LUNG-HOMING OF EPC AND ANGIOGENESIS IN ASTHMA: ROLE OF EOSINOPHILS

LUNG-HOMING OF ENDOTHELIAL PROGENITOR CELLS AND ANGIOGENESIS IN A MOUSE MODEL OF ASTHMA: ROLE OF EOSINOPHILS

By

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ABSTRACT:

Asthma involves a systemic element that includes the mobilization and lung-accumulation of bone marrow-derived endothelial progenitor cells (EPC). This traffic may be driven by the stromal cell derived factor-1α (SDF-1α)/CXCR4 axis, where SDF1-α is a potent progenitor cell chemoattractant.

Interfering with EPC lung-accumulation by administering AMD3100, a CXCR4 antagonist, was previously shown to be associated with the modulation of airway angiogenesis and airway hyperresponsiveness. However, since eosinophils express CXCR4, it is unknown whether AMD3100 acted directly on EPC or indirectly through its anti-inflammatory effects on eosinophils.

We investigated the role that eosinophilic inflammation plays in the lung-homing of EPCs and airway angiogenesis in allergic asthmatic response by utilizing eosinophil deficient (PHIL) mice.

Wild-type BALB/c (WT) and PHIL mice underwent a chronic house dust mite (HDM) exposure protocol. Treatment groups were administered AMD3100. Outcome measurements were made 24hrs post final exposure and included: flow cytometry to enumerate lung-extracted EPCs, immunostaining for von Willebrand factor to assess bronchial vascularity, bronchoalveolar lavage for airway inflammation, haematoxylin and eosin stain to enumerate eosinophils, picrosirius red stain to assess collagen deposition, and measurement of airway resistance to increasing intranasal doses of methacholine.

HDM exposed mice had a significant increase in EPC lung accumulation, bronchial vascularity, airway inflammation, collagen deposition and airway

hyperresponsiveness (AHR) in both WT and PHIL groups, with some indices at lower levels in PHIL mice. Concurrent treatment with AMD3100 significantly attenuated EPC lung homing, bronchial vascularity, eosinophil numbers in lung tissue and AHR, but not collagen deposition in WT mice. AMD3100 treatment significantly attenuated all indices in PHIL mice.

The findings of this study show that, EPC-driven angiogenesis and the development of AHR in allergic airway responses are independent of eosinophils, the presence of these cells, however, may have a role in worsening of the pathology of allergic airways disease.

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LIST OF ABBREVIATIONS

Ab	Antibodies
AHR	Airway Hyperresponsiveness
Ag	Allergen
ANOVA	Analysis of Variance
AREB	Animal Research Ethics Board
ASM	Airway Smooth Muscle
BAL	Bronchoalveolar Lavage
BM	Bone Marrow
Brd U	5'-bromo-2'deoxyuridine
CCR	C-C Chemokine Receptor
CFU	Colony Forming Unit
CDC	Center for Disease Control
Col	Collagen
COPD	Chronic Obstructive Pulmonary Disease
CXCR	C-X-C Chemokine Receptor
DC	Dendritic cell
EAR	Early Asthmatic Response
EC	Endothelial Cell
ECP	Eosinophil Cationic Protein
EDN	Eosinophil-derived Neurotoxin
EDTA	Ethylenediaminetetraacetic acid
EOG-EPC	Early Outgrowth EPC
EPO	Eosinophil Peroxidase
EPC	Endothelial Progenitor Cell
Eo/B	Eosinophil/Basophil
FACS	Fluorescent Activated Cell Sorting
FEV_1	Forced Expiratory Volume in 1 Second
FGF	Fibroblast Growth Factor
FVC	Forced Vital Capacity
FITC	Fluorescein Isothiocyanate
GCM	Goblet Cell Metaplasia
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GFP	Green Fluorescent Protein
HDM	House Dust Mite
H&E	Haematoxylin and Eosin
HLA	Human Leukocyte Antigen
HPC	Hematopoietic Progenitor Cell
HSPGs	Heparan Sulfate Proteioglycans
ICAM-1	Intracellular Adhesion Molecule 1
IgE	Immunoglobulin E
IL	Interleukin
i.n.	Intranasal

LAR	Late Asthmatic Response
LOG-EPC	Late Outgrowth EPC
LTC_4	Leukotriene C4
MACS	Magnetic-Activated Cell Sorting
MBP	Major Basic Protein
MCh	Methacholine
MIP	Macrophage Inflammatory Protein
MMP-1	Matrix Metalloproteinase 1
MSC	Mesenchymal Stem Cell
MVD	Microvessel Density
NGF	Nerve Growth Factor
NaN3	Sodium Azide
OVA	Ovalbumin
PECAM-1	Platelet Endothelial Cell Adhesion Molecule 1
PerCP	Peridinin Chlorophyll Protein
PBS	Phosphate Buffer Solution
PB	Peripheral Blood
PC_{20}	Provocative Concentration Causing a 20% fall in FEV_1
PDGFR	Platelet-derived Growth factor Receptor
PE	Phycoerythrin
PFA	Paraformaldehyde
PGD_2	Prostaglandin D2
PSR	Picrosirius Red Stain
RTK	Receptor Tyrosine Kinase
Sca-1	Stem-cell Antigen-1
SDF-1	Stromal-Derived Factor 1
SEM	Standard Error of the Mean
SSC	Side Light Scatter
TBS	Tris-buffered Saline
TGF	Transforming Growth Factor
T_H	T helper
TLR-4	Toll-like Receptor 4
TNF-a	Tumour Necrosis Factor alpha
TSLP	Thymic Stromal Lymphopoietin
VCAM-1	Vascular Cell Adhesion Molecule 1
vWF	Von Willebrand Factor
VEGF	Vascular Endothelial Growth Factor
ZO-1	Tight Junction Protein 1

CHAPTER 1: INTRODUCTION

1.1 Prevalence

Asthma is a chronic inflammatory disease affecting more than 300 million people worldwide (Urbano F, 2008) with annual deaths estimated at 250 000 (World Health Organization [WHO], 2007) and prevalence rates increasing by 50% every decade (Holmes AM, Solari R, Holgate ST, 2011). In Canada, numbers are rising with approximately 3 million individuals (8.5% of the population aged 12 and over) currently affected (Asthma, 2010) and 146 000 emergency room visits annually due to asthma exacerbations (Canadian Lung Association, 1994 Update). Asthma is also the most common childhood disease affecting at least 13% of Canadian children and is a major cause of hospitalizations (Garner R and Kohen D, 2008; Millar WJ and Gerry BH, 1998). Additionally, global economic costs associated with asthma exceed those of tuberculosis and HIV/AIDS combined (Braman SS, 2006). The increased economic and childhood impact of asthma emphasises the necessity for a better understanding of the mechanisms of asthma pathology to provide potential therapeutic targets to better treat this healthcare burden.

1.2 Clinical Manifestations of Asthma

The clinical symptoms of asthma include recurrent episodes of wheezing, breathlessness, chest tightness and coughing. Physiologically, it is characterized by reversible airflow obstruction which is associated with inflammation of the airways and airway hyperresponsiveness (AHR) (Murphy DM and O'Byrne PM, 2010).

Airway responsiveness refers to the ability of the airway to narrow following inhalation of a constrictor agonist (such as methacholine and histamine). AHR describes the combination of hypersensitivity and hyperreactivity; hypersensitivity being an increased sensitivity of the airway in developing this response, therefore requiring a smaller concentration of constrictor agonist to initiate bronchoconstriction, and hyperreactivity is characterized by a greater magnitude of constriction (O'Byrne PM and Inman MD, 2003). AHR is related to disease severity and is often used as a defining characteristic in asthma diagnosis (Townley and Horiba 2003).

Spirometry is a commonly used tool to diagnose asthma. In this test, an individual inhales or exhales volumes of air as a function of time providing multiple indices to assess lung function (Miller MR et al., 2005). The most important indices for assessing airway obstruction are forced expiratory volume in one second (FEV_1), the volume of air exhaled in the first second of a forced expiration from full inspiration, and forced vital capacity (FVC) which is the maximum volume of air that can be exhaled following full inspiration (Miller MR et al., 2005). A ratio of FEV_1/FVC less than 0.75 suggests airflow limitation (GINA, 2011). The provocative concentration $(PC)_{20}$ is the concentration of constrictor agonist required to cause a 20% fall in FEV_1 compared to baseline values (Lemanske RF and Busse WW, 2003). To measure AHR, a bronchoprovocation test is performed where measurements with a spirometer are made after the patient inhales incremental doses of methacholine (MCh). A PC_{20} of 16mg/ml or more in the absence of corticosteroid therapy indicates normal lung function whereas a PC_{20} less than 8mg/ml of

MCh suggests the patient is asthmatic (Lemanske RF and Busse WW, 2003; O'Byrne PM and Inman MD, 2003), however, individuals with COPD may also display these values.

To diagnose asthma, FEV_1/FVC is measured at a baseline and 15-20 minutes after administering a fast acting bronchodilator (such as 200µg albuterol). An improvement by more than 12% and 200mL indicates reversibility of the airflow obstruction and suggests the individual may have asthma and not irreversible obstructive diseases such as COPD (chronic obstructive pulmonary disease) (Bateman et al., 2008).

1.3 Inflammation and Asthma

A key feature of allergen-induced asthma is chronic inflammation of the airway (Boyce JA et al., 2009). Following inhalation, allergens encounter dendritic cells lining the airway subepithelium. These dendritic cells bind to the antigen (allergen) and migrate to draining lymph nodes, where antigen presentation to T-cells occurs. This results in an increased number of T-helper 2 cells (T_H2 cells) which produce cytokines including interleukin (IL)-4,5,9, and 13. T_H2 cytokines and co-stimulatory factors are involved in activating B-cells to produce Immunoglobulin E (IgE) antibody. Allergen-specific IgE can then circulate throughout the blood and bind to IgE receptors (FcεRI or FcεRII) found on inflammatory cells (mast cells, basophils, lymphocytes, eosinophils) stimulating the inflammatory activity of these cells during subsequent allergen exposure (Busse WW and Lemanske RF, 2001; Gauvreau GM and Evans MY, 2007).

Asthma can be divided into early and late phase responses:

1.3.1 Early Phase Response

Once allergen sensitization occurs, subsequent exposure to the sensitizing agent results in an inappropriate reaction to an otherwise innocuous agent; i.e. an allergic asthmatic response. Asthmatic responses are characterized by an 'early asthmatic response' (EAR) with a key feature being bronchospasm by smooth muscle cells following allergen exposure. When subjected to allergen challenge, the EAR occurs within 10 min of inhalation, reaches maximal bronchial contraction within 30 minutes and resolves within 3 hours. The EAR is characterized by a fall in FEV_1 by at least 15-20% from baseline (Gauvreau GM and Evans MY, 2007).

In EAR, the allergen binds to IgE antibodies produced by the initial sensitization phase (Boyce JA et al., 2009). The allergen initiates cross-linking of IgE anti-bodies which stimulate mast cells and basophils to release preformed mediators such as histamine, leukotrienes and prostaglandin D2 (PGD_2) which cause bronchoconstriction and vascular permeability (Gauvreau GM and Evans MY, 2007). In addition, these mast cell derived mediators can stimulate the influx of other pro-inflammatory cells, such as T-lymphocytes, eosinophils, basophils and neutrophils into the airways (Afshar R, Medoff BD, and Luster AD, 2008; Averbeck et al. 2007; Platts-Mills T, Leung DY, and Schatz M, 2007).

1.3.2 Late Phase Response

In 50% of individuals, a subsequent late asthmatic response (LAR) occurs between 4 and 8hrs after allergen challenge, measured by a fall in FEV_1 of 15% or greater (Gauvreau GM and Evans MY 2007; Robertson DG et al., 1974). More commonly

associated with chronic inflammation, LAR is characterized by increased bronchovascular permeability, increased mucus secretion, a significant increase in AHR as measured by a drop in methacholine PC_{20} which is associated with infiltration of the airway by inflammatory cells (Gauvreau GM and Evans MY, 2007; Afshar R, Medoff BD, and Luster AD, 2008; Gauvreau GM et al. 2000; Robertson DG et al., 1974). The main inflammatory cells involved are eosinophils and T_H2 cells, but bronchial biopsies from asthmatics also show increases in tissue numbers of mast cells and basophils (Busse WW and Lemanske RF, 2001).

1.3.3 Eosinophils

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 bone marrow (BM) by hemopoietic signals (Foster PS et al., 2001; Gauvreau GM et al., 2005). The primary activating signal of eosinophils is IL-5, which is released by T_H2 cells and promotes eosinophil proliferation and mobilization from the bone marrow (Wood LJ et al., 2002; Minshall E et al., 1998; Hogan MB et al., 2000). IL-5 promotes migration of eosinophils by up-regulating CCR3 expression on the eosinophil cell surface which is the receptor for eotaxin, the primary mediator of eosinophil migration (Conroy DM et al., 1997; Zeibecoglou K et al., 1999; Stirling RG et al., 2001).

Once recruited to the site of inflammation, eosinophils degranulate and release four functionally different cytotoxic granule basic proteins: eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN). ECP mediates toxic effects by inducing mast cell degranulation and

stimulating airway mucus secretion (Venge P et al., 1999; Hogan SP et al., 2008). MBP increases the release of acetylcholine by blocking vagal muscarinic M2 and M3 receptor, thereby intensifying bronchial responsiveness (Evans CM et al., 2000). MBP also stimulates mast cells and basophils. EPO makes up 25% of the proteins in eosinophil granules and promotes oxidative stress and subsequent cell death (Rothenberg ME and Hogan SP, 2006). All of these mediators promote tissue damage either directly or indirectly through activation of inflammatory cells.

Eosinophils also contribute to inflammation by releasing an array of cytokines (such as IL-4,-5,-13 and TGF-α/β) and chemokines (e.g. eotaxin, RANTES and MIP-1α) (Rothenberg ME and Hogan SP, 2006). Many of the cytokines released by eosinophils (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. Eosinophils have also been shown to directly activate T cells by acting as an antigen-presenting cell (Shi H, 2004).

Although bronchial inflammation is a feature of asthma exacerbation, anti-inflammatory therapies have been largely unsuccessful in completely reversing sustained airway hyperresponsiveness (AHR) and declining lung function in asthmatic subjects as patients continue to suffer with this disease (Jeffery PK, 2001). This emphasizes the necessity for a more complete understanding regarding the mechanisms and lung remodeling changes of this complex disease, which may reveal new potential therapeutic targets that would aid in relieving the public health burden of asthma.

1.4 Airway Remodeling

Although inflammation is a major contributor to asthma disease pathology, the inability for anti-inflammatory treatment to completely reverse asthmatic airway obstruction suggests that other pathological processes may be involved in producing changes in airway function (Leigh R et al., 2002). Other than airway infiltration of inflammatory cells, histopathological studies of resected lung and bronchial biopsies have demonstrated several characteristic structural changes within the airways of asthmatics which relate to the disease severity (Elias JA. et al., 1999; Jeffery PK, 2001; Pascual RM and Peters SP, 2005). These changes include variations in the composition, organization or quantity of cells in the airway wall and are collectively referred as 'airway remodeling' (Warner SM and Knight DA, 2008). Common remodeling features include airway fibrosis, airway smooth muscle hyperplasia and metaplasia, changes to the airway epithelium and changes to airway vascularity (Warner SM and Knight DA, 2008). These changes can contribute to airway obstruction, and have been correlated with loss of lung function in asthmatic individuals (Pascual RM and Peters SP, 2005).

1.4.1 Airway Fibrosis

Airway fibrosis in asthma is largely attributed to an increase in deposition of collagen I, III, fibronectin and proteoglycans (Roche WR et al., 1989). Fibroblasts are thought to be the source of this deposition, as these cells release a variety of cytokines and chemokines which facilitate extracellular matrix deposition in normal wound healing and, once this process is complete, disappear through mechanisms of apoptosis. However in a chronic disease such as asthma, fibroblasts persist and promote tissue remodeling,

resulting in significant sub-epithelial fibrosis, decreasing airway compliance, increasing thickening of the airway and contributing to AHR (Brewster CE et al., 1990; Kunzmann S et al., 2007; Sacco O et al., 2004). It has been proposed that these increased number of tissue fibroblasts may come from either, the trans-differentiation of lung resident epithelial cells to mesenchymal cells (Kim KK et al., 2006) or, as will be described in detail later in section 1.7.3, from bone marrow derived fibrocytes that may traffic and home to the lung where they adopt a fibroblastic phenotype, resulting in an increase of fibroblastic foci and airway fibrosis (Andersson-Sjoland A et al., 2008; Schmidt M et al., 2003; Reilkoff RA et al., 2011). Subepithelial fibrosis has been linked with disease severity, a decline in FEV_1, and AHR to methacholine in human subjects (Boulet LP et al., 1997).

1.4.2 Airway Smooth Muscle

An increase in airway smooth muscle (ASM) mass is commonly observed in asthmatic airways that has been shown through both, examinations of patient airway biopsies and in tissue samples from animal models of allergic asthma (An SS et al., 2007). Studies suggest that this increased ASM mass may be a resultant of hyperplasia which may be attributed to increased rates of division, decreased rates of apoptosis or trans-differentiation of mesenchymal cells to airway smooth muscle bundles (Ebina M et al., 1993; Halayko AJ et al., 2006; Schmidt M et al., 2003; Kuwano K, Bosken CH, and Pare PD, 1993). An increase in ASM cell diameter (hypertrophy) seen in asthmatic airways compared to non-asthmatics may also be a contributing factor to the increase in ASM mass in asthmatics (Hirst SJ et al., 2004; Woodruff PG et al. 2004; Kuwano K,

Bosken CH, and Pare PD, 1993). The increased thickness of the airway wall may contribute to damaging the lung parenchyma and decrease their tethering forces, reducing their resistance to subsequent smooth muscle contractions (Elias JA et al., 1999).

1.4.3 Airway Epithelium

A compromised airway epithelium is common in asthmatic individuals. Epithelial damage results in a more fragile airway epithelium and the inability to form an effective barrier against normally innocuous inhaled agents (Knight DA et al., 2010). Creola bodies (clusters of sloughed epithelial cells) have been observed in asthmatic sputa, increased numbers of epithelial cells in bronchoalveolar lavage (BAL) fluid and loss of surface epithelium in biopsy specimens (Jeffery PK, 2001; Knight DA et al., 2010). The asthmatic epithelium displays a loss of columnar, ciliated cells which is coincident with reduced expression of apical junction proteins such as E-cadherin and ZO-1 (Knight DA et al., 2010). The integrity of the airway epithelium is dependent on apical tight junctions and adherens junctions binding epithelial cells together. Reduced expression of E-cadherin, which is a major constituent of adherens junctions, could facilitate allergic sensitization by activating dendritic cells (by the lack of a tonic inhibitory signal commonly released by E-cadherin interactions with dendritic cells and epithelial cells) as well as enhanced thymic stromal lymphopoietin (TSLP) production (Lambrecht BN and Hammad H, 2012). T_H2 cytokines IL-4 and IL-13 have also been shown to disrupt epithelial barrier function, further perpetuating bronchial inflammation. The release of IL-33 and IL-25 by airway epithelial cells further promotes IL-13 production by nuocytes (innate lymphoid cells) (Lambrecht BN and Hammad H, 2012).

