is considered necessary for the persistence of asthma and consists of epithelial sloughing, mucous gland hyperplasia, extra cellular matrix deposition below the basement membrane and increased volume of airway smooth muscle. It may be that epithelial denudation of the epithelial cells allows these progenitor cells, resident in the epithelium, to escape into the airway lumen similar. Alternatively, chemokines may leak out into the lumen with the exudation of plasma and thereby induce chemotaxis of these cells. It will be important for future studies to both compare sputum $CD34^+$ cell numbers to biopsy and to examine their capacity to produce eosinophils *in situ.*

A limitation of this study is the subject selection needed to examine for sputum progenitors. FACS analysis on sputum samples requires large quantities of sputum to be collected in order to successfully stain these cells. This means that subjects unable to produce large quantities of sputum were excluded from this study. We are likely therefore to have selected subjects whose cells migrated into the airway lumen, representing increased severity of disease.

This thesis has also looked at the role of the homeostatic chemokine SDF-1α in progenitor trafficking. This chemokine has been studied extensively for its use in bone marrow transplants and in AIDS research, but relatively little has been done examining its function in airways disease. We have shown for the first time the reduction in the amount of SDF-1α in the bone marrow following allergen inhalation. This suggests that inflammatory cytokines co-operate with homeostatic cytokines in mediating a rapid transfer of progenitors from the bone marrow to the blood. Unlike IL-5, SDF-1α is produced by the bone marrow stroma and its reduction coincides with increasing bone marrow IL-5 levels, suggesting fundamental changes in the marrow microenvironment occur in concert to allow egress of eosinophils and their progenitors.

A somewhat surprising result from this thesis was the significant differences in bone marrow $CD34^+$ cell numbers measured between isolated early and dual responders. In the past, although there has been a trend for a difference between these groups, this has not reached statistical significance. This finding suggests that in dual responders, more progenitor cells are in the G_1 stage of cycling and are therefore primed to rapidly respond to allergic stimuli and produce new colonies of eosinophils. Interestingly, SDF-1α has

been shown to induce quiescence in bone marrow progenitors, via induction of a switch to the G_0 stage of cycling (Cashman *et al,* 2002). Although differences in baseline levels of SDF-1α were not detected between the groups, down-regulation of this cytokine may allow for more cells to enter a cycling stage.

Lastly, the results described in this thesis continue to support an important role for the activation of the bone marrow in allergic asthma and emphasizes the fact that the pathophysiology of asthma is a complex process. Thus, it is unlikely that any single molecule or cell type is capable of inducing or causing persistence of asthma on its own. This may explain why, to date, the most effective therapy in asthma remains inhaled corticosteroids - drugs capable of suppressing many different aspects of the cellular and immune response - and why therapies targeting only a single pathway in asthma have failed.

In summary, inhaled allergen challenge leads to increases in local and systemic mediator production and release which, in addition to their stimulatory effect on bone marrow progenitors, are also involved in the recruitment of these progenitor cells to the airways. The results from this thesis suggest that inhalation of allergen is also associated with activation of Th1 type cytokines as well Th2 type cytokines, particularly in subjects who develop both an early and late airway responses and more marked eosinophilic airway inflammation. Inhaled allergens also increase trafficking of eosinophil progenitors into the airways, which occurs in both isolated early and dual responders, but only persists in and expresses IL-5Rα in dual responders, who also develop increased IL-5 protein levels. These observations support *in situ* eosinophilopoesis in the airways.

Finally, the therapeutic implications of this research point to a strong requirement for systemic control of the immune response in regulating the changes occurring in the airways.

