CYTOKINE MODULATION OF PROGENITOR CELL MIGRATION

INTERLEUKIN-4 AND INTERLEUKIN-13 PRIME MIGRATIONAL RESPONSES OF HEMOPOIETIC PROGENITOR CELLS TO STROMAL CELL-DERIVED FACTOR-1α

By

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TITLE: Interleukin-4 and Interleukin-13 Prime Migrational Responses of Hemopoietic Progenitor Cells to Stromal Cell-Derived Factor-1α

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ABSTRACT

Rationale: Lung-homing of bone marrow (BM)-derived progenitor cells is associated with inflammatory and remodeling changes in asthma. Stromal cell derived factor-1α (SDF-1α) is a potent progenitor cell chemoattractant and its local production in the lung promotes lung homing of progenitor cells. The role of pro-inflammatory cytokines in promoting traffic of progenitor cells to the site of inflammation in asthma has not been investigated. The T_H2 cytokines, interleukin (IL)-4 and IL-13, are key regulators of asthma pathology.

Objective: To investigate the role of IL-4 and IL-13 in modulating the trans-migrational responses of hemopoietic progenitor cells (HPC).

Methods: HPC were isolated from cord blood (CB) and peripheral blood (PB) and migrational and adhesive responses were assessed using transwell migration assays and adhesion to fibronectin-coated wells, respectively. Responding cells were enumerated by flow cytometry.

Results: IL-4 and IL-13 had no direct effect on progenitor cell migration. Pre-incubation with each of these cytokines primed SDF-1α stimulated migration of CB and PB-derived HPC ($CD34^+45^+$ cells) but not eosinophil-lineage committed progenitors ($CD34^+45^+$IL-$5R\alpha^+$ cells) or mature eosinophils to SDF-1α. For HPC, priming effects of IL-4 (0.1ng/ml) and IL-13 (0.1ng/ml) were detectable within 1hr and optimal at 18hr post-incubation and IL-4 was the more effective priming agent. Disruption of lipid rafts inhibited IL-4 priming of SDF-1α stimulated migration of HPC indicating that increased

incorporation of CXCR4 into membrane lipid rafts mediates the cytokine primed migrational response of HPC. This was confirmed by confocal fluorescent microscopy.

Conclusions: IL-4 and IL-13 prime the migrational response of HPC to SDF-1α by enhancing the incorporation of CXCR4 into lipid rafts. The priming effect of these cytokines is specific to primitive HPC. These data suggest that increased local production of IL-4 and IL-13 within the lungs may promote increased SDF-1α mediated homing of BM-derived HPC to the airways in asthma.

ACHIEVEMENTS

Publications

1. **Lung-Homing of Endothelial Progenitor Cells in Human Asthmatics Following Allergen Challenge**. Imaoka H, Punia N, Irshad A, Ying S, Corrigan CJ, Howie K, O'Byrne PM, Gauvreau GM, Sehmi R.

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Ab	Antibodies
Ag	Allergen
AHR	Airway Hyperresponsiveness
AMP	Adenosine Monophosphate
APC	Allophycocyanin
BAL	Bronchoalveolar Lavage
BDIS	Becton Dickinson Instrument Systems
BM	Bone Marrow
CB	Cord Blood
CFU	Colony Forming Unit
CTL	Cytotoxic T-lymphocyte
DC	Dendritic Cell
DPBS	Dulbecco's Phosphate-Buffered Saline
E_O/B	Eosinophil/Basophil
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EPC	Endothelial Progenitor Cell
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FEV1	Forced Expiratory Volume in 1 second
FITC	Fluorescein Isothiocyanate
FSC	Forward Light Scatter
GM-CF	Granulocyte-macrophage colony-stimulating factor
HPC	Hemopoietic Progenitor Cells
HUVEC	Human Umbilical Endothelial Vein Cells
Ig	Immunoglobulin
IL	Interleukin
IL-4Rα	IL-4 Receptor Alpha
IL-13Rα	IL-13 Receptor Alpha
IMDM	Iscove's Modified Dulbecco's Media
LFA	Leukocyte Function Associated Antigen
mAb	Monoclonal Antibody
MACS	Magnetic-Activated Cell Sorting
MβCD	Methyl-β-Cyclodextrin
NAMNC	Non-Adherent Mononuclear Cells
NaN_3	Sodium Azide
PAF	Platelet Activating Factor
PB	Peripheral Blood
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
PFA	Paraformaldehyde
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted

RBC	Red Blood Cells
REB	Research Ethics Board
rh	Recombinant Human
SA	Streptavidin
SCF	Stem Cell Factor
SDF	Stromal Cell-derived Factor
SMFI	Specific Mean Fluorescence Intensity
SSC	Side Light Scatter
TGF	Transforming Growth Factor
Th	T helper
TSLP	Thymic Stromal Lymphopoietin
VC	Vital Capacity
VEGF	Vascular Endothelial Growth Factor
VLA	Very Late Antigen

CHAPTER 1: INTRODUCTION

1.1 Background of Asthma

1.1.1 Prevalence

Asthma is a serious global health problem, affecting approximately 300 million people worldwide (GINA, 2011). It is the most common chronic disease among children under the age of 12 (O'Connell EJ, 2004). The highest asthma prevalence is found in the United Kingdom (>15%), New Zealand (15.1%), Australia (14.7%), Canada (14.1%) and United States (10.9%) (Masoli M et al., 2004). Although asthma is most common in developed countries, the prevalence of asthma continues to rise sharply with increasing urbanization and westernization in developing countries. Annual worldwide deaths from asthma have been estimated at 250,000 and mortality does not correlate with prevalence (Masoli M et al., 2004; GINA, 2011). Globally, the economic costs associated with asthma exceed those of tuberculosis and HIV/AIDS combined (Braman SS, 2006). Asthma is a healthcare burden and a better understanding of the mechanisms of asthma pathology may provide potential therapeutic targets to better treat asthma.

1.1.2 Clinical Assessment of Lung Function

Asthma manifests clinically with recurrent episodes of wheezing, breathlessness, chest tightness and coughing. These symptoms of asthma overlap with various other conditions, requiring more advanced methods to diagnose asthma. The two key features of asthma, reversible airway obstruction and AHR, can be assessed using a spirometer

that measures the amount and speed of air inhaled or exhaled. Spirometry is the most widely used test for evaluating lung function (O'Byrne PM et al., 2003). Its two most informative measures with respect to asthma are vital capacity (VC) and forced expiratory volume in one second (FEV_1). The VC is the maximum volume an individual can exhale following maximum inhalation while FEV_1 measures how quickly air is exhaled in the first second. The FEV_1/VC ratio is the standard index used to help identify airway obstruction (O'Byrne PM et al., 2009). In asthmatics, bronchial constriction slows the rate at which air is exhaled/inhaled, resulting in a lower FEV_1 and FEV_1/VC ratio. A ratio less than 70% suggests a significant reduction in lung function.

In some patients, measuring airway responsiveness is required for diagnosis as they have asthma associated symptoms but no airway obstruction (O'Byrne et. al, 2003). Bronchoconstriction is mediated by smooth muscle cells which lie beneath the airway epithelium and airway hyperresponsiveness (AHR) is observed when these smooth muscle cells become more sensitive to constrictor stimuli. The extent of narrowing can be quantified by spirometry, which tracks the changes in FEV_1 following inhalation challenges with airway constrictor agonists. There are two types of airway constrictors: direct stimulators and indirect stimulators. Histamine, cholinergic agonists, cysteinyl leukotrienes (C_4 and D_4) and prostaglandin (D_2 and F_2) induce bronchoconstriction by directly acting on receptors present on the surface of airway smooth muscle cells (O'Byrne PM et. al, 2003). Whereas other stimuli such as allergens, adenosine monophosphate (AMP), exercise and hyperosmotic agents are indirectly induce bronchoconstriction via release of inflammatory mediators. AMP acts on the A2b

receptor on mast cells and exercise and hyper-osmotic agents increase osmolarity, both of which lead to mast cell degranulation and subsequent bronchoconstriction (Anderson SD, 2008). Even though indirect stimuli better mimic the pathways involved in asthma, direct stimuli allow a precise measure of the responsiveness of airway smooth muscle cells. Asthmatics have greater airway sensitivity and hence require a much smaller dose of the airway constrictor agonist to initiate bronchoconstriction. Airway responsiveness is most often expressed as the provocative concentration (PC) of the stimuli that causes a 20% reduction in FEV_1 (PC_{20}) (Cockcroft DW et al., 1977). In asthmatics PC_{20} is less than 8 mg/ml due to increased airway sensitivity and reactivity while in non-asthmatics it is generally greater than 16 mg/ml (Hargreave FE et al., 1981). A lower PC_{20} value indicates a greater degree AHR and is useful in determining the severity of asthma. Moreover, the dose response curve shows that not only does airway sensitivity and reactivity increase but the maximal response (i.e. extent of bronchoconstriction) also increases with severity of asthma (O'Byrne PM et. al, 2009).

1.2 Allergic Immune Responses

1.2.1 Allergic Sensitization & Subsequent Exposures

Sensitization to an allergen must first occur in order to develop immunity or hypersensitivity. There are four types of hypersensitivities, which are either antibody (i.e. IgE, IgG) or cellular mediated (i.e. T_H1 cells, T_H2 cells, CTL). Asthma is a combination of both type I and type IV hypersensitivity reactions, utilizing IgE and T_H2 cells as

primary immune reactants (Murphy DM et al., 2007). The sensitization process begins when a professional antigen-presenting cell (i.e. DCs, Macrophages and B cells) takes up an allergen and traffics to the secondary lymphoid tissue, where it presents the antigen along with the co-stimulatory molecule to naive T and B cells. Stimulation of naive T cells promotes proliferation and differentiation along a T_H1, T_H2 or cytotoxic T lymphocyte (CTL) phenotype. Activation of naive B cells promotes proliferation and production of IgM and IgD. Given that type I hypersensitivities are associated with IgE, B cells must undergo isotype class switching which requires two additional signals from T_H2 cells. The first signal is mediated by IL-4 or IL-13 which both lead to the activation of STAT-6 via a common receptor (i.e. type II IL-4R) (Busse et. al, 2001). The second signal involves the binding of CD40 on B cells to its ligand on T cells. Secreted IgE binds to high affinity epsilon receptors (FcεRI) on mast cells and basophils and to low affinity epsilon receptors (FcεRII) on eosinophils, lymphocytes, platelets and macrophages, making these cells sensitive to the allergen (Busse WW et. al, 2001).

A sensitized individual will go through the acute phase immediately following allergen exposure. The acute phase is a type I hypersensitivity reaction, primarily mediated by IgE and mast cells. This phase is initiated by cross linking of allergen specific IgE bound to sensitized mast cells leading to mast cell degranulation. In asthmatics, the release of mast cell derived products results in the early/immediate bronchoconstrictor response (i.e. > 15% drop in FEV_1). Cytokines (i.e. IL-3, IL-5, GM-CSF, IL-4 and IL-13) released by mast cells help recruit inflammatory cells such as T_H2 cells and eosinopohils, illustrating a possible mechanism by which mast cells may

facilitate the development of the delayed/late phase response (i.e. existence of a continuum between type I and IV hypersensitivity reactions). The late phase response involves type IV hypersensitivity reactions and usually peaks at 6 to12 hours post allergen challenge (Picado C, 1992). In asthmatics, the late phase is characterized by a 15-20% fall in FEV_1 and can last up to 24 hours. Although chronic inflammation is more commonly associated with the late phase, involving mainly eosinophils and T_H2 cells, bronchial biopsies from asthmatics show that a significant number of activated mast cells are also present (Busse WW et. al, 2001).

1.2.2 Mast Cells

Mast cells are relatively long-lived resident inflammatory cells found in mucosal and connective tissue. Mast cell progenitors arise in the BM and undergo tissue specific differentiation, which is mediated by stem cell factor (SCF) and its receptor, c-kit. Despite being terminally differentiated mast cells have the potential to proliferate (Abraham SN et al., 1997). The central role of mast cell derived products is to increase vascular permeability, recruitment of inflammatory cells and a putative role in driving tissue remodeling within the asthmatic lung (Krishnaswamy G et al., 2006).

Activation of mast cells can result in both degranulation and *de novo* synthesis of immune mediators. Degranulation involves the rapid (within seconds and minutes following stimulation) release of preformed mediators such as histamine, TNF-α, IL-4, IL-13, tryptase and chymase (Murphy KM et al., 2007; Hart PH, 2001). This strategy gives mast cell derived products a temporal advantage over those produced by other

immune surveillance cells (Abraham SN et al., 1997). Histamine is predominately produced by mast cells and exerts its effects via stimulation of 4 different G-protein coupled receptors (H1-H4 histamine receptors). Key effects of histamine are bronchoconstriction, vasodilation and secretion of pro-inflammatory cytokines (i.e. IL-1α, IL-1β and IL-6) and chemokines (i.e. RANTES) (Akdis CA et. al, 2006). Proteases (i.e. chymase and tryptase) make up 30% of the granule proteins in mast cells and play an important role in stimulating mucus secretion and tissue remodeling (Welle M et al., 1997). Chymase stimulates smooth muscle and fibroblast cell hyperplasia and synthesis of fibroblast collagen while tryptase inactivates the bronchodilatory peptide, vasoactive intestinal peptide (Welle M et al., 1997).

The newly synthesized lipid mediators, prostaglandins and leukotrienes, are synthesized from arachidonic acid and released within two minutes of mast cell activation (Hart PH, 2001). Mast cells release the prostaglandin, PGD_2, which induces vasodilation and increases vascular permeability (Bradding P et al., 2006). Mast cells also release leukotriene C_4 (LTC_4) and leukotriene B_4 (LTB_4). LTC_4 is a cysteinyl leukotriene which binds to the G protein coupled receptors, CysLT1 and CysLT2. Activation of CysLT1 results in bronchial constriction, goblet cell metaplasia, mucus secretion, airway edema, tissue fibrosis, collagen deposition and smooth muscle layer thickening while activation of CysLT2 increases vascular permeability and tissue fibrosis (Barret NA, 2009). Cysteinyl leukotrienes (i.e. LTC_4 and LTD_4) are responsible for majority of the bronchial constriction that occurs during the early asthmatic response (Gauvreau GM et al, 2007).

In addition to histamine, tryptase, chymase, PGD_2, LTC_4 and LTB_4, mast cells are a major source of cytokines, such as IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, GM-CSF and TNF-α (Hart PH, 2001). IL-3, IL-5, GM-CSF promote eosinophil production and activation while IL-4 and IL-13 play an important role in stimulating and amplifying the T_H2 cell response and IgE production (Gauvreau GM et al., 2005). Furthermore, mast cells release the chemokine, MIP-1α, which attracts monocytes, macrophages and neutrophils and the lipid mediator, platelet activating factor (PAF), which attracts leukocytes (Murphy KM et al., 2007).

1.3 Mechanisms of Asthma

1.3.1 Atopy, Allergy & Asthma

Bronchial inflammation plays a fundamental role in initiating and maintaining asthma pathogenesis. Inflammation of the airways can be induced or amplified by a number of factors, such as aeroallergens and respiratory viral infections (Lambrecht BN et al., 2003). Common aeroallergens associated with asthma are dust mite feces, plant pollens and dander of domestic animals (Murphy KM et al., 2007). Inhaled allergens are of great significance as allergic reactions are associated with the inappropriately activated T cell responses implicated in asthma pathology. This is further supported by evidence showing that more than 50% of asthmatic patients are sensitized to dust mite (Ulrik CS et al., 1999). Even though it is unknown why only a proportion of allergic individuals develop asthma, there is a strong association between allergy and asthma development.

As with allergy development, atopy (i.e. overactive T_H2 immune responses) is considered as the main risk factor for developing asthma (Gaffin JM et al., 2009). Atopic individuals are predisposed to the development of allergen-induced asthma due to the increased production of IgE, the key immune reactant in early inflammatory reactions (Lambrecht BN et al., 2003). The level of circulating IgE has been shown to directly correlate with the severity of atopic asthma (Busse WW et al., 2001). The activation of IgE producing B cells requires the aid of T_H2 cells, illustrating the role of the T_H2 arm in increasing production of IgE and subsequent predisposition to allergen induced asthma. The development of atopy is governed by both environmental and genetic factors. Environmental factors, such as the type of infection, route of entry, timing, dose and frequency of antigen exposure, all influence the response of the immune system. For instance, high levels of allergen exposure during infancy skew immune responses towards T_H2 (Prescott SL et al., 1998). The hygiene hypothesis further explores the ability of early infections to modulate the development of the immune system and subsequent development of immune disorders such as allergies and asthma (i.e. exposure to infection early in life influences the development of a child's immune system along a "nonallergic" pathway). Furthermore, an individual's response to environmental factors varies depending on one's genetic background. It has been found that 79 genes are expressed differently in asthmatics when compared to non-asthmatics; of which at least 18 have a potential biological role in asthma associated inflammation (Reed CE, 2006). A study by Prescott SL et al. demonstrated that newborn infants initially produce T_H2 dominated immune responses, consisting of IL-4, IL-5, IL-6, IL-9 and IL-13, in response to common

environmental antigens and that balance is achieved following increased expression of the anti-inflammatory cytokine, IL-10. Showing that the efficiency of immune deviation mechanisms present in an individual significantly affects the ability to establish a proper T_H1/T_H2 balanced immune system.

The presence of inflammatory cells, cytokines and other immune mediators, such as IgE, in the airways of asthmatics provides strong evidence for the involvement of inflammatory processes in the pathogenesis of asthma. In response to inappropriate immune activation, our immune system has adapted numerous mechanisms of immune tolerance. Disruptions of these mechanisms lead to destructive inflammation in response to otherwise harmless substances. The maintenance of mucosal homeostasis is critical for tolerance against aeroallergens. Mucosal homeostasis is maintained by the presence of a microenvironment suitable for the induction of regulatory T cells. Treg cells secrete regulatory cytokines (e.g. TGF-β and IL-10), which suppress systemic T cell responses and promote differentiation of B cells toward IgA production (Macpherson AJ et al., 2008). IgA is localized at mucosal surfaces, preventing the uptake of antigens and minimizing the probability of eliciting an immune response against an antigen and more importantly against an inhaled allergen. Other regulatory mechanisms include the requirement of multiple signals to permit the activation of immune responses. For instance, activation of naive T cells requires 2 signals: MHC antigen recognition and co-stimulation from dendritic cells. Up-regulation of co-stimulatory molecules on dendritic cells requires a pro-inflammatory microenvironment (Murphy KM et al., 2007). Such a microenvironment is present in the lungs of individuals with asthma, leading to additional

immune activation. These mechanisms and many others help to regulate immune responses, however, it is possible to overcome them resulting in allergies and allergen induced asthma. These mechanisms may be altered by factors such as T_H1/T_H2 imbalances and/or effector/regulatory imbalances (Macpherson AJ et al., 2008). It is important to remember that immune responses are shaped by several other factors, such as genetic susceptibility and environmental influences (e.g. dose, frequency and timing of allergen, co-exposure to infection, hygiene) (Murdoch JR et al., 2010). Consequently, under certain conditions, an individual may develop immunity to innocuous substances leading to excessive, abnormal immune responses.

1.3.2 Eosinophilic Inflammation

Although mast cells play a fundamental role in allergen induced asthma exacerbations, eosinophils are implicated as the primary effector cells of chronic inflammation in asthma. Allergen induced accumulation of eosinophils in the lungs is associated with the activation of the BM by hemopoietic signals (Foster PS et al., 2001; Gauvreau GM et al., 2005). An important activating signal is IL-5, which is released by T_H2 cells that mobilize to the BM following activation at the site of allergen challenge (Minshall EM et al., 1998; Wood LJ et al., 2002). In addition to T-lymphocytes, progenitor cells and stromal cells in the bone marrow also produce IL-5 (Minshall EM et al., 1998; Hogan MB et al., 2000). IL-5 is a critical activator of eosinophilopoiesis and also promotes the mobilization of eosinophils from the BM (Palframan RT et al., 1998; Rothenberg ME et al., 2006). In mice lacking the IL-5 gene, allergen induced lung eosinophilia does not occur indicating the importance of IL-5 in production and

localization of eosinophils (Wang J et al., 1998). Migration of eosinophils is largely mediated by the eosinophil selective chemokine, eotaxin (Conroy DM et al., 1997; Zeibecoglou K et al., 1999). Eotaxin binds to the receptor, CCR3, which is predominantly expressed on the surface of eosinophils. IL-5 facilitates migrational responses of eosinophils by up-regulating CCR3 expression (Stirling RG et al., 2001). Moreover, IL-5 and eotaxin co-operate to induce production of IL-13 in the lungs, which along with IL-4 is a potent inducer of eotaxin by epithelial cells and macrophages (Zimmermann N et. al, 2003).

Eosinophils release 4 functionally different cytotoxic granule basic proteins: eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin. ECP mediates toxic effects by inducing mast cell degranulation and stimulating airway mucus secretion (Venge P et al., 1999). MBP increases the release of acetylcholine by blocking vagal muscarinic M2 and M3 receptor, thereby intensifying bronchial responsiveness (Evans et. al, 2000). MBP also stimulates mast cells and basophils. EPO makes up 25% of the proteins in eosinophils granules and promotes oxidative stress and subsequent cell death (Rothenberg ME et al., 2006). All of these mediators either directly or indirectly through activation of inflammatory cells promote tissue damage.

Eosinophils further propagate inflammation by releasing an array of cytokines (e.g. IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-16, IL-18, TGF-α/β, GM-CSF, TNF-α and INF-γ) and chemokines (e.g. eotaxin, RANTES and MIP-1α) (Rothenberg ME et. al, 2006). Many of the cytokines released by eosinophils such as IL-

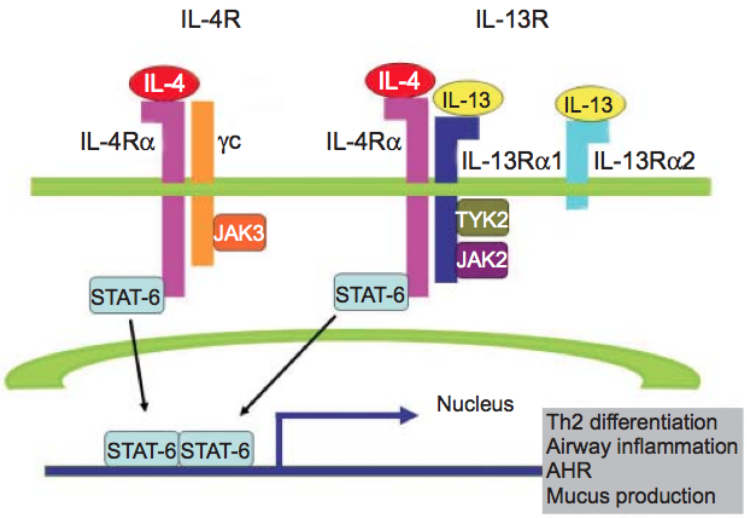
5, IL-3, GM-CSF, IL-4, IL-13 and eotaxin play an important role in eosinophil development and localization, thereby further promoting lung eosinophilia. In addition, cytokines such as IL-4 amplify T_H2 immune responses by enhancing T cell proliferation, activation and polarization towards T_H2. Moreover, it has been recently shown that eosinophils have the ability to directly activate T cells by acting as an antigen-presenting cell (Shi H, 2004).

1.3.3 T_H2 Cytokines: IL-4, IL-13 and IL-5

Allergic diseases are characterized by a skewed production of T_H2 cytokines, such as interleukin (IL)-4, 5, and 13. These cytokines released by T_H2 cells initiate, maintain and perpetuate allergic responses. Differentiation of naïve $CD4^+$ T cells is primarily influenced by two cytokines, the T_H2 cytokine, IL-4, and the anti-inflammatory cytokine, IL-12. IL-4 favors differentiation towards a T_H2 phenotype by promoting the expression of the transcription factor, GATA3, while IL-12 inhibits T_H2 differentiation by blocking the actions of GATA3 with T-bet (Amsen D et al., 2009). GATA3 up-regulates the transcription of genes encoding the T_H2 cytokines, IL-4, IL-13 and IL-5, by reorganizing chromatin structure (Barnes PJ, 2008). In addition, IL-5 suppresses T_H1 differentiation by inhibiting the IL-12 receptor β2 chain (Amsen D et al., 2009).