It has also been observed that the greater the loss of surface epithelium in biopsy specimens the greater the degree of airway responsiveness, though the loss of epithelium observed in biopsies of mild asthmatics is highly variable and an unreliable end point of determination of extent of injury or the response to treatment (Jeffery PK, 2001).

Goblet cell metaplasia (GCM) and excessive mucus production is also observed, which contribute to mucus plugging and narrowing of the airway lumen (Knight DA et al., 2010). GCM occurs from the proliferation of extant goblet cells as well as the trans-differentiation of ciliated epithelial cells and Clara cells (Lambrecht BN and Hammad H, 2012). As GCM occurs, they abnormally appear in the small airways of asthmatics, compromising mucociliary clearance and contributing to airflow obstruction (Rogers DF, 2003; Warner SM and Knight DA, 2008; Kim KC et al., 1997). GCM is induced by the T_H2 cytokine IL-13 (Zhu Z et al., 1999).

1.5 Vascular Remodeling and Asthma

Airway vasculature necessitates several functional roles in maintaining homeostasis. These roles include provision of oxygen and nutrients, temperature regulation, humidification of inspired air and allowing a conduit for immune cells to defend against airway pathogens (Walters EH et al., 2008). During chronic inflammatory diseases, the airway vasculature promotes the migration of inflammatory cells to the site of inflammation. As the inflammatory disease evolves, the microvasculature undergoes progressive changes to further supply oxygen and nutrients to the proliferating inflamed tissue. These changes include vasodilation, microvascular leakage (a resultant of

increased vascular permeability), and increased bronchial vascularity through angiogenesis (Pandya NM, Dhalla NS, and Santani DD, 2006; Walters EH et al., 2008; Wilson JW and Hii S, 2006; Puxeddu I et al., 2005b).

1.5.1 Vascular Permeability and Vasodilation

Increased microvascular permeability leading to oedema is a common feature of airway vascular remodeling in asthma (Puxeddu I et al., 2005a). This increased permeability is thought to be due to the formation of intercellular gaps between endothelial cells (EC) along the bronchial vasculature (McDonald DM, Thurston G, and Baluk P, 1999). This increased vascular permeability may be from the interaction of endothelial cells with inflammatory mediators such as histamine released by activated mast cells (Bradding P and Holgate ST, 1999; Chetta A, et al., 2007). Mast cell activation during an asthmatic exacerbation has been identified to result in plasma leakage and oedema which may contribute to the thickening of the airway, obstruction of air flow and directly increase AHR (Bradding P and Holgate ST, 1999; Wilson JW and Hii S, 2006).

Increased vessel calibre has also been identified in asthmatic lungs (Puxeddu I et al., 2005a; Carroll NG, Cooke C, and James AL, 1997) which, when combined with increased bronchial vascularity, vascular permeability and other indices of airway remodelling mentioned previously (airway fibrosis and airway smooth muscle thickening), contribute to airway occlusion (Wilson JW, Li X, and Pain MC, 1993). Mediators associated with vasodilation include prostanoid mediators released by mast cells, particularly PGD_2 and leukotriene C4 (LTC_4) (Holgate ST, et al., 1991).

1.6 Airway Angiogenesis

Angiogenesis (or neovascularisation) is the sprouting of new blood vessels from extant blood vessels. Classically, this consists of the proliferation of resident endothelial cells which are reorganized into a three-dimensional tubular structure for new vessel outgrowth (Puxeddu I et al., 2005b). However, it has been found that bone marrow-derived endothelial progenitor cells (EPC) may also contribute to angiogenesis, as will be further discussed in section 1.8.2 (Asahara T, et al., 1997). During homeostatic conditions, angiogenesis is essential for tissue repair, foetal development and the female reproductive cycle. When uncontrolled, angiogenesis can promote tumor, retinopathies and coronary artery disease (Pandya NM, Dhalla NS, and Santani DD, 2006).

Angiogenesis requires the activity of angiogenic factors (such as the pro-angiogenic cytokine vascular endothelial growth factor [VEGF] and anti-angiogenic factor angiostatin), extracellular matrix proteins, adhesion receptors, and proteolytic enzymes (Puxeddu I et al., 2005a; Pandya NM, Dhalla NS, and Santani DD, 2006). Under physiologic conditions, this is regulated by a balance between positive and negative angiogenic mediators within the vascular microenvironment where an excess of pro-angiogenic factors promotes new vessel growth whilst a tip in the balance towards anti-angiogenic factors results in the inhibition of angiogenesis.

Angiogenic mediators include members of the FGF family, angiogenin, transformation growth factor (TGF)-α, TGF-β, platelet derived growth factor, tumor necrosis factor (TNF)-α, GM-CSF, angiopoietin 1, angiopoietin 2, and VEGF (Puxeddu I et al., 2005a). Of these mediators, VEGF is the most potent regulator of angiogenesis.

Once mature endothelial cells bind to VEGF (or other pro-angiogenic mediators), they release proteases which allow for the degradation of the basement membrane and escape of endothelial cells from the parent vessel. These endothelial cells then proliferate, migrate and interact with several matrix elements including collagen IV, laminin, fibronectin and vitronectin. These elements facilitate additional endothelial cell proliferation and migration as well as re-construction of the vascular basement membrane and tube formation (Carmeliet P, 2005; Wilson JW and Robertson CF, 2002; Wilson JW and Hii S, 2006).

1.6.1 VEGF

VEGF is secreted from pre-existing endothelial cells, a number of inflammatory cells involved in asthma including eosinophils, macrophages and mast cells, as well as from structural cells such as airway smooth muscle (Feltis BN, et al., 2006; Lee CG, et al., 2004; Hasaneen NA et al., 2007; Simcock DE, et al., 2007).

There are 6 isoforms of VEGF ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, $VEGF_{206}$), the major forms being $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$. These differ primarily in their bioavailability where $VEGF_{121}$ may diffuse freely in tissues and are easily available for activity whereas half of the secreted $VEGF_{165}$ binds to cell-surface heparan sulfate proteoglycans (HSPGs). HSPGs also provide a reservoir of $VEGF_{189}$ and $VEGF_{206}$ which are completely sequestered, but can be mobilized through proteolysis (Puxeddu I et al., 2005a).

The ability of VEGF to cause endothelial cell proliferation is mediated by signals generated by binding to receptor tyrosine kinases (RTKs) on endothelial cells. These

high-affinity receptors include VEGFR-1 (Flt-1), VEGFR-2 (Flk/KDR), and VEGFR-3. VEGFR-2 is primarily expressed on endothelial cells and other structural and hematopoietic stem cells. It is the primary receptor transmitting VEGF signals in endothelial cells by binding to VEGF, VEGF-C, and VEGF-D. Sequestration of VEGF results in down-regulation of VEGFR-2 and in apoptotic death of some capillary endothelial cells *in vivo* (Puxeddu I et al., 2005a; Ferrara N, 2002).

In endothelial cells, VEGF promotes secretion of MMP-1, von Willebrand factor (vWF), and expression of ICAM-1, VCAM-1, and E-selectin (Leung DW et al., 1989). VEGF also acts as a potent survival factor (induces EC expression of anti-apoptotic proteins) and causes vasodilation through the induction of the endothelial nitric oxide synthase and subsequent increase in nitric oxide production. VEGF stimulates monocyte chemotaxis and contributes to hematopoietic stem cell survival and recruitment of bone marrow-derived endothelial cells in angiogenesis. (Puxeddu I et al., 2005a).

Airway angiogenesis has been correlated with airway hyper-responsiveness and is thought to contribute to disease pathology by either supporting the growth of other hyperplastic cells, such as the airway smooth muscle, or contributing to airway thickness by providing additional permeable vasculature, leading to oedema and airflow obstruction (Asosingh K, Swaidani S, and Aronica M, 2007; Carmeliet P, 2005; Chetta A, et al., 2007; Walters EH et al., 2008; Wilson

JW and Hii S, 2006).

1.6.2 Quantification of Blood Vessels

Quantification of airway vessels can be achieved by counting the number of vessels per mm^2 of bandwidth area surrounding the airway; this ratio is used to describe microvessel density (MVD) (Asosingh K, Swaidani S, and Aronica M, 2007). In vessel enumeration vascular endothelial cells express a variety of markers which can provide a means for their identification, the most commonly used being CD31, collagen IV, CD34 and von Willebrand factor (vWF) (Pusztaszeri MP, Seelentag W, and Bosman FT, 2006). Unfortunately, the expression of these markers is not uniform throughout all vascular beds, and this has been the cause for conflicting findings between studies (Wilson JW and Kotsimbos T, 2003; Wilson JW and Hii S, 2006).

CD31, or PECAM-1 (platelet endothelial cell adhesion molecule – 1) is a consistent endothelial cell marker that has particular expression in junctions between adjacent cells. CD31 is a part of the immunoglobulin super-family and has varying functions including roles in angiogenesis, thrombosis and leukocyte migration (Woodfin A, Voisin MB, and Nourshargh S, 2007). Although CD31 is a marker which effectively identifies vessels throughout the vascular tree, it is also expressed on platelets, macrophages, granulocytes, lymphocytes, megakaryocytes, fibroblasts and neutrophils and therefore can illicit confounding variables when used for vessel quantification (Muller AM, et al., 2002a; Woodfin A, Voisin MB, and Nourshargh S, 2007).

Collagen IV, found primarily in the basal lamina has also been used as a consistent marker for vessel identification. Its potential for this use was discovered while examining scar-type collagen in the asthmatic airway (collagens I, III, V) (Li X and

Wilson JW, 1997). Collagen IV, has also been found to detect a larger number of vessels when compared to vWF, but it is possible that this is due to its constitutive expression in the asthmatic airway (Kawanami O, et al., 2000). Despite the reported effectiveness of using Collagen IV in vessel enumeration, we opted not to use this marker in the present study. This decision was based in our objective to reproduce results from previous studies which used von Willebrand factor staining for vessel quantification (Asosingh K, Swaidani S, and Aronica M, 2007; Doyle TM, et al., 2011).

CD34, as will be discussed in detail in section 1.7.1, is a 110-kDA transmembrane glycoprotein which is a well-known progenitor cell marker expressed in endothelial cells (Sutherland DR et al., 1993). It is commonly used as a marker for leukaemia diagnosis (Muller AM et al., 2002a). In the lungs, studies have shown that alveolar capillaries strongly stain for CD34 and the staining intensity does not vary by vessel type or caliber (Pusztaszeri MP, Seelentag W, and Bosman FT, 2006).

1.6.3 von Willebrand Factor

Von Willebrand factor (vWF) is a large glycoprotein that is essential for blood coagulation and vessel wall repair. It is stored in Weibel-Palade bodies within the cytoplasm of endothelial cells, synthesized by both endothelial cells and megakaryocytes and is expressed in both, the sub-endothelial extracellular matrix and in blood plasma (Moroose R and Hoyer LW, 1986). Comparative immunohistochemical staining and confocal laser microscopy of vWF throughout varying calibers of pulmonary vasculature reveal that vWF is a consistent positive marker for all vessels greater than 10μm. This

marker is undetectable in alveolar capillary endothelial cells, but is highly expressed in venules (Kawanami O, et al., 2000; Muller AM et al. 2002b).

The inability of normal alveolar capillary endothelial cells to react with vWF could be related to the increased fenestrations throughout these vessels. These fenestrations are diaphragm-like in structure and contain very little cytoplasm. Since vWF is stored in Weibel-Palade bodies within the cytoplasm of endothelial cells, it follows that, in the absence of cytoplasm, vWF will fail to be stored and expressed (Moroose R and Hoyer LW 1986; Muller AM, et al., 2002a).

vWF has been consistently used for quantification of MVD in asthma studies, and as such was considered to be a good marker for detecting the formation of new vessels in the lungs in the present study (Asosingh K, Swaidani S, and Aronica M, 2007; Kuwano K, Bosken CH, and Pare PD, 1993; Wilson JW and Kotsimbos T, 2003; Doyle TM, et al., 2011).

1.7 Progenitor Cells and Asthma

The adult bone marrow contains pluripotent progenitor cells with the capacity to differentiate into a variety of cell types. These cells can be divided into hematopoietic progenitor cells (give rise to leukocytes, red blood cells and platelets) and non-hematopoietic progenitor cells such as mesenchyme stem cells (MSC), fibrocytes and endothelial progenitor cells (EPC) (Jones CP and Rankin SM, 2011). Under homeostatic conditions non-hematopoietic progenitor cells contribute to tissue repair, however, under

inflammatory conditions their mobilization and activation lead towards lung remodelling (Jones CP and Rankin SM, 2011).

1.7.1 Hematopoietic Progenitor Cells (HPC)

Hematopoietic progenitor cells (HPC) are highly proliferative, pluripotent cells which can differentiate into granulocytic, erythroid, macrophage or megakaryocyte colony forming units (CFU) (Sehmi R and Denburg JA, 2000). These cells are characterized by high cell surface expression of CD34, an O-sialylated glycophosphoprotein. CD34 is a well-known progenitor cell marker expressed in endothelial cells, BM stromal progenitors and a subset of fibroblasts and dendritic cells (Sutherland DR et al., 1993). While found on progenitor cells, their cell surface expression is progressively lost during cell maturation (Krause DS et al., 1996). To identify HPC through flow cytometry, high CD34 expression is assessed in combination with low CD45 expression, small cell size and low granularity (Carion A, et al., 2002).

$CD34^{+}CD45^{+}$ progenitor cells in the peripheral blood and bone marrow are increased in asthmatics when compared to normal individuals (Sehmi R, et al., 1996). It has been suggested that these HPC may contribute to asthma disease pathology through BM hemopoeitic events that result in the release of both mature inflammatory cells and HPC. These cells then traffic to the lung and contribute to airway inflammation by either differentiating immediately *in situ* or by preparing a priming mechanism for future exacerbations (Inman MD et al., 1999; Sehmi R, et al., 1996; Sehmi R and Denburg JA, 2005; Wood LJ et al., 1998).

Studies suggest that events within the BM contribute to bronchial inflammatory cell accumulation. Research using canine models of allergen challenge showed that airway neutrophilic inflammation and AHR were associated with an increase in BM progenitor cells of the granulocyte-macrophage colony forming units (GM-CFU) line, which gives rise to neutrophils (Woolley MJ et al., 1994). In another canine model of allergen induced asthma, 5'bromo-3'deoxyuridine (BrdU) was injected into bone marrow to label actively dividing cells. Here it was found that 24hrs following allergen challenge there was a significant increase in $BrdU^+$ cells found in the peripheral blood and BAL fluid, suggesting that airway inflammation and AHR was associated with an increase in newly formed cells which may have originated from the BM (Wood LJ et al., 1998).

In mouse models of allergy-induced asthma, airway eosinophilic inflammation and AHR were accompanied by an increase in BM Eosinophil/Basophil (Eo/B)-CFU. Interestingly this study found that despite an increase in airway eosinophilia, 6hrs after allergen challenge BM eosinophils decreased. This increased airway eosinophilia and corresponding reduction in BM eosinophils following allergen challenge further supports the notion that the BM provided a reservoir for bronchial eosinophils which was depleted as demand for these inflammatory cells exceeded the supply (Inman MD, 2000; Ohkawara Y et al., 1997). Additional support for the role of BM-derived HPC in airway inflammation was observed in another mouse model of allergen-induced asthma. Here, airway eosinophilia increased after allergen exposure and BM HPC also increased 24-48hrs after challenge. This suggests that, HPC proliferation may be occurring to restore the depleted supply of eosinophils (Inman MD et al., 1999).

The BM may contribute to asthma disease pathology by releasing progenitor cells which traffic and home to the lung. Stromal derived factor (SDF)-1α and eotaxin are important mediators driving this process. SDF-1α, which is described in detail in section 1.8.4, is a chemokine involved in the retention of $CD34^+$ cells by interacting with the $CXCR4^+$ cell surface receptor and has been shown to be down regulated in the BM of asthmatics, resulting in the egress of progenitors from the BM (Dorman SC et al., 2005; Kucia M et al., 2004). Eotaxin, a chemokine interacting with the CCR3 receptor is also increased in the airways of asthmatics (Brown JR et al., 1998). Together these mediators may interact to promote progenitor cell egress, trafficking and retention within the airways of asthmatics where HPC may differentiate *in situ* into effector cells.

The proposed retention and differentiation of BM-derived HPC within asthmatic airways is supported by studies where $CD34^+$ cells which were isolated from atopic nasal tissue differentiated and proliferated *in vitro* into Eo/B-CFU, indicating that progenitors have the potential to differentiate *in situ* (Cameron L et al., 2000). This supports the view that *in situ* differentiation can occur by tissue-resident HPC, that can arrive as a result of the trafficking of $CD34^+$ hematopoietic lineage committed progenitors may be released from the bone marrow and traffic to the lung where they may contribute to increasing inflammatory cell populations. This has been supported in human studies which report the activation of progenitor cells within the BM and a resultant increase in progenitor cell numbers within the airways after allergen challenge (Sehmi R, et al., 1997; Wood LJ et al., 1998; Wood LJ et al., 1999; Dorman SC et al., 2005).

The trafficking and *in situ* differentiation of BM derived HPC in the lung has similarly been demonstrated in a mouse model of allergic asthma where $CD34^+CD45^+$IL-$5R\alpha^+$ eosinophil-basophil lineage committed progenitor cells increased within the lung before the development of airway eosinophilia. Interestingly, non-lineage committed $CD34^+CD45^+$ primitive progenitor cells were found to be increased in the lung well after the resolution of inflammatory response (14 days post-allergen challenge). These data suggest that $CD34^+CD45^+$ progenitor cells may not only be supporting airway inflammation, but these cells might be important contributors to structural changes in asthma disease pathology (Southam DS et al., 2004). This aspect was addressed in the current project where we investigated the contribution of bone marrow derived endothelial progenitor cells in the development of airway remodeling and functional changes.

1.7.2 Mesenchymal Stem Cells (MSC)

Mesenchymal stem cells (MSC) are a population of multipotent progenitor cells that can be isolated from many different tissues, including adipose tissue, placenta, skeletal muscle, and the most accessible source, bone marrow (Shi Y et al., 2012; Iyer SS, Co C, Rojas M, 2009). Though they are found in small numbers in the bone marrow (estimated to be 10 MSC per 1 million total bone marrow cells), these cells can be expanded *in vitro* and isolated from non-adherent hematopoietic stem cells and macrophages via trypsinization (Iyer SS, Co C, Rojas M, 2009). MSCs exhibit trilineage differentiation into chondrocytes, osteoblasts, and adipocytes; which is a feature used in their identification (Shi Y et al., 2012; Dominici M et al., 2006). These cells also have

the ability to differentiate into neurons, myocytes, and skeletal muscle (Jones CP and Rankin SM, 2011).