LABORATORY TECHNIQUES

For all four studies, subjects were selected based on their airway responses and then allocated as either isolated early or dual responders. In the first study, subjects had 6 bone marrow aspirates in total spread out over 48 hours. Because of the number of bone marrow samples required, the study was divided into 2 arms. The first arm had baseline bone marrow aspirates taken in the morning before allergen challenge, and then again 5 and 24 hours after allergen inhalation. The second arm had a baseline aspirate taken as well as aspirates at 12 and 48 hours after allergen inhalation. Each arm was separated by one month. The benefit of this design was that the arms of the study could be randomized and the pre-allergen aspirates served as the subject's own control. The drawback however, was the timeframe over which external, environmental factors could affect the bone marrow responses. We controlled for these factors by measuring baseline methacholine before allergen inhalation, ensuring the subject was within 2 doubling doses of their normal PC_{20} and by instilling the same dose of allergen in each arm of the study. The cost and discomfort associated with aspirate prevented us from including a control arm with diluent challenge, however the fact that measurable differences occurred between groups, argues against that potential for diurnal variation alone to cause the changes seen post-allergen.

The methylcellulose colony forming assay used to identify bone marrow Eo/B CFU has been extremely instrumental in characterizing the role of the bone marrow and the progenitor cell in asthma. However, the length of time required to grow colonies (14 days), and the assumption that colony growth under artificial *in vitro* conditions truly reflects the number of lineage-specific progenitors *in vivo* are inescapable problems. Having said this, our lab and others have shown this technique to be reliable and repeatable. This is hi-lighted by the fact that we were able to measure similar results *within* individuals in the two arms of study number one.

Complementing the methylcellulose data and extending this work, we looked at the surface expression of IL-5Rα, CCR3 and CXCR4 on $CD34^+$ cells using antibodies directed against these molecules, in collaboration with Dr. Roma Sehmi. Flow cytometry is a rapid and reproducible means of qualifying surface marker expression. A drawback of flow cytometry is that often only a small number of cells are present that are positive for the marker of interest and it can be difficult to distinguish a true positive from non-specific or artificial staining. This can be overcome by the use of isotype control antibodies, which have no reactivity with the marker being examined. Isotype control antibodies were used in all flow cytometric analyses performed as part of this thesis. In addition, 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, 1994) was employed. All measurements were made by a third, blinded party.

In the second and third study, migrational assays were employed to examine responsiveness of progenitor cells to chemokine. The drawbacks of this technique are that the cells are stimulated artificially *in vitro*. This problem was overcome in study three by removing cells before and after allergen inhalation and comparing migrational responses within individuals. In addition, all assays included a diluent control arm to exclude random, non-specific migration.

The final study used flow cytometry on sputum derived cells, which is a new, and useful technique. However, until confirmed and expanded upon, results from this study need to be interpreted with care. Sputum induction and processing have been carefully evaluated and optimized to maximize cell collection, but, apoptosis and degranulation of cells within the sputum and repeated sputum induction and/or inhalation of methacholine to determine PC_{20} preceding sputum induction may alter cell collection and induce variability. To reduce the effects of apoptosis and degranulation all samples were processed immediately after induction. To control for potential sources of variability we used a diluent-controlled cross-over study design. In addition, supporting data from biopsy studies which show both the presence of $CD34^+$, $CD34^+$/IL-5RαmRNA$^+$ cells as well as increased overall numbers in asthmatic compared to normal subjects support our interpretation of this data. As previously mentioned in order to measure CD34 and IL-5Rα expression using control antibodies, adequate quantities of sample is required. Subjects unable to produce sputum or whom produce very small amounts of sputum, were by necessity disqualified from participating in the study.

STATISTICS

Each subject used in this study acted as their own control. Repeated samples were obtained from each subject during each arm of the study. All results in this thesis were graphed for distribution, and all normally distributed data was analysed using repeated measures (rm) ANOVA, with between group comparisons: isolated early; dual; and normal subjects and within group comparisons: over time; or with treatment. RmANOVA is a powerful statistical tool in a randomized cross-over study design, since the error term is based only on the variability within subjects. This allows for practical sample sizes to demonstrate changes within groups over time. Results that were not normally distributed were analysed with Mann Whitney U for between group differences and with Friedman's followed by Wilcoxon for within group differences. All correlations were performed using spearman's rho and Pearson correlation coefficient and significance was accepted at $p<0.05$.