A degree of functional redundancy exists between IL-4 and IL-13 because both of these cytokines signal via the IL-4 receptor α-chain. IL-13 mimics IL-4 in inducing IgE secretion but does not promote T_H2 cell differentiation due to the lack of IL-13Rα1 expression on T cells (Kawakami K et al., 2001). Both IL-4 and IL-13 induce the release

of potent eosinophil chemoattractants, eotaxin 1, 2 and/or 3 by cultured airway smooth muscle (Moore PE et al., 2002), lung fibroblasts (Teran LM et al., 1999), airway epithelial cells (Komiya A et al., 2003), alveolar epithelial cells (Abonyo BO et al., 2005) and vascular endothelial cells (Shinkai A et al., 1999; Kuperman DA et al., 2008). Blocking IL-13 prevents AHR in mice following antigen challenge, suggesting that induction of allergen induced AHR is an IL-13 specific or dominant effect (Wills-Karp M et al., 1998). IL-13 is also commonly associated with structural changes seen in chronic asthma, including goblet cell hyperplasia, airway smooth muscle proliferation, and sub-epithelial fibrosis (Wills-Karp M, 2004; Zhu Z et al., 1999). Blocking the actions of IL-4 and IL-13 by using a mutated form of IL-4 reduces the late response, showing the significance of IL-4 and IL-13 in mediating immune responses in the late phase (Barnes, 2008). The involvement of IL-4 and IL-13 to the pathogenesis of asthma has encouraged clinical trails of IL-4 (i.e. Nuvance & Pascolizumab), IL-13 (Tralokinumb & Lebrikizumab) and IL-4/IL-13 dual (Patrakinra & AMG 317) antagonists. The current data available suggest that inhibiting these cytokines may be more effective in patients with severe asthma, however, further clinical investigation is needed to better understand the clinical efficacy of specific cytokine antagonists (Corren J, 2011).



Adapted from Oh CK, Geba GP, Molfino N. *Eur Respir Rev.* 2010;19;46-54

Figure 1. IL-4 and IL-13 Receptor Structure

Another T_H2 cytokine critically involved in the disease process of asthma is IL-5. In addition to T_H2 cells, activated mast cells also produce significant amounts of IL-5. IL-5 acts through a heterodimeric receptor (IL-5R) composed of a ligand specific α-chain and the signal transducing β-chain (Tavernier J et al., 1991). IL-5 plays a key role in inflammation mediated by eosinophils, as it is a critical activator of eosinophilopoiesis (Refer to section 1.4.3.1 - IL-5: Critical Activator of Eosinophilopoesis). IL-5 prolongs eosinophil survival by inhibiting apoptosis and also promotes eotaxin-stimulated migration by up-regulating CCR3 (Rankin SM, 2008). Moreover, IL-5 has been shown to stimulate a dose dependent release of eosinophils from the BM (Palframan RT et al., 1998; Rankin SM, 2008). Intravenous injection of IL-5 into guinea pigs has been shown to lead to a 10-fold increase in the circulating levels of eosinophils within one hour with a

corresponding decrease in eosinophil numbers in the BM (Collins PD et al., 1995). IL-5 is not only involved but also necessary for the development of airway eosinophilia as mice either lacking IL-5 or treated with a neutralizing anti-IL-5 antibody do not develop airway eosinophilia and have an attenuated allergen induced eosinophilic inflammatory response (Matthaei KI et al., 1997; Egan RW et al., 1997). In addition to its effects on eosinophils, IL-5 induces terminal differentiation of activated B cells into antibody forming cells in mice.

1.3.4 Pro-inflammatory Chemokines

Chemokines are cytokines that guide the migration of cells. Cells that are attracted by chemokines follow a signal of increasing chemokine concentration towards the source of the chemokine (i.e. chemotaxis). As mentioned earlier, asthma is associated with increased recruitment of eosinophils, T_H2 cells and mast cells to the lungs. Localization of these cells to increasing concentrations of the chemokine is mediated by G protein coupled chemokine receptor(s) on the cell surface. Eotaxin binds to CCR3 and is essential for eosinophil migration. CCR3 is predominately expressed on eosinophils and to a much lesser degree on mast cells and T cells (Gauvreau GM et al., 2009). RANTES and MCP-4 also mediate CCR3 dependent chemotaxis. In addition to CCR3, T_H2 cells also express CCR2 and CXCR4, which are activated by MCP-1 and SDF-1α, respectably (Gauvreau GM et al., 2009). SDF-1α, the ligand for CXCR4 is a potent progenitor cell chemoattractant that is involved in the retention and lung-homing of HPC (Doyle TM et al., 2011). SDF-1α stimulated migration has been shown to be enhanced following incorporation of its receptor into lipid rafts (Wysoczynski M et al., 2005). Lipid rafts are

cholesterol and sphingolipid enriched microdomains within the plasma membrane that organize signaling molecules and complexes, resulting in enhanced signaling (Patel HH et al., 2008).

1.3.5 Growth Factors

Cytokines that are classified as growth factors stimulate cellular growth, proliferation and differentiation of inflammatory cells. Important growth factors in asthma are GM-CSF, SCF, TGF-β, EGF and VEGF (Barnes PJ, 2008). GM-CSF plays a role in differentiation and survival of neutrophils, eosinophils and macrophages and is primarily secreted by macrophages, epithelial cells and T cells. SCF, on the other hand, stimulates the c-kit receptor, which promotes differentiation, survival, activation, adhesion and chemotaxis of mast cells. SCF is produced by epithelial cells, airway smooth muscle cells, endothelial cells, fibroblast, mast cells and eosinophils. SCF acts on many inflammatory cells but is a critical growth factor for mast cells (Hart PH, 2001). TGF-β induces proliferation of fibroblasts, airway smooth muscle cells, deposition of ECM and epithelial repair (Barnes PJ, 2008). However, TGF-β also suppresses T_H1 and T_H2 cells by activating T regulatory cells. TGF-β1 increases sub-epithelial fibrosis, contributing to airway remodeling (Barnes PJ, 2008). EGF activates EGFR, which increases expression of mucin genes leading to excessive mucus secretion while VEGF promotes angiogenesis, both defining features of airway remodeling (Barnes PJ, 2008).

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1.4 Hemopoietic Progenitor Cells in Allergic Asthmatic Responses

Both the inflammatory and remodeling components of asthma are associated with the lung homing of BM derived progenitor cells (Gauvreau GM et al., 2005). During an asthma exacerbation, the BM is activated and hemopoietic progenitor cells (HPC) traffic to the lung (Wood LJ et al., 1998; Wood LJ et al., 2002; Dorman SC et al., 2005). Once within the lung, HPC have the potential to differentiate *in situ* into pro-inflammatory cells (eosinophils (Southam DS et al., 2005) and mast cells (Hallgren J et al., 2007) and tissue structural cells (fibrocytes (Schmidt M et al., 2004) and vascular endothelial cell (Asosingh K et al., 2008; Doyle TM et al., 2011; Imaoka et al., 2011)) directed by the local cytokine milieu (Denburg et al., 2006).

1.4.1 CD34: Marker of HPC

HPC are characterized by high surface expression of a transmembrane, O-sialylated glycophosphoprotein known as CD34 (Sutherland DR et al., 1992). CD34 is a stage specific antigen, which is progressively lost with cell maturation (Krause DS et al, 1996). Other cell types including endothelial cells, BM stromal progenitors and a subset of fibroblasts and dendritic cells also express CD34. However, within the hemopoietic system CD34 is the only cell surface antigen whose expression is restricted to progenitor cells and is absent from terminally differentiated cells (Sutherland DR et. al, 1992). To ensure that HPC are accurately identified by flow cytometry, dull CD45 expression and small cell size and low granularity determinants are assessed in combination with high CD34 expression. The functionality of CD34 is not clearly understood. However,

previous studies suggest that it may facilitate the mobilization of progenitor cells from the BM by down regulating integrin mediated cell adhesion to stromal components (Carion et.al, 2002). A role for progenitor cells in allergic airway disease is suggested by the presence of a significantly higher number of $CD34^{+}$ progenitor cells in the PB and BM of atopics compared to normals (Sehmi R et al, 1996).

1.4.2 Differentiation of HPC along the Eo/B lineage

Pluripotent and lineage restricted HPC are capable of forming colonies *in vitro*. A colony-forming unit (CFU) has distinct morphological characteristics and can be identified as granulocyte/macrophage (GM)-CFU, eosinophil/basophil (Eo/B)-CFU, erythroid-CFU, megakaryocytic-CFU or lymphocyte-CFU (Sehmi R & Denburg JA, 2000). Both Eo/B-CFU and GM-CFU differentiate from a common myeloid lineage committed progenitor, CFU-GEMM, characterized by $CD34^{low}CD33^{high}$ (Sehmi R and Denburg JA, 2000). Although the BM is a source for a large number of progenitors, it is principally the neutrophil-macrophage progenitor (i.e. GM-CFU) that differentiates from the myeloid lineage committed pool in the BM. On the contrary, the blood tends to be a repository of basophil-eosinophil progenitors even though the total number of progenitors in the blood is 10 times less than the BM (Denburg JA et al., 1994).

The differentiation of progenitor cells towards one or the other lineage appears to be stochastic but exposure to a particular microenvironment may reinforce or override the selection (Sehmi R and Denburg JA, 2000). Commitment of bone marrow derived progenitors to the Eo/B lineage is regulated by three pro-inflammatory cytokines

commonly found in allergic tissue: IL-3, IL-5 and granulocyte-macrophage colony stimulating factor (GM-CSF) (Denburg JA et al, 1994). GM-CSF and IL-3 regulate the early stage of eosinophil differentiation while IL-5 is responsible for the terminal differentiation of eosinophils. A study by Chihara J et al. found that sequential incubation of human eosinophilic cell line, EoLO-3 cells, with IL-3, GM-CSF and then IL-5 at 6 day intervals induced a significant expression of FcεRII and CD25, whereas incubation with either cytokine alone had no effect (1992). Sequential exposure is important because GM-CSF and IL-3 must first differentiate $CD34^{+}CD33^{+}$ cells to $CD34^{-}CD33^{+}$ cells, in order for the cells to be susceptible to the effects of IL-5 (Ema H et al., 1990). Both of these studies indicate that Eo-CFU does not occur with IL-5 alone because IL-5 acts on the appropriate, committed progenitor to induce differentiation along the Eo/B lineage (Ema H et al, 1990). In addition to these three cytokines, IL-9 is a growth factor for eosinophils and has been shown to enhance IL-5Rα surface expression, differentiation and survival of eosinophils (Gounni AS et al., 2000). While T_H1 cytokines, such as IL-12 and IFN-γ, are negative regulators of eosinophil differentiation. IL-12 has been shown to selectively inhibit eosinophilopoiesis from bone marrow stem cells through IFN-γ induction in a mouse model of asthma (Rais M et al, 2002). An increase in bone marrow IFN-γ levels 48 hours after allergen challenge in dual responders is associated with a reduction in IL-5 levels and eosinophilopoesis in the bone marrow, suggesting that IFN-γ may mediate the termination of allergen induced eosinophilopoesis by down-regulating IL-5 production (Dorman SC et al., 2004).

1.4.3 Allergen Induced Activation of Eosinophilopoiesis in the Bone Marrow

A cardinal feature of asthma is the elevated number of inflammatory cells, particularly eosinophils, within the respiratory mucosa. Whilst this increase is likely due to the increased recruitment of mature eosinophils from the periphery, it is now postulated that the traffic of progenitor cells from the BM with the potential to differentiate locally into mature eosinophils may also contribute to this process. The localization of primitive eosinophil progenitors to the airways is supported by the presence of elevated levels of proliferating myeloid cells in the lung within hours of airway allergen challenge (Herzog EL et al, 2003). A strong, positive relationship between numbers of circulating Eo/B progenitors and histamine airway responsiveness in asthmatics further supports selective activation of eosinophil differentiation pathways in the BM and the role these progenitor cells play in the development of airway inflammation (Gibson PG et al, 1991). A study conducted by Johansson AK et al. showed that following adoptive transfer of $BrdU^+$ BM cells, 54% of the bronchoalveolar lavage fluid (BAL) eosinophils originated from BM of the donor mice after allergen challenge (2004). This study demonstrates that newly produced eosinophils originated from the BM contribute extensively to allergen induced airway inflammation and most importantly shows that the bone marrow is activated in response to allergen exposure.

A number of studies have shown that allergen induced hemopoietic signals from the airways selectively activate eosinophil differentiation pathways in the bone marrow. The three pro-inflammatory cytokines, IL-5, IL-3 and GM-CSF involved in eosinophilopoesis, the cysteinyl leukotrienes, LTC_4, LTD_4 and LTE_4 and the eosinophil

selective chemokine, eotaxin, are all important inflammatory mediators shown to facilitate the recruitment of eosinophil/basophil progenitor cells from the bone marrow to the airways following an allergen challenge (Gauvreau GM et al, 2005). IL-5 is perhaps one of the most studied and critical activators of eosinophilopoesis. IL-5 is increased in the bronchial lavage fluid and bronchial biopsies of asthmatics and has been shown to have a positive relationship with clinical features of asthma (Hamid Q et al, 1991; Wood LJ et al, 2002). The role of IL-5 as an allergen induced hemopoietic signal is indicated by studies in which systemic and local administration of IL-5 to asthmatic patients resulted in an increase in circulating eosinophils and $CD34^+$ eosinophil progenitors (Stirling RG et al., 2001). Intravenous injection of IL-5 into guinea pigs led to a 10-fold increase in the circulating levels of eosinophils within one hour with a corresponding decrease in eosinophil numbers in the bone marrow (Collins PD et al., 1995). Moreover, IL-5 has been shown to stimulate a dose dependent release of eosinophils from the BM (Palframan RT et al., 1998; Rankin SM, 2008). Many studies have further shown that IL-5 is, in fact, necessary for the development of airway eosinophilia. Mice either lacking IL-5 or treated with a neutralizing anti-IL-5 antibody do not develop airway eosinophilia and have an attenuated allergen induced eosinophilic inflammatory response (Matthaei KI et al., 1997; Egan RW et al, 1997). Even though bone marrow stromal cells and microvascular endothelial cells produce IL-5 in response to pro-inflammatory signals, the increase in IL-5 expression within the bone marrow after an allergen challenge is primarily attributed to $CD3^+$ T lymphocytes (Minshall E et al., 1998; Wood et al., 2002).

1.4.3.1 IL-5: Critical Activator of Eosinophilopoesis

In non-sensitized mice, IL-5 mRNA is expressed predominantly by BM-derived $CD34^+$ progenitor cells. Following sensitization and allergen challenge, $CD3^+$ T lymphocytes become the major source of IL-5 as 20% of the IL-5 mRNA in the BM is co-localized to $CD3^+$ T lymphocytes (Minshall E et al., 1998). A study conducted by Isogai S et al. shows that $CD4^+$ T cells travel from the trachea to the bone marrow after allergen exposure and that this may be facilitated by eotaxin and IL-16 expression in the bone marrow (2004). Even though the numbers of $CD3^+$ cells in the BM are significantly higher in both isolated early and dual responders following an allergen challenge, the percentage of $CD3^+$ cells expressing IL-5 mRNA is significantly increased in only dual responders, who developed AHR and a greater degree of blood and airway eosinophilia compared to isolated early responders (Wood LJ et al, 2002). Adoptive transfer of $CD3^+$ lymphocytes over-expressing IL-5 mRNA results in increased eosinophils and $CD34^+$ cells in the bone marrow, blood and airways of sensitized and challenged mice (Johansson AK et al., 2004). These studies suggest that inhaled allergen induces trafficking of IL-5 producing T lymphocytes to the bone marrow to further promote eosinophilopoiesis through IL-5 receptor signaling. The ability of T_H2 lymphocytes from the airways to regulate events in the BM confirms the existence of bi-directional communication between the airways and the BM in allergic diseases (Gauvreau GM et al., 2009). In addition to T cells, progenitor cells are believed to aid in their own differentiation. 50% of $CD34^+$ cells from asthmatics have been shown to express intracellular IL-5 protein, suggesting that autocrine IL-5 expression may auto-regulate

eosinophilic colony formation from these progenitor cells (Kuo HP et al., 2001). Increases in IL-5 responsive progenitors at 12 and 24hr, coincident with increased IL-5 protein levels in the BM confirm allergen-induced activation of an eosinopilopoietic process (Dorman SC et al., 2004). Such evidence further supports the role of IL-5 in eosinophilopoesis and as a potent signal capable of activating the bone marrow.

It has been shown that bone marrow from asthmatic subjects developing airway eosinophilia is more responsive to IL-5 than the bone marrow from control subjects with minimal airway inflammation and that a higher portion of $CD34^+$ cells express IL-5Rα after allergen challenge. *In vitro* experiments on CB and BM-derived human progenitor cells show that IL-5 enhances its own signaling by increasing the expression of the alpha subunit of the IL-5 receptor (Sehmi R et al., 1997). IL-5 does this by skewing the splicing of the IL-5Rα gene towards the membrane bound isoform, TM-IL-5Rα, as oppose to the antagonist variant, SOL-IL-5Rα (Tavernier J et al., 2000). A significantly lower expression of IL-5Rα on bone marrow $CD34^+CD45^+$ progenitor cells is observed in IL-5 deficient mice, confirming that IL-5 is indeed responsible for the up-regulation of the IL-5Rα chain (Saito H et al., 2002). Considering that $CD34^+$IL-5Rα$^+$ is the phenotype of the earliest eosinophil lineage committed progenitor these findings show the importance of IL-5 in mediating eosiophilopoesis (Sehmi R et al, 1997). These studies indicate that progenitor cells in asthmatics are primed to respond to IL-5 more readily due to the increased expression of IL-5Rα and the skewing of $CD34^+$ cells towards eosinophilopoesis after an allergic event contributes to the subsequent development of blood and tissue eosinophilia.

The importance of IL-5 for eosinophil differentiation has also been verified and further studied using anti-IL-5 therapy. Administration of an IL-5 antibody prior to an allergen challenge prevents airway eosinophil infiltration and bronchial hyperreactivity in mice, suggesting that IL-5 is in fact a key factor in the development of AHR (Hamelmann E et al., 1997). However in humans, administration of a humanized monoclonal antibody (mepolizumab) only prevents blood and airway eosinophilia with only a partial reduction in both the level of eosinophils in bronchial biopsies and degranulation of these cells within the airways (Flood-Page PT et al, 2003). Moreover, anti-IL-5 therapy decreases eosinophil myelocytes and mature eosinophils but has no effect on the numbers of $CD34^+$ cells, $CD34^+$/IL-5Rα^+ cells and Eo/B-CFU, suggesting that the effects of anti-IL-5 therapy in the bone marrow are downstream from undifferentiated $CD34^+$ cells (Menzies-Gow A et al., 2003). This finding is consistent with the fact that IL-5 acts on late stage progenitors to promote eosinophil differentiation. Even though these findings suggest that IL-5 is required for normal bone marrow eosinophilopoiesis, suppression of IL-5 does not completely suppress eosinophilia likely due to the presence of redundant/overlapping cytokine progenitor pathways. For instance, the ability of IL-3 and GM-CSF to compensate for IL-5 is supported by studies in which BM clonogenic responses to IL-3 and GM-CSF in methycellulose cultures are normal in IL-5 deficient mice, suggesting that both IL-3 and GM-CSF are capable of and hence may control progenitor cell lineage commitment and maturation in absence of IL-5 (Saito H et al., 2002).

Primitive and Eo/B lineage committed progenitor cells (e.g. $CD34^+$/IL-5Rα^+ and $CD34^+$/$CCR3^+$) are present in the bone marrow, circulation and sputum of allergic

asthmatics. Increases in mature eosinophils and BM eosinophil progenitors after allergen challenge provide evidence that the increase in eosinophil production is also occurring as a result of the expansion of relevant eosinophil progenitor population in the bone marrow and not strictly from an increase in the release of eosinophils from the bone marrow compartment. The ability of the BM to respond to an allergen challenge, primarily by IL-5 driven eosinophilopoesis is detected as early as 5 hours post allergen (Dorman SC et al, 2004). The activation of the BM during asthma exacerbations suggests a significant role for BM derived pluripotent progenitor cells (i.e. HPC) in maintaining inflammation in chronic allergic diseases.

1.4.4 Egress of Hemopoietic Progenitor Cells from the Bone Marrow

The association between increased levels of HPC in the BM and PB and the presence of proliferating myeloid progenitors in the lungs following allergen exposure suggests that BM-derived progenitor cells may be the source of the precursor cells detected in the airways (Sehmi R et al., 1996; Herzog EL et al., 2003). Studies suggest that the release of progenitor cells from the BM is due to a reduction in the adhensive interactions to the BM microenvironment. Adhesive forces between HPC and the BM may become weaker due to changes in the expression of surface proteins on progenitor cells and/or the BM microenvironment (Catalli et al., 2008; Sehmi R et al, 2009).

Changes in the expression of chemokine receptors, such as CXCR4 and CCR3, and adhesion molecules, particularly β1 integrins, have been shown to be principle phenotypic changes in progenitor cells that facilitate the mobilization of these cells. The

migrational responsiveness of cells to stromal derived factor (SDF)-1α and eotaxin is dependent on the expression of CXCR4 and CCR3, respectably. SDF-1α is a potent chemoattractant for hemopoietic progenitor cells as these cells have been shown to express its receptor, CXCR4 (Aiuti A et al., 1997). SDF-1α is produced by stromal cells and is retained within the bone marrow by binding specifically to heparin sulfate on associated proteoglycans (Sweeney EA et. al, 2001). Many studies have suggested that the CXCR4/SDF-1α axis is critical for the retention of stem cells in hemopoietic compartments while the CCR3/eotaxin axis directs cells to the target tissue. Increased expression of CCR3 on BM $CD34^+$ cells is only induced in dual responders who developed a significant sputum and blood eosinophilia and increased methacholine airway responsiveness in response to an allergen challenge, indicating a significant role of the CCR3/eotaxin axis in the disease process of asthma (Sehmi et al, 2003). *In vitro* eotaxin has been shown to stimulate $CD34^+$ progenitor cell migration in a dose dependent manner with inhibition by anti-CCR3 monoclonal antibody (Sehmi et al, 2003). A study conducted by Dorman SC et al, has shown that the expression of the SDF-1α receptor, CXCR4, on BM $CD34^+$ cells is greater in normal subjects compared with atopic asthmatics (2005). Due to a significantly higher expression of CXCR4, progenitor cells from normal subjects showed a greater migrational responsiveness to SDF-1α than progenitor cells from atopic asthmatics and hence will likely be more strongly retained within the bone marrow. Moreover, it has been shown that progenitor cells from normal subjects compared to asthmatics had a greater migrational response to SDF-1α than eotaxin, indicating that $CD34^+$ cells in normal individuals were more attracted to SDF-1α

than eotaxin. This is the case because allergen inhalation has been shown to attenuate the intensity of CXCR4 expression and SDF-1α levels but increase the intensity of CCR3 expression on BM $CD34^+$ cells in dual responders. These findings suggest that allergen inhalation triggers the release of progenitor cells from the bone marrow by decreasing the CXCR4/SDF-1α axis while at the same time increasing the responsiveness to eotaxin. Furthermore, IL-5 has been shown to enhance the response of BM-derived $CD34^+$ cells to eotaxin while lowering the response to SDF-1α, suggesting that in addition to its role as a hemopoietic signal, IL-5 may also regulate the mobilization of BM-derived $CD34^+$ cells in response to an allergen challenge.

The ligation of chemokines to specific receptors on hemopoietic cells is not only a migrational stimulus but also regulates the function of adhesion molecules present on the surface of the cells. Adhesion molecules expressed by human $CD34^+$ cells are the β1 integrins, very late antigen (VLA)-4 and VLA-5, and the β2 integrins, Mac-1 and leukocyte function associated antigen (LFA)-1. Under normal conditions, VLA-5 is the most highly expressed adhesion molecule whereas VLA-4 and Mac-1 are moderately expressed. Administration of anti-VLA-4, anti-VLA-5 and anti-Mac-1 antibody in mice resulted in rapid accumulation of progenitor cells in the circulation, suggesting that adhesion molecules are critical players in retaining progenitor cells within the BM (Gigant C et al, 2001). A study by Catalli AE et al. shows that the expression of β1 integrin VLA-4 and VLA-5 is significantly reduced on mobilized progenitor cells in peripheral blood compared to steady state bone marrow with VLA-4 having a greater functional impact (2008). Allergen induced integrin down-regulation decreases the

adhesion of progenitor cells to the BM microenvironment, thus facilitating the release of progenitor cells. Moreover, it has been confirmed that the CXCR4/SDF-1 axis enhances the adhesive responses of progenitor cells to fibronectin and VCAM-1. In combing these observations, it can be concluded that allergen challenge induced down-regulation of CXCR4 expression decreases retention of progenitor cells as a result of reduced responsiveness of progenitor cells to SDF-1α and subsequent attenuation of adhesive forces. All of these factors along with the up-regulation of the CCR3/eotaxin axis contribute to the egress of progenitor cells from the bone marrow to peripheral blood.