MSCs are of particular interest in asthma as it has been suggested that they may significantly contribute to collagen deposition and airway smooth muscle hyperplasia (Schmidt M et al., 2003; Singh SR and Hall IP, 2008). Although resident MSC in the bronchial wall were initially thought to be the primary contributors to these features of airway remodeling, recent data suggest that during an asthmatic exacerbation MSC may be migrating from the bone marrow to the lung and also contributing to this pathology (Brewster CE et al., 1990; Bucala R et al., 1994; Gizycki MJ et al., 1997; Phillips RJ et al., 2004; Sacco O et al., 2004).

Though there is not a proper classification system for MSCs, the International Society for Cellular Therapy has proposed that MSCs must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD18 and human leukocyte antigen (HLA)-DR surface molecules (Dominici M et al., 2006). Also, murine MSCs have recently been shown to be identified as platelet-derived growth factor receptor (PDGFR)α^{+}Sca-$1^{+}$$CD45^{-}$$TER119^{-}$ cells (designated PαS cells) (Jones CP and Rankin SM, 2011).

1.7.3 Fibrocytes

Fibrocytes are mesenchymal progenitor cells found in the BM. Once in the lungs, these cells differentiate into fibroblasts and myofibroblasts, contributing to an increase of fibroblastic foci and airway fibrosis (Andersson-Sjoland A et al., 2008; Schmidt M et al., 2003). Fibrocytes are recruited to the lungs during chronic inflammation and contribute

to airway remodelling by producing extracellular matrix molecules such as collagen I and III, vimentin, and fibronectin (Jones CP and Rankin SM, 2011). *In vitro* studies of fibrocytes isolated from peripheral blood mononuclear cells show they produce an array of pro-inflammatory cytokines, including TNF-α, IL-6, IL-8, IL-10, macrophage inflammatory protein(MIP)-1α/β, and VEGF, and they can secrete metalloproteinases (Jones CP and Rankin SM, 2011). In healthy individuals, fibrocytes compose a minor population of the circulating pool of leukocytes (0.1%-0.5% of total leukocytes). Patients with mild-to-severe asthma exhibit increased levels of circulating fibrocytes in PB when compared with control groups (Jones CP and Rankin SM, 2011).

Fibrocytes express hematopoietic cell markers (CD34, CD11b, and CD45) as well as produce procollagen I, express CCR2, CCR3, CCR5, CCR7, and CXCR4 (Jones CP and Rankin SM, 2011). Interestingly they have also been reported to express CXCR4 (Mehrad B, Burdick MD, and Strieter RM, 2009; Phillips RJ et al., 2004), a receptor for Stromal derived factor -1 (SDF-1), a potent chemokine up regulated in the airways and down regulated in the BM of asthmatics (Dorman SC et al., 2005). This factor is suggested to be a key regulator in the trafficking and homing of progenitors to the lung in asthma. The expression of CXCR4 on fibrocytes indicates the potential for these cells to be released from the bone marrow and attracted to lung tissue where they may contribute to disease pathology (Aiuti A et al., 1999; Aiuti A et al., 1997; Lapidot T and Petit I, 2002).

1.8 Endothelial Progenitor Cells

Angiogenesis is essential in repairing tissue damage and in establishing reperfusion of ischemic tissue. It was classically thought to only involve terminally differentiated mature endothelial cells, which have the capacity to migrate, proliferate and form vasculature (Risau W, 1997). However, in 1997 Asahara *et al.* isolated a subpopulation of $CD34^{+}VEGFR\text{-}2^{+}$ cells from human peripheral blood. These cells adhered to fibronectin and proliferated with the capacity to differentiate into endothelial cells *in vitro*. After 7 days of culture, these cells began to express markers typical of mature endothelial cells, such as vWF and also began to form tube like structures; these cells have been subsequently referred to as endothelial progenitor cells (EPC) (Asahara T, et al. 1997).

1.8.1 EPC Phenotypes and Identification

EPC are phenotypically difficult to isolate from HPC because they express similar markers such as CD34, CD31 and VEGFR-2 (Khakoo AY and Finkel T, 2005). Initially, in order to attempt to characterize EPC, cells were isolated through selection for VEGFR-2 and CD34; however, these markers are also expressed on mature endothelial cells and, when used alone, are ineffective in identifying the population of interest (Levesque JP et al., 2007; Miettinen M, Lindenmayer AE, and Chaubal A, 1994). CD133 is a 5 transmembrane glycoprotein that is expressed in cell populations with pluripotent hemopoetic stem cell properties and is not expressed on mature endothelial cells (Yin AH et al., 1997). In addition, several groups have demonstrated that $CD133^{+}CD34^{+}VEGFR\text{-}2^{+}$ cells have a high proliferative capacity and develop into endothelial like colonies.

Since mice have been found not to express CD133 other markers in murine models are used to identify EPC. These markers are stem-cell antigen-1 (Sca-1) which has consistently been used as a primitive marker for stem cells in mice only (Holmes C and Stanford WL, 2007), c-kit which is a cytokine receptor for stem cell factor and VEGFR-2 (Vascular Endothelial Growth Factor Receptor-2). Cells expressing Sca-1^+c-kit^+VEGFR-2^+ have been consistently characterized as EPC (Asosingh K, Swaidani S, and Aronica M, 2007; Peichev M et al., 2000; Doyle TM, et al., 2011) and this phenotype was used to identify EPC in the current study, whilst Sca-1^+c-kit^+ cells were identified as primitive progenitor cells.

EPCs can also be divided into two subsets: early outgrowth EPCs (EOG-EPCs) derived from hematopoetic lineage, and late outgrowth EPCs (LOG-EPCs). EOG-EPCs are detected as colonies after 5 days of culture, exhibiting spindle-shaped morphology and have characteristics of both endothelial and monocytic cells. These cells express the leukocyte markers CD45, CD11c, and CD14, and the endothelial markers CD31 and VEGFR-2. LOG-EPCs are observed in colony assays after 21 days in culture and exhibit cobblestone morphology that is characteristic of mature endothelial cells. These express the endothelial markers VEGFR-2, vWF, CD34, and CD31, but do not express monocytic markers (Jones CP and Rankin SM, 2011).

1.8.2 EPC and Angiogenesis

The contribution of EPCs to angiogenesis have primarily been studied with respect to tumor angiogenesis, models of ischemia/reperfusion injury, and in bone marrow transplantation studies (Jones CP and Rankin SM, 2011; Khakoo AY and Finkel

T, 2005). Recently, the role of bone marrow derived EPCs in increased bronchial vascularity has also been assessed in human and mouse models (Asosingh, Swaidani, Aronica, and Erzurum, 2009; Doyle TM et al., 2011).

In human studies, $CD133^{+}CD34^{+}VEGFR\text{-}2^{+}$cells have been implicated as a contributing factor in angiogenesis both *in vitro* and *in vivo*. When first investigated, cultured isolated cells from human peripheral blood not only demonstrated morphological characteristics of mature endothelial cells after one week, but also expressed vWF and decreased expression of CD133 thus suggesting a differentiation of EPC into mature cells (Hristov M, Erl W, and Weber PC, 2003). In addition, when plated on fibronectin, these cells began to form tube like structures which resembled vasculature. This differentiation and contribution to angiogenesis was further demonstrated *in vivo*, by injecting a bolus of human $CD133^{+}CD34^{+}VEGFR\text{-}2^{+}$ cells into a mouse model of hind-limb ischemia. Here it was found that a significant fraction of capillaries in the reperfused limb were of human origin, suggesting an essential role of EPCs in post natal vasculogenesis (Asahara T et al., 1999).

EOG-EPCs are not believed to directly form new vessels, but they have been shown to secrete key pro-angiogenic factors, such as VEGF-A, SDF-1, and insulin-like growth factor-1, and is therefore suggested that they may promote angiogenesis/vasculogenesis through a paracrine mechanism (Yoder MC and Ingram DA, 2009). LOG-EPCs possess the ability to form vessels *in vitro* and *in vivo* (in lung and heart models) (Yoder MC and Ingram DA, 2009). Thus, EPCs may contribute to angiogenesis in two ways: 1) by *in situ* differentiation and incorporation into new vessel

formation (LOG-EPCs) or 2) by promoting angiogenesis through the release of pro-angiogenic factors (EOG-EPCs).

1.8.3 Mobilization of EPC

It has been further demonstrated that EPC can be mobilised from the bone marrow and the traffic to sites of injury. This was first examined by transplanting bone marrows from transgenic mice which expressed β-galactosidase encoded by *lacZ* under the endothelial cell specific tyrosine-kinase promoter, Tie-2 (Schlaeger TM et al., 1995) into FVB/N wild-type mice. These mice were then given injections of VEGF, and experienced injury to the cornea. 6 days following ischemia β-galactosidase expression was observed in the cornea indicating migration of endothelial lineage-committed cells from the bone marrow to the site of tissue injury (Leung DW et al. 1989). Also, a significantly greater number of BM-derived EPC were observed in the corneas of VEGF treated mice, suggesting an essential role of this growth factor in the migration of EPC to injured sites (Asahara T et al., 1999).

VEGF has also been found to be increased in hypoxic tissues, tumours, and recently the brochoalveolar lavage (BAL) fluid of asthmatics (Banai S et al., 1994; Hoshino M, Nakamura Y, and Hamid QA, 2001; Lee CG, et al., 2004; Lee YC and Lee HK, 2001). VEGF has been shown to contribute to endothelial cell survival by inducing the expression of anti-apoptotic proteins and increase vascular permeability (Brown LF et al., 1995; Gerber HP, Dixit V, and Ferrara N, 1998). In addition, as mentioned above, VEGF has also been shown to promote EPC chemotaxis, however recent studies examining the ability of VEGF to recruit bone marrow derived EPC to tumour

vasculature have suggested that increasing secreted amounts of VEGF was not sufficient to mobilise bone marrow derived EPC, this suggests that other chemotactic factors are necessary for adequate mobilisation and recruitment (Larrivee B et al., 2005).

SDF-1α has also been demonstrated to be a potent chemotactic factor for EPC (Yamaguchi J et al., 2003) which (along with vascular cell adhesion molecule-1 [VCAM-1/CD106]) has been shown to be essential in retention of EPC and other $CD34^+$ progenitor cells in the bone marrow (Aiuti A et al., 1997; Ratajczak MZ and Kim C, 2012). However, in times of asthma exacerbations SDF-1 is down-regulated in the BM (Dorman SC et al., 2005; Balabanian K et al., 2005; Aiuti A et al., 1997) and correspondingly increased in the blood (Dorman SC et al., 2005) and airways (Southam et al., 2004).

1.8.4 SDF-1α/CXCR4 Axis

SDF-1, a CXC chemokine, has two forms- SDF-1α and SDF-1β of which SDF-1α is a proven potent chemoattractant for $CD34^+$ cells (Faber A et al., 2007; Aiuti A et al., 1999). Many studies have suggested that the interaction of SDF-1α and its primary receptor, the G-protein coupled, seven-transmembrane-spanning receptor CXCR4, is critical for the retention of progenitor cells in the bone marrow. During an asthmatic exacerbation, however, the SDF-1α/CXCR4 axis is downregulated in the BM initiating the mobilization and release of progenitor cells into peripheral circulation. Progenitor cells are then directed towards the lungs via the eotaxin/CCR3 axis, where their resultant lung-homing takes place in the presence of up-regulated SDF-1α expression in the airways. (Sehmi R

et al., 2003; Catalli AE et al., 2008; Dorman SC et al., 2005; Lapidot T and Petit I, 2002; Southam DS et al., 2004; Doyle TM et al, 2011).

Emerging evidence also suggests a possible heterodimerization of CXCR7 and CXCR4 which also produces a functional receptor to SDF-1α (Levoye A et al., 2009). However, CXCR7 has been shown not to be a primary receptor, but instead regulates access of SDF-1α to CXCR4 via scavenger activity (Ratajczak MZ and Kim C, 2012).

1.8.5 EPC and Asthma

Studies have shown that there is an increase of circulating EPC in the peripheral blood of asthmatics with a greater proliferative potential than EPC from normals. As well, a previous study by our lab had shown that in a mouse model of asthma, following chronic ovalbumin (OVA) challenge there was a correlation between lung-extracted EPC, airway angiogenesis and airway resistance Also, modulation of EPC traffic into the lungs resulted in a decrease of these indices (Doyle TM et al., 2011). In an acute OVA challenge mouse model, EPC accumulation within the lungs was also found to be an early remodelling (as early as 12 hours after OVA challenge) event and preceded angiogenesis (24-48 hours) and airway eosinophilia (>2 days) (Asosingh K, Swaidani S, and Aronica M, 2007). These data support the view that bone marrow derived EPC are involved in asthma disease pathology, and that modulating EPC activity may result in the attenuation of this pathology.

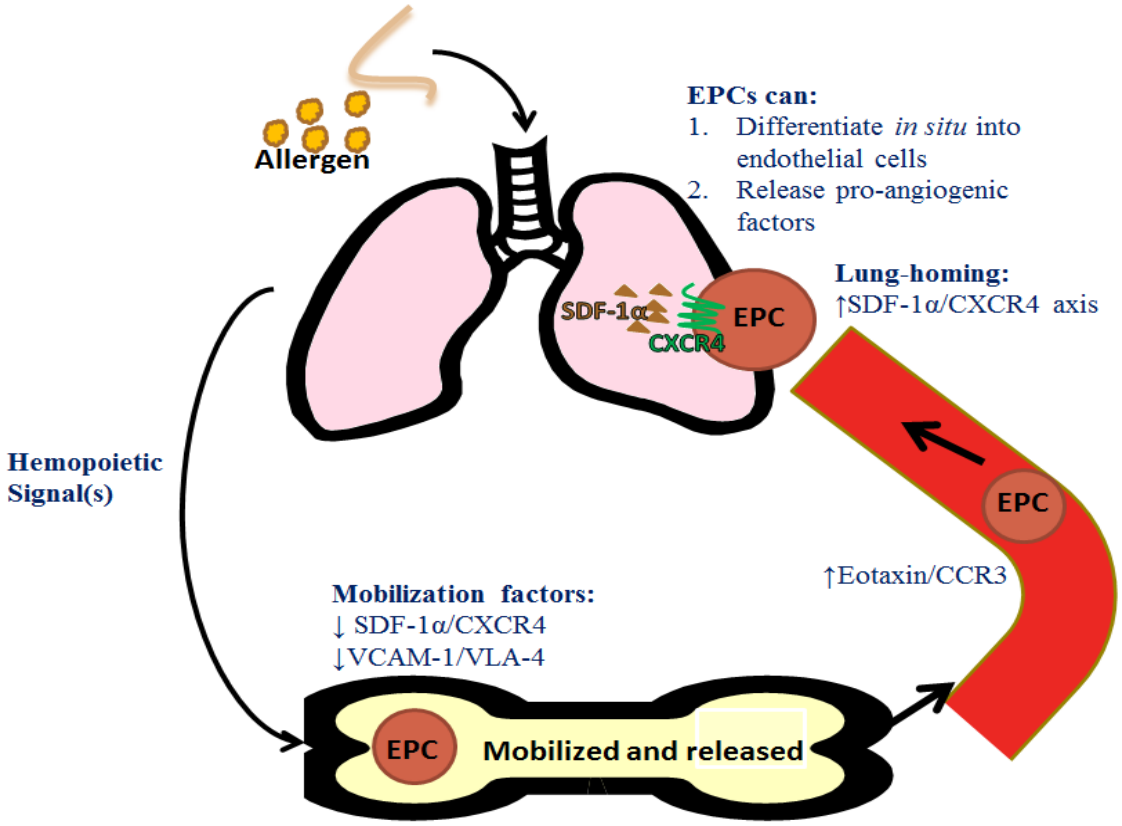


Figure 1: Lung Recruitment of Endothelial Progenitor Cells in an Allergic Asthmatic Response

1.9 Proposed Treatment and Confounding Factors

1.9.1 AMD 3100

AMD3100 is a CXCR4 antagonist which was originally developed as a treatment to prevent the entry of HIV into T-cells (De Clercq E et al., 1994; Schols D et al., 1997). It has since been shown to only interact with CXCR4 and effectively prevent SDF-1α/CXCR4 interactions, thereby inhibiting the migration of $CXCR4^{+}$ cells to this chemokine (Hatse S et al., 2002).

When administered as intraperitoneal bolus injections of 10mg/kg to allergen challenged sensitized mice, AMD 3100 attenuated lung inflammation and AHR (Lukacs NW et al., 2002). In addition, since EPC are $CXCR4^+$, AMD 3100 mediated blocking of this receptor has been shown to inhibit EPC migration, proliferation and survival *in vitro* (Zheng H et al., 2007).

Our lab has previously shown that intranasally administered AMD3100-mediated blocking of the $CXCR4^+$ receptor resulted in inhibition of EPC accumulation in the lung as well as attenuation in lung inflammation, bronchial vascularity and AHR (Doyle TM et al., 2011). However, since SDF-1α is also an eosinophil chemoattractant (Lukacs NW et al., 2002), it remains unclear whether AMD3100 works directly to attenuate EPC lung accumulation or indirectly through its anti-inflammatory effects on eosinophils.

1.9.2 Potential role of Eosinophils in EPC mobilization and Angiogenesis

In addition to their roles in airway inflammation, eosinophils have also been observed to regulate airway remodelling. In severe asthmatics, thickening of the subepithelial basement membrane was associated with increases in bronchial eosinophils (Puxeddu I et al., 2005b). Also, when treated with anti-IL-5 to reduce eosinophil numbers, asthmatics had a reduced expression of ECM proteins in the reticular basement membrane (Flood-Page P et al., 2003).

Eosinophils also have the potential to induce neovascularization. Indirectly, they synthesize and store in their granules several pro-angiogenic mediators, such as VEGF, FGF-2, TNF-α, GM-CSF, nerve growth factor (NGF), IL-8, and eotaxin and are positively stained for VEGF and FGF-2 (Puxeddu I et al., 2005a).

Most studies have focused on the correlation between VEGF levels and eosinophilia (Hoshino M, Nakamura Y, Hamid QA, 2001; Asai K et al., 2003; Nishigaki Y et al., 2003). However, a study by Puxeddu *et al*. has also observed a direct contribution of eosinophils to angiogenesis without VEGF where they enhance endothelial proliferation and induce a strong angiogenic response. In this study assessing rat aortic endothelial cell proliferation *in vitro* and aorta sprouting *ex vivo* as well as angiogenesis in the chick embryo chorioallantoic membrane *in vivo*, it was found peripheral blood eosinophils enhanced endothelial cell proliferation and induced angiogenesis. Pre-incubation of eosinophil sonicates with anti-vascular endothelial growth factor antibodies only partially reduced the angiogenic response in the chorioallantoic membrane suggesting that eosinophil-derived granule proteins (EPO and LTC_4) may play a role in promoting angiogenesis (2005b).