FUTURE DIRECTIONS

We have demonstrated bone marrow activation and mobilization is required for allergic asthmatics developing late responses in association with airway hyperresponsiveness and airway eosinophilia. However, these studies have not answered a central question, "is the activation and mobilization of the bone marrow progenitors an essential component for persisting asthma?"

It will be important to further investigate whether currently available anti-inflammatory therapies are effective in attenuating the bone marrow's responses by

acting directly on the marrow, or indirectly through effects on airway mediator production, This is particularly relevant for inhaled corticosteroids, as the mechanism of steroid action on bone marrow responses, is, at yet, unknown.

It will also be important in the future to establish the effect of directed therapy, for example with monoclonal antibodies against IL-5 or drugs blocking the eotaxin receptor (CCR3 antagonists) on bone marrow responses, as well as airway eosinophilia in asthma and whether clinical benefit can be obtained with these treatment approaches.

Further work is also needed to examine the role of sputum progenitor cells in persisting airway eosinophilia. $CD34^+$ cells need to be isolated from sputum and shown to have the capacity to produce colonies of cells. $CD34^+$ numbers need to be compared to biopsy samples and it may be of some use to compare $CD34^+$ cell numbers in subjects with other airway diseases, such as cystic fibrosis or chronic obstructive pulmonary disease to see if this is a feature of asthma, or a feature of airways disease.

Lastly, more work needs to be done on the role of IFN-γ in resolving eosinophilopoiesis. Until now, we have not examined mechanisms in resolving bone marrow activation. This thesis has given a useful time-course in predicting when bone marrow responses decrease and one potential component initiating this response. This work needs be expanded.

In conclusion, this thesis has demonstrated a role for bone marrow inflammatory progenitor cells in allergic asthma and has highlighted the potential for these cells to become activated and migrate out of the bone marrow and into the airways. Further research in this area should be aimed at establishing the importance of this pathway in

asthma, in particular at examining therapies which would prevent or reduce the activation and trafficking of these cells. A more complete understanding of these fundamental concepts will provide a basis for the development of novel therapeutic interventions and potentially explain discrepancies in current interventions in allergy and asthma.

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APPENDIX 1

MATERIALS AND METHODS

Subjects

In all studies, subjects had stable asthma, with a greater than 70% predicted baseline FEV_1, were non-smokers, none used medication other than intermittent inhaled β_2-agonists which was withheld 8 hours before each laboratory visit, and none had experienced a respiratory tract infection nor were knowingly exposed to altered levels of allergen two weeks prior to allergen inhalation. 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. Subjects were instructed to refrain from rigorous exercise and caffeine the morning of scheduled visits and to refrain from alcohol consumption the night before bone marrow aspiration. Subjects 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 20% from baseline. All human studies were approved by the Ethics Committee of the McMaster University Health Sciences Centre and subjects gave their written, informed consent to participate prior to study entry.

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-sealed spirometer and kymograph (Warren Collins, Braintree, MA, USA).

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 FEV1 (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 histamine based control (0.01 mg/ml) were administerd. The reactions were read after 20 min and recorded as the largest diameter of the whel elevation produced by the diluent control subtracted from that produced by the allergen. The results were expressed in millimetres of wheal.

Methacholine Inhalation Test

Methacholine inhalation challenge was performed as described by Cockcroft *et al,* (Cockcroft *et al,* 1987). Subjects inhaled normal saline and then increasing, doubling concentrations of methacholine phosphate (0.03 to 32mg/ml) from a Wright nebulizer (output: 0.13ml/min; airflow:8L/min; particle size aerodynamic mass median diameter 1 to 1.5μm)(Roxon Medi-Tech Ltd., Montreal, QC, Canada) containing 3 ml of solution for 2 minutes each. FEV_1 was initially measured in triplicate, and then repeated at 30, 90, (and at 180 and 300 if necessary) seconds after each inhalation period. The test was terminated when a fall in FEV_1 of 20% from baseline occurred. The percent reduction in FEV_1 was calculated for each concentration using the lowest post-saline 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 non-cumulative concentration-response curve.