In addition to cytokines and chemokines, proteases also facilitate the release of progenitor cells from the bone marrow by altering the bone marrow microenvironment. Proteases have been shown to promote the egress of progenitor cells from the bone marrow by cleaving VCAM-1 expressed by stromal cells and by reducing the levels of SDF-1 (Kucia M et al., 2004).

1.4.5 In situ Differentiation of Progenitor Cells

i) Inflammatory Effector Cells

Allergen induced increases in HPC within the BM and peripheral circulation suggests that in addition to maturing within the BM, progenitor cells may also differentiate locally within inflamed tissues. The trafficking of BM derived progenitor cells to the lungs via peripheral circulation in response to allergen challenge has been verified in dogs using $BRDU^{+}$ progenitor cells (Wood LJ et al., 1998). Moreover, it has been shown that bronchial and nasal mucosal biopsies from atopic subjects compared to

normal have significantly higher numbers of eosinophil lineage committed progenitor cells (e.g. $CD34^+IL\text{-}5R\alpha^+$ cells) (Robinson DS et al., 1999; Minshall E et al, 1998). The cytokine environment of the challenged tissue dictates the differentiation of progenitor cells towards either pro-inflammatory cells (e.g. eosinophils) or structural cells involved in tissue repair or remodeling. Blocking differentiation with the administration of steroids to the upper airway increases $CD34^+CD45^+$ myeloid progenitors in nasal polyp tissue, suggesting that progenitor cells can mature within an inflamed tissue in the presence of pro-inflammatory cytokines (Kim YK et al., 1999).

An array of cytokines produced by structural (stroma and epithelial) and inflammatory cell components of inflamed tissues make the microenvironment of the target tissue suitable for eosinophilopoiesis (Denburg JA, 1995). A significant increase in IL-3, IL-4, IL-5 and GM-CSF producing cells at allergen challenged sites with only occasional detection of IL-2 and IFN-γ, indicates that T_H2 rather than T_H1 cytokines contribute to allergic inflammation (Durham SR et al., 1992). A study by Cameron L et al. used an IL-5 receptor antagonist to illustrate that differentiation of progenitor cells in the nasal mucosa is primarily mediated by the T_H2 cytokine, IL-5, and that IL-5 promotes local eosinophil differentiation (2000). T cells secrete majority of the IL-5 in inflamed tissues with a small percent derived from eosinophils. Moreover, in absence of IL-5, IL-3 and GM-CSF, eotaxin has been shown to induce differentiation of eosinophils from CB derived $CD34^+$ progenitor cells. Such evidence proposes the presence of an alternative pathway by which lung eosinophilia might occur in absence of systemic IL-5 (i.e. eotaxin mediated mobilization and subsequent *in situ* differentiation) (Lamkhioued B et al.,

2003). Allergen induced increases in $CD34^{+}IL\text{-}5R\alpha^{+}$ cells and the eosinophilopoietic factors IL-5 and eotaxin in the lung, may promote *in situ* differentiation of eosinophils that contribute to ongoing allergic airway inflammation.

ii) Structural Cells

In addition to increased mobilization of mature granulocytes and HPC, fibrocytes, mesenchymal stem cells (MSC) and endothelial progenitor cells (EPC) are also released from the bone marrow in response to allergen challenge (Schmidt M et al., 2004; Asosingh K et al., 2008). Under normal conditions, stem cells contribute to tissue repair but in inflamed tissues these cells promote tissue remodeling. Airway remodeling is a key feature of asthma, which results in persistent and irreversible airway obstruction. Permanent structural changes in asthmatics include increased sub-epithelial fibrosis, angiogenesis, smooth muscle hyperplasia and hypertrophy, proliferation of mucus secreting goblet cells and desquamation of the epithelium (Barnes PJ, 2008; Murdoch JR et al., 2010). Fibrocytes and MSC contribute to these structural changes primarily by being a valuable source of structural cells at the site of inflammation. TGF-β1 stimulates the differentiation of fibrocytes into fibroblasts and myofibroblast, both of which synthesize increased amounts of collagen and fibronectin (Abe R et al., 2001). A study Schmidt M et al. shows that allergen exposure induces the accumulation of fibrocyte-like cells in the areas of collagen below the epithelium, suggesting that circulating fibrocytes may function as myofibroblast precursors and hence contribute to the genesis of sub-epithelial fibrosis (2003). MSC also circulate in the blood and play a vital role in tissue regeneration. These cells have the capacity to differentiate into an array of cell types

including endothelial cells, epithelial cells and myocytes. Increased presence of endothelial cells and myocytes in the inflamed tissue may contribute to the formation of new blood vessels and smooth muscle, respectably.

EPC are implicated as a critical source of cells required for angiogenesis in asthmatics. EPC and HPC are derived from a common precursor cell, the hemangioblast, phenotypically characterized as $CD34^{+}CD133^{+}$ cells. BM-derived EPC have been detected in human PB and umbilical CB (Asahara T et al., 1997; Murohara T et al., 2000). However, the rate of endothelial cell turnover is very low and the frequency of EPC in circulating blood is also low, representing only 0.01% of all the cells (Zammaretti P, 2005). These $CD133^{+}$ cells that are then mobilized from the circulation have been shown to differentiate into hematopoietic and endothelial cells (Loges S et al., 2004). Phenotypically, human EPCs express CD34, CD133, vascular endothelial growth factor receptor-2 (VEGFR-2) and KDR/ Flk-1.In addition to the ability of EPC to differentiate into mature endothelial cells, it has been shown that these progenitors can transdifferentiate into smooth muscle cells *in vitro* (Frid MG, 2002). Studies have shown that EPC can also differentiate from myeloid cells as these cells have the ability to transdifferentiate into the endothelial lineage. Increased angiogenesis is a key feature of asthma as the total number of vessels and vascular area in the airways of asthmatics is greater compared to control subjects (Li X et al., 1997). Increased number of vessels, vasodilatation and vascular leakage contribute to thickening of the airway wall resulting in narrowing of the lumen (Moreno RH et al, 1986).

Pathological angiogenesis is due to increased expression of angiogenic factors and/or down-regulation of inhibitors, such as endostatin. VEGF is one of the most studied pro-angiogenic factors as the level of VEGF has been shown to correlate with the number of blood vessels in the asthmatic lung (Chetta A et al., 2005). The role of VEGF and angiogenesis in asthma has been supported by studies that show the spontaneous development of asthma like phenotype (e.g. inflammation, angiogenesis, edema, mucus metaplasia, myotcyte hyperplasia and airway hyperresponsiveness) in mice over-expressing VEGF (Lee CG et al, 2004). Both inflammatory and structural cells produce VEGF. Angiogenesis and airway inflammation in asthmatics are equally supported by each other. Inflammatory cells, such as eosinophils and mast cells, produce pro-angiogenic factors and induce angiogenesis while increased bronchial vascularization facilitates the recruitment of inflammatory cells. The recruitment of BM-derived EPC into the lungs and subsequent vascular remodeling occurs within few hours after allergen challenge while the peak influx on eosinophils is 4-6 days after allergen exposure (Asosingh K et al., 2007). At large, angiogenesis augments airway remodeling and bronchial inflammation by providing inflammatory and structural cells a greater opportunity to be recruited into the lung tissue.

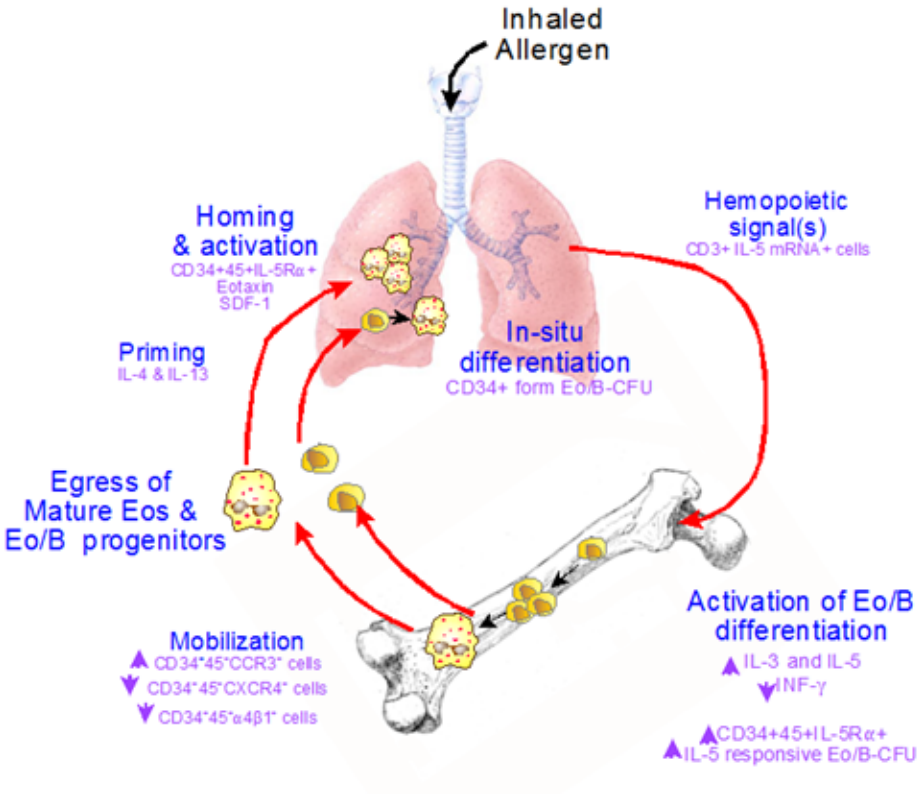

Figure 2. Hemopoietic Progenitor Cells in Allergic Asthmatic Responses

1.5 Scientific impact/Significance

Asthma is a chronic disorder of the airways characterized by bronchial inflammation, AHR and variable airway obstruction. The disease course of asthma is variable, ranging from complete recovery to persistent and/or progressive loss of lung function (Panettieri RA, 2009; Sears MR et al., 2003). Mechanisms that may contribute to progression or persistence of asthma symptoms may include airway inflammation, airway remodeling, or a combination of both processes (Panettieri RA, 2009).

Both the inflammatory and remodeling components of asthma are associated with the lung homing of BM derived progenitor cells. During an asthma exacerbation, the BM is activated and HPC traffic to the lung (Wood LJ et al., 2002; Dorman SC et al., 2005; Gauvreau GM et al., 2005). Once within the lung, HPC have the potential to differentiate in situ into pro-inflammatory cells (eosinophils (Southam DS et al., 2005; Dorman SC et al., 2004) and mast cells (Hallgren J et al., 2007)) and tissue structural cells (fibrocytes (Schmidt M et al., 2004) and vascular endothelial cell (Doyle TM et al., 2011)) directed by the local cytokine mileau (Denburg et al., 2006). Moreover, HPC can directly contribute to allergic inflammation by releasing high levels of pro-inflammatory cytokines and chemokines (Allakhverdi Z et al., 2009). Our studies have shown that in asthma exacerbations stromal cell derived factor-1 (SDF-1), a potent progenitor cell chemoattractant, is down-regulated in the BM (Gauvreau GM et al., 2005; Catalli AE et al., 2008) and correspondingly increased in the airways in asthma (Southam DS et al., 2005) thus stimulating HPC egress to the circulation and lung-homing in asthma. Investigating the directional cues that promote the migration of HPC will provide novel targets for controlling the lung-homing of HPC and an understanding of the role that these cells play in the pathology of asthma.

Allergic diseases are characterized by a skewed production of T_H2 cytokines, such as interleukin (IL)-4, 5, and 13. These cytokines released by T_H2 cells initiate, maintain and perpetuate allergic responses. IL-4 is critical in promoting the differentiation of naïve T cells into T helper 2 cells and isotype class switching to IgE in B cells. A degree of functional redundancy exists between IL-4 and IL-13 because both

of these cytokines signal via the IL-4 receptor α-chain. The type I IL-4 receptor (IL-4Rα and γc) is selectively activated by IL-4 while the type II IL-4 receptor (IL-4Rα and IL-13Rα1) is activated by both IL-4 and IL-13. IL-13 likely mediates it effects entirely through the type II IL-4 receptor as the alternative IL-13 receptor, IL-13Rα2, is a decoy receptor (Kawakami K et al., 2001). IL-13 mimics IL-4 in inducing IgE secretion but does not promote T_H2 cell differentiation due to the lack of IL-13Rα1 expression on T cells (Wills-Karp M et al., 2008). Both IL-4 and IL-13 induce the release of potent eosinophil chemoattractants, eotaxin 1, 2 and/or 3 by cultured airway smooth muscle (Moore PE et al., 2002), lung fibroblasts (Teran LM et al., 1999), airway epithelial cells (Komiya A et al., 2003), alveolar epithelial cells (Abonyo BO et al., 2005) and vascular endothelial cells (Shinkai A et al., 1999; Kuperman DA et al., 2008). Blocking IL-13 prevents AHR in mice following antigen challenge, suggesting that induction of allergen induced AHR is an IL-13 specific or dominant effect (Wills-Karp M et al., 1998). IL-13 is also commonly associated with structural changes seen in chronic asthma, including goblet cell hyperplasia, airway smooth muscle proliferation, and sub-epithelial fibrosis (Wills-Karp M, 2004; Zhu Z et al., 1999). Given that increased amounts of IL-4 and IL-13 have been found in the BAL of asthmatic subjects post-Ag challenge, we investigated the potential involvement of IL-4 and IL-13 in the lung homing of HPC in asthma (Robinson DS et al., 1992; Kroegel C et al., 1996).

1.6 Hypothesis

We hypothesize that interleukin-4 and interleukin-13 promote the lung homing of hemopoietic progenitor cells by increasing the migrational responsiveness of these cells to pro-inflammatory chemokines such as SDF-1α and eotaxin.

1.7 Specific Questions

1. Can IL-4 and IL-13 modulate the migrational responses of hemopoietic progenitor cells towards SDF-1α or eotaxin, *in vitro*?

2. What is the mechanism by which IL-4 and IL-13 prime the migrational response of hemopoietic progenitor cells?
    i. Change in receptor expression?
    ii. Enhance signaling?

3. Can IL-4 and IL-13 prime SDF-1α or eotaxin stimulated adhesion of hemopoietic progenitor cells?

CHAPTER 2: METHODS

2.1 Participants

CB samples were obtained from consenting expectant mothers in the Labour and Delivery ward of St. Joseph's Healthcare and PB samples were obtained from atopic asthmatic subjects. All experimental procedures were reviewed and approved by the Research Ethics Board at St. Joseph's Healthcare (REB Project #06-2692), and all participants gave their informed written consent prior to sample collection (Appendix E).

2.2 Progenitor Cell Isolation

2.2.1 Blood Collection

Immediately after delivery, umbilical cord blood (CB) was collected into a 60ml syringe (Becton Dickinson, Mississauga, ON) containing heparin (1000 units/ml; Sigma Aldrich, Oakville, ON). CB was depleted of red blood cells by gravity sedimentation in 1% (vol/vol) dextran (Sigma Aldrich, Oakville, ON) at a 1:5 dilution at 37ºC, 5% carbon dioxide (CO_2) for 45 min in 50ml conical tubes (Becton Dickinson, Mississauga, ON).

2.2.2 Enrichment of Non-adherent Mononuclear Cells

The CB-derived plasma fraction was diluted at a 1:1 ratio with McCoy's 5A (Gibco, Burlington, ON) and low-density mononuclear cells (MNC) were isolated by sedimentation on 60% Percoll (VWR International, Edmonton, AB) gradients (2000rpm, 20 mins). The interface mononuclear cell layer was collected, washed with McCoy's 5A and subject to adherence on plastic to deplete monocytes/macrophages. After 1.5 hr

(37ºC, 5% CO_2), cells adhered to plastic were discarded and the non-adherent MNC fraction (NAMNC) was collected.

2.2.3 *$CD34^+$ Cell Enrichment*

Prior to CD34 enrichment, NAMNC were resuspended in McCoy's 5A supplemented with 10% FBS and a total cell count was performed using a Hausser Scientific haemocytometer. The cells were diluted in a 1:100 dilution with trypan blue (Sigma) and two quadrants were counted. The total number of cells was calculated as follows: Number of cells/ml = (number of cells counted x dilution factor x 1000)/(volume under the coverslip of the haemocytometer x number of quadrants counted). The number of cells/ml was then multiplied by the volume of supplemented McCoy's 5A used to resuspend the cells.

Enriched $CD34^+$ cells were obtained by positive selection using the magnetic activated cell selection technique (MACS®) (Miltenyi Biotech, Auburn, CA). NAMNC were washed and then resuspended with MACS® buffer (Dulbecco's phosphate-buffered saline (DPBS; Gibco, Burlington, ON) with 0.5% bovine serum albumin (BSA; fraction V; Sigma Aldrich, Oakville, ON) and 2mM EDTA (Sigma Aldrich, Oakville, ON). To the resuspended cells, 100µl/10^8 cells of FcR Blocking Reagent and 100µl/10^8 cells of hapten-labeled antibody directed against CD34 (Miltenyi Biotech) were added and the cells were incubated for 15 mins at 4ºC. The cells were then washed (1200rpm, 10 minutes, 4ºC), resuspended in 400µl of MACS buffer, 100µl/10^8 cells of anti-hapten microbeads (Miltenyi Biotech) were added and the cells were incubated (15 mins, 4ºC). The labeled cells were loaded onto an MS separation column (Miltenyi Biotech) that was

placed in the magnetic field of a VarioMacs™ (Miltenyi Biotech). The column was then washed 3 times with 500µl of MACS buffer before being removed from the VarioMacs™. The $CD34^+$ cell fraction was then eluted by plunging the column with 1ml of MACS buffer. The resulting cells were centrifuged (1000rpm, 10 minutes, 4ºC) and resuspended in Iscove's modified Dulbecco's media (IMDM; Gibco, Burlington, ON) supplemented with 10% FBS.

2.3 Transmigration Assay

2.3.1 Priming of HPC

To investigate the role of various cytokines (IL-4, IL-13 and TGF-β) in priming the migrational responsiveness of HPC, $5x10^4$ cells/100µl of $CD34^+$ enriched CB cells were incubated with IL-4 (0.01, 0.1, 1, 10ng/ml), IL-13 (0.1ng/ml), TGF-β (0.05, 0.5, 5, 50ng/ml) or diluent for 1 or 18hr (37˚C, 5% CO_2) prior to being added to the upper transwell insert.

The mechanism of IL-4 and IL-13 priming was studied by pre-incubating cells with IL-4Rα and IL-13Rα1 neutralizing antibodies (R&D Systems, Minneapolis, MN) for 15 min prior to 1hr incubation with IL-4 or IL-13.

To determine whether CXCR7 plays a role in SDF-1α stimulated migration, cells were pre-incubated with a previously optimized dose (10 µg/ml) (Tarnowski M et al., 2010) of CXCR7 neutralizing antibody (clone 9C4; MBL, Woburn, MA) for 15 min prior to 1hr incubation with IL-4.

To evaluate the influence of lipid raft formation on transmigration, cells were pre-incubated with molecules that disturb lipid raft formation for 15 min prior to 1hr incubation with IL-4 or IL-13: 2.5 mM methyl-β-cyclodextran (MβCD), 25 μg/ml nystatin or 10 μg/ml amphotericin B (Sigma, St Louis, MO), as described by (Wysoczynski M et al., 2005).

2.3.2 Transwell Migration assay

The migrational response of the enriched $CD34^+$ cell populations *in vitro* was assessed using transwell chambers (24-well cell clusters, 6.5mm Transwell® with 5μm pore polycarbonate membrane insert filters; Corning Costar, Corning, NY). Into the lower well of the transwell plate, 600μl dilutions of the chemoattractants SDF-1α (100ng/ml; R&D Systems, Minneapolis, MN), eotaxin (500ng/ml; R&D Systems, Minneapolis, MN), IL-4 (0.1 ng/ml; R&D Systems, Minneapolis, MN), IL-13 (0.01, 0.1, 1, 10ng/ml; PeproTech, Rocky Hill, NJ) or diluent were loaded. Each transwell insert was washed 2 times with IMDM plus 10% FBS and $5x10^4$ $CD34^+$ cells/100μl were loaded into each transwell insert. The inserts were then placed into the lower wells containing stimulants or diluent and the transwell plates were placed in an incubator (37ºC, 5% CO_2) for 4hr. After 4hr, the transwell inserts were carefully removed and discarded. The cells in the lower well representing migrated cells were aspirated, immunostained for progenitor cell markers and enumerated by flow cytometry as described below.

2.4 Adhesion Assay

Forty-eight well plates were coated with 200µl of PBS containing 20µg/ml of human fibronectin and incubated overnight at 4°C. The next day, each well was washed 3 times with warmed PBS, blocking buffer was added and the plate was incubated for one hour at room temperature. The plates were then washed three times with IMDM supplemented with 10% FBS. Enriched CD34 cells (1 x 10^6 cells/500µl) were incubated with SDF-1α, eotaxin or diluent in serum-free medium (37°C, 5% CO_2, 30 min) and then added to fibronectin-coated wells (50,000 cells/well). After 30 mins incubation (37°C, 5% CO_2) the supernatant from each well was aspirated and adhered cells were dislodged with cell dissociation buffer and immunostained for flow cytometry as described below. Adhered cells were calculated as a percentage of the total number of cells added to the well.

2.5 Immunofluorescence Staining

2.5.1 CD34 and CD45 Staining

Cells were washed with 2ml sodium azide (NaN_3) before being resuspended in FACS staining buffer (PBS plus 0.1% NaN_3; 2.5% each of mouse serum and human serum) and stained with phycoerythrin (PE)-conjugated CD34 monoclonal antibody (mAb) and fluorescein (FITC)-conjugated CD45 mAb in the dark on ice for 30 minutes. The cells were subsequently washed with NaN_3 and fixed in 250µl PBS plus 1% paraformaldehyde (PFA). Cells were stored at 4°C in the dark until enumerated by flow cytometery.

2.5.2 CD34, CD45 and IL-5Rα Staining

Cells were washed with 2ml sodium azide (NaN_3) before being resuspended in FACS staining buffer and stained with allophycocyanin (APC)-conjugated CD34 mAb, FITC-conjugated CD45 mAb and PE-conjugated IL-5 Rα mAb in the dark on ice for 30 minutes. The cells were subsequently washed with NaN_3 and fixed in 250µl PBS plus 1% PFA. Cells were stored at 4°C in the dark until enumerated by flow cytometery.

2.5.3 CD34, CD45 and CXCR4 Staining

Cells were washed with 2ml sodium azide (NaN_3) before being resuspended in FACS staining buffer and stained with PE-conjugated CD34 mAb, FITC-conjugated CD45 mAb and APC-conjugated CXCR4 mAb in the dark on ice for 30 min. The cells were subsequently washed with NaN_3 and fixed in 250µl PBS plus 1% PFA. Cells were stored at 4°C in the dark until enumerated by flow cytometery.

2.6 Flow Cytometric Analyses

2.6.1 *Identifying and Quantifying HPC* (*$CD34^+45^+$ cells)*

Cells were analyzed using a FACScan flow cytometer equipped with an argon ion laser (Becton Dickinson Instrument Systems (BDIS)). Hemopoietic progenitor cells were identified on the basis of their unique cell size, granularity and immunofluorescence characteristics. To obtain comparable values of migrated cells, flow cytometric counts of

cells were run for 120 seconds. The migrated cells were expressed as a percentage of the total $CD34^+45^+$cells added to the transwell insert.

Analysis of FACS data was performed using a four step gating strategy previously described in detail by Dorman et al., 2005. True $CD34^+$ blast cells were identified as cells with forward $scatter^{low}$ /side $scatter^{low}$ and $CD34^{high}/CD45^{dull}$ staining (see Appendix C).

2.6.2 Identifying and Quantifying Eosinophil-lineage Committed Progenitor Cells ($CD34^+45^+IL\text{-}5R\alpha^+$ cells)

Within this gated population of $CD34^+45^+$ cells, PE-conjugated IL-5 Rα mAb or control Ab was detected and data were collected with confidence thresholds set at 99% using the matched IgG isotype control. Specific staining for IL-5Rα was determined as the number of positive events over this threshold.