We propose that the mechanism of bone marrow-derived EPC recruitment into the lungs is mediated through the SDF-1α/CXCR4 axis and this precedes and contributes to airway angiogenesis, development of AHR and airway eosinophilia. Treatment with AMD3100 is expected to attenuate these factors of airway remodelling by directly inhibiting EPC recruitment into the lungs. However, there is the possibility that AMD3100 mediated inhibition of SDF-1α chemotactic activity directly attenuates airway eosinophilia resulting in an indirect attenuation in EPC lung accumulation and angiogenesis. To investigate the role that eosinophils may play in EPC-derived angiogenesis the current study utilized a well described transgenic mouse strain that is devoid of eosinophils (PHIL +/+ mice) (Lee JJ, et al., 2004), subjected this strain to a

chronic allergen mouse model and assessed for the indices of airway inflammation, lung-extracted EPC, bronchial vascularity, and airway responsiveness.

1.10 Mouse Model of Asthma

Asthma is a uniquely human disease with the exception of asthma-like diseases in cats (eosinophilic bronchitis) and horses (heaves) (Holmes AM, Solari R, Holgate ST, 2011). Though human models would be the ideal experimental standard to elucidate this multi-factorial disease, obvious ethical restrictions prevent this from happening. Although not identical to mechanisms in human asthma, the mouse model of asthma has become the most widely used alternative in disease research. Mice are easy to breed, maintain and handle, have a vast array of specific reagents available for analysis of cellular and mediator responses, and have genetically engineered transgenic or gene-knockout varieties available for modelling airway disease (Holmes AM, Solari R, Holgate ST, 2011).

1.10.1 Mouse Strain

In this study we utilized female wild-type (WT) BALB/c mice as well as eosinophil deficient PHIL (+/+) mice bred on a BALB/c background. To create PHIL mice, Lee *et al*. selected the promoter region of eosinophil peroxidase (EPO) to incorporate the expression of a cytocidal protein. This promoter region was chosen as the upstream sequences from the mouse EPO gene were found to be capable of supporting high-level expression unique to eosinophil lineage-committed cells. In these transgenic mice, an open reading frame for the cytocidal protein, diphtheria toxin A, was inserted

into the promoter region of EPO. Expression of the DTA chain results in the catalytic degradation of elongation factor-2 and subsequent collapse of protein synthesis, in turn causing eosinophil ablation. Assessment of circulating leukocytes in PHIL mice confirm they have a full complement of hemopoietic cells (lymphocytes, monocytes, and neutrophils) aside from eosinophils (Lee JJ et al., 2004).

1.10.2 Allergens utilized in Mouse Models of Asthma

Antigen sensitisation and challenge protocols inducing T_H2-type inflammation in the lung can be customized to specific endpoints to assess inflammation and lung function, and by acute or chronic aspects of asthma to be modelled (Holmes AM, Solari R, Holgate ST, 2011). Traditional protocols used ovalbumin (OVA) with adjuvant (usually intraperitoneal alum) followed by inhalation antigen challenge to produce an acute asthma-like phenotype that focuses on T_H2 inflammatory mechanisms that links to manifestations of established asthma in humans (Holmes AM, Solari R, Holgate ST, 2011). OVA is commonly used as a surrogate allergen in research because it is easily available (Zosky et al., 2007). OVA exposure (*i.n.*) to naïve mice requires incorporation into a chemical adjuvant (aluminum hydroxide) and systemic administration during the sensitization phase. Once subjects are sensitized to the OVA+adjuvant, subsequent airway challenge to OVA can elicit the asthma-like phenotype (airway remodeling, airway inflammation, and AHR) (Nials AT and Uddin S, 2008). A great disadvantage to using OVA is that it fails to model the natural process of asthma as adjuvants may alter the mechanism of sensitization to allergen (Zosky GR and Sly PD, 2007). Also, when sensitisation and challenge is attempted only though the airways, tolerance to OVA is

observed with only slight bronchial inflammation and mild AHR, which are not sufficient to imply an asthma-like phenotype (Nials AT and Uddin S, 2008).

In an effort to better simulate the chronic nature of human asthma, models have been developed using more physiologically relevant antigens, such as house dust mite (HDM) which is utilized in the current study. HDM, an aeroallergen, has a multitude of components that contribute to its allergenicity and promotion of airway inflammation. The major allergen, Der p 1, is a cysteine protease which cleaves intracellular tight junctions of the bronchial epithelium, allowing for the delivery of allergen from airway lumen to submucosal antigen-presenting cells (dendritic cells) and allergen sensitization (Gregory LG and Lloyd CM, 2011). Once HDM particles are in the lung, macrophages are stimulated to produce IL-6 (enhances T_H2 differentiation and proliferation of memory T_H2 cells (Robinson DS, 2010)), TNF-α (a chemoattractant for neutrophils and eosinophils (Berry et al., 2007)), and nitric oxide (Sjöbring U and Taylor JD, 2007). *In vitro* studies have also shown purified HDM extracts Der p 1, 3, and 9 together induce production of GM-CSF which can promote eosinophil production (Gauvreau GM et al., 2005), IL-6, IL-8, and eotaxin from bronchial epithelial cells (Sjöbring U and Taylor JD, 2007).

1.11 Hypothesis

We hypothesize that the mobilization, lung-homing and in-situ differentiation of endothelial progenitor cells in allergic asthmatic response is independent of eosinophilic inflammation.

1.12 Specific Questions:

1. Are eosinophils required for allergen-induced lung accumulation of EPC and angiogenic responses in a mouse model of asthma?
2. Does AMD3100 act independently of eosinophils to attenuate lung accumulation of EPC, angiogenesis, and airway hyperresponsive lung function?

1.13 Aims:

1. In a mouse HDM exposure model of asthma we will determine whether allergen challenge induces progenitor cell traffic, airway inflammatory and remodelling changes comparable to the previously established OVA challenge model.
2. Using eosinophil deficient (PHIL) mice, the role of eosinophils in lung homing of EPC angiogenic responses and airway dysfunction will be investigated.
3. Using PHIL mice, we will determine the effect of treatment with AMD3100 on lung homing of EPC and angiogenic responses in the absence of eosinophils.

1.14 Scientific Impact

Asthma is a multifaceted disease associated with recurrent inflammatory episodes and tissue remodeling within the lung. Although inflammation of the airway is an essential component in initiating and perpetuating an asthma exacerbation, current anti-inflammatory therapies have been largely unsuccessful in completely reversing disease airflow obstruction. There is now considerable evidence that asthma is not merely a local inflammatory disease confined to the lung, but has a systemic element that includes activation of hemopoeitic events in the bone marrow leading to the mobilization of progenitors and recent evidence suggests that these progenitors may contribute to structural changes of the airway. Although it is further suggested that vascular remodelling is a critical component of asthma disease pathology, a current lack of understanding regarding the mechanisms of airway angiogenesis prevents utilizing this knowledge towards treatment development.

In this study, the information gained from using AMD3100 to modulate endothelial progenitor cell homing and differentiation will reveal the effect of local anti-angiogenic delivery in the development of lung pathology and airway dysfunction in asthma. Furthermore, this study will provide information pertaining to the role of eosinophils a major effector cell in asthma, in progenitor cell accumulation within the lung, relevant tissue remodelling changes, and airway hyperresponsiveness. In addition, this project will also allow further understanding of the mechanisms involved in airway angiogenesis and the role of bone marrow derived progenitor cells in asthma pathology and development of airway dysfunction.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animals

Female BALB/c and *PHIL* eosinophil deficient mice (BALB/c background) were bred and maintained in a pathogen-free environment. *PHIL* eosinophil-deficient male mice (8^{th} generation BALB/c background; courtesy of Dr. J.J. Lee) were mated with WT BALB/c females (8 week old; Charles River Laboratories, Ottawa, ON). Female PHIL eosinophil-deficient mice (8 week old) and WT littermate controls were kept in a pathogen-free environment. All procedures were reviewed and approved by the Animal Research Ethics Board at McMaster University (Hamilton, ON, Canada).

2.2 Allergen exposure and Treatment Intervention

2.2.1 Chronic Allergen Exposure

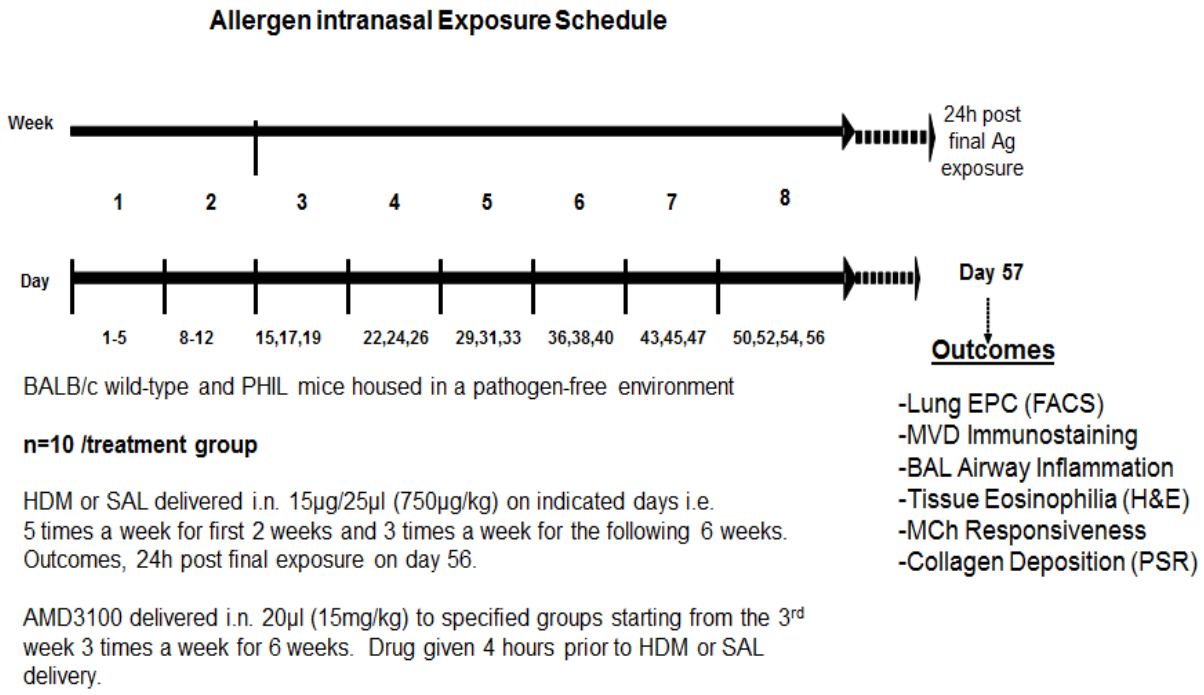
Mice (PHIL or WT; n≈10/group) were sensitized with intranasal (*i.n.*) doses of 15µg/µl house dust mite (HDM) administered for 2 weeks, 5 consecutive days each week as described by Southam et al., 2007. Mice were subsequently challenged with *i.n.* doses of 15µg/µl HDM 3 times a week for 6 weeks. Control mice followed an identical model of exposure but were administered 25µl saline (SAL) *i.n.* instead of HDM (Figure 2).

2.2.2 Treatment Regimen

Mice were treated *i.n.* with an optimal dose of 15mg/kg AMD3100 previously established in pilot experiments by Doyle et al (see Appendix B) as the lowest dose capable of preventing allergen-induced increases in airway vascularity in a brief ovalbumin (OVA) exposure protocol. Treatment was administered to mice (n= 7-10 per group) during the challenge period of the chronic exposure period 4 h before each

allergen challenge 3 times a week for 6 weeks (Figure 2A). Refer to figure 2B for all treatment groups.

A



B

		Treatment	
Group	**Mouse**	**Sensitization**	**Challenge**
A	WT	SAL	SAL
B	WT	HDM	HDM
C	PHIL	SAL	SAL
D	PHIL	HDM	HDM
E	WT	HDM	AMD/HDM
F	PHIL	SAL	AMD/SAL
G	PHIL	HDM	VEH/HDM
H	PHIL	HDM	AMD/HDM

Figure 2: Allergen exposure protocol. (A) Intranasal (i.n.) sensitization with HDM or SAL for the first 2 weeks is followed by i.n. challenges with HDM or SAL for the remaining 6 weeks as indicated. During the challenge period, AMD3100 is administered i.n. 4 hours prior to HDM or SAL delivery for selected groups. Outcomes are made 24h following final exposure. (B) Table of specific groups indicating the treatments given during sensitization (Week 1-2) and challenge (Week 3-8).

2.3 Airway Responsiveness

Airway responsiveness to intranasal methacholine was measured based on the response of airway resistance using the FlexiVent ventilator system (SCIREQ, Montreal, QC, Canada).

Mice were given 20mg/ml Xylazine by IP injection at an approximate dose of 10mg/kg. Following 10 minutes, mice were administered 54.7mg/ml pentobarbital by IP injection at $10ml/g_{mouse}$. After 10 minutes, mice were placed on the FlexiVent ventilator system. Pancuronium bromide at 2mg/ml was then used as a paralytic, given through IP injection at 20mg/kg.

2.3.1 Airway Physiology

Airway responsiveness was assessed by measuring total respiratory resistance to saline and progressively increasing intranasal doses of 0, 3.1, 6.3, 12.5, 25, and 50mg/ml of methacholine (MCh) which were continuously recorded with a FlexiVent ventilator system.

The mouse trachea was exposed, cannulated with an 18 gauge needle and connected to the ventilator. An ear probe was placed on the hindlimb of the mouse and heart rate and oxygen saturation were monitored via infrared pulse oxymetry. A script for data collection was then started with PEEP levels set at 2cmH2O. Once ventilated and stabilized, progressive doses of 0, 3.1, 6.3, 12.5, 25, and 50mg/ml of MCh were delivered as a mist inhaled into the lungs via nebulizer.

Utilizing QuickSnap-150, the total resistance and elastance were calculated and airway responsiveness was assessed by the maximal resistance produced by the MCh

challenge. Airway reactivity was measured by the slope of the linear regression between peak respiratory system resistance and log10 of the MCh using 3.1, 6.3, 12.5, and 25mg/ml doses.

2.4 Bronchoalveolar Lavage

2.4.1 BAL Fluid Collection

In anaesthetized mice, the trachea was exposed and cannulated using a blunted 18 gauge needle. Two boluses of 250µl phosphate-buffered saline (PBS) were injected using a 16G needle. Following 5 seconds, the cell emulsion was withdrawn and BAL fluid centrifuged for 10 minutes at 150g.

The cell pellet was then resuspended in PBS and a sample of cells were stained using a 1:1 ratio of trypan blue (Sigma Oakville, Ontario, Canada). Total cell count was performed using a hemocytometer with calculations as follows:

Number of Cells/ml= (# cells counted) x (dilution factor) x 1000 x (# quadrants counted)

2.4.2 Cytospin Preparation and Cell Staining

The cell emulsion was then diluted with PBS to 5 x 10^5 cells/ml and cytocentrifuge slides were prepared as two slides per mouse and dried for 24 hours. Cells were then fixed using methanol, air dried and stained with 2 drops of Wright Giemsa (Sigma Oakville, Ontario, Canada) for 2 minutes and thoroughly mixed with 3 drops of distilled water and left for 3 ½ minutes. Slides were rinsed with distilled water and air

dried. Following this, cell differential counts were carried out using morphologic and histologic criteria. As each mouse had 2 slides created, 200 cells were counted per slide and the average of the two were determined per mouse. Cells were morphologically identified as eosinophils, neutrophils, lymphocytes or macrophages.

2.5 Progenitor Cell Isolation

2.5.1 Cell Isolation from Lung Tissue

In anesthetized mice the thoracic cavity was opened by blunt dissection exposing the pulmonary organs. The lungs were then perfused with a 10µl/ml heparin solution via injection of the right ventricle closest to the apex of the heart. Following this, the descending aorta was cut and perfusion of the lung was allowed for either 1 minute or until lungs became whitish.

After perfusion was complete, the thymus and bronchiolar lymph nodes were removed and the heart and lungs were removed from the chest cavity. The right lung was tied off with 3.0 suture string (Johnson and Johnson Medical, Markham ON), separated and was transferred into 3ml warm digestion buffer containing 300U/ml collagenase, 50U/ml DNAse I, and 10% fetal bovine serum (FBS) (Sigma Oakville, Ontario Canada) diluted into RPMI (Gibco Burlington, Ontario Canada). The right lung was then homogenized further by mincing and incubated in the digestion buffer for 90 minutes at 37^0C. Cells released from the lung digest were then filtered through a 52µm nylon mesh to rid the filtrate of larger tissue and debris. The filtrate was then topped up to 5ml with cold wash buffer consisting of RPMI enriched with 1% FBS and 1%

Penicillin/Streptomycin to promote cell viability and prevent bacterial growth respectively. The cells where then collected in a pellet by centrifugation at 1300rpm (Heraeus Instruments Megafuge 1.0R), 4^0C for 10 minutes. The cell pellet was then resuspended in 6ml wash buffer and layered onto 3ml of Histopaque 1083 (Sigma Oakville, Ontario, Canada) at room temperature which then isolated the mononuclear cells were isolated by density gradient centrifugation at 2400rpm (Heraeus Instruments Megafuge 1.0) , 25^0C for 20 minutes, no brakes. The mononuclear cell interface was removed and washed with wash buffer at 1300rpm (Heraeus Instruments Megafuge 1.0R), 4^0C, 10 minutes. The supernatant was discarded and the pellet was resuspended in 0.5-3ml wash buffer determined by the pellet size.

A cell count was performed on a sample of the isolated mononuclear cells using a 1:1 dilution of trypan blue. The Countess Cell Counter system (Invitrogen Burlington, Ontario Canada) was used to determine the total number of cells/ml as well as percent viability.

2.5.2 Cell Staining and Flow Cytometric Analysis

Cells were washed in 1ml PBS/NaN3 at 1300rpm (Heraeus Instruments Megafuge 1.0R), 4^0C, 5 minutes and were incubated with Fc block dilution (eBioscience, San Diego, CA) for 20 minutes at 4^0C. The cells were then stained and incubated for 40 minutes at 4^0C with Sca-1-FITC, c-kit-PE, VEGFR2-APC or isotype control. Following this, the cells were washed in 2ml PBS/NaN3 at 1300rpm (Heraeus Instruments Megafuge 1.0R), 4^0C, 5 minutes and resuspended/fixed in 300µl 1% PFA and kept in the

dark at 4^0C until enumerated by flow cytometry. Specific stains and compensation tubes were organized as described in Table 1.

Table Fc Block and Antibody (Mab) proportions used in compensation tubes and specific stains for FACS staining

Tube	Fc Block	Mab
PBS	100 µl	-
c-kit – PE	95 µl	5µl
Sca-1-FITC/ VEGFR2- APC	92.5µl	2.5µl FITC+ 5µl APC
IgG2a-FITC/2b-PE/IgG2a – APC	87.5µl	2.5µl FITC + 5 µl APC + 5µl PE
Sca-1-FITC/c-kit-PE/VEGFR2-APC	87.5µl	2.5µl FITC + 5 µl PE + 5µl APC

Stained Sca-1^+c-kit^+ cells and Sca-1^+c-kit^+VEGFR-2^+ cells were analyzed by using a FACS Caliber flow cytometer. A four step gating strategy was used which began by tracing region 1 (R1) on a dot plot of Sca-1+ cells vs. Linear side light scatter (SSC) around all nucleated white blood cells while excluding red blood cells and contaminants. Quadrants were arranged based on isotype-matched control staining. Double positive cells were defined as gate R2 and total primitive Sca-1^+c-kit^+ cells were calculated by multiplying this percentage by the total amount of cells counted after histopaque separation. Sca-1^+c-kit^+ cells that also positively expressed VEGFR-2^+ were defined in gate R3. Absolute numbers of cells were calculated from this percentage multiplied by total cell count after separation using the histopaque gradient.