Allergen Inhalation Challenge

Allergen inhalation challenge was performed as described by O'Byrne *et al.* using a Wright nebulizer (O'Byrne *et al,* 1987). The allergen extract was selected and diluted for inhalation at a concentration determined from a formula using both the results from the skin test and the methacholine PC_{20} (Cockcroft *et al,* 1987). 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 was chosen to be three doubling doses below this predicted dose. Baseline measurements of FEV_1 were followed by inhalation of allergen for 2 minutes, using the same method as for methacholine challenge. The FEV_1 was measured at 10 minutes and doubling concentrations of allergen were inhaled until a decrease of 20% or more occurred in the FEV_1 from baseline. The percent reduction in FEV_1 was calculated for each concentration using the highest baseline value to the post-allergen value. At this point FEV_1 was measured at 10, 20, 30, 45, 60, 90 and 180 minutes and then every hour for 7 hours. The allergen-induced early response was defined as the maximal decrease in FEV_1 between 0 and 2 hours and the late response was defined as the maximal decrease between 3 and 7 hours after allergen inhalation.

Sputum Induction

Sputum was induced and processed according to Popov *et al.* (Popov *et al,* 1994). Subjects inhaled 3, 4 and 5% aerosolized saline for 7 minutes each until sufficient sample was obtained or FEV_1 dropped by 20% from baseline. Cell plugs were removed, placed

in an eppendorf tube and the weight was recorded. Then 0.1% dithiothreitol (Sputolysin, Calbiochem) and Dulbecco's phosphate buffered saline (GIBCO, Grand Island, NY, USA) were added sequentially to the sample in volumes equal to four times the weight of the sputum portion (in mg) and mixed mechanically with a Pasteur pipette. The cell suspension was filtered through 52μm 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 sample was then centrifuged for 10 min the supernatant was removed with a pipette and stored at -70°C. The cell pellet remaining was adjusted to a concentration of $1x10^6$ cells/ml with DPBS and cytospins were prepared onto glass slides (4 total) and on Aptex (3-aminopropyltriethoxysilane)(Sigma Chemical Co., St. Louis, MO, USA) coated slides (8 total) using a Shandon III cytocentrifuge (Shandon Southern Instruments, Sewickley, PA). Differential counts were performed in a blinded fashion on Diff-Quick stained slides. Mean counts from duplicate slides were obtained (400 cells counted per slide) and expressed as a percentage or absolute counts (10^4 cells/ml). Aptex coated slides were fixed for 10 min in periodate-lysine-paraformaldehyde (McLean & Nakane, 1974) and washed in 15% sucrose (Sigma Chemicals, St Louis, MO) for 10 min and then stored at -70°C. These slides were used for immunocytochemical staining which was performed with murine monoclonal antibodies to the activated form of human eosinophil cationic protein (ECP) at 1.0 μg/ml (EG_2)(Pharmacia Upjohn, Uppsala, Sweden)(Girgis-Girbado *et al,* 1994). For staining, 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 threes 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)(1μg/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 (AAPAAP) 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 min 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.

Sputum processed for flow cytometry underwent the same collection criteria, however, only 2 slides were prepared for Diff-Quik staining. The remaining cell suspension was divided equally in to 5 tubes with an optimum of $1x10^6$ cells/tube.