To obtain comparable values of migrated cells, flow cytometric counts of cells were run for 120 seconds. The migrated cells were expressed as a percentage of the total $CD34^+45^+IL\text{-}5R\alpha^+$ cells added to the transwell insert.

2.6.3 CXCR4 Expression on HPC

Within the gated population of $CD34^+45^+$ cells, APC-conjugated CXCR4 mAb or control Ab was detected and data were collected with confidence thresholds set at 99% using the matched IgG isotype control. Specific staining for the receptors was determined as the number of positive events over this threshold. Receptor expression of

$CD34^{high}/CD45^{dull}$ cells was expressed as the intensity of receptor expression (specific mean fluorescence intensity, SMFI) on $CD34^{+}45^{+}$ cells.

2.7 GM1/ CXCR4 Staining & Confocal Imaging

Cells were washed with 2ml sodium azide (NaN_3) and then fixed with 400µl of 4% paraformaldehyde/ Ca and Mg-free phosphate-buffered saline (PBS) for 15 min at 4ºC. Cells were washed with 2ml sodium azide (NaN_3) and resuspended in FACS staining buffer (PBS plus 0.1% NaN_3; 2.5% each of mouse serum and human serum) before being stained with cholera toxin B–subunit conjugated with fluorescein isothiocyanate (FITC) (Sigma, St Louis, MO) and biotin-conjugated mouse monoclonal anti-hCXCR4 (R&D Systems, Minneapolis, MN) for 30 min. The cells were then rinsed with 2ml sodium azide (NaN_3), resuspended in FACS staining buffer and stained with Phycoerythrin(PE)-conjugated streptavidin (R&D Systems, Minneapolis, MN) for 30 min. Stained cells were resuspended in 100µl of PBS and examined using a Leica DMI 6000 B confocal microscope. Images were taken at a 60x magnification and separate pictures were merged using Image-Pro Plus software (Media Cybernetics Inc., Silver Spring, MD).

2.8 Eosinophil Isolation & Transmigration Assay

2.7.1 Eosinophil Isolation

Heparinized venous blood was diluted 1:1 in McCoy's 5A and subject to density gradient centrifugation on accuprep© (Accurate Chemical & Scientific Corporation, Westbury, NY, USA). Eosinophils were purified using a MACS column $CD16^{+}$

neutrophil depletion assay (Miltenyi Biotec, Auburn, CA, USA) and resuspended in RPMI complete (10% FBS, 1M HEPES in RPMI 1640).

2.7.2 Eosinophil Transmigration

Eosinophils were pre-incubated for 2hrs with diluent (RPMI complete), IL-13 (0.1ng/ml), or IL-4 (0.1ng/ml) and subsequent migration to diluent, Eotaxin (8.4ng/ml) or SDF-1α (100ng/ml) was assessed. Chemoattractants (30µl) were placed into the lower wells of the 48 well microboyden chamber (Neuro Probe Inc., Gaithersburg, MD, USA), separated from the upper wells by a nitrocellulose filters (8µm pore size). Fifty microlitres of cells ($3x10^6$cells/ml) was placed into the upper wells and the chamber was incubated (1.5hrs at 37°C). The filter was fixed with saturated mercuric chloride and stained with hematoxylin, HCl, Lithium chloride, and Chromotrope 2R. The filter was then fixed in xylene and mounted on a glass slide and cover slipped with permount glue (Fisher Scientific, Pittsburgh, PA, USA). Ten random fields from the underside of the filter were counted using a light microscope at 400x magnification.

2.9 Statistical Analysis & Power Calculations

Data are presented as mean ± SEM. Statistical analyses of all data sets were performed using analysis of variance (ANOVA) from the STATISTICA Software package. Post-hoc analyses of significant overall relationships were performed using a LSD (Fisher's least significant difference) test. Data were deemed significant with an alpha value < 0.05.

This study is powered based on previously observed significant increases in the migrational response of HPC ($CD34^{+}45^{+}$cells) (Thomson J. MSc Thesis, 2008). The sample size required to detect the "minimal important differences" baseline and primed responses was calculated using the power calculation module of Minitab. We set $\beta=0.20$ (power=80%) and $\alpha=0.05$ (likelihood of type I error=5%).

CHAPTER 3: RESULTS

3.1 IL-4 & IL-13 stimulated migration of CB-derived HPC

The ability of IL-4 and IL-13 to directly stimulate the migration of CB-derived HPC was assessed *in vitro* using transwell chambers. Compared to diluent control, both IL-4 (0.1ng/ml) and IL-13 (0.1ng/ml) had no effect on CB-derived HPC migration whilst the positive control, SDF-1α (100ng/ml), stimulated a significant migrational response (Dil: 5.0 ± 0.9%, IL-4: 3.4 ± 1.8%, IL-13: 5.5 ± 0.8% and SDF-1α: 29.8 ± 4.6%, $p<0.05$) (Figure 3). The concentration of SDF-1α (100ng/ml) used has previously been determined to stimulate an optimal migratory response of CB-derived HPC (Appendix B; Thomson J. MSc Thesis, 2008). The optimal priming doses of IL-4 and IL-13 were used to confirm that these cytokines at this concentration did not stimulate direct migration.

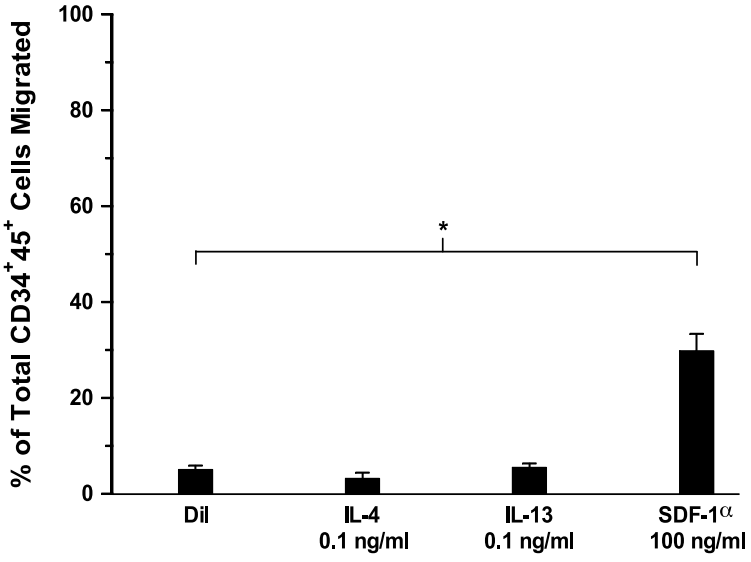

Figure 3. IL-4, IL-13 & SDF-1α stimulated migration of CB-derived HPC (n=3). IL-4 and IL-13 did not stimulate a significant migrational response of HPC while SDF-1α did compared to diluent (*$p<0.05$). Data are expressed as mean ± SEM.

3.2 IL-4 priming of CB-derived HPC migration

The role of IL-4 in priming the migrational responses of HPC was assessed by incubating CB-derived HPC with IL-4 prior to the transmigration assay. Pre-incubation with a wide concentration range of IL-4 (0.01, 0.1, 1, 10ng/ml) for 18 hr (37°C, 5% CO_2) significantly increased the migratory response of CB-derived HPC to an optimal concentration of SDF-1α (100ng/ml) (Figure 4B). In contrast, pre-incubation with IL-4 had no effect on the migrational response of CB-derived HPC to a sub-optimal dose of eotaxin (200ng/ml) when compared to pre-incubation with diluent (Figure 4B). The priming effect of IL-4 (0.1ng/ml) on SDF-1α stimulated migration of CB-derived HPC was detectable as early as 1 hr (Figure 4A) but this effect was significantly greater 18hr post-incubation (IL-4 increased the migratory response of HPC compared to diluent by 14.31 $\pm$ 7.26% at 1 hr vs. 47.1 $\pm$ 9.2% at 18 hr, $p<0.05$).

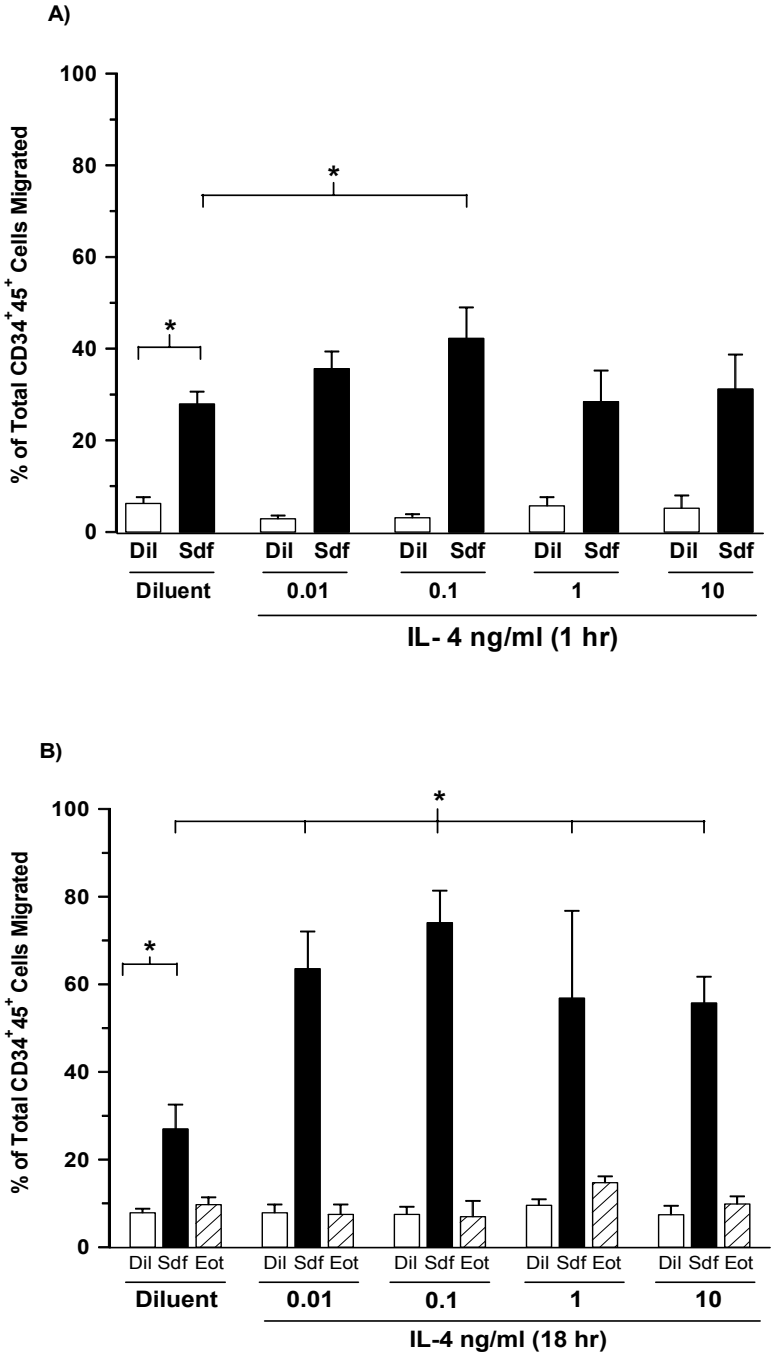

Figure 4. IL-4 priming of CB-derived HPC transmigration (n=8). (A) Pre-incubation with IL-4 for 18hr primes the migrational response of HPC towards the optimal dose of SDF-1α (100ng/ml) but not eotaxin (500ng/ml) (B) IL-4 priming of SDF-1α stimulated migration is detectable as early as 1hr at the optimal dose of IL-4 (0.1ng/ml). (*$p<0.05$) Data are expressed as mean ± SEM.

3.3 Inhibition of IL-4 priming of SDF-1α stimulated migration

IL-4 initially binds to the IL-4Rα chain, which then dimerizes with the common γ chain (cγ) or the IL-13Rα1 chain to form the type I IL-4 receptor or type II IL-4 receptor, respectively. To determine the receptor by which IL-4 mediates its priming effects, CB-derived HPC were incubated with neutralizing IL-4Rα antibody (0.1, 1, 10ng/ml) or IL-13Rα1 antibody (0.1, 1ng/ml) for 15 minutes prior to incubation with IL-4 for 1 hr. All three doses of IL-4Rα antibody significantly inhibited IL-4 priming of SDF-1α stimulated migration of HPC, suggesting that binding of IL-4 to the IL-4Rα subunit is essential for IL-4 priming (Figure 5A). In contrast, IL-13Rα1 antibody (0.1, 1ng/ml) did not effect IL-4 priming of HPC to SDF-1α, implying that either inhibition of the IL-13Rα1 subunit does not interfere with its dimerization to IL-4Rα or IL-4 mediates its priming effects solely through the type I IL-4 receptor (Figure 5B).

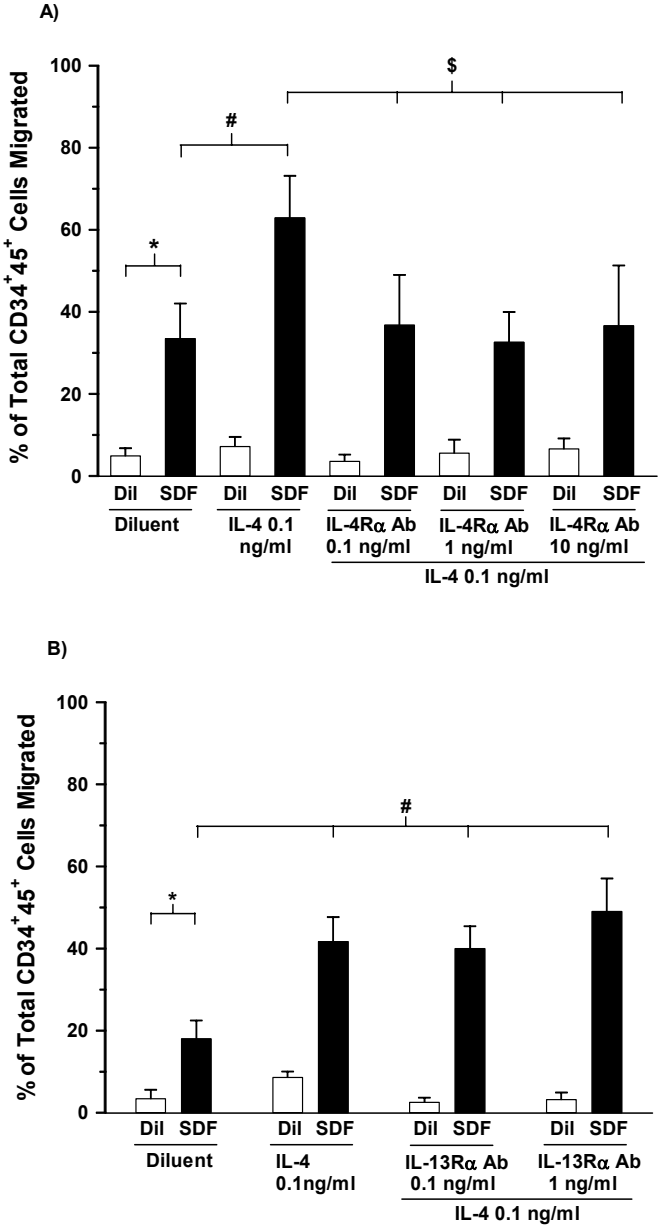

Figure 5. Inhibition of IL-4 Priming of HPC Migration (n=8). (A) Pre-exposure to an optimal dose of IL-4 (0.1ng/ml; 1hr) in presence of an IL-4Rα antibody blocks the increased migratory response of HPC to SDF-1α (100ng/ml). (B) Pre-exposure to an optimal dose of IL-4 (0.1ng/ml; 1hr) in presence of an IL-13Rα1 antibody did not block the increased migratory response of HPC to SDF-1α (100ng/ml). Data are expressed as mean ± SEM. *$p < 0.05$ comparison between response to diluent and SDF-1α; #$p<0.05$ represents comparison of HPC response to SDF-1α following incubation with diluent compared to various doses of cytokine and neutralizing antibody. \$$p<0.05$ represents comparison of HPC response to SDF-1α following incubation with cytokine compared to various doses of neutralizing antibody.

3.4 IL-13 Priming of CB-derived HPC migration

Pre-incubation with IL-13 (0.1 ng/ml) for 1 or 18 hrs., (37°C, 5% CO_2) significantly primed the migrational response of CB-derived HPC to SDF-1α (100ng/ml) (Figure 6). Although the priming effect was detectable within 1 hr., optimal priming responses were observed 18 hrs. post-incubation (IL-13 increased the migratory response of HPC by 6.3 $\pm$ 3.3% at 1 hr. vs. 20.5 $\pm$ 11.1% at 18 hr.). In contrast to SDF-1α stimulated migration, pre-incubation with IL-13 for 18 hrs. (37°C, 5% CO_2) did not prime the migratory response of CB-derived HPC to eotaxin (200ng/ml) (Figure 6B).

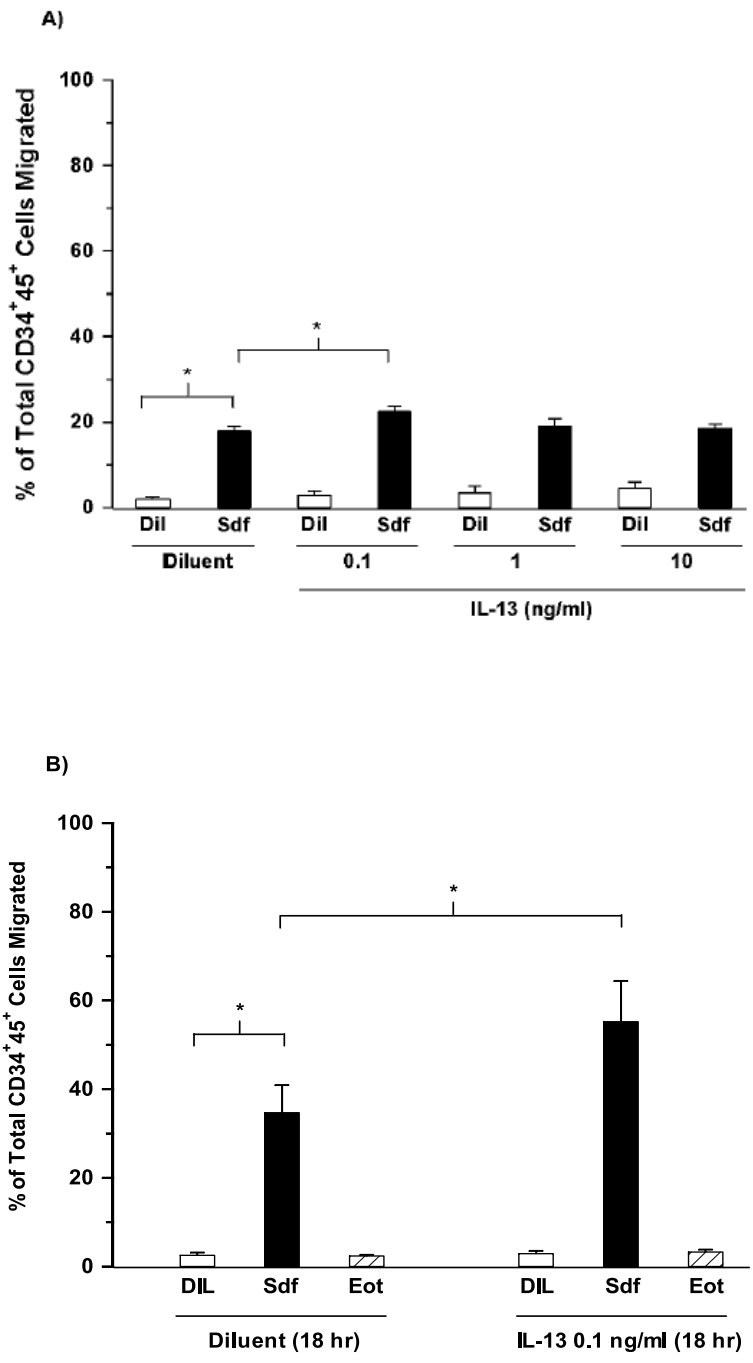

Figure 6. IL-13 priming of CB-HPC transmigration (n=8). A) IL-13 priming of SDF-1α stimulated migration is detectable as early as 1hr. B) Pre-incubation with IL-13 for 18hrs primes the migrational response of HPC towards an optimal dose of SDF-1α (100ng/ml) but not eotaxin (200ng/ml). (*$p<0.05$) Data are expressed as mean ± SEM.

Although both IL-4 and IL-13 primed SDF-1α stimulated migration of HPC, IL-4 was a significantly more potent priming agent compared to IL-13 (Table 1, $p<0.05$). Since both IL-4 and IL-13 prime the migrational responsiveness of HPC to SDF-1α, we wanted to determine whether exposing HPC to both these cytokines had an additive or synergistic priming effect, when the optimal priming doses were tested together. CB-derived HPC incubated with the optimal dose of IL-4 (0.1 ng/ml) and IL-13 (0.1 ng/ml) had no additive or synergistic effect on the subsequent migrational response to SDF-1α (100 ng/ml) (Table 1).

	Diluent	**SDF-1α (100 ng/ml)**
DILUENT	1.69 ± 1.43 %	14.57 ± 2.47 % #
IL-4 0.1 ng/ml	2.27 ± 0.55 %	55.94 ± 8.29 % *
IL-13 0.1 ng/ml	3.35 ± 2.51 %	31.59 ± 4.82 % *
IL-4 0.1 + IL-13 0.1 ng/ml	2.59 ± 1.59 %	36.48 ± 5.19 % *

Table 1. Additive or synergistic IL-4 and IL-13 priming of SDF-1α stimulated migration of HPC. #$p<0.05$ comparison between diluent and SDF-1α stimulated migration; *$p<0.05$ represents comparison of HPC response to SDF-1α following incubation with diluent compared to various doses of cytokine.

3.5 Inhibition of IL-13 priming of SDF-1α stimulated migration

IL-13 initially binds to the IL-13Rα1 chain, which then dimerizes with the IL-4Rα chain to form the signaling complex known as the type II IL-4 receptor. To determine whether the priming effect of IL-13 is mediated through the IL-13Rα1, CB-derived HPC were incubated with IL-13Rα1 Ab (0.1, 1ng/ml) or IL-4Rα Ab (0.1, 1, 10ng/ml) for 15 min prior to incubation with IL-13 (0.1ng/ml; 1 hr.). IL-13Rα1 antibody at a dose of 1ng/ml significantly inhibited IL-13 priming of SDF-1α stimulated migration, implying that IL-13 binding to IL-13Rα1 is required for the priming effect of IL-13 (Figure 7A). However, pre-exposure to IL-4Rα Ab (0.1, 1, 10ng/ml) did not inhibit IL-13 priming of HPC to SDF-1α, suggesting that blocking the IL-4Rα binding site has no effect on the dimerization of IL-4Rα with IL-13Rα1 and the IL-13 mediated effects (Figure 7B).