2.6 Immunohistochemistry

Following perfusion with the previously noted heparin solution, the right lung was tied off with suture string and removed for EPC collection while the left lung was formalin fixed for 24 hours and paraffin embedded. Transverse lung sections of 3µm were then mounted onto poly-l-lysine coated slides and dried overnight. Slides were then backed at 72^0C for 40 minutes within 24 hours prior to staining.

2.6.1 von Willebrand Factor

To remove wax and rehydrate tissue, slide were passed through 3 changes of xylene (5 minutes each) followed by 3 changes of 100% ethanol (1 minute each). The tissue was incubated for 30 minutes in a methanol solution (200ml methanol, 10ml 30% H_2O_2 and 0.5ml concentrated HCL) and then brought to water by passing the tissue through 100%, 95%, and 70% ethanol (1 minute each) and washed in running tap water (5 minutes). Tissue was rinsed in distilled water for 1 minute and circled with a hydrophobic pen to keep solutions on the tissue and kept hydrated with tris-buffered saline (TBS). Following this, the tissue was incubated for 6 minutes with protease K solution for antigen retrieval, washed and incubated with protein block serum-free solution for 5 minutes. Slides were drained and incubated for 60 minutes with the primary antibody, von Willebrand factor (DAKO Burlington, Ontario, Canada) in a dilution of 1:600 in PBS. Slides were then washed with TBS (3 times for 5 minutes) and incubated for 20 minutes with the secondary biotinylated goat anti-rabbit antibody (Invitrogen Oakville, Ontario, Canada), washed again with TBS (3 times for 5 minutes) and incubated for 20 minutes with streptavidin peroxidise conjugate (Invitrogen Oakville,

Ontario, Canada). Slides were then washed with TBS (3 times for 5 minutes) and had AEC chromogen added for 10 minutes, washed in running tap water and counterstained with Mayer's Haematoxylin for no more than 30 seconds. Slides were then washed with running tap water for 2 minutes, placed in TBS for 1 minute to blue nuclei, washed in running tap water for 1 minute, and transferred to distilled water. Slides were cover slipped with Faramount liquid media and allowed to dry.

All slides were analyzed using Northern Eclipse software. The main airway in the lung section was identified and traced to create a 50μm bandwidth area from the airway in which to count vessels. Only vessels 10μm diameter or less were included in vessel quantification and microvessel density (MVD) was calculated by dividing the total number of counted vessels by the bandwidth area.

2.6.2 Picrosirius red staining (Collagen Staining)

Slides of lung tissue were prepared as for immunohistochemistry. Slides were then rehydrated through successive passes through xylene followed by ethanol. Slides were then stained with Celestine blue for 5 minutes followed by a rinse and staining in hematoxylin for 5 minutes. Slides were then washed in Tris buffer and stained in Sirius red for 1 hour. They were then washed in acidified water and dried. Slides were then dehydrated through three changes of ethanol and xylene and then mounted with permount resinous medium.

Lung sections were examined using a polarized lens and analyzed with Northern Eclipse software. A 29.7μm bandwidth surrounding the main airway was traced and images were converted to black and white, where the positive stained area appeared

white. The Northern Eclipse software then analyzed the percentage of white area within the traced bandwidth.

2.6.3 Haematoxylin and Eosin (enumeration of lung eosinophils)

Slides of lung tissue were prepared as for immunohistochemistry. Slides were then stained with Haematoxylin for 10 minutes, rinsed and stained in Tris buffer for 1 minute to blue nuclei. Slides were then washed again and quickly dipped into 1% acid alcohol followed by another rinse and wash in Tris buffer. Slides were then rinsed in distilled water and stained in 0.5% Eosin for 5 minutes, washed, dehydrated through three changes of ethanol and xylene and then mounted with permount resinous medium.

2.7 Statistical Analysis

In the text and figure legends, data are presented as mean ± SEM. Data analysis was completed using STATSTICA software (Statsoft, Tulsa, OK, USA) to perform ANOVAs; post-hoc analyses for between groups comparisons were performed using Duncan's test. Type 1 error probability (α) was set at 0.05.

CHAPTER 3: RESULTS

3.1 HDM Chronic Exposure Model in WT Mice

Our group has previously utilized an ovalbumin (OVA) exposure model to investigate the role of bone marrow-derived progenitor cells in the development of lung remodeling changes in a mouse model of asthma. House dust mite (HDM) is a more relevant allergen to human asthma and thus the aim of my study was to investigate the effect of HDM exposure in BALB/c mice on lung accumulation of progenitor cells and tissue remodeling events. Treatment with AMD3100, previously shown to inhibit progenitor cell lung-homing (Doyle TM et al., 2011) was included to investigate effects of modulating progenitor cell traffic on, bronchial vascularity, inflammation and methacholine (MCh) airway resistance following allergen challenge. Airway fibrosis was also assessed as a secondary outcome measure.

3.1.1 Lung-extracted progenitor cell numbers and drug effects

Lung-extracted progenitor cell numbers were enumerated by flow cytometry. Primitive progenitor cell (PC; Sca-1^+c-kit^+) populations were significantly increased in HDM group compared to SAL group (HDM: 42 716±7 458 vs. SAL: 12 306±2 495 cells, $p<0.0001$). Treatment with AMD3100 significantly attenuated PC levels compared to HDM group bringing the numbers down to SAL levels (HDM: 42 716±7 458 vs. AMD: 12 402±4 002 cells, $p<0.0001$) (Figure 3A). Similar trends were also seen in EPC (Sca-1^+c-kit^+VEGFR-2^+), where HDM exposure significantly increased EPC numbers (HDM: 33 156±5 596 vs. SAL: 7 597±1 813 cells, $p<0.0001$) and treatment with AMD3100

attenuated these values to SAL levels (HDM: 33 156±5 596 vs. AMD: 7 020±2 627cells, $p<0.0001$) (Figure 3B).

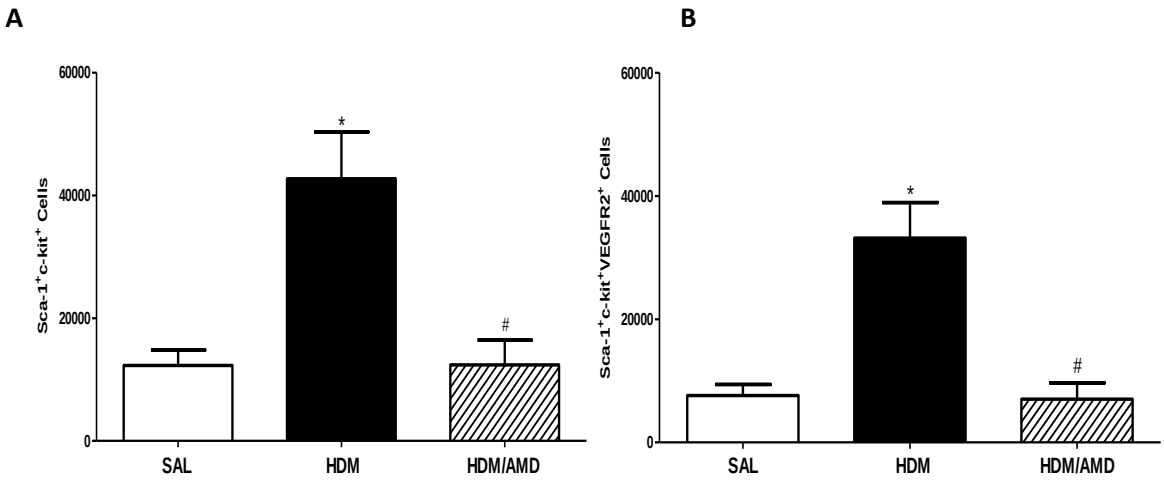


Figure 3: WT (n=10/group) (A) Total primitive progenitor (Sca-1^+c-kit^+) and (B) Total endothelial progenitor (EPC) (Sca-1^+c-kit^+VEGFR-2^+) cell populations in lung samples 24hrs post allergen challenge enumerated by flow cytometry. HDM exposed mice had a significantly increased number of primitive progenitor cells which was attenuated to SAL levels in the AMD3100 group. EPCs were also significantly increased in the HDM group when compared to SAL and treatment with AMD3100 significantly attenuated this to SAL levels. (*$p<0.05$ compared to saline and #$p<0.05$ compared to HDM) Data expressed as mean± standard error of the mean.

3.1.2 Bronchial vascularity and drug effects

Bronchial vascularity was assessed by immunostaining for von Willebrand factor (vWF), quantifying the number of vessels within a 50µm diameter width around the main airway and determining the microvessel density (MVD) including vessels of ≤10µm. MVD was significantly increased in the HDM group compared to SAL group (HDM: 166.137±21.71 vs. SAL: 59.879±10.66 vessels/mm^2, $p<0.0001$). Treatment with AMD3100 attenuated MVD compared to HDM group (AMD: 110.811±17.34 vessels/mm^2 vs. HDM: 166.137±21.71, $p<0.01$) but were not completely reversed to SAL levels (AMD: 110.811±17.34 vessels/mm^2 vs. SAL: 59.879±10.66, $p<0.05$) (Figure 4).

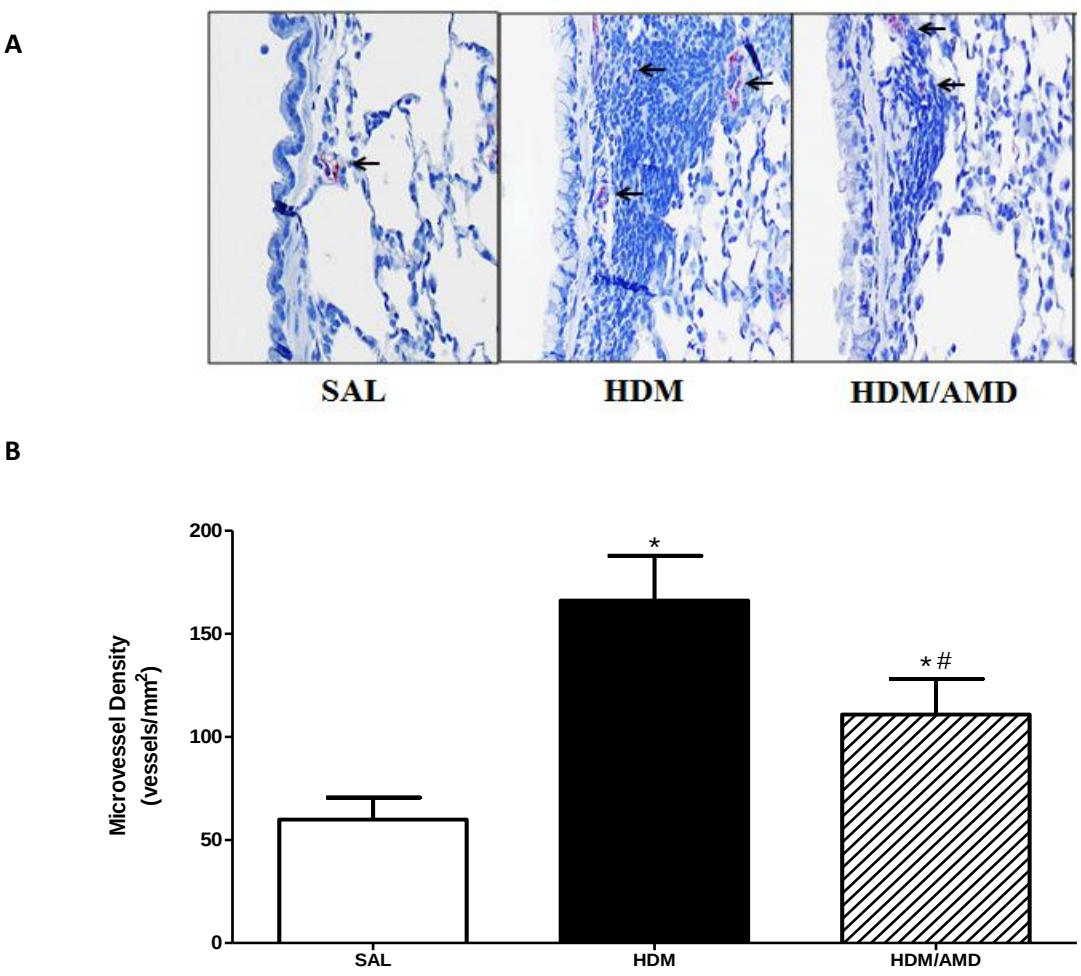


Figure 4: WT mice (n=10/group) (A) Microvessel Density (MVD) as measured by von Willebrand factor staining (vWF) - images from 24hrs post-final allergen challenge at 40X magnification. (B) Vessels counted were 10µm or less (arrows) in a 50µm bandwidth area around the main airway. MVD was significantly higher in the HDM group compared to SAL. Treatment with AMD3100 attenuated MVD, though not to SAL levels (*$p<0.05$ compared to saline and #$p<0.05$ compared to HDM) Data expressed as mean± standard error of the mean.

3.1.3 Airway inflammation and drug effects via BAL

Airway inflammatory cells were enumerated by cell differential counts of bronchial alveolar lavage (BAL) fluid. Compared to SAL, HDM treated mice had significantly elevated total cell counts (SAL: 3.37± 0.54 vs. HDM: 9.20± 1.60 ($x10^4$) cells, $p<0.0001$), eosinophils (SAL: 0.01± 0.01 vs. HDM: 2.85± 0.47($x10^4$) cells, $p<0.0001$), neutrophils (SAL: 0.19± 0.06 vs. HDM: 1.15± 0.32 ($x10^4$) cells, $p<0.01$), and lymphocytes (SAL: 0.52± 0.06 vs. HDM: 2.36± 0.59 ($x10^4$) cells, $p<0.001$) (Figure 5).

Compared to the HDM group, treatment with AMD3100 had no significant effect on any cell type. (HDM vs. AMD3100 total cells: 9.20± 1.60 vs. 8.88±1.61($x10^4$) cells; eosinophils: 2.85± 0.47 vs. 3.29±0.76($x10^4$) cells; neutrophils: 1.15± 0.32 vs. 1.48±0.20($x10^4$) cells; lymphocytes: 2.36± 0.59 vs.2.20±0.47($x10^4$) cells) (Figure 5).

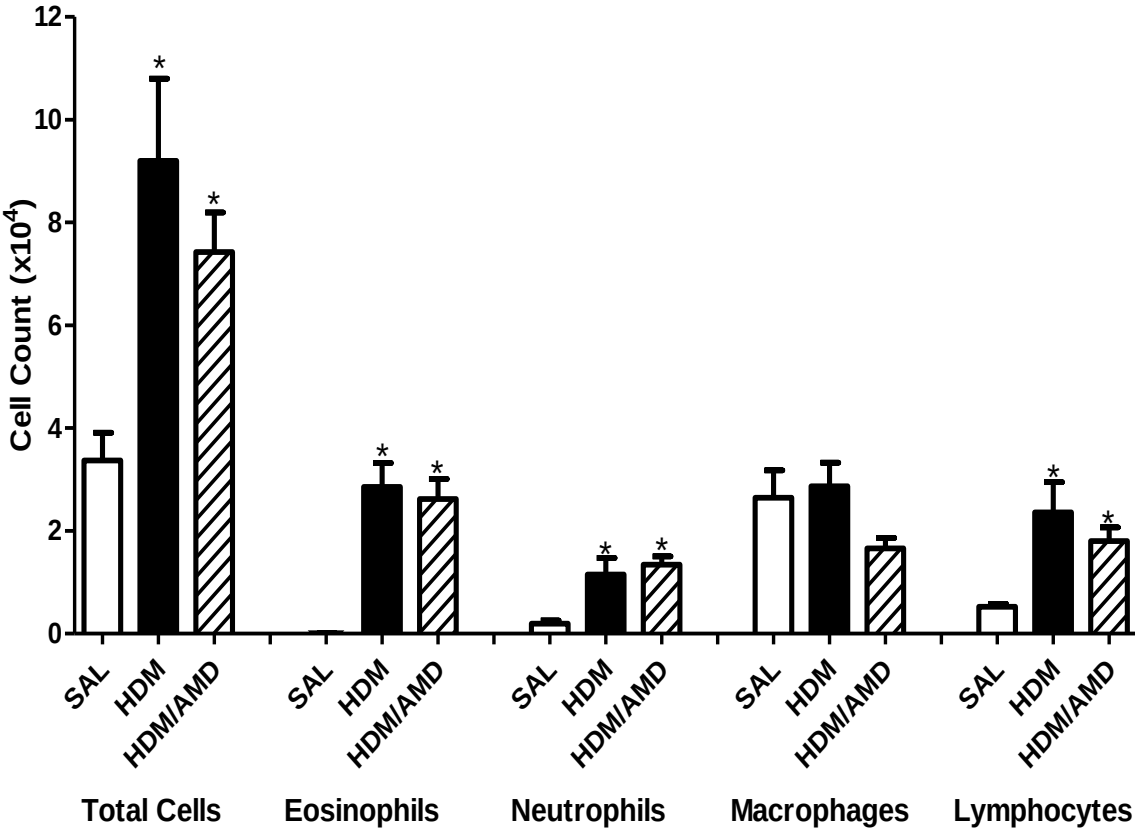


Figure 5: Airway inflammation as measured by bronchial alveolar lavage (BAL) for WT mice (n=10/group). HDM group had a significantly increased number of inflammatory cells (excluding macrophages) compared to SAL. Treatment with AMD3100 (HDM/AMD) did not decrease cell numbers compared to the HDM group and inflammatory cell numbers were still significantly higher than SAL. (*$p<0.05$ compared to saline and #$p<0.05$ compared to HDM) Data expressed as mean± standard error of the mean.

3.1.4 Lung tissue eosinophilia and drug effects

Lung eosinophilia was assessed by haematoxylin & eosin (H&E) staining and enumerating the number of eosinophils within a 50µm bandwidth around the main airway. Eosinophil numbers significantly increased following HDM exposure (HDM: 1.21±0.18vs. SAL: 0.13±0.04 cells/mm^2, p<0.0001). Concurrent treatment with AMD3100 significantly attenuated this (HDM: 1.21±0.18vs. AMD: 0.87±0.11 cells/mm^2, p<0.01), though not to SAL levels (SAL: 0.13±0.04vs. AMD: 0.87±0.11 cells/mm^2, p<0.0001) (Figure 6).