Blood Samples

Venous blood samples were collected into vacu-containers containing nothing, ethylenediaminetetraacetic acide (EDTA) and sodium heparin for serum collection, smear preparation and flow cytometry respectively. Vacu-containers with blood only were left to sit for 20 minutes to allow for complete clotting before being centrifuged for 10 min. Serum was collected with a sterile pipette and 1 ml samples were placed in labelled eppendorf tubes and then stored at -70°C. Total cell counts were performed using a Neubauer hemocytometer (Neubauer Chamber; Hausser Scientific, Blue Bell, PA, USA) and differential cell counts were made from blood smears stained with Diff-Quik (American Scientific Products, McGaw Park IL). Mean counts from duplicate slides were obtained (400 cells counted per slide) in a blinded fashion and expressed as a percentage. Cells were classified using standard morphologic criteria. Blood collected for flow cytometry was diluted to 50 ml with McCoy's 5A medium and mononuclear cells were separated using 65% Percoll density gradients (Pharmacia Upjohn, Uppsala, Sweden). The cells were then washed with McCoy's 5A medium followed by 2-hour incubation on plastic to remove adherent cells (at 37°C in McCoy's 5A medium supplemented with 15% foetal bovine serum (GIBCO), 1% penicillin/streptomycin (GIBCO) and 5×10^{-5} M 2-mercaptoethanol). The remaining non-adherent mononuclear cells (NAMCs; containing progenitor cells and lymphocytes) are then enumerated using a Neubauer hemocytometer and 1 x 10^6 cells/ml are aliquoted into tubes for staining.

Bone Marrow Aspirates

After administering local anaesthetic (Xylocaine), 3-6 mls of bone marrow was aspirated from the posterior iliac crest by a qualified physician, using an aspiration needle (16×2"; Sherwood Medical, St Louis, MO) into a 10ml syringe containing 1ml hepalean (1000U/ml)(Leo Laboratories, Canada). Prior to processing, smears were prepared on glass slides. Smears were stained with Diff-Quik and mean counts from duplicate slides were obtained in a blinded fashion (1000 cells counted per slide). Cells were classified using standard morphologic criteria and were expressed as percentage and total number of eosinophils (immature and mature forms). Next total leukocyte counts were performed using a hemocytometer and bone marrow aspirates were centrifuged. Bone marrow Supernatant was collected using a sterile pipette and 0.5ml samples were stored at -70°C.

Bone Marrow Culture

Heparinized bone marrow was diluted to 50 ml with McCoy's 5A medium (GIBCO, Grand Island, NY) and mononuclear cells were separated using 65% Percoll density gradients (Pharmacia Upjohn, Uppsala, Sweden). The interface of the mononuclear-rich cell fraction was then washed with McCoy's 5A medium followed by 2-hour incubation on plastic to remove adherent cells (at 37°C in McCoy's 5A medium supplemented with 15% foetal bovine serum (GIBCO), 1% penicillin/streptomycin (GIBCO, Grand Island, NY) and 5×10^{-5} M 2-mercaptoethanol (final concentration) (SIGMA Chemicals, St Louis, MO). Non-adherent mononuclear cells (NAMCs; containing progenitor cells and lymphocytes) were then cultured (2.5 x 10% 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 5×10^{-5} M 2-mercaptoethanol (final concentration), 0.9% methylcellulose (Sigma Chemical Co., St. Louis, MO) and 20% foetal bovine serum. To evaluate allergen-induced changes in Eo/B CFU, NAMC were grown *in vitro* in the presence of diluent, rhIL-5 (1 ng/ml), rhIL-3 (1 ng/ml) and rhGM-CSF (10 ng/ml) (Pharmingen, Markham, ON, Canada). Cultures were incubated for 14 days at 37°C and 5% CO_2 and then enumerated by light microscopy. Eo/B CFUs were identified by a distinct morphology, previously described as tight granulated, compact round refractile cell aggregations (Denburg *et al,* 1985).