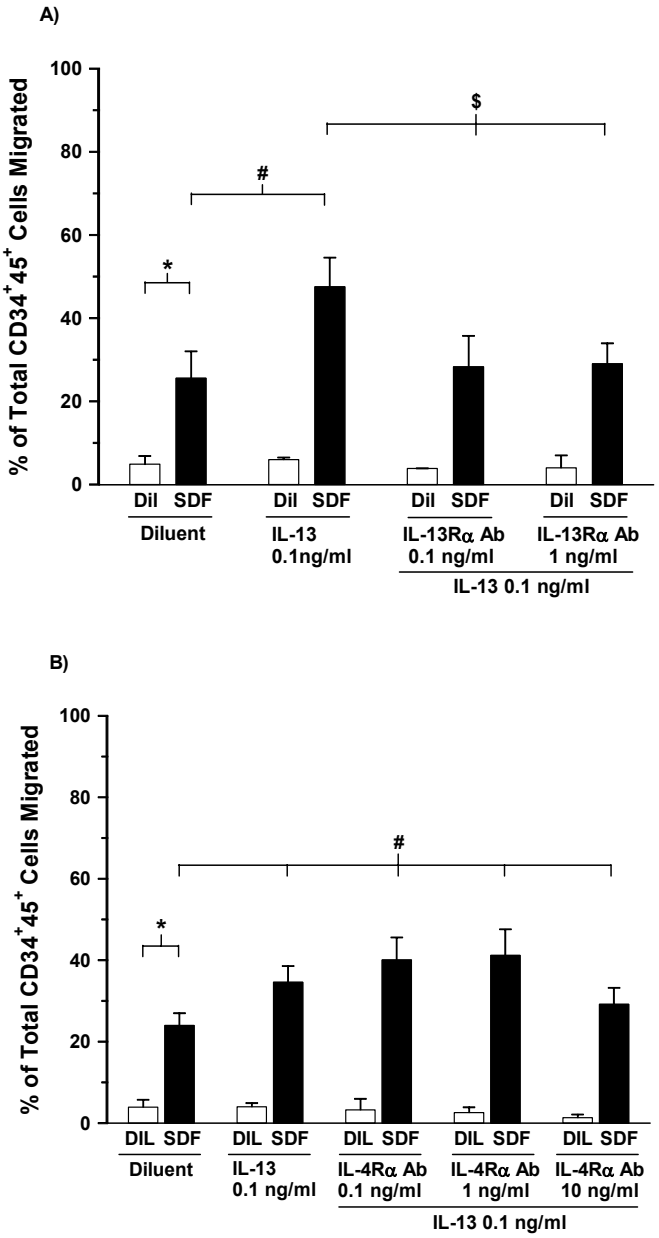

Figure 7. Inhibition of IL-4 Priming of HPC Migration (n=6). (A) Pre-exposure to an optimal dose of IL-13 (0.1ng/ml; 1hr) in presence of an IL-13Rα1 antibody blocks the increased migratory response of HPC to SDF-1α (100ng/ml). (B) Pre-exposure to an optimal dose of IL-13 (0.1ng/ml; 1hr) in presence of an IL-4Rα antibody did not block the increased migratory response of HPC cells to SDF-1α (100ng/ml). Data are expressed as mean ± SEM. *$p < 0.05$ comparison between response to diluent and SDF-1α; #$p<0.05$ represents comparison of HPC response to SDF-1α following incubation with diluent compared to various doses of cytokine and neutralizing antibody. \$$p<0.05$ represents comparison of HPC response to SDF-1α following incubation with cytokine compared to various doses of neutralizing antibody.

3.6 IL-4 & IL-13 priming of PB-derived HPC migration

The primary source of progenitor cells in this study was cord blood. However, to show that these findings are applicable to adult asthmatics, priming of the migrational responsiveness was performed on HPC from PB of atopic asthmatic subjects. Our data show that pre-exposure of PB-derived HPC to IL-4 or IL-13 (0.1ng/ml, 18hr) enhanced the migrational response to SDF-1α (100ng/ml) by 18.9±11.8% and 9.12±3.24%, respectively (Figure 8). This effect was significant compared to diluent and comparable to the priming effect of IL-4 & IL-13 on CB-derived HPC.

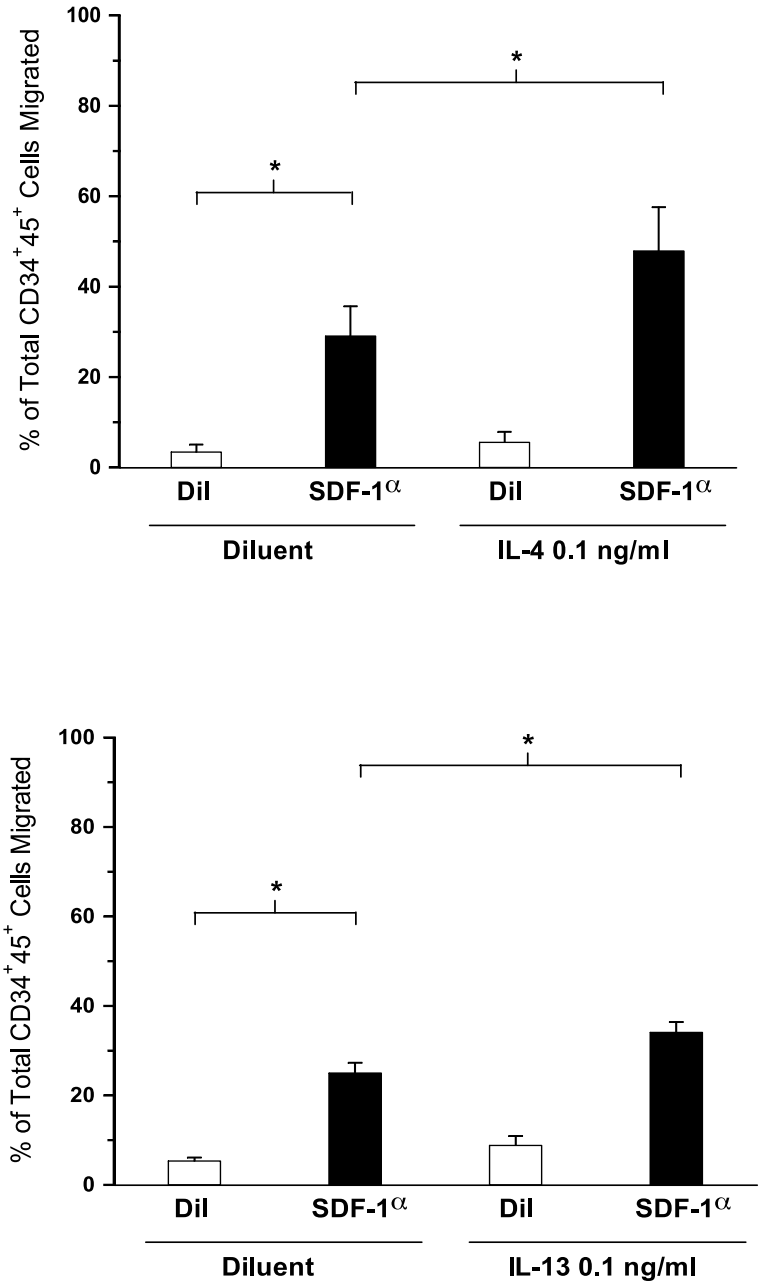

Figure 8. A) IL-14 & B) IL-13 priming of PB-HPC transmigration (n=8). IL-4, at the optimal concentration of 0.1ng/ml, primes the migrational response of PB-derived HPC towards the optimal dose of SDF-1α (100 ng/ml) (*$p<0.05$). Data are expressed as mean ± SEM.

3.7 IL-4 & IL-13 priming of eosinophil-lineage committed progenitor cells

So far we had concentrated on studying the priming of primitive HPC. However, studies in human asthmatics and animal models of asthma have shown that there is a local increase in eosinophil-lineage committed progenitors at the site of allergen challenge (Sehmi R et al., 1997; Southam DS et al., 2005). We investigated whether IL-4 and IL-13 could prime the migrational responsiveness of eosinophil-lineage committed progenitor cells ($CD34^+45^+$ IL-5Rα+ cells). Under optimal priming conditions, IL-4 (0.1ng/ml; 18hr) and IL-13 (0.1ng/ml; 18hr) primed the migration of CB-derived HPC ($CD34^+45^+$ cells) to SDF-1α (100 ng/ml) but had no significant effect on the migration of CB-derived eosinophil-lineage committed progenitor cells ($CD34^+45^+$IL-5Rα^+ cells) to SDF-1α (100 ng/ml) (Figure 9). Analyses of receptor expression by flow cytometry for CXCR4, IL-4Rα and IL-13Rα1 on HPC and eosinophil-lineage committed progenitor cells is shown in appendix D.

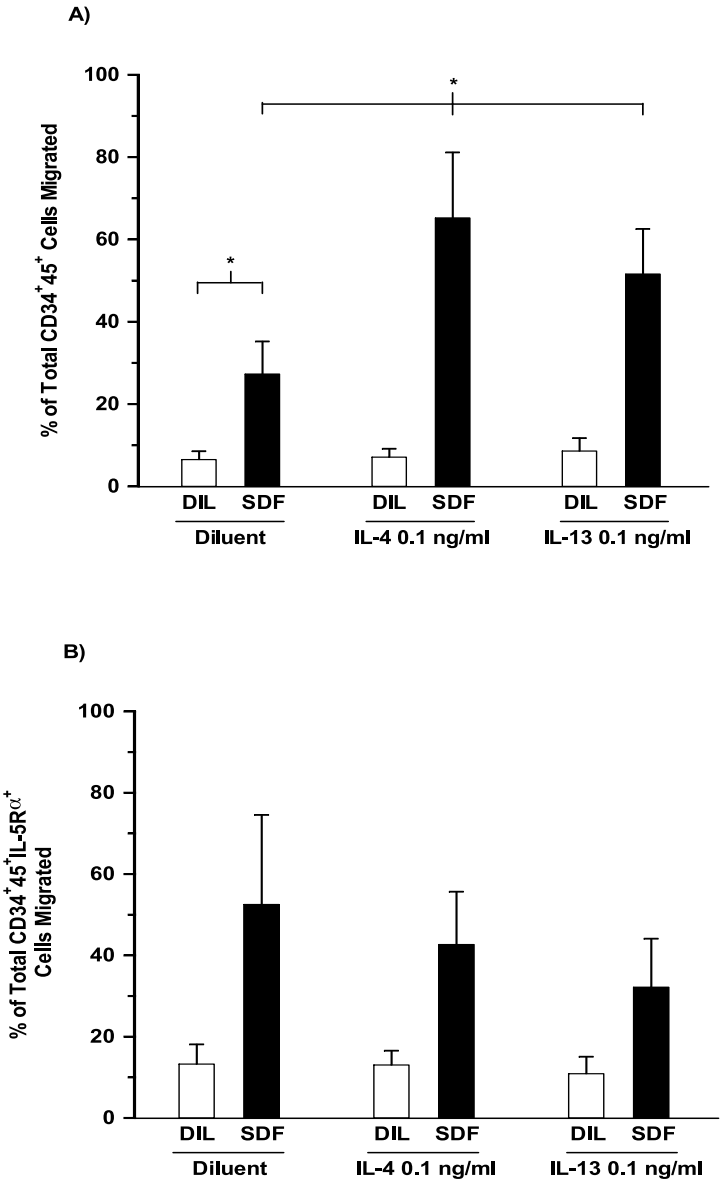

Figure 9. IL-4 and IL-13 priming of CB-derived eosinophil lineage committed progenitor cells (n=6). Both IL-4 (0.1ng/ml) and IL-13 (0.1ng/ml) primed **(A)** HPC ($CD34^+45^+$ cells) but not **(B)** eosinophil-lineage committed progenitor cells ($CD34^+45^+IL\text{-}5R\alpha^+$ cells) migration to SDF-1α (100ng/ml) (*$p<0.05$). Data are presented as mean ± SEM.

3.8 IL-4 & IL-13 priming of mature eosinophils

To determine whether IL-4 and IL-13 can prime SDF-1α stimulated migration of mature eosinophils, PB-derived eosinophils were pre-incubated with IL-4 (0.1ng/ml), IL-13 (0.1ng/ml) or diluent for 18 hrs at 37°C prior assessment of migrational responsiveness. In contrast to the effect observed on CB- and PB- derived HPC, IL-4 and IL-13 had no significant effect on the migration of mature eosinophils to SDF-1α (100ng/ml). However, these cytokines did prime eosinophil migration to a sub-optimal dose of eotaxin, *in vitro*. IL-4 and IL-13 increased the number of migrated eosinophils to eotaxin by 29.5 $\pm$ 4.0 and 28.0 $\pm$ 12.2 eosinophils, respectively (Figure 10).

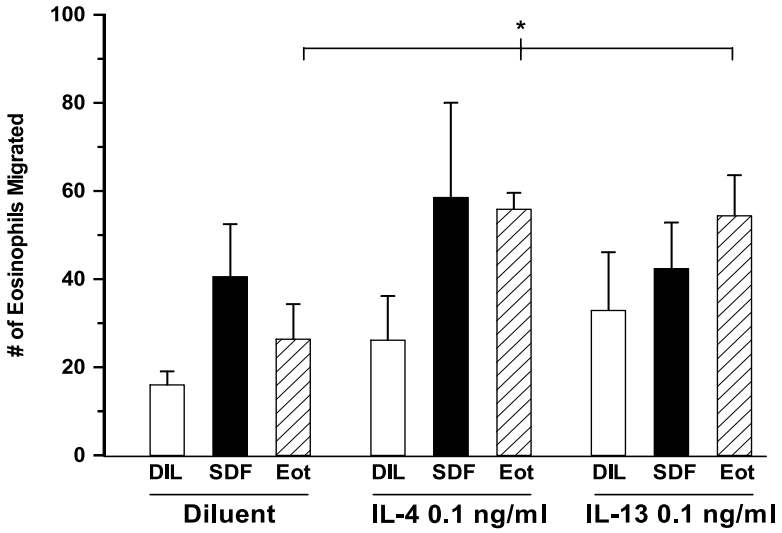

Figure 10. IL-4 and IL-13 priming of eosinophil transmigration (n=4). IL-4 and IL-13 did not prime the migrational response of PB-derived eosinophils towards the optimal dose of SDF-1α (100 ng/ml) but did prime eotaxin (8.4 ng/ml) mediated migration. Data are presented as mean ± SEM (n=4) (*$p<0.05$). Data are presented as mean ± SEM.

3.9 Role of CXCR7 in mediating migrational responses of HPC

A second receptor for SDF-lα, CXCR7, has recently been described (Balabanian K et al., 2005). Although SDF-lα binds to CXCR7 with high affinity, characteristic chemokine signaling has not been demonstrated (Thelen M et al., 2008). To determine whether CXCR7 is involved in SDF-1α stimulated migration or IL-4 priming, CB-derived HPC were incubated with CXCR7 Ab for 15 min. prior to 1 hr. incubation with IL-4 (0.1 ng/ml). In the presence of previously optimized dose of CXCR7 Ab (Tarnowski M et al., 2010), we found that inhibition of CXCR7 had no effect on SDF-1α stimulated migration or on IL-4 priming of SDF-1α stimulated migration of HPC (Table 2). In contrast, our lab has previously shown the CXCR4 Ab, AMD3100 (15mg/ml), to significantly inhibit SDF-1α stimulated migrational responses (Dil: 6 $\pm$ 2.5% vs SDF-1α: 22 $\pm$ 1.5% vs SDF-1α + AMD 3100: 5 $\pm$ 1.0%, $p<0.05$) (Doyle et al., ERJ 2011). This indicates that the priming effect of IL-4 is mediated through the SDF-1α/CXCR4 axis and not through CXCR7 ligation.

	Diluent	**SDF-1α (100 ng/ml)**
DILUENT	5.15 $\pm$ 0.60 %	27.23 $\pm$ 8.56 % #
IL-4 0.1 ng/ml	6.05 $\pm$ 1.30 %	51.62 $\pm$ 13.53 % *
IL-4 (0.1 ng/ml) + Anti-CXCR7 (10 µg/ml)	7.88 $\pm$ 1.54 %	55.19 $\pm$ 14.91 % *

Table 2. Role of CXCR7 in mediating SDF-1α stimulated migrational responses of HPC. #$p<0.05$ comparison between diluent and SDF-1α stimulated migration; *$p<0.05$ represents comparison of HPC response to SDF-1α following incubation with diluent compared to various doses of cytokine.

3.10 Effect of IL-4 & IL-13 on CXCR4 surface expression

To investigate the mechanism by which IL-4 and IL-13 prime the migrational response to SDF-1α, we examined the cell surface expression of CXCR4. Incubating CB-derived HPC with IL-4 (0.01, 0.1, 1, 10ng/ml) for 1 or 18hr at 37°C had no effect on the intensity of CXCR4 expression on HPC compared to diluent (Figure 11). At 18 hr incubation, our data showed a significant increase in the levels of CXCR4 expression on HPC incubated with diluent or IL-4 compared to baseline (pre-incubation levels). This suggests that there may be a spontaneous non-specific temperature dependent up-regulation of CXCR4, as previously described, which may not contribute to the subsequent IL-4 or IL-13 mediated priming effect (Wang J et al., 1998). In addition, priming effects by IL-4 were seen as early as 1hr of pre-incubation at which time point there was no change in the intensity of CXCR4 expression on HPC suggesting that the increased migrational response to SDF-1α is not dependent on the level of CXCR4 expression on HPC. Similarly, our lab has previously shown that IL-13 (0.1ng/ml) has no effect on the intensity of CXCR4 expression on HPC compared to baseline or diluent (Thomson JV, MSc thesis published 2008).

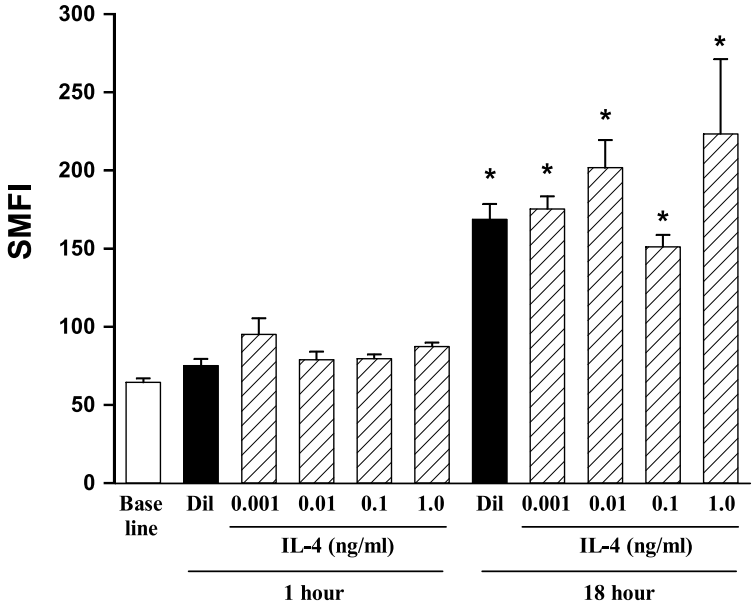

Figure 11. Effect of IL-4 on CXCR4 Expression (n=6). Effect of IL-4 on CXCR4 surface expression on HPC. Pre-incubation with IL-4 (0.1 ng/ml) for 1 hr or 18 hr did not alter the intensity of CXCR4 expression. However, there was a temperature dependent increase in CXCR4 intensity after 18 hr compared to baseline (*$p<0.05$). Data are expressed as mean ± SEM.

3.11 Effect of lipid raft disruption on IL-4 priming

To determine whether the incorporation of CXCR4 into lipid rafts is responsible for the priming effect of IL-4, we examined the effect of membrane lipid raft disruption on SDF-1α stimulated migration of HPC. CB-derived HPC were incubated with polyene antibiotics, amphotericin B (10 μg/ml) and Nystatin (25 μg/ml), and methyl-β-cyclodextrin (2.5 mM) for 15 minutes prior to 1 hr incubation with IL-4 (0.1 ng/ml). All three of these compounds inhibit the priming effect of IL-4, reducing the migrational response of HPC to SDF-1α to pre-incubation levels (Figure 12).

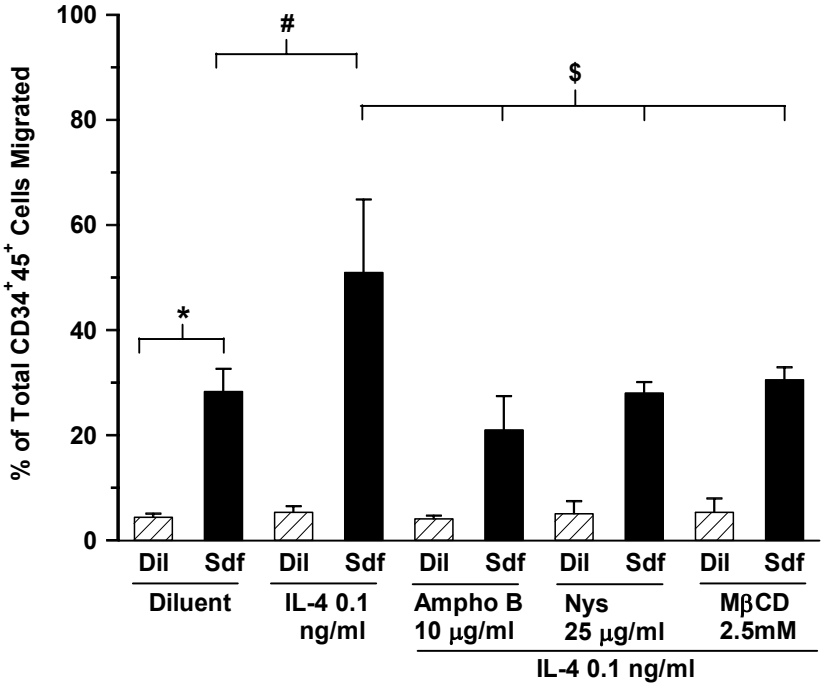

Figure 12. Effect of Lipid Raft Disruption on IL-4 Priming of HPC (n=6). IL-4 priming of SDF-1α stimulated migration of CB derived HPC is inhibited following lipid raft disruption by Amphotericin B (10 μg/ml), Nystatin (25 μg/ml) and methyl-β-cyclodextrin (2.5mM). Data are presented as mean ± SEM. *$p < 0.05$ comparison between response to diluent and SDF-1α; #$p<0.05$ represents comparison of HPC response to SDF-1α following incubation with diluent compared to IL-4. \$$p<0.05$ represents comparison of HPC response to SDF-1α following incubation with IL-4 compared to Ampho B, Nys & MβCD.

3.12 Co-localization of CXCR4 with membrane bound lipid rafts

Confocal microscopy was used to visualize lipid rafts in $CD34^+$ cells enriched from cord blood. Cells were incubated with either diluent, IL-4 (0.1 ng/ml) or IL-13 (0.1 ng/ml) for 18hr. Cells were then stained for the lipid raft marker, GM1, and CXCR4 and examined using a Leica DMI 6000 B confocal microscope. Cells incubated with IL-4 and IL-13 had a higher degree of overlap between CXCR4 and GM1 compared to cells incubated with diluent, suggesting that both these cytokines promote co-localization of lipid rafts and CXCR4 (Figure 13).

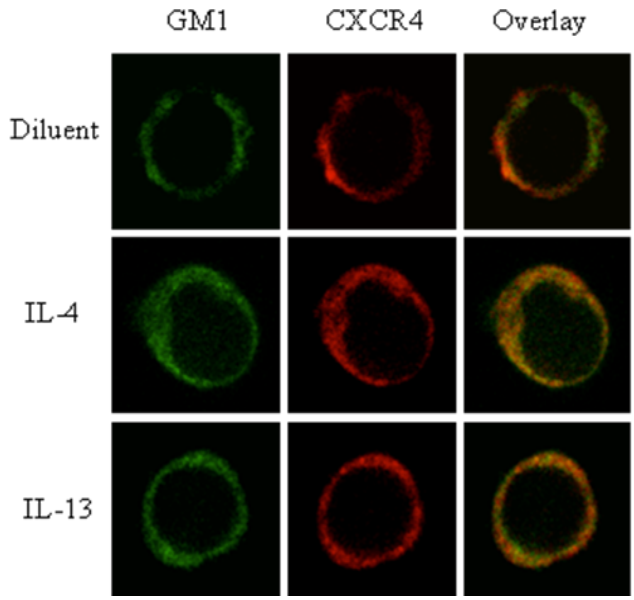

Figure 13. IL-4 and IL-13 Priming is dependent on the incorporation of CXCR4 into GM1- enriched lipid rafts. Cells incubated with IL-4 (0.1 ng/ml) and IL-13 (0.1 ng/ml) have a higher degree of overlap between CXCR4 and GM1 compared to cells incubated with diluent. Primary antibodies used for raft analysis were cholera toxin B–subunit conjugated with fluorescein isothiocyanate (FITC) and biotin-conjugated mouse monoclonal anti-hCXCR4. Magnification x60.

3.13 IL-4 & IL-13 priming of HPC adhesion

To determine whether IL-4 and IL-13 primed SDF-1α or eotaxin stimulated adhesion to human fibronectin, HPC were incubated with IL-4 and IL-13 prior to the adhesion assay. IL-4 over a wide concentration (0.01, 0.1, 1, 10ng/ml) range had no priming effect on SDF-1α (100ng/ml) or eotaxin (200ng/ml, 500ng/ml) stimulated adhesion of CB-derived HPC to human fibronectin (Figure 14A). Similarly, IL-13 (0.1ng/ml) did not prime SDF-1α stimulated adhesion to human fibronectin (Figure 14B).