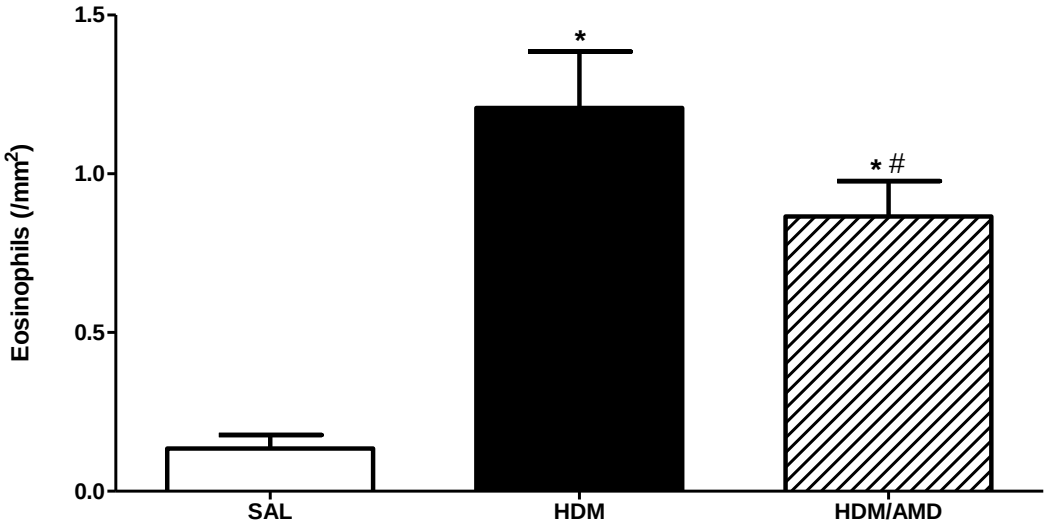


Figure 6: WT mice (n=10/group) (A) Airway eosinophilia as measured by haematoxylin and eosin (H&E) stain- images from 24hrs post-final allergen challenge at 40x magnification. (B) HDM exposed mice had a significantly increased number of eosinophils compared to SAL. Treatment with AMD3100 (HDM/AMD) significantly decreased cell numbers compared to the HDM group, though not to SAL levels (*p<0.05 compared to saline and #p<0.05 compared to HDM) Data expressed as mean± standard error of the mean.

3.1.5 Collagen deposition and drug effects

Collagen deposition was assessed by staining lung sections with picrosirius red (PSR), a stain for collagen deposition and enumerating the percentage of stain within a 29.7μm bandwidth around the central airway. HDM group had a significantly greater level of collagen deposition compared to SAL (HDM: 13.20±1.30 vs. SAL: 9.16±0.75%, $p<0.05$). Mice treated with AMD3100 *i.n.* did not show a significant decrease of stain compared to HDM (AMD: 10.03±1.06 vs. HDM: 13.20±1.30%, $p=0.107$), though the AMD treated group was not significantly different from the SAL (Figure 7).

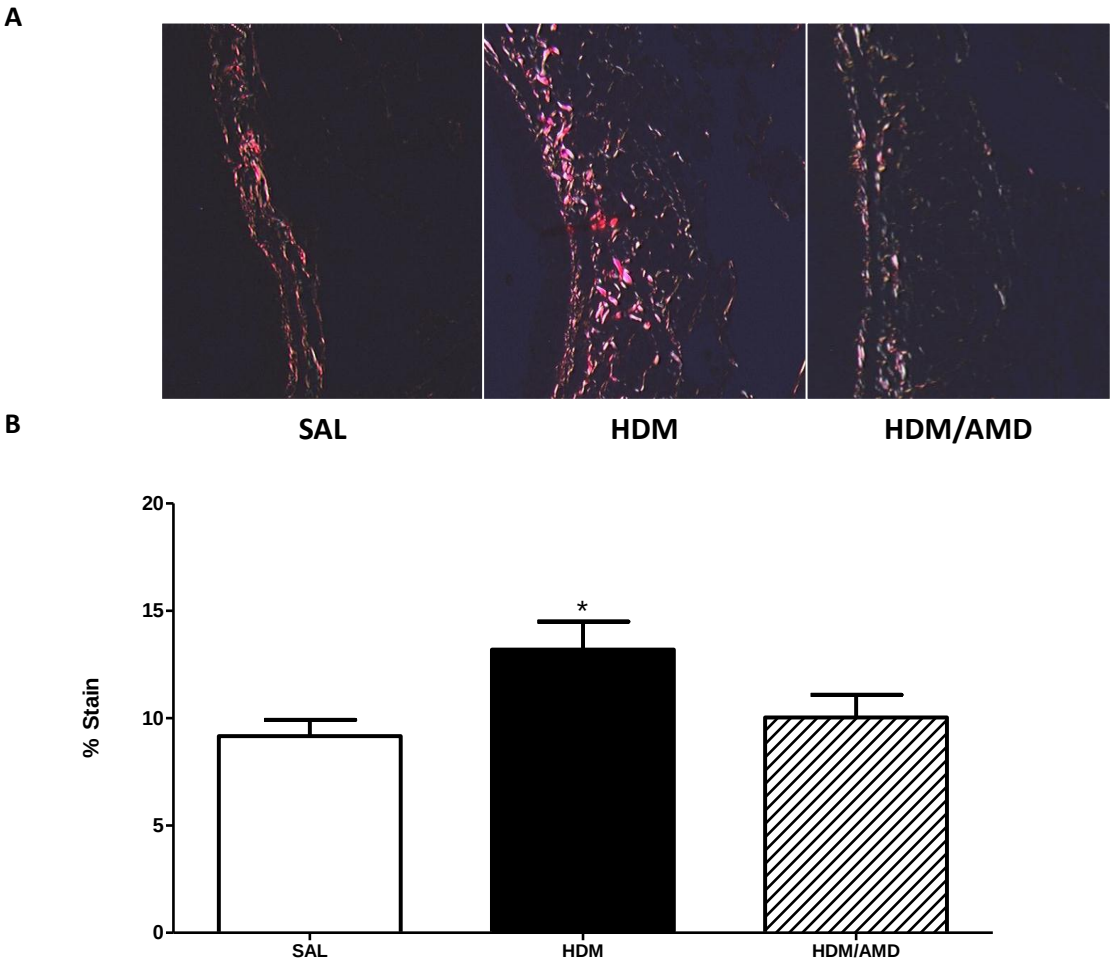


Figure 7: WT mice (n=10/group) (A) Collagen deposition as measured by picrosirius red (PSR) stain- images from 24hrs post-final allergen challenge at 20x magnification. (B) HDM exposed mice had a significantly greater collagen deposition compared to their corresponding SAL group. Treatment with AMD3100 (HDM/AMD) did not decrease collagen deposition. (*$p<0.05$ compared to saline) Data expressed as mean± standard error of the mean.

3.1.6 Airway Responsiveness and drug effects

Airway resistance was assessed by measuring the maximal resistance to progressively increasing intranasal (*i.n.)* doses of MCh. In the dose-response curve, HDM group expressed the greatest response to MCh. Treatment with AMD3100 attenuated airway responsiveness to SAL levels (Figure 8A).

Doses used to determine maximal resistance were 3.1, 6.3, 12.5, and 25mg/ml MCh. Maximal airway resistance was significantly increased in the HDM group compared to SAL (HDM: 5.65±1.90 vs. SAL: 1.39±0.45 $cmH_2O/ml/s$, $p<0.05$). Treatment with AMD3100 completely attenuated this (HDM: 5.65±1.90 vs. AMD: 1.50±0.18 $cmH_2O/ml/s$, $p<0.001$) to SAL levels ($p=0.91$) (Figure 8B).

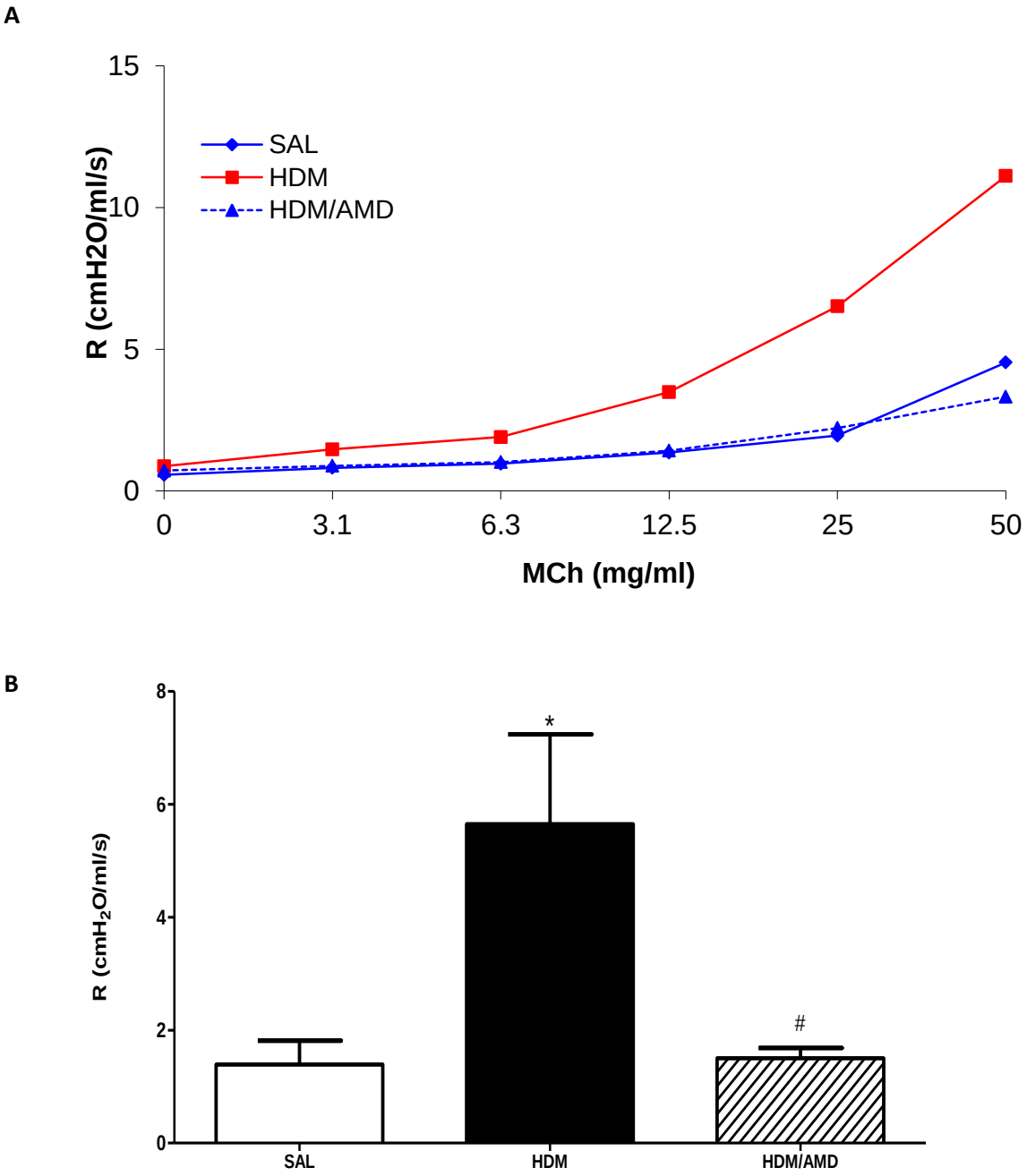


Figure 8: WT (n=10/group) (A) Airway resistance curve and (B) Maximal airway resistance 24hrs post allergen challenge. HDM exposed mice had a higher resistance compared to saline (SAL) exposed mice. Treatment with AMD3100 (AMD) attenuated airway resistance to SAL levels (*p<0.05 compared to saline and #p<0.05 compared to HDM) Data expressed as mean± standard error of the mean. Note: values up to 25mg/ml were used in the analysis.

3.2- HDM Chronic Exposure Model in eosinophil deficient PHIL Mice

To determine if eosinophils are required for HDM-induced lung accumulation of EPC, bronchial vascularity, and airway hyperresponsiveness, we utilized a transgenic strain of eosinophil deficient PHIL mice and assessed for changes in these outcome measures following allergen challenge and concurrent treatment with AMD3100.

3.2.1 Lung-extracted progenitor cell numbers

PC (Sca-1^+c-kit^+) numbers were significantly increased in HDM group compared with SAL (HDM: 24 257±5 470 vs. SAL: 6 614±3 117 cells, $p<0.05$). Treatment with AMD3100 attenuated PCs to SAL levels (AMD: 7 504±2 927 vs. HDM: 24 257±5 470 cells, $p<0.05$) (Figure 9A). Similar trends were also seen in EPC (Sca-1^+c-kit^+VEGFR-2^+), where HDM exposure increased EPCs compared to SAL (HDM: 18 362±4 723 vs. SAL: 5 859±2 887 cells, $p<0.05$) and treatment with AMD3100 significantly attenuated these values to SAL levels (HDM: 18 362±4 723 vs. AMD: 6 501±2 538 cells, $p<0.05$) (Figure 9B). In the drug control groups, HDM/VEH was not significantly different from HDM group (HDM/VEH vs. HDM for PC: 33 787±12 998 vs. 24 257±5 470 cells; EPC: 23 989±9 128 vs. 18 362±4 723 cells) and SAL/AMD was not significantly different from SAL group (SAL/AMD vs. SAL for PC: 10 376± 7 351 vs. 6 614±3 117 cells; EPC: 6 333±4 293 vs. 5 859±2 887 cells). These data indicate no placebo effect.

Between group comparisons showed PHIL HDM exposed mice had a comparatively lower number of PC compared to WT mice (PHIL HDM: 24 257±5 470 vs. WT HDM: 42 716±7 458 cells, $p<0.05$) (Figures 9A & 3A) and EPC (PHIL HDM: 18 362±4 723 vs. WT HDM: 33 156±5 596 cells, $p<0.01$) (Figures 9B & 3 B).

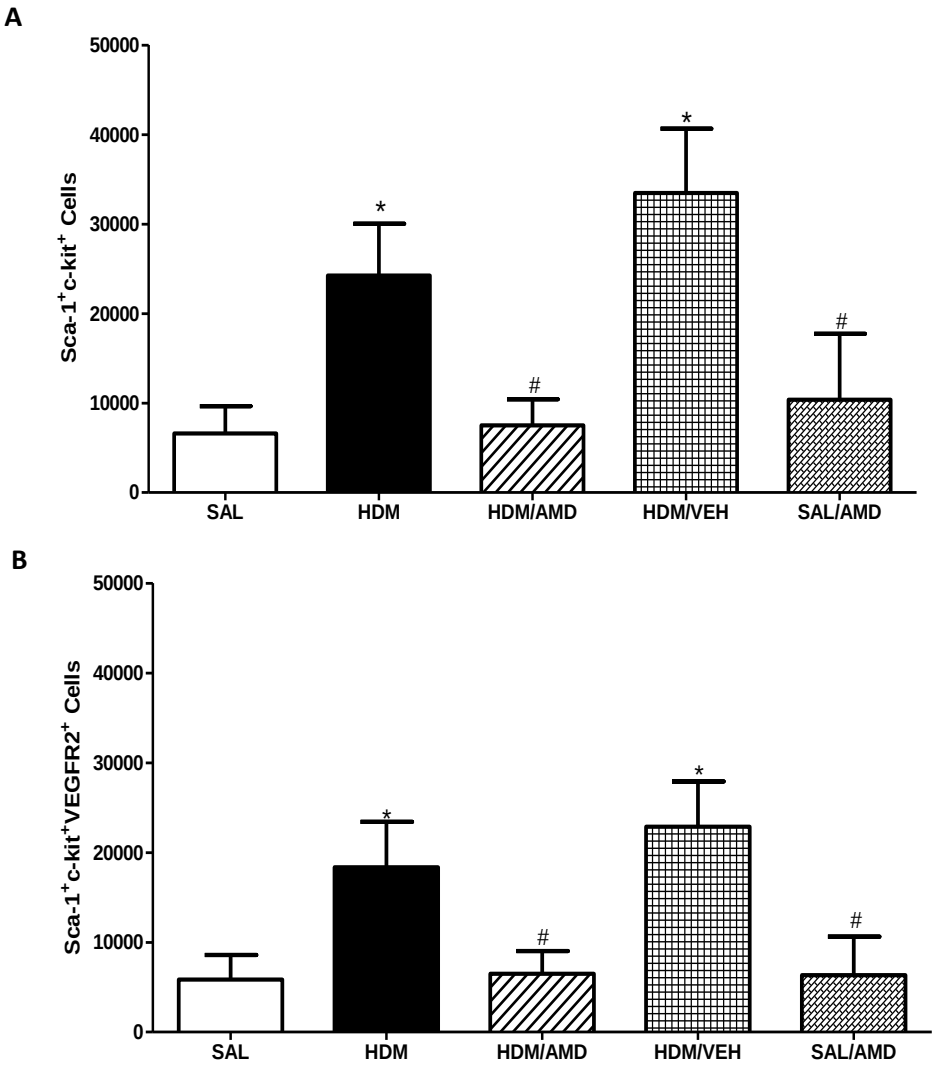


Figure 9: PHIL (n≈10/group) (A) Total primitive progenitor (Sca-1$^+$c-kit$^+$) and (B) Total endothelial progenitor (EPC) (Sca-1$^+$c-kit$^+$VEGFR-2$^+$) cell populations in lung samples 24hrs post allergen challenge enumerated by flow cytometry. HDM exposed mice had a significantly increased number of primitive progenitor cells compared to SAL which was significantly attenuated when treated with AMD3100. EPC numbers were increased in the HDM group compared to SAL which was also attenuated to SAL levels in the AMD group. Control groups did not show placebo effects for primitive progenitor and EPC numbers. (*$p<0.05$ compared to saline and #$p<0.05$ compared to HDM) Data expressed as mean± standard error of the mean.

3.2.2 Bronchial vascularity

MVD was significantly elevated in the HDM group when compared with SAL mice (HDM: 123.95±16.65 vs. SAL: 37.465±9.94 vessels/mm^2, p<0.001). Treatment with AMD3100 significantly reduced MVD to SAL levels (HDM: 123.95±16.65 vs. AMD: 45.279±10.66 vessels/mm^2, p<0.001) (Figure 10). In the drug control groups, HDM/VEH was not significantly different from HDM group (HDM/VEH: 87.97±16.42 vs. HDM: 123.95±16.65 vessels/mm^2) and SAL/AMD was not significantly different from SAL group (SAL/AMD: 54.322±13.51 vs. SAL: 37.465±9.94 vessels/mm^2). These data indicated no vehicle effect.

Between group comparisons showed a trend towards lower MVD in PHIL HDM exposed mice compared to WT HDM group (PHIL HDM: 24 257±5 470 vs. WT HDM: 42 716±7 458 cells, p=0.58) (Figures 10 & 4).