Serum and supernatant analysis

Sputum supernatant, blood serum and bone marrow supernatant were separated by centrifugation and stored at -70°C as described above. Cytokine protein levels were quantified using commercially available ELISAs (Pharmingen, San Diego, CA, USA). All samples for a given cytokine and from each of the 3 sources were run simultaneously. As instructed by the manufacturer, wells were coated with capture antibody (1:250 dilution at 100μl) and stored overnight at 4°C. One row of wells was left uncoated as a negative control. The next morning, plates were washed 3 times with cold wash buffer, followed by cold PBS and all wells were coated with 200μl blocking agent (PBS + 3% Bovine serum albumin) for 2 hours at 37°C. Wells were then rinsed with cold PBS and standards and samples were added at 100μl for 2 hours at room temperature. Standards were added to the negative control row plus 2 other positive control wells. Next, wells were washed 5 times with cold wash buffer and rinsed with cold PBS, followed by

addition of working detector (detection antibody:enzyme reagent 250/250 ratio in diluent) mixed just before applying. 100μl was placed in the wells for 90 minutes at room temperature. Wells were then washed again 5 times with cold wash buffer followed by rinsing with cold PBS and 100μl of substrate solution was added for 30 minutes at room temperature in the dark. This was followed by the addition of 100μl of stop solution (2N H_2SO4). Plates were immediately read for absorbance at 450nm with correcting wavelength of 540nm. The two rows of standards were compared for repeatability and the negative control wells were confirmed to be at or near zero. This row was averaged and subtracted from all other readings to adjust for background noise. The two rows of standards were averaged and used to plot a graph of absorbance versus known concentrations of cytokine protein in pg/ml. Calculating the slope of this graph, we then used this equation to convert the absorbance values for the samples into pg/ml. The lower limits of detection were given by the manufacturer and values below this were assigned a value of 2 pg/ml for statistical analysis. Readings were blinded.

Transmigration assays

Bone marrow NAMC, separated as described above are washed with magnetic cell sorting (MACS) buffer (phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin and 5mM EDTA) and incubated for 20 minutes on ice in the dark, with antibody-conjugated MicroBeads targeting CD3 and CD16 surface receptors (Miltenyi, Biotec). The cell suspension is then run though a magnetic cell sorting system (MACS,) (Miltenyi, Biotec), which binds the magnetically labelled cells and eliminates

them from the suspension, and allows the non-magnetic, untouched cell fraction to pass though, leaving an enriched population of progenitor cells (purity>85%).

The migrational response of progenitors *in vitro* was then assessed using transwell chambers (24-well cell clusters with 5-μm pore polycarbonate filters, Costar, Boston, MA, USA). The enriched population of bone marrow $CD34^+$ cells were resuspended in RPMI media (GIBCO, Grand Island, NY) plus 4% BIT (Stem cell technology) at 2 x 10^6 cells/ml. Filters were washed with RPMI+4%BIT and progenitor enriched cells were loaded into each filtered transwell insert ($1x10^5/100\mu l$). These transwell inserts were then placed into larger wells containing either 600μl of diluent or chemoattractants (SDF-1α and eotaxin) in serum-free medium, at concentrations of 200, 100, 50 and 25μg/ml which were previously incubated in the wells for 15 min at 37°C in 5% CO_2. The transwell system was then incubated for 4h at 37°C and 5% CO_2. After which, the filtered transwell insert was carefully removed and the cells in the bottom chamber (representing migrated cells) were aspirated. These cells were resuspended in RPMI media (GIBCO, Grand Island, NY) plus 4% BIT and stained with PE-conjugated CD34 mAb and FITC-conjugated CD45 mAb, as described below. To obtain absolute values of migratory cells, flow cytometric counts for each sample were obtained during a constant predetermined time period. Data for $CD34^+$ cell enumeration are expressed a percent of total numbers of $CD45^+$ cells counted in the bottom chamber.