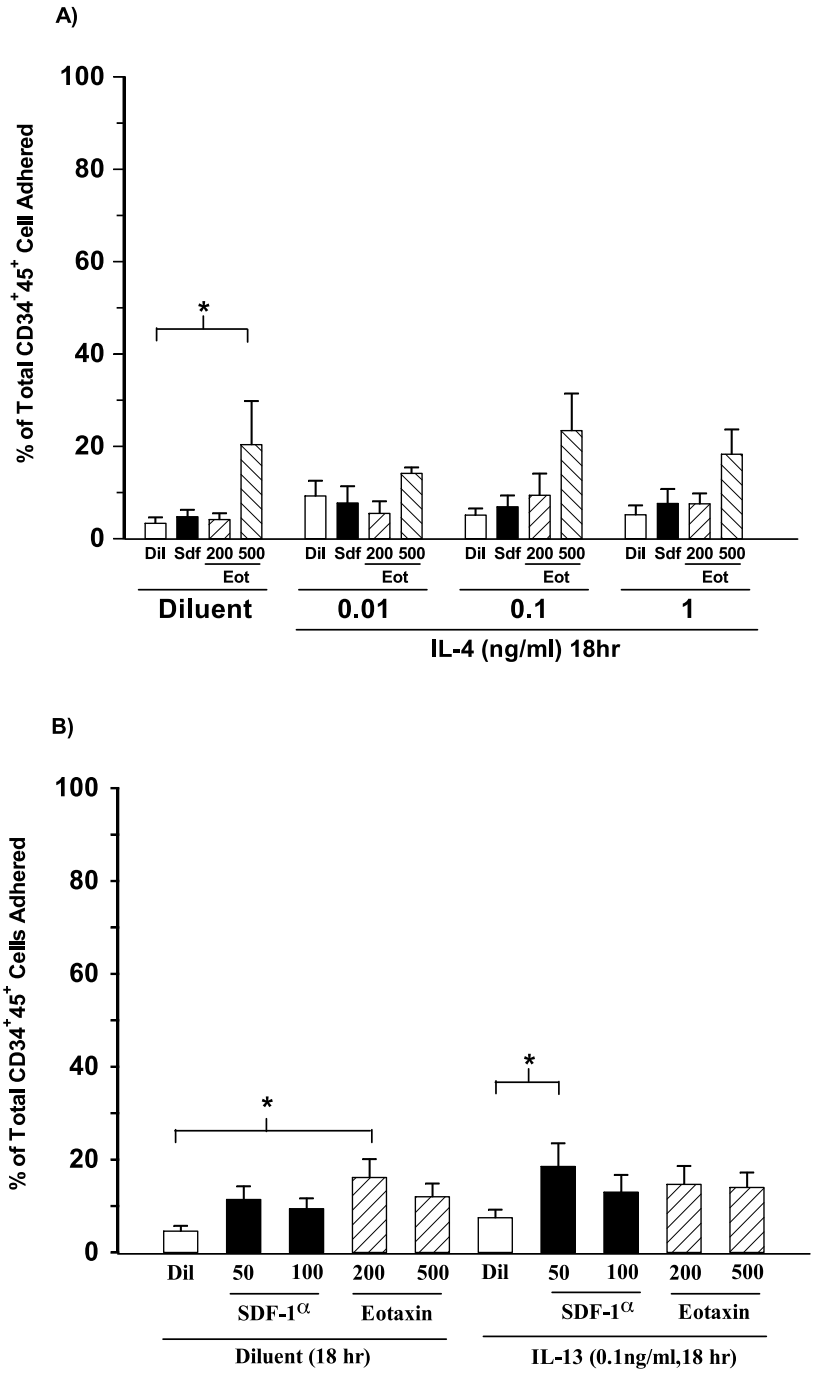

Figure 14. IL-4 and IL-13 priming of CB- derived progenitor cell adhesion (n=8). (A) IL-4 and (B) IL-13 did not prime SDF-1α or eotaxin stimulated adhesion of CB derived HPC to human fibronectin.

CHAPTER 4: DISCUSSION

Allergen induced increases in $CD34^+$ hemopoietic progenitor cells in the bone marrow (Southam DS et al., 2005; Johansson AK et al., 2004) and airways (Dorman SC et al., 2004) suggest that a component of the pathophysiology of asthma involves the traffic of hemopoietic progenitors from the bone marrow to the lung. Once within the lung, HPC have the potential to differentiate *in situ* into pro-inflammatory cells (eosinophils (Southam DS et al., 2005; Dorman SC et al., 2004) and mast cells (Hallgren J et al., 2007)) and tissue structural cells (fibrocytes (Schmidt M et al., 2003) and vascular endothelial cell (Asosingh K et al., 2008; Doyle TM et al., 2011)) directed by the local cytokine milieu (Denburg JA et al., 2006). In addition to providing an ongoing source of inflammatory and structural cells, HPC can directly contribute to allergic inflammation by releasing pro-inflammatory cytokines and chemokines such as IL-5 and IL-13 (Allakhverdi Z et al., 2009). Previous studies have shown that in asthma exacerbations SDF-1, a potent progenitor cell chemoattractant, is down-regulated in the BM (Dorman SC et al., 2005; Catalli AE et al., 2008) and correspondingly increased in the airways in asthma (Southam DS et al., 2005) thus stimulating HPC egress to the circulation and lung-homing in asthma. Investigating the directional cues that promote the migration of HPC will provide novel targets for controlling the lung-homing of HPC and an understanding of the role that these cells play in the pathology of asthma.

In the current thesis we investigated the role of pro-inflammatory T_H2 cytokines, IL-4 and IL-13, in modulating the trans-migrational responses of HPC. We found that although IL-4 and IL-13 did not directly stimulate the directional migration of HPC,

these cytokines prime SDF-1α stimulated migration of CB and PB-derived HPC. The priming effects of IL-4 and IL-13 were specific to HPC as no effects were seen on eosinophil-lineage committed progenitors or mature eosinophils to SDF-1α. This priming effect was not mediated through up-regulation of surface CXCR4 expression but rather through increased incorporation of the SDF-1α receptor, CXCR4, into lipid rafts. These findings suggest that IL-4 and IL-13 may promote SDF-1α mediated homing of bone marrow-derived HPC to the airways in asthma.

4.1 SDF-1α stimulated migration of HPC

SDF-1α is a potent chemoattractant for BM, PB and CB-derived HPC (Auiti et al., 1997) and exerts its chemoattractive and activating functions by binding to its G-protein–coupled receptor, CXCR4. A second receptor for SDF-1, CXCR7, has recently been discovered (Balabanian K et al., 2005). Although signaling through this new receptor is still controversial, recent studies suggest that decoy and scavenger activity of CXCR7 might be important for fine tuning mobility of progenitors in the bone marrow and in the lymphoid organs (Thelen M et al., 2008). Nonetheless, CXCR7 is expressed at very low levels (approximately 3-6%) on human HPC (Tarnowski M et al., 2010) and our findings confirm that CXCR7 has no direct or significant effect on SDF-1α stimulated migration of CB-derived HPC (Table 2).

The SDF-1/CXCR4 axis plays a pivotal role in the homing, trafficking and retention of HPC. While a reduction in the SDF-1/CXCR4 axis is important in the mobilization of progenitor cells from the BM in allergic inflammatory responses (Dorman

SC et al., 2005), increased levels of SDF-1α in the BAL of mice post allergen challenge (Dorman SC et al., 2004) suggest that SDF-1 may contribute to the recruitment of CXCR4 positive HPC to the lung. The concentration of SDF-1α in the BAL fluid of asthmatics is 15 fold higher compared to normal subjects (Negrete-Garcia MC et al., 2010). Furthermore, asthmatic subjects have a greater number of SDF-1-positive cells in the airway mucosa, which inversely correlate with airway caliber and airway hyperresponsiveness (Hoshino M et al., 2007). The most significant cellular sources of SDF-1 in the airway mucosa of asthmatic subjects are endothelial cells, T-lymphocytes and macrophages (Hoshino M et al., 2007). In addition, Hoshino et al. have previously reported increased SDF-1 immunoreactivity in bronchial biopsies from patients with asthma and associated this chemokine with angiogenesis and airway remodeling (2003). The role of the SDF-1/CXCR4 axis in the lung-homing of HPC is strengthened by the ability of the CXCR4 antagonist, AMD 3100, to significantly inhibit allergen-induced accumulation of progenitor cells in the lung (Doyle TM et al., 2011). AHR and BAL eosinophilia were also down regulated following treatment with AMD3100 thus highlighting the effect of this antagonist on asthma-like symptoms. These studies suggest a central role for the SDF-1α/CXCR4 axis in stimulating lung-homing of HPC in allergic diseases such as asthma.

4.2 IL-4 & IL-13 priming of SDF-1α stimulated migration of HPC

Given that increased amounts of IL-4 and IL-13 are found in the BAL of asthmatic subjects post-Ag challenge, we investigated the potential involvement of these T_H2

cytokines in modulating the lung homing of HPC in asthma (Robinson DS et al., 1992; Kroegel C et al., 1996). We found that CB and PB-derived HPC exposed to IL-4 and IL-13 exhibit an enhanced migrational response to SDF-1α, *in vitro*. The priming effect of IL-4 can be mediated by the type I and type II IL-4 receptor while IL-13 mediates its effect via the type II IL-4 receptor. By comparison, IL-4 was a more potent priming agent compared to IL-13 for HPC migration to SDF-1α. The greater potency of IL-4 can be explained by the greater relative abundance of IL-4Rα compared to IL-13Rα1 in cells expressing both type I and II IL-4 receptors (Wills-Karp M et al., 2008). In addition to this, both the type I and II IL-4 receptor consist of the IL-4Rα chain allowing IL-4 to activate both receptors while IL-13 can only signal via the type II receptor. Even though IL-4 is a more effective priming agent, IL-13 may have a greater role in promoting the migration of HPC *in vivo* because IL-13 is produced in considerably greater quantity, by more cells and for longer period of time than IL-4 during a T_H2 dependent response (Wills-Karp M et al., 2008; Munitz A et al., 2008; Huang SK et al., 1995). Although, HPC will likely be exposed to both IL-4 and IL-13 *in vivo*, our findings show that these cytokines do not have an additive or synergistic effect on SDF-1α stimulated migration of HPC.

4.3 IL-4 & IL-13 priming of SDF-1α stimulated migration is selective to primitive progenitor cells

Our data suggests that IL-4 and IL-13 priming of SDF-1α stimulated migration is specific to primitive non-lineage committed progenitor cells, as these two cytokines did

not prime the migrational response of eosinophil-lineage committed progenitor cells ($CD34^+45^+IL\text{-}5R\alpha^+$ cells) or mature eosinophils to SDF-1α. In this thesis we did not investigate why this difference exists but as cells differentiate the response of cells to a stimulus may be altered. For instance, while we found that IL-4 and IL-13 do not effect surface expression of CXCR4 on HPC, these cytokines have been shown to down-regulate the expression of CXCR4 on mature eosinophils (Hiroyuki N et al., 2000). This down-regulation of CXCR4 expression may in part explain the inability of IL-4 and IL-13 to enhance the migrational responsiveness of mature eosinophils to SDF-1α. On the contrary, IL-13 did prime eotaxin-stimulated migration, which is a stronger chemoattractant than SDF-1α for eosinophils. Even though IL-4 has been shown to up-regulate the expression of CCR3 on eosinophils, it did not prime the migrational response to eotaxin (Jinquan T et al., 1999). However, IL-4 has been shown to prime migrational responses of eosinophils to RANTES (Regulated upon activation normal T cell expressed and secreted) (Dubois GR et al., 1998). Although IL-4 and IL-13 do not prime SDF-1α stimulated migration of eosinophils, this does not rule out the potential of these cytokines to prime migration of eosinophils to other chemokine. We have identified a unique pathway by which IL-4 and IL-13 may work to enhance the selective recruitment HPC to the site of inflammation.

4.4 Mechanism of IL-4 & IL-13 priming of SDF-1α stimulated migration of HPC

To determine the mechanism by which IL-4 and IL-13 prime SDF-1α stimulated migration of HPC, we first examined the effect of these cytokines on the expression of the SDF-1α receptor, CXCR4. We found that both IL-4 and IL-13 do not cause a specific up-

regulation of surface CXCR4 expression, suggesting that these cytokines must be increasing post-receptor signaling events. Recent studies have shown that for optimal signaling CXCR4 requires association with lipid rafts (Ratajczak MZ et al., 2006; Wysoczynski M et al, 2005; Nguyen DH et al, 2002). Wysoczynski et al. have shown that the complement cleavage fragment, C3a anaphylatoxin, can increase the migrational responsiveness of HPC to an SDF-1 gradient by promoting the association of CXCR4 and Rac1 with membrane lipid rafts (Wysoczynski M et al., 2005; Wysoczynski M et al., 2007). Thus, an alternative explanation for IL-4 and IL-13 priming is that these cytokines increase SDF-1α stimulated migration by enhancing the incorporation of CXCR4 into lipid rafts.

Our results show that priming by IL-4 and IL-13 of HPC migration to SDF-1α is mediated by the increased incorporation of CXCR4 into lipid rafts as this can be inhibited by disrupting the cholesterol content of the cell membrane. Previous studies have shown that stimulation of CXCR4 by SDF-1α results in rapid translocation of Rac1 to the cell plasma membrane and activation of Rac1 in HPC (Chae HD et al., 2008). Rac1 is essential for dynamic remodeling of actin cytoskeleton elements and for the formation of a cortical F-actin structure at the cell periphery, which is critical for directed cell migration (Small JV et al., 2002). Lipid rafts allow CXCR4 and Rac1 to co-localize in the plasma membrane and thereby facilitate activation of Rac-1 by CXCR4 (Wysoczynski M et al., 2005; Cancelas JA et al., 2005). It has been shown that enhanced activation of Rac1

by CXCR4 increases SDF-1α induced chemotaxis of HPC (Chae HD et al., 2008) (Figure 15).

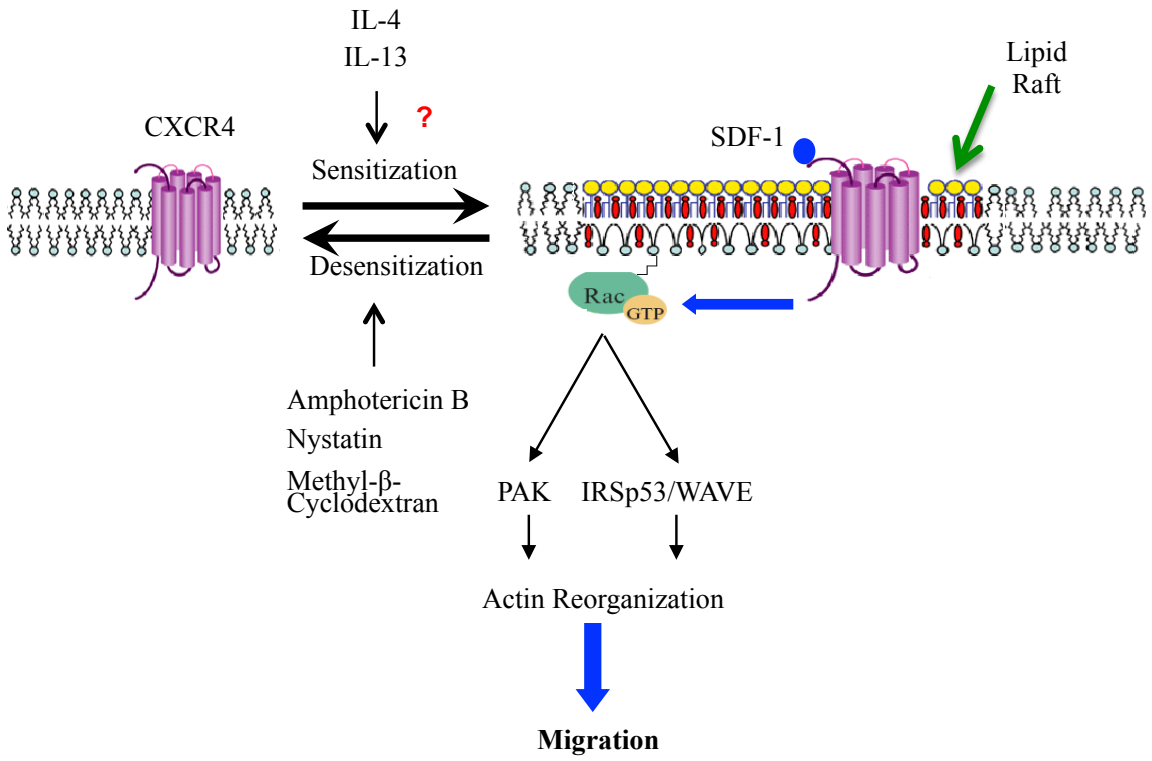

Figure 15. Mechanism of IL-4 & IL-13 Priming

The mechanism by which IL-4 and IL-13 promote the incorporation of CXCR4 in lipid rafts is not known but it has been proposed that increased binding of hydrophobic acids such as palmitate and myristic acid to G-protein coupled receptors promotes the localization to lipid rafts and association with signaling moieties (Qanbar R et al., 2003; Resh MD, 2006; Patel HH et al., 2008).

4.5 IL-4 & IL-13 priming of SDF-1α and eotaxin stimulated adhesion of HPC

In addition to being a potent progenitor cell chemoattractant, SDF-1α stimulates adhesion of progenitor cells to extracellular matrix (ECM) components and vascular endothelial cells. SDF-1 modulates the firm adhesive interactions of progenitor cells to ECM components such as fibronectin by the selective activation of LFA-1/ ICAM-1 and VLA-4/VCAM-1 interactions (Chan JY et al., 2001). Moreover, SDF-1 expressed on vascular endothelial is crucial for translating rolling adhesion of $CD34^+$ cells into firm adhesion by increasing the adhesiveness to the endothelial ligands, VCAM-1 and ICAM-1 (Peled A et al., 1999). Given the involvement of SDF-1 in modulating adhesive interactions, we investigated whether IL-4 and IL-13 prime SDF-1α stimulated adhesion of HPC to human fibronectin. We found that both IL-4 and IL-13 had no effect on SDF-1α stimulated adhesion of CB-derived HPC to fibronectin. These findings suggesting that although IL-4 and IL-13 have a role in promoting migration of HPC to SDF-1α, these cytokines do not prime SDF-1α stimulated adhesion of HPC to fibronectin. Eotaxin stimulated adhesion was also investigated as selective activation of VLA-4 by eotaxin is a mechanism for more efficient recruitment of eosinophils to the lung (Lintomen L et al., 2008). However, both IL-4 and IL-13 did not prime eotaxin-stimulated adhesion of HPC to human fibronectin.

4.6 Limitations

4.6.1 Primary Source of HPC

The three main sources of HPC are bone marrow (BM), peripheral blood (PB) and umbilical cord blood (CB). Hematopoiesis takes place in the BM, where majority of HPC reside (1-3% of nucleated WBC in BM; Sutherland et al., 1994). Under steady state, approximately 1% of the HPC in the BM are mobilized to PB (Aiuti et al., 1997). The number of immature $CD34^+$ progenitor cells released from the BM is significantly higher in infants immediately after birth, making CB a rich source of HPC (0.5% of nucleated WBC in CB; Lapidot et al., 2002). The fact that CB yields a greater number of HPC, is readily available and is the most cost effective source makes it a preferable source of HPC.

The aim of this current thesis is to show that IL-4 and IL-13 promote the lung homing of mobilized PB HPC by enhancing SDF-1α stimulated migration. Since the priming of SDF-1α stimulated migration by IL-4 on PB-derived HPC was comparable to the priming effect on CB-derived HPC, the primary source of HPC used was CB. Voermans et al. compared the migrational responses of $CD34^+$ cells isolated from PB and CB and suggest that differences exist between progenitor cells derived from these two sources (1999). The first observation was that the spontaneous migration of CB-derived $CD34^+$ cells was greater compared to PB-derived $CD34^+$ cells (Voermans et al., 1999). This difference is unlikely to affect our results as the priming effect of IL-4 and IL-13 is described as the enhanced migrational response of CB-derived HPC incubated with IL-4 and IL-13 compared to CB-derived HPC incubated with diluent. In addition, our data

shows that IL-4 and IL-13 prime SDF-1α and not eotaxin stimulated migration, suggesting that a greater spontaneous migration does not influence the specific priming effects of IL-4 and IL-13. Voermans et al. also found CB-derived $CD34^+$ cells to have a higher expression of CXCR4 compared to PB-derived $CD34^+$ cells. Although the baseline migrational responses of CB-derived HPC to SDF-1α may be higher than PB-derived HPC, the enhanced migration towards SDF-1α by IL-4 and IL-13 should be detectable in HPC from both sources. Moreover, the finding that IL-4 and IL-13 mediate the priming effect by enhancing incorporation of CXCR4 into lipid rafts suggest that priming is not associated with receptor expression but rather with enhanced signaling of CXCR4. The lower expression of CXCR4 on PB-derived HPC may however limit the priming effect, as there are less CXCR4 receptors that can be incorporated into lipid rafts. Since we assessed the enhanced and not the absolute migrational response of CB-derived HPC to SDF-1α and have shown that IL-4 has a comparable priming effect on the migrational response of HPC derived from PB of atopic asthmatic subjects, our findings using CB derived HPC should be applicable to mobilized PB HPC.

4.6.2 Sample Variability

In analyzing over 100 CB samples on the flow cytometry, it was evident that $CD34^+$ cell populations are noticeably different amongst samples. Although this had no effect on our ability to accurately identify HPC, it indicated that differences exist between samples. In several birth cohort studies, CB analyses have shown that the functional phenotype of $CD34^+$ CB cells is altered in infants at high atopic risk compared to low-risk

infants (determined by parental history and maternal allergy skin tests) (Fernandes R et al., 2008). Dr. Denburg's group has shown that CB progenitors of high-risk infants have a reduced expression of IL-5Rα, IL-3Rα, GM-CSFRα, TLR-2, TLR-4 and TLR-9 compared to low risk infants (Reece P et al., 2011). Furthermore, $CD34^+$ cells isolated from CB of high-risk infants give rise to more Eo/B CFU following IL-3, IL-5 and GM-CSF stimulation compared to low risk infants (Reece P et al., 2011). These studies suggest that the maternal atopic milieu may affect the functional phenotype of CB HPC. Although the differences in CXCR4 expression or responses to SDF-1α between high atopic risk and non-atopic infants have not been studied, the migrational responses between theses groups could potentially be altered.

For the ease of obtaining an adequate supply of CB samples, we required no medical information from our participants. It is possible that had our participants been assigned groups based on their atopic status, we may have obtained different results. In the future, a study comparing the migrational responses of CB HPC obtained from high atopic risk and low-risk infants will allow us to determine whether difference in the migrational responses of HPC re present at birth.

4.6.3 Limitations of in vitro studies

The process of progenitor cell isolation requires cells to be removed from their physiological environment for at least 5 hrs prior to the priming experiments. CXCR4 expression maybe altered during the separation process and may influence the migrational response of HPC to SDF-1α. It has previously been noted that positively selected BM

$CD34^+$ cells have a spontaneous increase in CXCR4 expression compared to NAMNC (Thomson et al., 2007 unpublished observations). Furthermore, our current data shows that there is a temperature dependent increase in CXCR4 expression on HPC incubated at 37˚C for 18hr (Figure 11). A spontaneous or temperature dependent change in CXCR4 expression during the separation process may not significantly influence our results as we studied the enhanced and not absolute migrational response of HPC to SDF-1α.

4.6.4 CD34 Enrichment

Fifty thousand $CD34^+$ cells were added to each transwell insert to assess the migrational response of HPC to SDF-1α. However, not all $CD34^+$ cells are HPC as CD34 is also be expressed by endothelial cells, mast cells and a subset of fibroblast and dendritic cells. Although we can further negatively select these cells for non-HPC specific surface markers (i.e. CD3, CD10, CD14, CD19, CD20, CD40, CD56, CD83, CDw125 (IL-5 receptor) and FceR1), it is difficult to exclude the possibility that other $CD34^+$ cells were present in our population and could have manipulate the results we observed. It is difficult to overcome this limitation but after the transmigration assay, migrated cells were analyzed on the flow cytometry to ensure that only the migrational response of true HPC was assessed.