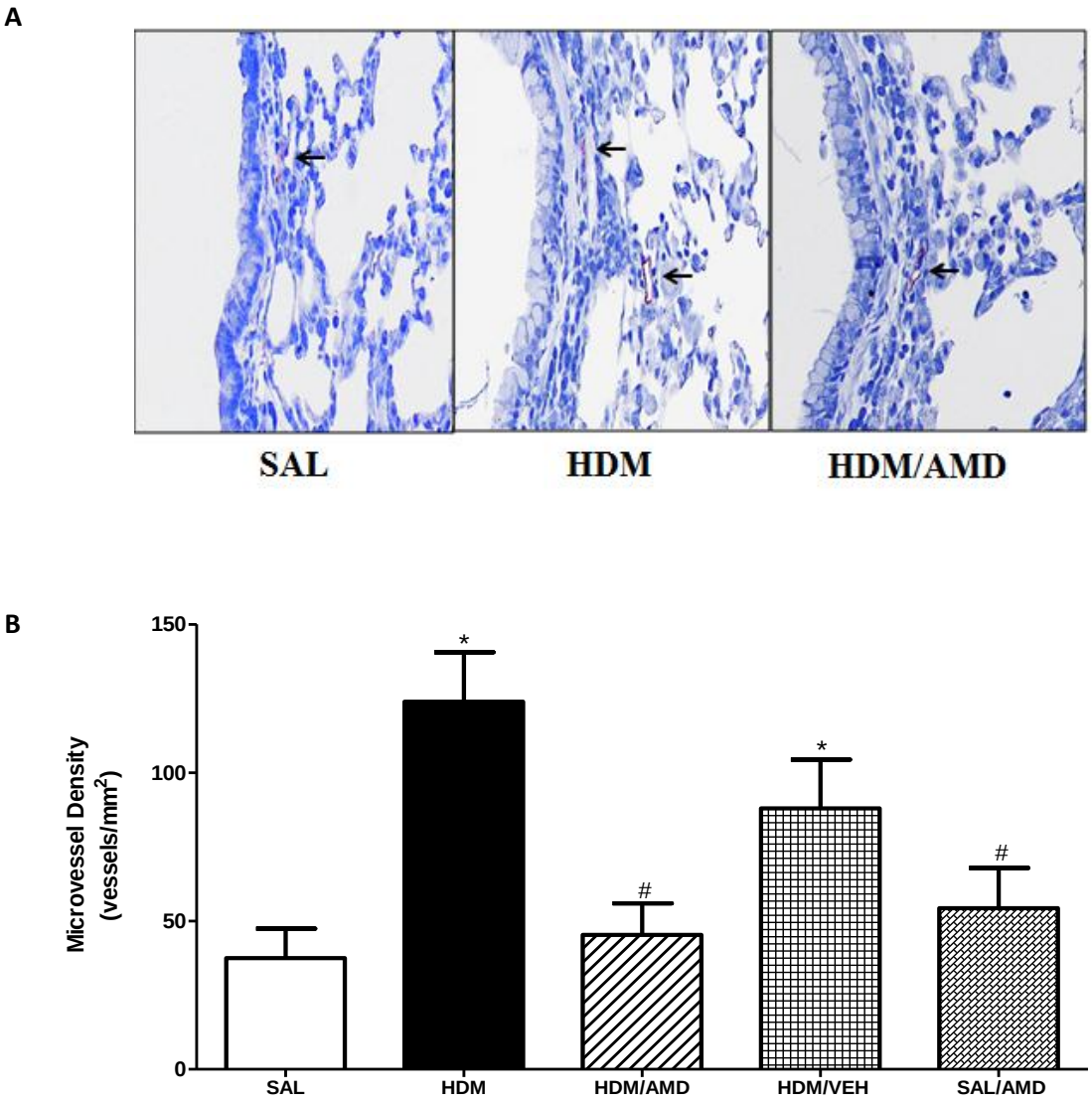


Figure 10: PHIL mice (n≈10/group) (A) Microvessel Density (MVD) as measured by von Willebrand factor staining (vWF) - images from 24hrs post-final allergen challenge at 40X magnification. (B) Vessels counted were 10µm or less (arrows) in a 50µm bandwidth area around the main airway. MVD was significantly higher in the HDM group compared to SAL. Treatment with AMD3100 attenuated MVD to SAL levels. Control groups did not show placebo effects (*$p<0.05$ compared to saline and #$p<0.05$ compared to HDM) Data expressed as mean± standard error of the mean.

3.2.3 Airway inflammation via BAL

BAL was used to assess for airway inflammatory cells. Compared to SAL groups, HDM treated mice had significantly elevated total cell counts (SAL: 4.13±0.48 vs. HDM: 7.64± 0.93 ($x10^4$) cells, $p<0.01$), neutrophils (SAL: 0.18±0.04 vs. HDM: 1.47± 0.31 ($x10^4$) cells, $p<0.0001$), and lymphocytes (SAL: 0.59±0.13 vs. HDM: 2.71±0.36 ($x10^4$) cells, $p<0.0001$). As this strain is eosinophil deficient, no eosinophils were detected in BAL fluid (Figure 11).

Compared to HDM group, AMD treated PHIL mice had significantly lower total cell counts (HDM: 7.64±0.93 vs. AMD: 5.20±0.33($x10^4$) cells, $p<0.05$), macrophages (HDM: 3.45±0.48 vs. AMD: 2.24±0.19 ($x10^4$) cells, $p<0.05$), and lymphocytes (HDM: 2.71±0.36 vs. AMD: 1.71± 0.24 ($x10^4$) cells, $p<0.05$). In the control groups no significant effect of vehicle was observed (HDM/VEH vs. HDM Total cells: 6.92±0.75 vs. 7.64± 0.93 ($x10^4$) cells ; neutrophils: 2.27±0.26 vs. 1.47± 0.31 ($x10^4$) cells; macrophages: 2.34±0.39 vs. 3.45±0.48 ($x10^4$) cells; lymphocytes 2.30±0.35 vs. 2.71±0.36 ($x10^4$) cells and SAL/AMD vs. SAL: Total cells: 5.44±0.61 vs. 4.13±0.48 ($x10^4$) cells ; neutrophils: 0.17±0.06 vs. 0.18±0.04 ($x10^4$) cells; macrophages: 4.78±0.64 vs. 3.37±0.44 ($x10^4$) cells; lymphocytes 0.49±0.08 vs. 0.59±0.13 ($x10^4$) cells).

Between group comparisons for HDM exposed WT and PHIL mice showed no significant differences in inflammatory cell numbers (Figures 5 & 11).

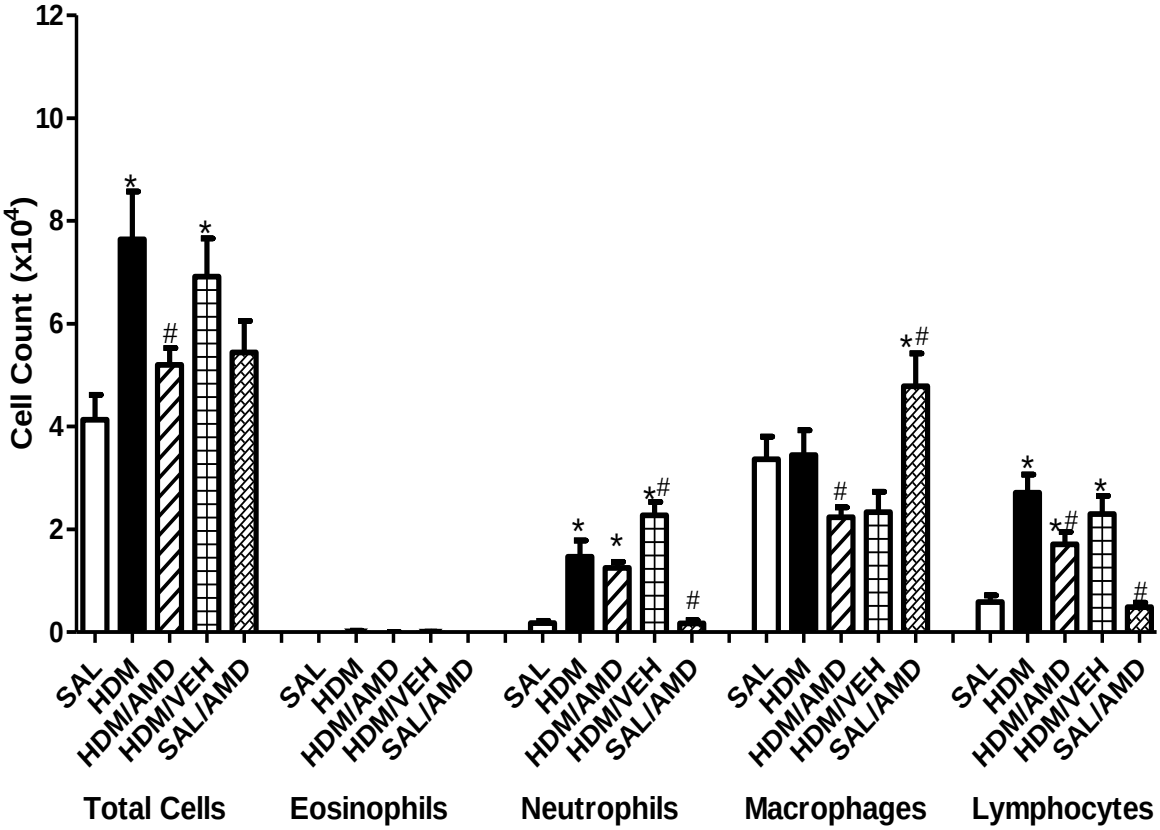


Figure 11: Airway inflammation as measured by bronchial alveolar lavage (BAL) for PHIL mice (n≈10/group). HDM group had a significantly increased number of inflammatory cells (excluding macrophages) compared to SAL. Treatment with AMD3100 (HDM/AMD) decreased cell numbers compared to the HDM group (trend towards decreased neutrophils, though not significant). (*p<0.05 compared to saline and #p<0.05 compared to HDM) Data expressed as mean± standard error of the mean.

3.2.4 Lung eosinophilia

To further confirm PHIL mice are eosinophil deficient, an H&E stain was performed on lung sections. Few eosinophils were observed in SAL, HDM, and AMD groups (SAL: 0.00± 0.00, HDM: 0.03± 0.01, AMD: 0.03± 0.01eosinophils/mm^2) (Figure 12).

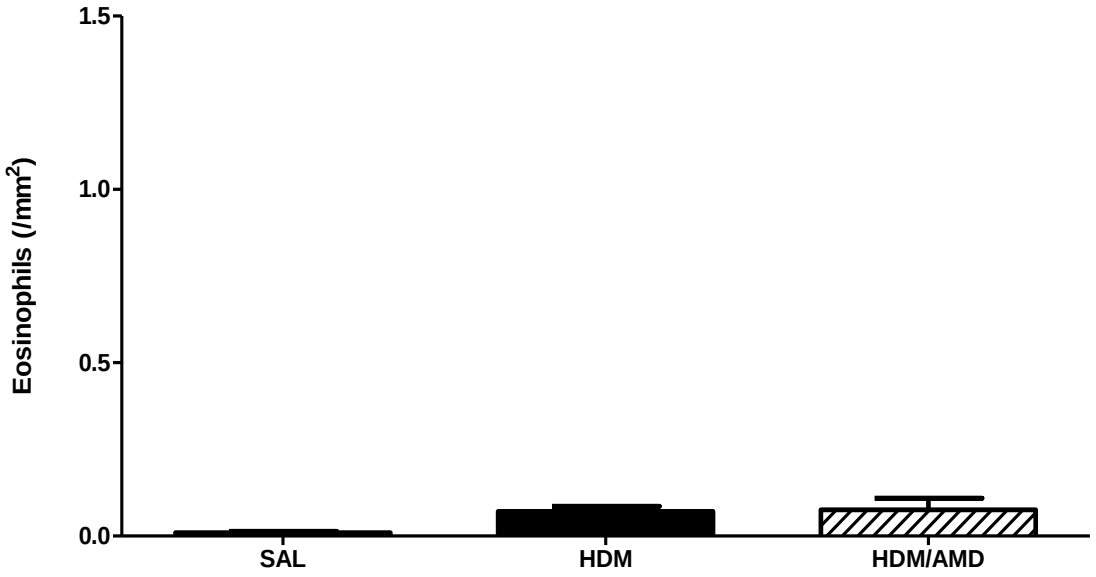


Figure 12: PHIL (n≈10/group) (A) Airway eosinophils as measured by hematoxylin and eosin (H&E) stain- images from 24hrs post-final allergen challenge at 40x magnification. (B) Little to no eosinophils were observed in SAL, HDM and HDM/AMD groups. (*p<0.05 compared to saline and #p<0.05 compared to HDM) Data expressed as mean± standard error of the mean.

3.2.5- Collagen deposition

HDM exposed mice had a significantly higher collagen deposition compared to SAL group (HDM: 19.25±2.48 vs. SAL: 13.2±1.30%, $p<0.01$). Mice treated with AMD3100 showed a significant decrease in collagen deposition compared to HDM to SAL levels (AMD: 10.92±0.85 vs. HDM: 19.25±2.48%, $p<0.0001$) (Figure 13).

The HDM/VEH group had a significantly higher collagen deposition than SAL (HDM/VEH: 12.25±1.37 vs. SAL: 13.2±1.30%, $p<0.01$), but was significantly lower than HDM exposed mice (HDM: 19.25±2.48 vs. HDM/VEH: 12.25±1.37%, $p<0.01$) suggesting a vehicle effect.

Between group comparisons for HDM exposed WT and PHIL mice showed a significant increase in collagen deposition for the PHIL HDM group (WT HDM: 13.2±1.30 vs. PHIL HDM: 19.25±2.48%, $p<0.01$) (Figure 7 & 13).

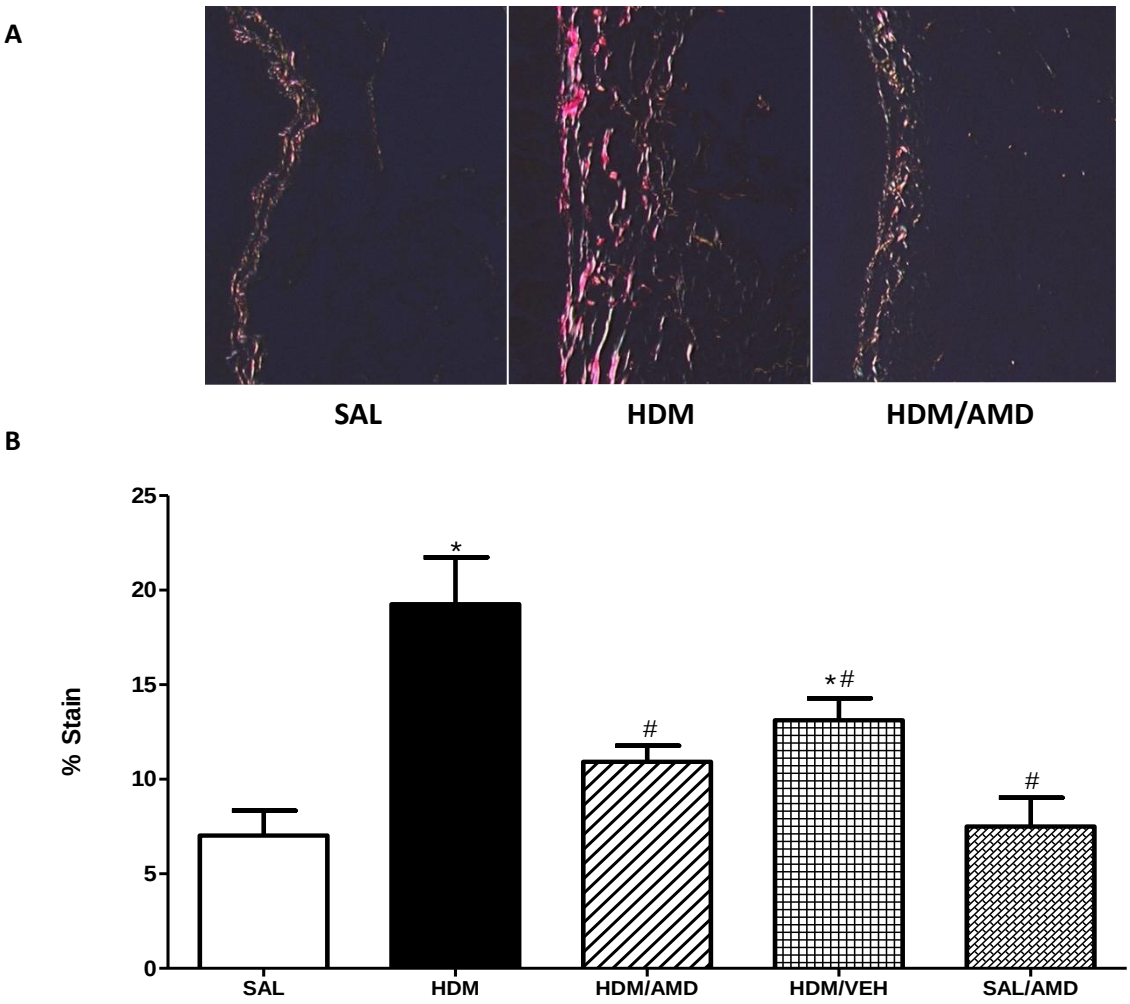


Figure 13: PHIL mice (n≈10/group) (A) Collagen deposition as measured by picrosirius red (PSR) stain- images from 24hrs post-final allergen challenge at 20x magnification. (B) HDM exposed mice had a significantly greater collagen deposition compared to their corresponding SAL group. Treatment with AMD3100 (HDM/AMD) significantly attenuated collagen deposition. The HDM/VEH group, though significantly higher than SAL, was significantly lower than HDM. (*$p<0.05$ compared to saline and #$p<0.05$ compared to HDM) Data expressed as mean± standard error of the mean.

3.2.6 Airway Responsiveness

In the dose-response curve, HDM group expressed the greatest response to increasing doses of MCh. Treatment with AMD3100 attenuated airway responsiveness to SAL levels. HDM/VEH group expressed an airway responsiveness similar to HDM group and SAL/AMD responded to increasing doses of MCh similar to SAL group (Figure 14A).

Maximal resistance was significantly elevated in the HDM group compared to SAL (HDM: 5.51±0.99 vs. SAL: 1.10±0.28 $cmH_2O/ml/s$, p=0.001). Treatment with AMD3100 completely attenuated this increase to SAL levels (AMD: 1.79±0.40 vs. HDM: 5.51±0.99 $cmH_2O/ml/s$, p<0.01). Control groups (HDM/VEH: 3.76±0.77 vs. HDM: 5.51±0.9928 $cmH_2O/ml/s$ and SAL/AMD: 0.83±0.19 vs. SAL: 1.10±0.28 $cmH_2O/ml/s$) showed no vehicle effects (Figure 14B).

Between group comparisons for WT and PHIL HDM groups showed no significant differences in maximal airway resistance to MCh (Figures 8 & 14).