Flow cytometry

All samples were run by a third blinded party. Cells were first stained with saturating amounts of biotin-conjugated anti-IL-5Rα, anti-CCR3, anti-CXCR4 or isotype

control antibody (determined in preliminary studies) in PBS plus 0.1% NaN_3, 2.5% each of mouse serum and human serum followed by simultaneous staining with streptavidin PerCp, PE-conjugated CD34 mAb (HPCA-2) and FITC-conjugated CD45 mAb (anti-HLE-1) (Becton Dickinson, Canada)^{TM} or PE-linked IgG_1 isotype control (Bectin Dickinson, Canada) in a final volume of 100μl PBSNaN3 for 30 min at 4°C. Lysis buffer (Becton Dickinson, Canada) was then added to the cells and incubated for 5 min after which the cells were washed twice with 3 ml PBS NaN3 and finally fixed in 500 μl PBS plus 1% paraformaldehyde (BDH Inc, Toronto, ON).

Antibodies.

Phycoerythrin (PE)-conjugated IgG_1 CD34 antibody (HPCA-2), fluorescein isothiocyanate (FITC) conjugated IgG_1 CD45 antibody (anti-HLE1), PE conjugated isotype control antibody (i.e. anti IgG_1- PE) and streptavidin conjugated peridinin chlorophyll protein (PerCp) were purchased from Becton Dickinson, Canada (Mississauga, ON). Non-neutralizing monoclonal antibodies directed against the α-subunits of IL-5R (IL5Rα; α16) was a kind gift from Dr. Jan Tavernier (University of Ghent, Belgium). Cytokine receptor antibodies and isotype matched controls were biotinylated using a long-arm biotin procedure (Coligan *et al,* 1991) 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, ON).

Flow Cytometry and Gating Strategy

Cells were analysed using a FACScan flow cytometer equipped with an argon ion laser and using the Cell Quest programme (Becton Dickinson Instrument Systems, BDIS). 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. There parameters identified progenitor cells based on their unique cell size, granularity and immunofluorescence characteristics. True $CD34^{+}$ blast cells were identified as cells with $CD34^{high}/CD45^{dull}$ staining as previously described. 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 quantify total leukocytes and distinguish contamination 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, analysis 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%.

In Situ Hybridization and Immunohistochemistry

To confirm the cell surface expression of CCR3 on $CD34^+$ progenitor cells detected by flow cytometry, we co-localized CCR3 messenger RNA to CD34 immunopositive cells by sequential non-radioactive *in situ* hybridization (ISH) and immunocytochemistry (ICC), as previously described (Ying *et al,* 1997). A population of unstimulated $CD34^+$ cells was enriched from a bone marrow aspirate by positive selection using a magnetic cell separation technique (MACS) (purity determined by flow cytometry, >85%). 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. Peripheral blood

eosinophils, purified by negative selection using CD16 coated MACS beads as described above, were used as a positive control for detection of CCR3 mRNA. The cell phenotypes were first identified by ICC using an alkaline phosphatase antialkaline phosphatase (APAAP) technique and phenotype-specific murine anti-human mAb (CD34, Dako, High Wycombe, UK and $EG2^{+}$, Pharmacia, Uppsala, Sweden). After developing with Fast Red for $CD34^{+}$ immunostaining, slides were hybridized with 200ng of digoxigenin-labelled riboprobes for CCR3 (antisense and sense) in hybridization buffer at 50°C overnight, then washed in 2 x SSC, 1 x SSC and 0.5 SSC, respectively; unhybridized probe was removed by digestion with RNase A(Ying, S., Robinson, D. S., Meng, Q., Rottman, J., Kennedy, R., Ringler, D. J. et al. 1997). After blocking with 2% normal sheep serum, the slides were incubated with sheep anti-digoxigenin Fab fragment conjugated AP (1:200, Boehringer Mannheim, Mannheim, Germany) at room temperature for 3 h. The signals were developed with BCIP/NBT (X-phosphate-5-bromo-4-chloro-3-indoly phosphate/ nitro-blue tetrazolium). Positive cells expressing phenotypic markers (red colour), mRNA for CCR3 (dark blue colour), or both (mixed colours) were counted (Panel 1). A minimum of 200 cells were counted per slide.

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