4.6.5 Transwell Migration Assay

The tranwell migration assay is an excellent method to assess the migrational responsiveness of cells to a chemokine. The 5μm pores in the polycarbonate membrane

filter allow transmigration of only migratory/active HPC towards the chemoattractant in the lower well. A limitation of this assay, however, is that the migration of cells from the blood vessel to the lung is a much more complex process than that simulated by transwell inserts. The components of *in vivo* transendothelial migration absent in this model are the adhesive interactions between migratory cells and endothelial cells and a subendothelial basement membrane. To better mimic *in vivo* transendothelial migration, human umbilical vein endothelial cells (HUVEC) can be grown on transwell filters prior to the transmigration experiment. However, given the variability at which CB samples are collected it will be difficult to coordinate sample availability with a confluent monolayer that ready to be used for a migration assay. An achievable alternative to this is to coat transwell filters with a basement membrane extract (laminin I, type IV collagen, entactin, and heparin sulfate proteoglycan; R&D Systems), which will then limit transmigration to invasive cells (i.e. capable of degrading the basement membrane matrix). Although coating transwell filters with both the basement membrane extract and endothelial monolayer will best mimic *in vivo* transmigration, addition of anyone of these techniques will improve our transmigration assay.

4.7 Conclusions

In conclusion, our findings suggest that the pro-inflammatory T_H2 cytokines, IL-4 and IL-13, may promote SDF-1α mediated homing of bone marrow-derived HPC to the airways in asthma.

1) We found that although IL-4 and IL-13 are not chemoattractants for CB-derived HPC, pre-incubating CB and PB-derived HPC with these cytokines significantly enhanced SDF-1α stimulated migration, *in vitro*.

2) The priming effect of IL-4 and IL-13 on SDF-1α stimulated migration was specific to HPC as no effects were seen on eosinophil-lineage committed progenitors or mature eosinophils.

3) Priming of SDF-1α stimulated migration of HPC was not mediated through up-regulation of surface CXCR4 expression but rather through increased incorporation of the SDF-1α receptor, CXCR4, into lipid rafts.

4) IL-4 and IL-13 did not prime SDF-1α or eotaxin stimulated adhesion of HPC to human fibronectin, suggesting that these cytokines do not promote transendothelial migration of HPC.

4.8 Future Directions

4.8.1 How does co-localization of CXCR4 and lipid rafts enhance migration of HPC to SDF-1α?

The finding that T_H2 cytokines such as IL-4 and IL-13 prime SDF-1α stimulated migration of HPC suggests a novel role for these cytokines in promoting the lung-homing of progenitor cells. Even though this thesis clearly shows that IL-4 and IL-13 priming is mediated through enhanced co-localization of CXCR4 and lipid rafts, further investigation is needed to understand how increased incorporation of CXCR4 into lipid rafts increases migrational responsiveness to SDF-1α. As mentioned in the discussion section above, SDF-1α stimulation results in rapid translocation of Rac1 to the cell membrane, where Rac1 co-localizes with lipid rafts and induces F-actin polymerization (Chae HD et al., 2008). We hypothesize that increased incorporation of CXCR4 into lipid rafts by IL-4 and IL-13 enhances Rac1 signaling, which then by its effects on actin cytoskeleton elements results in increased SDF-1α stimulated migration.

Future studies will investigate whether IL-4 and IL-13 increase SDF-1α induced activation of Rac1 and localization to the cell membrane. The Rac1 pull down assay (Rac activation assay kit 17-283; Upstate Biotechnology, Lake Placid, NY) will be used to determine the percent of Rac1 proteins activated (i.e. GTP-bound Rac) in response to SDF-1α stimulation. If IL-4 and IL-13 enhance SDF-1α induced Rac1 activation, the percent of activated Rac1 proteins should be higher in cells incubated with IL-4 and IL-13 prior to SDF-1α stimulation. To study the effect of IL-4 and IL-13 on SDF-1α induced

localization of Rac1, we will transduce cells with a retroviral vector expressing EGFP-tagged Racl, which can then be visualized using a fluorescent microscope. If IL-4 and IL-13 promote SDF-1α induced localization of Rac1 to the cell membrane, cells incubated with IL-4 and IL-13 prior to SDF-1α stimulation will have increased localization of Rac1 to the cell periphery compared to cells incubated with diluent.

Rac1 regulates cell directed migration by its effect on actin polymerization (Chae HD et al., 2008). If IL-4 and IL-13 enhance Rac1 signaling, the formation of filamentous actin in HPC following IL-4 and IL-13 incubation should be increased. In the actin polymerization assay, CD34 enriched cells exposed to IL-4, IL-13 or diluent for 10-120 min with 10 min intervals will be stained for CD34, CD45 and Alexa 488 phalloidin (Molecular Probes to visualize the F-actin) and the mean fluorescent intensity (MFI) of F-actin will be measured using flow cytometry. According to our hypothesis, cell exposed to IL-4 and IL-13 should have a higher intensity of F-actin compared to diluent.

4.8.2 Other potential priming agents of HPC migration?

Allakhverdi et al. have recently shown that circulating $CD34^+$ cells express receptors for thymic stromal lymphopoietin (TSLP) and IL-33 and respond to these cytokines by rapidly releasing high levels of pro-inflammatory T_H2-like cytokines and chemokines (i.e. IL-5, IL-13, GM-CSF, IL-6, CXCL8, CCL1, CCL17 and CCL22) (Allakhverdi et al., 2009). Both TSLP and IL-33 are released predominately by epithelial cells and are expressed more abundantly in the lung of asthmatics compared healthy

individuals (Bulek K et al., 2010). Mice expressing a TSLP transgene in the airway epithelium develop a spontaneous, progressive inflammatory disease with cardinal features of asthma while a disruption of IL-33 signaling during the course of experimental asthma or anaphylaxis reduces the severity of disease, *in vivo* (Fang C et al., 2010; Smith DE et al., 2009). Given that these cytokines are abundantly present in asthmatics and $CD34^+$ progenitor cells can respond to these cytokines, future studies will investigate the effects of TSLP and IL-33 on SDF-1α stimulated migration of HPC. TSLP and IL-33 activate the pro-inflammatory activity of $CD34^+$ cells in a synergistic manner, hence priming experiments will also examine the effects of TSLP and IL-33 in combination.

Furthermore, the idea that $CD34^+$ cells release significant amounts of pro-inflammatory T_H2 cytokines such as IL-13 suggest that progenitor cells can promote their own SDF-1α stimulated migration by releasing IL-13 or other potential priming agents. If TSLP and IL-33 prime migrational responses of HPC, it will be important to determine whether TSLP and IL-33 have a direct priming effect or is it due to IL-13 or other potential priming agents released by HPC in response to TSLP and IL-33.

4.8.3 Can growth factors such as TGF-β prime HPC migration? (Preliminary data)

To determine whether TGF-β can prime HPC migration, CB-derived HPC were pre-incubated with TGF-β (0.05, 0.5, 5 ng/ml) or diluent for 18 hr at 37°C prior to the transmigration assay. At the optimal priming concentration of 0.5 ng/ml, TGF-β increases the migratory response to SDF-1α by 18.61 $\pm$ 7.76% (Figure 16).

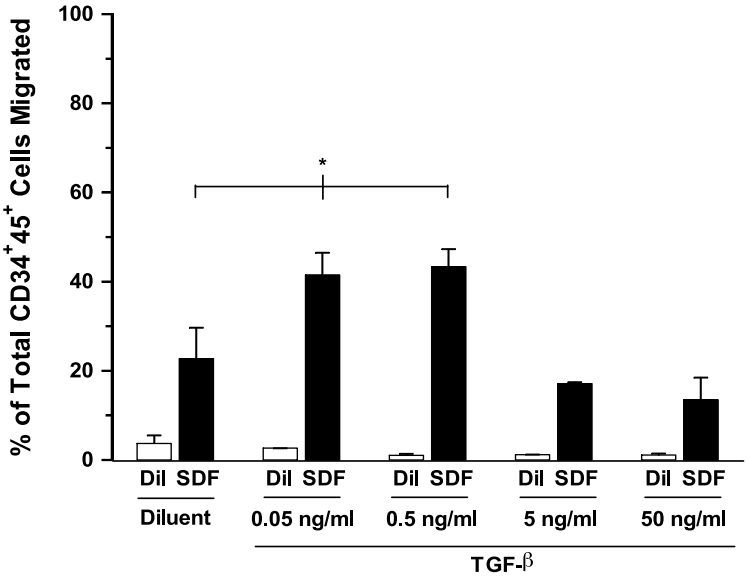

Figure 16. TGF-β priming of CB-derived HPC transmigration. Pre-incubation with TGF-β (0.05 & 0.5ng/ml) for 18hr primes the migrational response of HPC towards the optimal dose of SDF-1α (100 ng/ml).

In addition to IL-4 and IL-13 the growth factor, TGF-β, primes SDF-1α stimulated migration of HPC. Studies involving monocytic dendritic cells, T cells and eosinophils have reported that TGF-β primes migratory response to SDF-1 by up-regulating the expression of CXCR4 (Sato K 2000; Nagase H 2000; Franitza S 2002). In addition to being a priming agent, a study by Basu and Broxmeyer suggests that TGF-β1 helps retain the responsiveness of $CD34^+$ progenitor cells to SDF-1 in the presence of sustained exposure to SDF-1 (2005). This is important because prolonged exposure of cells to SDF-1 induces desensitization and internalization of CXCR4. These findings suggest that TGF-β not only promotes SDF-1 mediated migration but also retention of HPC at sites of increased SDF-1 expression (i.e. bone marrow, inflamed tissues).

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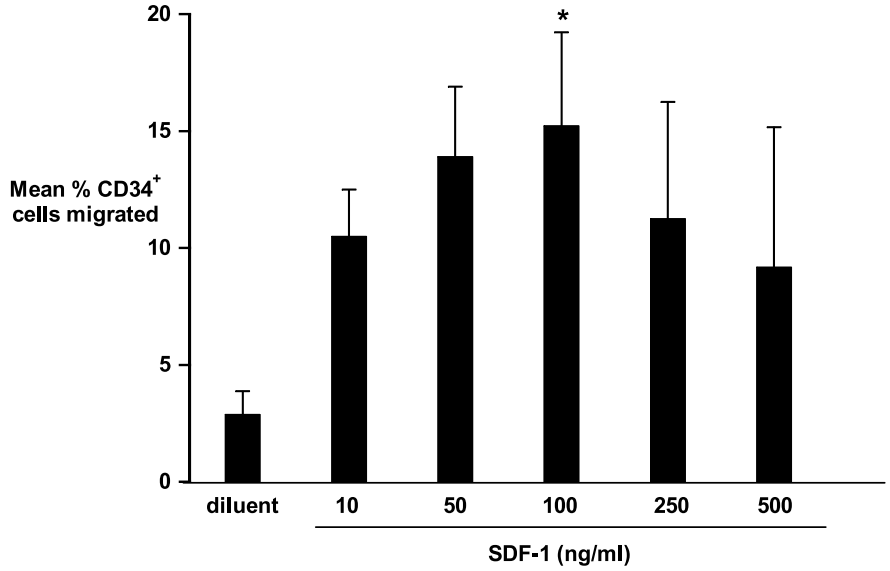
APPENDIX A – IL-4 PRIMING OF PB-DERIVED HPC FROM NON-ATOPIC SUBJECTS

File Name	Dil + Dil	Dil + Sdf	IL-4 0.01 + Dil	IL-4 0.01 + Sdf
Normal #1	2.72	53.19	5.44	66.99
Normal #2	1.12	29.22	3.67	39.1
Normal #3	0.76	25.95	1.91	29.01
Normal #4	0.66	28.29	1.32	46.71
Average	1.32	34.16	3.09	45.45

Peripheral blood was collected from n=4 normal healthy subjects and the priming effect were similar to those seen for HPC from atopic bloods (Figure 8). No difference between these groups was observed.

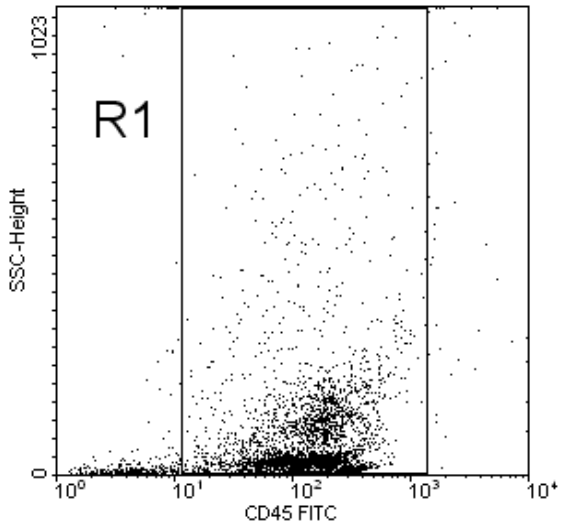
APPENDIX B – SDF-1α STIMULATED MIGRATION OF CB-DERIVED HPC



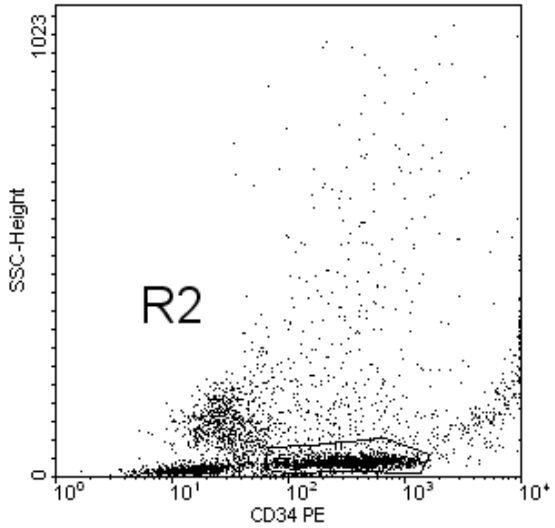
SDF-1α-stimulated migration of CB HPC (n=8). SDF-1α stimulated the dose-dependent migration of HPC optimal at 100ng/ml (*$p<0.05$). Data are expressed as mean ± standard error. (Thomson J. MSc Thesis, 2008)

APPENDIX C – FACS HPC GATING STRATEGY

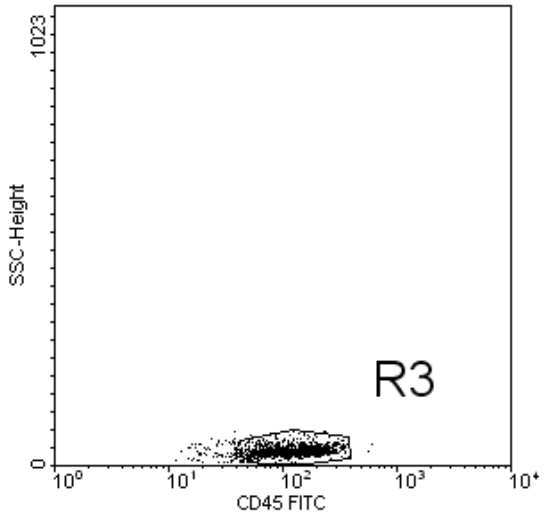
Plot 1.



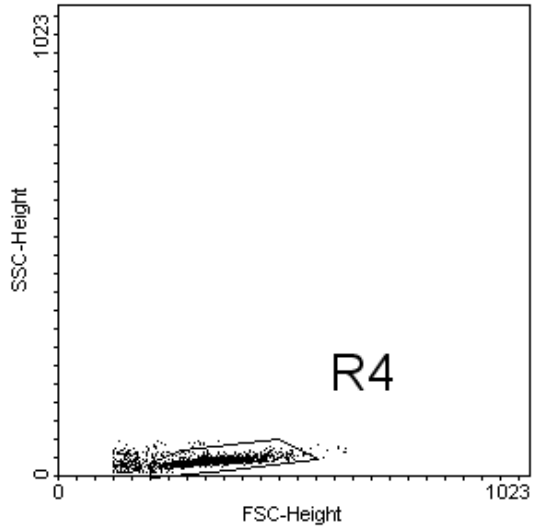
Plot 2.



Plot 3.



Plot 4.

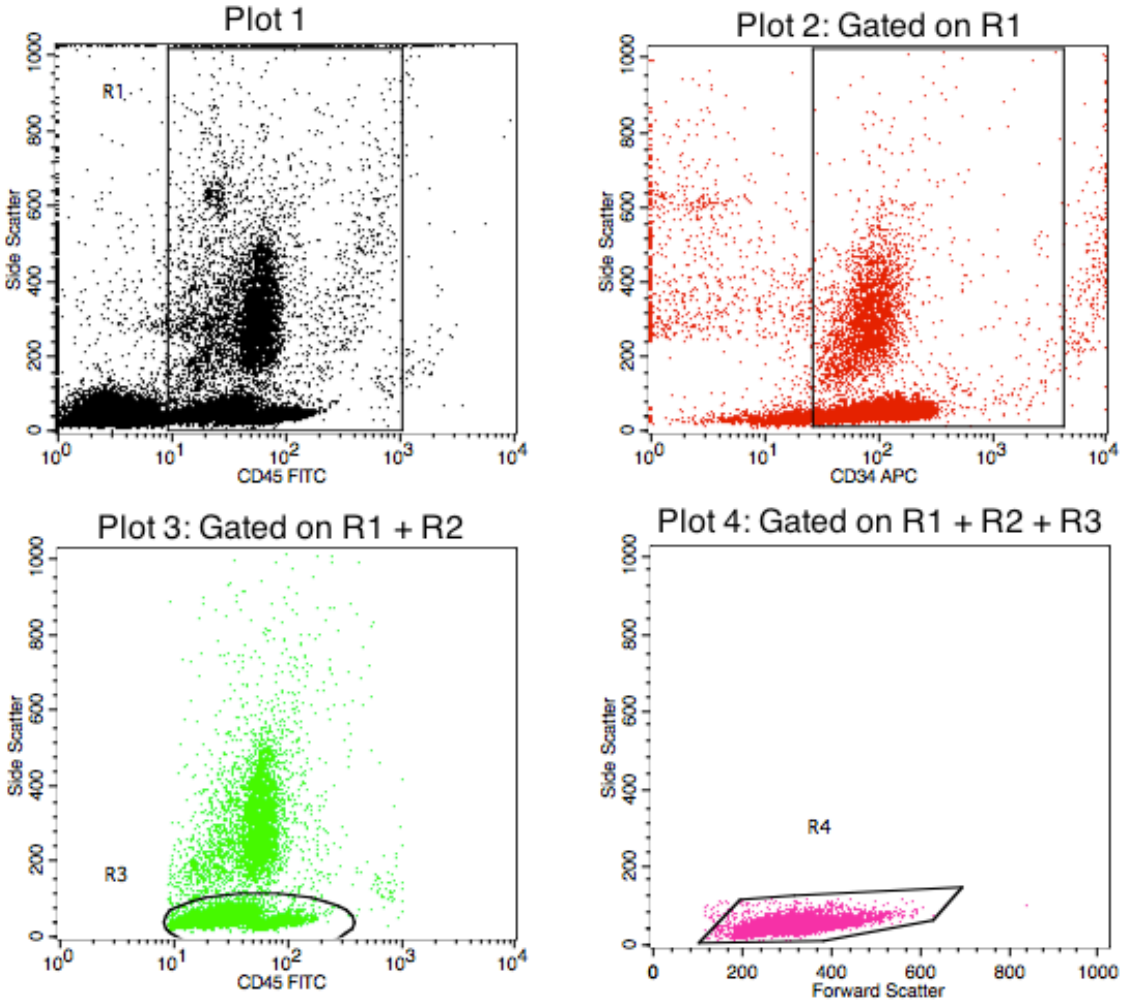


Four-step FACS gating strategy for CB-derived $CD34^+45^+$ cells as described in section 2.3.5. Briefly, region (R1) represents all nucleated WBCs. R2 frames $CD34^+CD45^+$ cells. $CD34^+$ progenitor cells were further identified based on their low granularity and low CD45 expression (region R3). The progenitor cells in R4 display low to medium cell size and low granularity. Enumeration data were obtained from the gate statistics for regions R1 to R4.

APPENDIX D - CXCR4, IL-4Rα & IL-13Rα1 EXPRESSION ON PROGENITORS

D1) CXCR4 Expression on HPC & Eosinophil Lineage Committed Progenitors

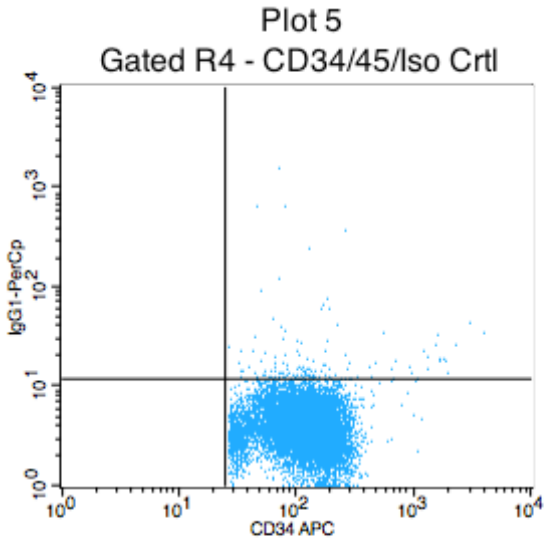
Plot 1-4. Gating of HPC ($CD34^+CD45^+$). True $CD34^+$ blast cells were identified as cells with $CD45^{low}/CD34^{high}$ staining and low forward (FSC) and side scatter (SSC).



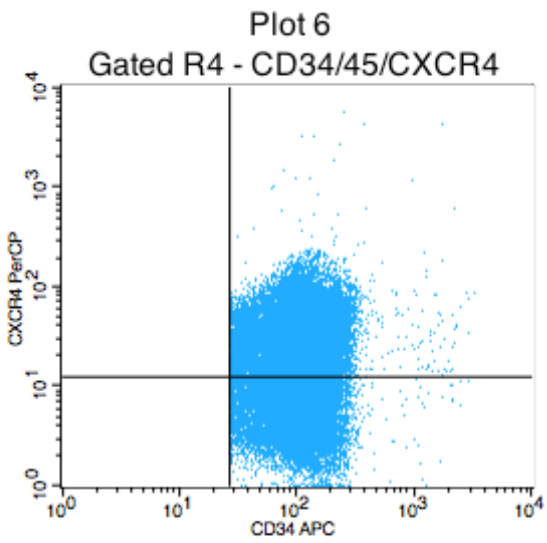
Region Statistics

Region	Events	% Gated	% Total	Px,Py
R1	69330	68.92	68.92	3, 2
R2	69100	68.69	68.69	7, 2
R3	57112	56.78	56.78	3, 2
R4	84994	84.50	84.50	1, 2
R5	5268	5.24	5.24	7, 4

Plot 5-6: Expression of CXCR4 on HPC ($CD34^+CD45^+$). Events in region R4 (true progenitor cells) were further analysed for staining with PerCp-linked isotype control or CXCR4 mAb (Plot 5&6). 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). Data show 65.35% of Cord blood-derived $CD34^+45^+$ cells express CXCR4.

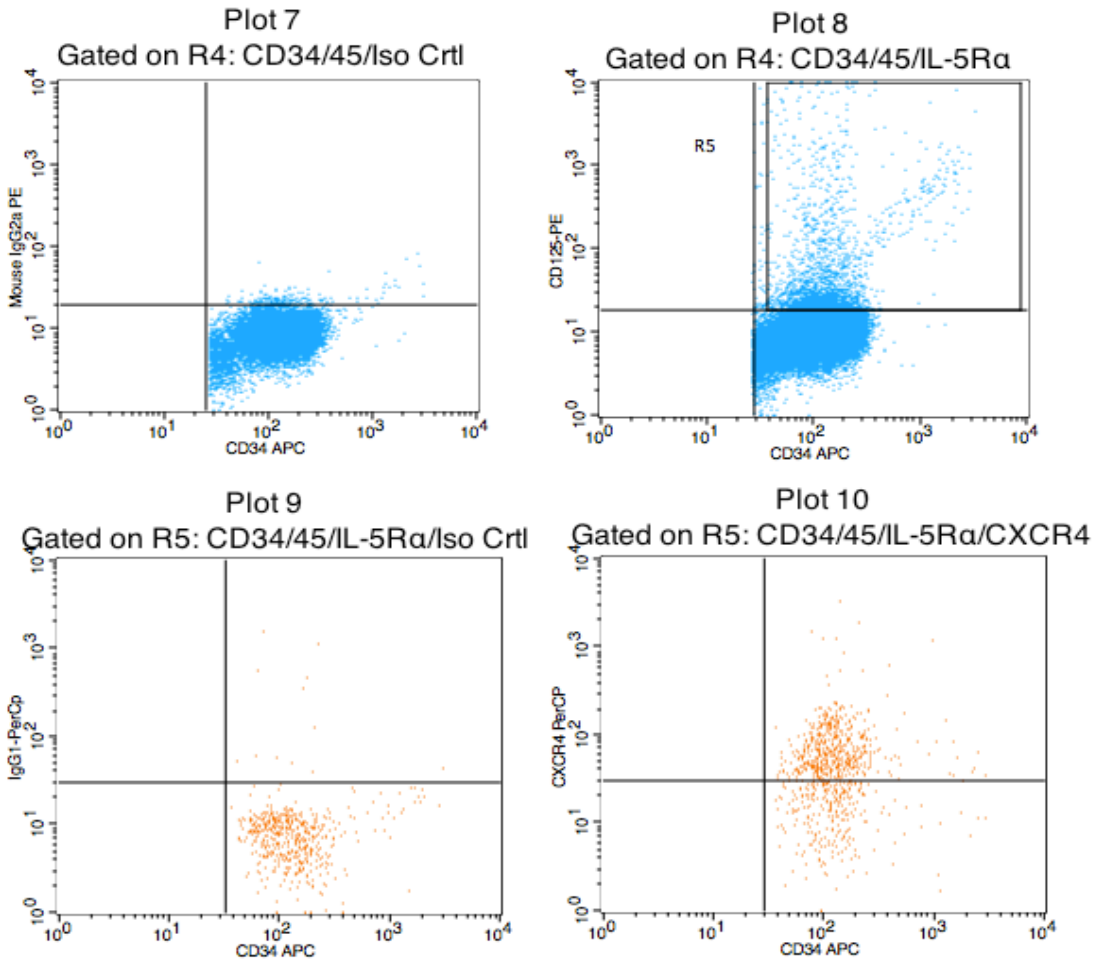


Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	358	0.99	0.49
LL	0	0.00	0.00
LR	35786	99.01	49.07



Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	33681	65.35	33.48
LL	0	0.00	0.00
LR	17856	34.65	17.75

Plot 7-10: Expression of CXCR4 on Eosinophil Lineage Committed Progenitors ($CD34^+CD45^+IL\text{-}5R\alpha^+$). Events in region R4 (true progenitor cells) were also analysed for staining with PE-linked isotype control or IL-5Rα mAb (Plot 7&8). Region 5 identified $CD34^+45^+IL\text{-}5R\alpha^+$ (Plot 8: eosinophil-lineage committed progenitors) which were then further analysed for expression of PerCp-linked isotype control or CXCR4 mAb (Plot 9&10). 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). Data show 66.97% of Cord Blood-derived $CD34^+45^+$ cells express CXCR4.

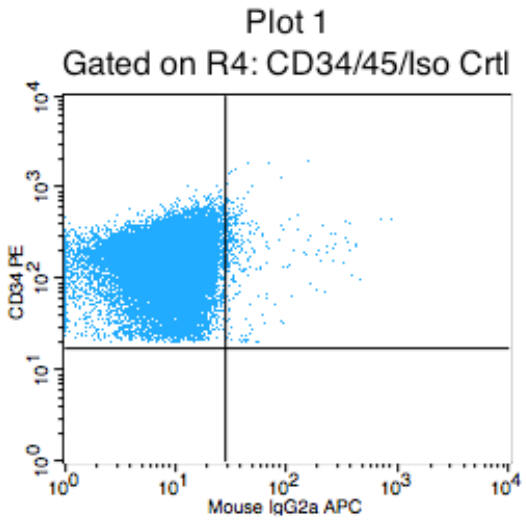


Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	53	3.12	0.07
LL	0	0.00	0.00
LR	1648	96.88	2.26

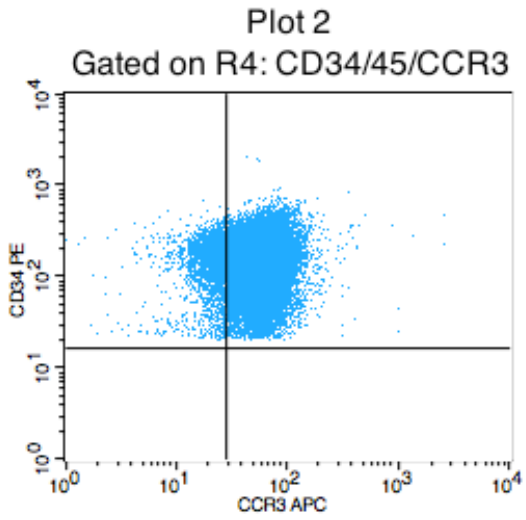
Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	1740	66.97	1.73
LL	0	0.00	0.00
LR	858	33.03	0.85

D2) Expression of CCR3 on HPC

Plot 1-2. Expression of CCR3 on HPC ($CD34^+CD45^+$). Events in region R4 (true progenitor cells) were further analysed for staining with APC-linked isotype control or CCR3 mAb (Plot 1&2). 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). Data show 88.24% of cord blood-derived $CD34^+45^+$ cells express CCR3.



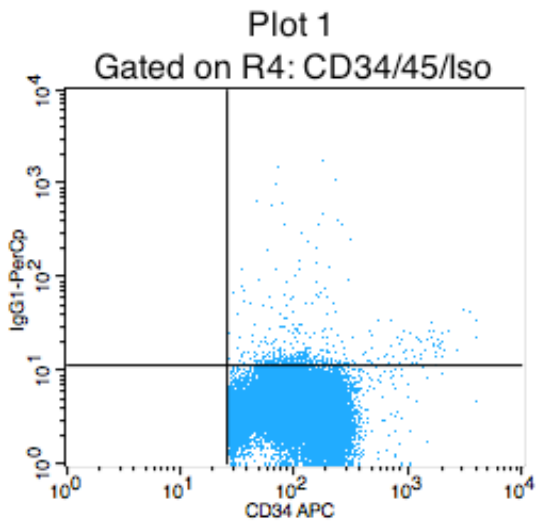
Quad	Events	% Gated	% Total
UL	29567	98.92	60.10
UR	323	1.08	0.66
LL	0	0.00	0.00
LR	0	0.00	0.00



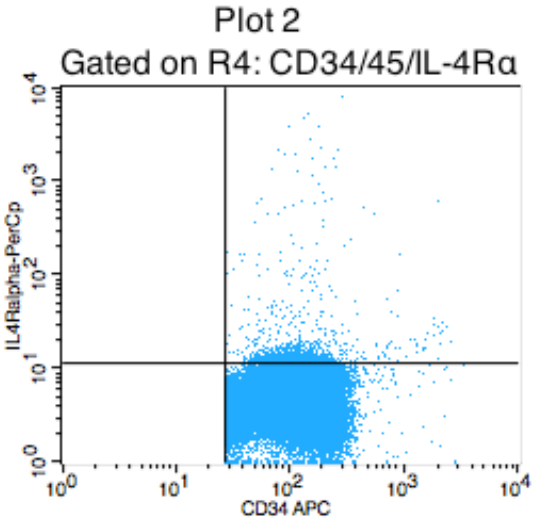
Quad	Events	% Gated	% Total
UL	3438	11.76	7.08
UR	25808	88.24	53.15
LL	0	0.00	0.00
LR	0	0.00	0.00

D3) Expression of IL-4Rα on HPC & Eosinophil Lineage Committed Progenitors

Plot 1-2. Expression of IL-4Rα on HPC ($CD34^+CD45^+$). From the previous figure, events in region R4 (HPC cells; Plot 1&2) were analysed for staining with PerCp-linked isotype control or IL-4Rα mAb. 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). Data show 1.897% of cord blood-derived HPC ($CD34^+45^+$) express IL-4Rα.

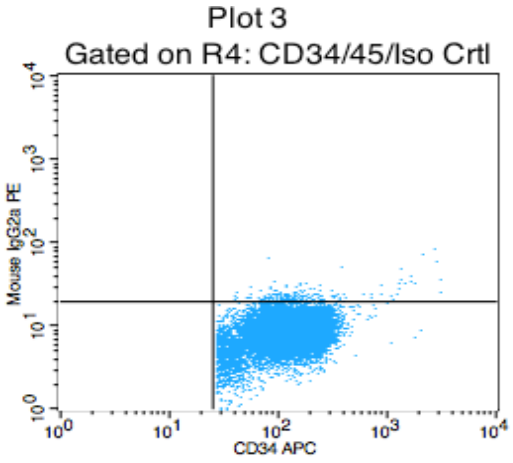


Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	436	1.21	0.60
LL	0	0.00	0.00
LR	35708	98.79	48.96

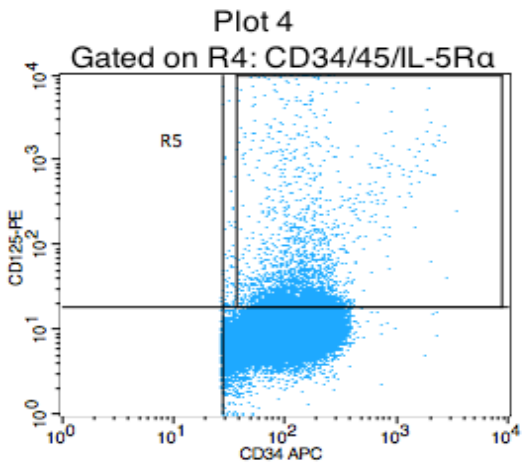


Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	1128	1.89	1.01
LL	0	0.00	0.00
LR	58526	98.11	52.25

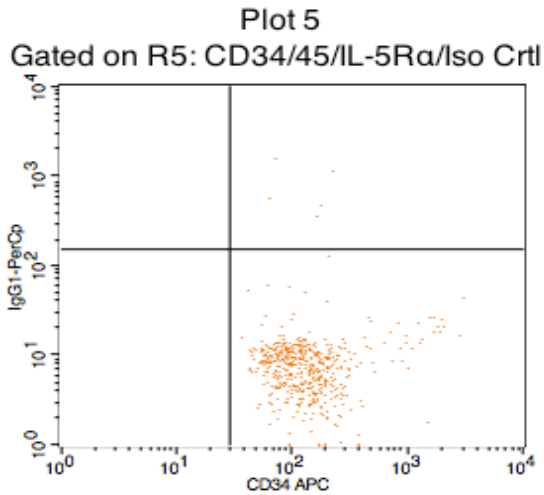
Plot 3-6. Expression of IL-4Rα on Eosinophil Lineage Committed Progenitors ($CD34^+CD45^+IL\text{-}5R\alpha^+$). From the previous figure**,** events in region R5 (eosinophil lineage committed progenitors; Plots 3&4) were analysed for staining with PerCp-linked isotype control or IL-4Rα mAb. Data were 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). Data show 1.43% of eosinophil-lineage committed progenitors express IL-4Rα.



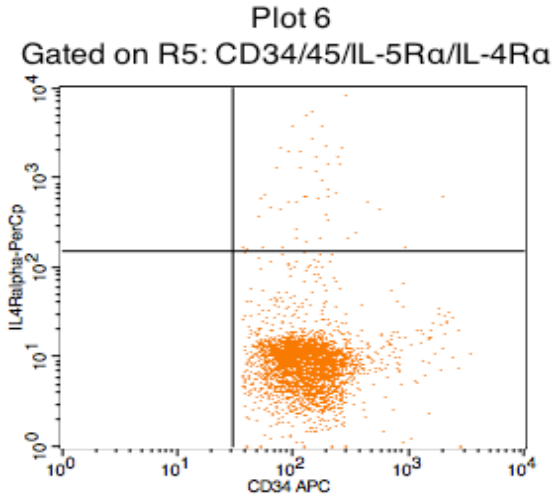
Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	362	0.94	0.48
LL	0	0.00	0.00
LR	38161	99.06	51.10



Quad	Events	% Gated	% Total
UL	5	0.01	0.00
UR	3317	5.56	2.96
LL	250	0.42	0.22
LR	56082	94.01	50.07



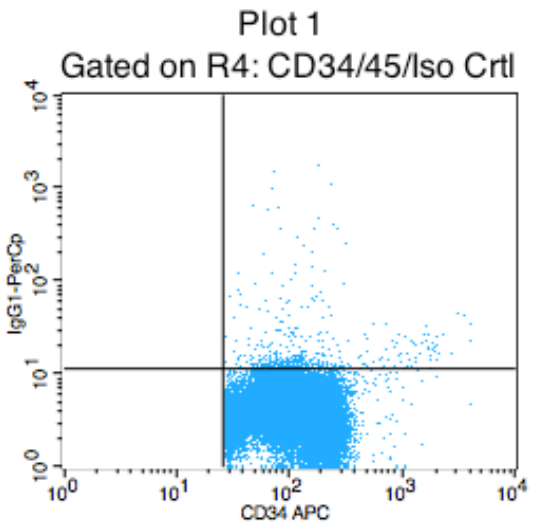
Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	17	1.00	0.02
LL	0	0.00	0.00
LR	1684	99.00	2.31



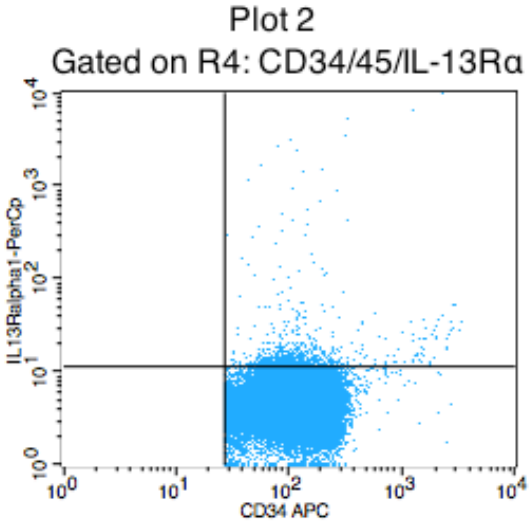
Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	48	1.43	0.04
LL	0	0.00	0.00
LR	3302	98.57	2.95

D4) Expression of IL-13Rα1 on HPC & Eosinophil Lineage Committed Progenitors

Plot 1-2. Expression of IL-13Rα1 on HPC ($CD34^+CD45^+$). From the previous figure, events in region R4 (HPC cells; Plot 1&2) were analysed for staining with PerCp-linked isotype control or IL-13Rα1 mAb. 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). Data show 2.33% of cord blood-derived HPC ($CD34^+45^+$) express IL-13Rα1.

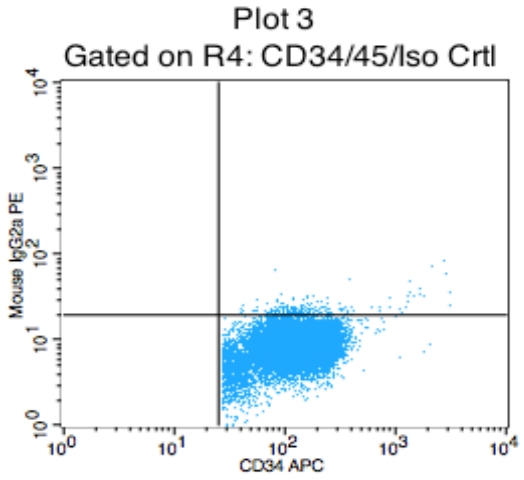


Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	436	1.21	0.60
LL	0	0.00	0.00
LR	35708	98.79	48.96

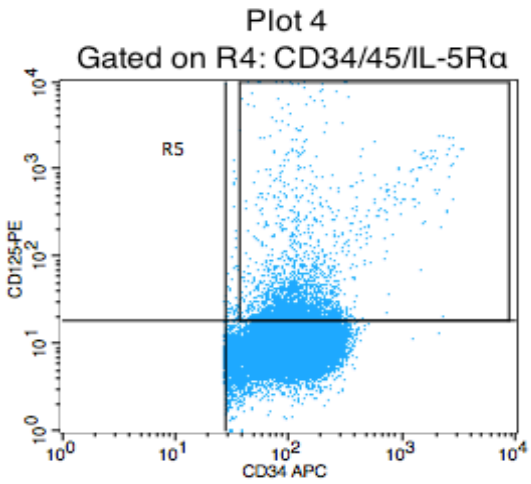


Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	900	2.33	1.24
LL	0	0.00	0.00
LR	37756	97.67	52.20

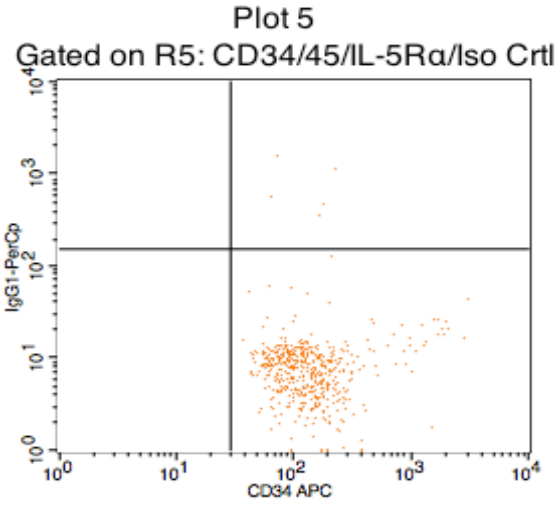
Plot 3-6. Expression of IL-13Rα1 on Eosinophil Lineage Committed Progenitors ($CD34^+CD45^+IL\text{-}5R\alpha^+$). From the previous figure, events in region R5 (eosinophil lineage committed progenitors; Plots 3&4) were analysed for staining with PerCp-linked isotype control or IL-13Rα1 mAb. Data were 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). Data show 0.91% of eosinophil-lineage committed progenitors express IL-13Rα1.



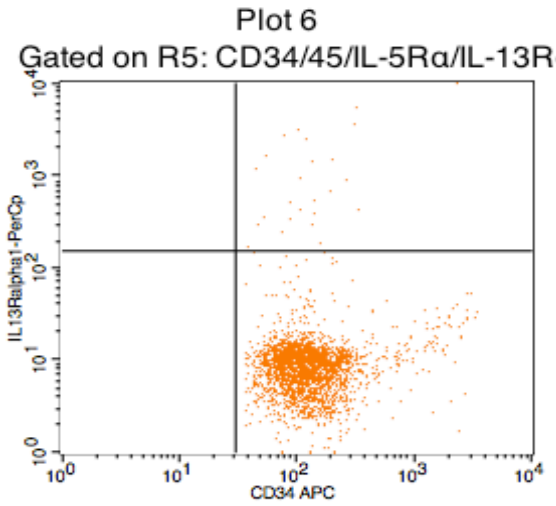
Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	362	0.94	0.48
LL	0	0.00	0.00
LR	38161	99.06	51.10



Quad	Events	% Gated	% Total
UL	3	0.01	0.00
UR	2732	7.07	3.78
LL	151	0.39	0.21
LR	35770	92.53	49.45



Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	17	1.00	0.02
LL	0	0.00	0.00
LR	1684	99.00	2.31



Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	25	0.91	0.03
LL	0	0.00	0.00
LR	2732	99.09	3.78

APPENDIX E – INFORMATION AND CONSENT FORM

PARTICIPANT INFORMATION SHEET

Title of Study: Modulation of cord blood-derived CD34+ progenitor cell migrational and adhesive responses

Local Principal Investigator/Department/School/Programme/Hospital/Institution:

Dr. Roma Sehmi/Medicine/McMaster University/SJHH/Firestone Institute for Respiratory Health (FIRH)

Co-investigator(s)/Department/School/Programme/Hospital/Institution:

Navneet Punia/Medical Sciences/McMaster University/ St.Joseph's Healthcare/FIRH

Sponsor: Canadian Institutes of Health Research (CIHR)

You are being invited to participate in a research study conducted by Dr. Sehmi and Navneet Punia because you have indicated that you do not wish to save the umbilical cord blood for banking. This is a student research project conducted under the supervision of Dr. Sehmi. The study will help Ms. Punia learn more about the role of progenitor cells in asthma and develop skills in research design, collection and analysis of data, and writing a research paper.

In order to decide whether or not you want to be a part of this research study, you should understand what is involved and the potential risks and benefits. This form gives detailed information about the research study, which will be discussed with you. Once you understand the study, you will be asked to sign this form if you wish to participate. Please take your time to make your decision. Feel free to discuss it with your friends and

family, or your family physician. The Firestone Institute for Respiratory Health at St. Joseph's Healthcare, McMaster University and Dr. Sehmi are under contract with the Sponsor of this study and are receiving compensation to cover the costs of conducting this study.

Why is this research being done?

Our studies show that precursor cells (immature blood cells) play a role in asthma, a disease of the airways. In newborns these immature cells are located in the umbilical cord blood and therefore, this sample would provide us with the desired cells for future studies.

What is the purpose of this study?

The purpose of this study is to improve our understanding of the way in which the precursor cells move to the lungs during asthma.

What will my responsibilities be if I take part in this study?

If you agree to participate we will ask you to:

- Following the birth of your baby, allow a nurse to withdraw 30-60ml of blood from the umbilical vein in the placenta.
- No patient medical information is necessary.
- This study is completely anonymous and there are no follow up questions or procedures.

What will be done with my cord blood during and after the study?

Precursor cells will be separated from the cord blood sample and experiments to study cell movement will be performed. No other tests will be performed on the blood from your umbilical cord, and as soon as this study is completed, the cells will be disposed of

in the usual manner. No samples will be kept and all findings and study records will be kept private.

What are the possible risks and discomforts?

By giving your consent to participate there will be no additional risks or discomforts to your health.

How many people will be in this study?

We will be approaching 2 to 3 expectant mothers per week to obtain cord blood samples for the duration of one year. The total number obtained depends on the number of consenting expectant mothers.

What are the possible benefits for society and me?

As this is a basic research study there will be no immediate benefits to you or society. However, in future this research will extend our knowledge of asthma and perhaps lead to the development of better drugs to treat this condition.

If I do not want to take part in the study, are there other choices?

It is important for you to know that you can choose not to take part in this study. If you choose not to participate in the study, this will not influence your current or future medical care in any way. If your doctor has concerns about risks during delivery, the collection of a research sample will not be performed.

What information will be kept private?

Your data will not be shared with anyone except with your consent or as required by law. All personal information such as your name will be removed from the data and will be replaced with a number. A list linking the number with your name will be kept in a secure

place, separate from your file. The data, with identifying information removed will be securely stored in a locked office in the research laboratory. The data for this research study will be retained for 10 years.

For the purpose of ensuring proper monitoring of the research study, it is possible that a member of the St. Joseph's Healthcare Hamilton Research Ethics Board and representatives of Canadian Institutes for Health Research may consult your research data. However, no records that identify you by name or initials will be allowed to leave the hospital. By signing this consent form, you or your legally acceptable representative authorize such access.

Should you choose to participate in this study and the results of the study are published, your identity will remain anonymous.

Can participation in the study end early?

You may withdraw from this study at any time and this will not influence the quality of care you receive from this institution. You also have the option of removing your cord blood sample or data collected from the sample from the study at any time.

Will I be paid to participate in this study?

You will not be paid for your participation in this study.

Will there be any costs?

Your participation in this study will not involve any additional costs to you or your healthcare provider.

What happens if I have a research-related injury?

If you are injured as a direct result of taking part in this study, all necessary medical treatment will be made available to you at no cost. Financial compensation for such things as lost wages, disability or discomfort due to this type of injury is not routinely available. However, if you sign this consent form it does not mean that you waive any legal rights you may have under the law, nor does it mean that you are releasing the investigator(s), institution(s) and/or sponsor(s) from their legal and professional responsibilities.

If I have any questions or problems, whom can I call?

If you have any questions about the research now or later, you may contact Dr. Roma Sehmi at (905) 522-1155 ext. 35927 or Navneet Punia at (905) 522-1155 ext. 33869.

Also, if you have any questions regarding your rights as a research participant, you may contact the Office of the Chair of the Research Ethics Board, St. Joseph's Healthcare Hamilton, and (905) 522-1155 ext. 33537.

CONSENT FORM

Signature of research participant

I have read the preceding information thoroughly. I have had the opportunity to ask questions, and all of my questions have been answered to my satisfaction. I agree to participate in this study. I understand that I will receive a signed copy of this form. I consent to participate in this study.

_____

Name of Participant (please print)

_____ _____

Signature of Patient Date

Consent form administered and explained in person by:

_____

Name and title

_____ _____

Signature Date

Signature of Witness to Participant's Signature:

My signature as witness, certifies that I witnessed the participant (or the participant's legally authorized representative) voluntarily sign this consent form in my presence.

_____ _____

Signature Date

SIGNATURE OF INVESTIGATOR:

In my judgment, the participant is voluntarily and knowingly giving informed consent and possesses the legal capacity to give informed consent to participate in this research study.

_____ _____

Signature of Investigator Date