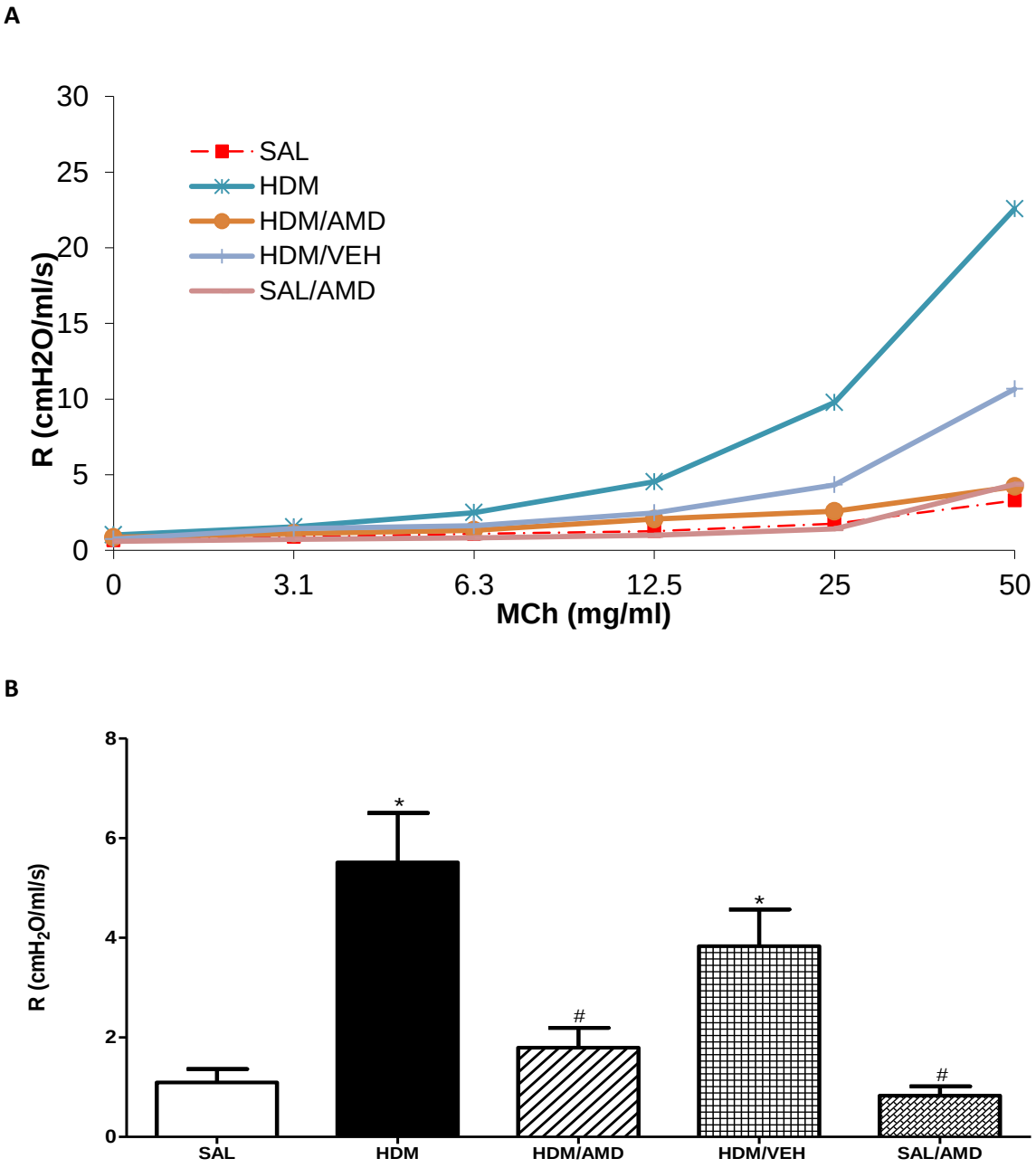


Figure 14: PHIL (n≈10/group) (A) Airway resistance curve and (B) Maximal airway resistance 24hrs post allergen challenge. HDM exposed mice had a higher resistance compared to saline (SAL) exposed mice. Treatment with AMD3100 (AMD) attenuated airway resistance to SAL levels. Control mice showed no placebo effect (*$p<0.05$ compared to saline and #$p<0.05$ compared to HDM) Data expressed as mean± standard error of the mean. Note: values up to 25mg/ml were used in the analysis

CHAPTER 4: DISCUSSION

Asthma is characterized by reversible airway obstruction and AHR (Jeffery PK, 2001; Wilson JW and Hii S, 2006; Knight DA et al., 2010). Histologically, tissue eosinophilia and tissue remodeling are a hallmark of asthmatic lungs. A central focus of this thesis is understanding the role of eosinophils in the traffic of bone marrow-derived endothelial progenitor cells in to the lungs and development of bronchial vascularity in allergic asthmatic responses.

Asthmatic responses are associated with a systemic element that includes activation of the bone marrow leading to the mobilization and lung-homing of myeloid populations of progenitor cells, which are then manipulated by local cytokines and chemokines (Denburg JA et al., 2006) to differentiate *in situ* into pro-inflammatory cells (eosinophils, basophils, mast cells) (Kim et al., 1999; Dorman SC et al., 2004; Southam DS et al., 2004; Hallgren J et al., 2007) and/or structural cells (myofibroblasts, vascular endothelial cells) (Schmidt M et al., 2003). This traffic is thought to be driven by the down-regulation of retentive forces within the BM microenvironment including cell-associated adhesion molecules (namely VLA-4) and receptors for chemoattractants such as SDF-1 (CXCR4) on progenitor cells, allowing for the egress of cells to the peripheral circulation. Homing into the lungs is assisted by an up-regulation of the SDF-1α/CXCR4 axis (Sehmi R et al., 1997; Catalli AE et al., 2008; Wood LJ et al., 1998; Dorman SC et al., 2004; Southam DS et al., 2004).

We have previously shown in a mouse model of asthma that chronic exposure to OVA results in a significant increase in lung-extracted EPC to the lungs, increased

bronchial vascularity, and airway eosinophilia. By modulating the migration of EPC to the lungs with AMD3100 (a CXCR4 antagonist) all of these indices of remodeling were attenuated (Doyle TM et al., 2011). However, SDF-1α is an eosinophil chemoattractant (Lukacs NW et al., 2002) and eosinophil-derived factors (such as VEGF, TNF-α, GM-CSF, NGF) and granule-associated proteins (EPO and LTC_4) (Puxeddu I et al., 2005a; Kay AB, Phipps S, and Robinson DS, 2004) are known to be EPC chemoattractants (Asahara T et al., 1999) and pro-angiogenic factors. Therefore, it was unclear from our earlier findings whether AMD3100 acted directly to attenuate EPC lung accumulation or indirectly through it's anti-inflammatory effects on eosinophils.

The hypothesis of this thesis was: the mobilization, lung-homing and in-situ differentiation of bone marrow-derived endothelial progenitor cells in asthma is independent of eosinophilic inflammation. The aims of my project were: i) determine if HDM exposure induces EPC traffic and lung vascular changes comparable to the established OVA mouse model, ii) investigate if eosinophils are required in Ag-induced lung homing of EPC and increased lung vascularity, and iii) determine if AMD3100 acts independently of eosinophils to attenuate HDM-induced lung accumulation of EPC, increased lung vascularity, and airway dysfunction.

4.1 HDM Chronic Exposure Model in WT Mice

4.1.1 HDM vs. OVA

The current study showed that chronic allergen challenge with HDM results in a significant increase in lung-extracted EPC, bronchial vascularity, airway eosinophilia,

AHR and collagen deposition compared to SAL group (Figures 3-8) which was in keeping with our labs previous study utilizing an OVA exposure model (Doyle TM et al., 2011). However, while treatment with AMD3100 attenuated lung-extracted EPC (Figure 3) and bronchial vascularity (Figure 4), the drug did not attenuate airway eosinophilia in BAL fluid (Figure 5), which was not in agreement with the reduction reported by Doyle *et al*. (Doyle TM et al., 2011).

To confirm the data obtained by BAL fluid, we investigated eosinophilia directly in lung tissue obtained from our study subjects. Eosinophils were enumerated from 3µm sections of the left lung lobe (transverse cross-sections of the first generation airway) (Ellis R et al., 2003) stained with hematoxylin and eosin. We found that eosinophil numbers in the peribronchial region were significantly increased from SAL levels in HDM group, and treatment with AMD3100 significantly attenuated this, though not to SAL group (Figure 6). These results were similar to the information obtained from BAL data in the OVA-based study (Doyle TM et al., 2011).

Differences were observed between OVA and HDM models in the effect of drug on airway eosinophilia which may be because HDM exposed mice have a more robust eosinophilia that could not be as easily attenuated by the same dosage of AMD3100 that was effective for OVA in the OVA model. In a study comparing OVA and HDM chronic exposure models in BALB/c mice, eosinophilia was found to be approximately 3 times greater in HDM mice 24hours following final allergen exposure (DiGiovanni FA et al., 2009).

Also, the dosage of AMD3100 utilized in our HDM model was optimized as the minimum dose to attenuate lung traffic of progenitor cells, bronchial vascularity and airway eosinophilia for a brief-exposure OVA model (Appendix B, Figures 21-23; Doyle T. MSc Thesis, 2009). Though this concentration of drug was sufficient to assess for changes in the other indices of airway remodelling, a greater concentration of drug may have been required to express an attenuation of eosinophilia in BAL fluid for the HDM exposed group.

The robust eosinophilia seen in HDM exposed mice (DiGiovanni FA et al., 2009) may be due to an airway epithelial cell-based mechanism of allergenicity for HDM in addition to antigen interaction and presentation by DCs which is commonly used to promote T_H2 cytokine production in OVA and HDM exposure models as described in detail in section 1.3. In this pathway, HDM triggers the airway epithelium to promote a T_H2-cytokine profile. HDM-associated ligands (such as proteases Der p 2,7 and endotoxin) trigger TLR-4s (toll-like receptor 4) found on airway epithelial cells which produce TSLP, GM-CSF, IL-25, and IL-33. These TLR-4 products further promote inflammatory cell (i.e. eosinophil) accumulation into the lungs, activate DCs for a T_H2 phenotype, and promote the production of IL-5, IL-13, and IL-4. A feedback loop is also achieved which promotes epithelial cell production of cytokines such as IL-8 which recruit eosinophils into the airways (Lambrecht BN and Hammad H, 2012). Thus, the airway epithelium's reaction to HDM may provide an extra driving force for increased eosinophilia compared to OVA.

As HDM has multiple routes through which eosinophilia can occur, it is also possible that 24 hours post final allergen challenge is not an adequate time frame to observe changes in BAL eosinophil levels from drug treatment. Since AMD3100 was administered *i.n.*, the subepithelium along the primary airway may provide the fastest response to drug, thus resulting in an attenuation in eosinophil numbers as observed by H&E staining. However, in BAL fluid eosinophils may first need to leak out from lung tissue into the alveolar sacs and bronchial columns. Thus, any changes in these numbers from treatment with AMD3100 would be indirect, following after changes seen in the lung tissue, and this delayed effect may require BAL to be collected at 48 hours or later in order to observe changes in eosinophilia. As studies have not looked at the time course of eosinophilia in an HDM model, this may be a suitable next step in further understanding this mechanism.

4.2 PHIL Mice

Our group has shown that, in WT BALB/c mice, bronchial vascularity and airway resistance are associated with the modulation of EPC homing into the lungs (Doyle TM et al., 2011). As well, a kinetic study by Asosingh et al. found that, in a brief OVA exposure model, EPCs are immediately recruited into the lungs (6-12hrs), followed by increased bronchial vascularity (24-48hrs) and a delayed, yet rapid increase in eosinophilia (>2 days) (Asosingh K et al., 2007). These findings, along with our current data in PHIL mice using an HDM exposure protocol, suggest that by modulating EPC traffic into the lungs, we can reduce bronchial vascularity. In asthma, increased bronchial

vasculature provides a portal of entry for inflammatory cells through the local up-regulation of adhesion glycoproteins (Wilson JW & Robertson C, 2002). As we reduce bronchial vascularity, this would in turn decrease bronchial circulation of inflammatory cells.

Increased bronchial vascularity can promote oedema and development of increased airway wall thickness, which then may contribute to AHR (Wilson JW & Robertson C, 2002). Thus, with the attenuating bronchial vascularity a reduction of AHR also occurs. Our data supports the view that by modulating EPC traffic into the lungs we can attenuate bronchial vascularity which in turn may contribute to attenuation of airway remodelling changes by affecting the recruitment of pro-inflammatory cells to the lungs and development of lung dysfunction.

Eosinophils express CXCR4 and can be recruited to the lungs via the SDF-1α/CXCR4 axis (Lukacs NW et al., 2002; Dorman SC et al., 2005). Studies have previously shown, following an allergen induced asthmatic exacerbation, these primary effector cells are activated by IL-5, mobilize from the bone marrow (BM) and then traffic to the lung, which is mediated by eotaxin binding to the up-regulated CCR3 receptor on eosinophils (Zimmermann N et al., 2003; Stirling RG et al., 2001; Conroy DM et al., 1997; Zeibecoglou K et al., 1999; Wang et al., 1998; Palframan RT et al., 1998; Rothenberg ME and Hogan SP, 2006; Minshall E et al., 1998; Wood et al., 2002). It has also been shown that once in the lungs, eosinophils release pro-angiogenic factors (such as angiogenin, VEGF, and NGF) which can contribute to bronchial vascularity (Puxeddu I et al., 2005a**;** Kay AB, Phipps S, and Robinson DS, 2004). Therefore, it is possible that

AMD3100 attenuates lung remodelling factors by inhibiting eosinophil traffic into the lungs, which in turn decreases EPC and bronchial vascularity, as well as, airway inflammatory cell circulation and homing into the lungs. The reduction in bronchial vascularity and inflammatory cell accumulation within the lungs would in turn reduce AHR. In order to test our hypothesis that EPC lung-homing is independent of eosinophilic inflammation and confirm AMD3100 acts primarily by modulating EPC traffic into the lungs, we utilized eosinophil deficient PHIL mice and assessed for changes in remodelling factors with HDM exposure and drug treatment.

4.2.1 WT vs. PHIL chronic HDM exposure model

In PHIL mice, our study found that HDM exposure significantly increased the number of lung-extracted primitive progenitors and EPC (Figure 9), bronchial vascularity (Figure 10), airway inflammation (Figure 11), collagen deposition (Figure 13), and maximal airway resistance (Figure 14). When compared with WT HDM group, PHIL HDM had significantly lower lung-extracted progenitor cells (Figure 3 & 9), lower (though not significant) bronchial vascularity (Figures 4 & 10), and significantly higher collagen deposition (Figures 7 & 13). Airway inflammation and maximal resistance to incremental doses of MCh were similar in both WT and PHIL HDM groups (Figures 5 & 11 and 8 & 14 respectively).

As allergen-induced lung homing of EPC and increased vascularity have been observed in PHIL mice, eosinophils may not be required for these changes in airway remodeling. Because by comparison WT mice had a significantly greater level of EPC recruitment to the lungs and level of vascularity following allergen challenge, we propose that

eosinophils and eosinophil-derived products may worsen these disease outcome measures.

4.2.2 WT vs. PHIL drug treatment- progenitor cells, bronchial vascularity and AHR

Concurrent treatment of PHIL transgenic mice with AMD3100 significantly attenuated lung-extracted primitive progenitor and EPC populations to SAL levels (Figure 9). Between group comparisons also showed no significant difference between PHIL HDM/AMD and WT HDM/AMD groups (Figure 3). Thus, AMD3100 was able to inhibit progenitor cell accumulation in the lungs in both WT and eosinophil deficient models.

Unlike WT HDM/AMD group (Figure 4), bronchial vascularity was significantly attenuated to SAL levels in the PHIL HDM/AMD group (Figure 10). Since eosinophils release pro-angiogenic factors such as VEGF and NGF (Puxeddu I et al., 2005a), their presence in WT mice may have attributed to the increased MVD in HDM/AMD group. In WT mice, AMD3100 was able to inhibit EPC accumulation in the lungs which significantly reduced their contribution to bronchial vascularity via differentiation into endothelial cells (Asahara T et al., 1997). As a result, HDM/AMD group had a significantly lower MVD than HDM treated group, but the presence of eosinophils (Figures 5 & 6) may have been sufficient to release enough pro-angiogenic factors to maintain MVD levels above the SAL group. In the transgenic eosinophil deficient PHILs, both the inhibition of EPC recruitment into the lungs and lack of eosinophils may have contributed to the attenuation of lung vascularization in the HDM/AMD group to the level of the SAL treated group.

Treatment with AMD3100 significantly attenuated maximal airway resistance to SAL levels in PHIL mice (Figure 14) and, when compared to WT HDM/AMD group (Figure 8), there was no significant difference. This is in agreement with previous studies that have shown the ability of AMD3100 to block development of AHR (Doyle TM et al., 2011; Lukacs NW et al., 2002).

As there were no differences between HDM and HDM/VEH as well as SAL and SAL/AMD group, we can confirm there was no placebo effect.

4.2.3 WT vs. PHIL drug treatment- airway inflammation and lung tissue eosinophilia

In the BAL fluid of PHIL mice, treatment with AMD3100 significantly attenuated total cell number and macrophages to SAL as well as attenuated lymphocyte numbers, though not to SAL (Figure 11). These values were significantly lower than WT HDM/AMD (Figure 5).

In PHIL mice, HDM and HDM/AMD groups were found to have low but no significant numbers of eosinophils in the peribronchial region of the left lung (Figure 12). In a study by Ramzi Fattouh *et al.* eosinophils were also detected following HDM exposure in BAL fluid (Fattouh R et al., 2011). Flow cytometric analysis of whole lung digests were also done, identifying eosinophils as $CD45^{+}Gr\text{-}1^{low/int}SSC^{int/high}$ which were detected at low levels in both SAL and HDM groups (Fattouh R et al., 2011). An acute OVA exposure model was used in the study that first introduced PHIL mice. This study also noted trace amounts (<0.5% eosinophils) in the BAL fluid of one of four PHIL mice as well as mentioned a few eosinophils observed in blood films and bone marrow smears

(Lee JJ et al., 2004). These studies further support our results and suggest that while eosinophils are present in some PHIL mice, their effects on airway remodelling are negligible.

4.3 Collagen Deposition

Collagen deposition was assessed as a secondary outcome measure because fibrocytes have been found to express CXCR4 (Mehrad, Burdi, and Strieter 2009; Phillips RJ et al., 2004). We cannot make any statements from our data with confidence due to a vehicle effect which was observed in our control HDM/VEH group (Figure 13).

Based on previous studies and knowledge, we would expect HDM exposed WT mice to have greater collagen deposition compared to eosinophil deficient PHIL mice (Fattouh R et al., 2011, Puxeddu I et al., 2009). Eosinophil granule basic proteins modulate tissue remodeling by acting on the extracellular matrix components and on some fibroblast properties, further promoting a profibrogenic environment in WT mice which PHIL mice lack. For example, eosinophil cationic protein inhibits proteoglycan degradation and increases intracellular accumulation of glycosaminoglycans in human lung fibroblasts. Major basic protein acts in collaboration with IL-1 and TGF-β to increase production of pro-fibrogenic cytokines (IL-6, IL-11, and LIF-1) (Puxeddu I et al., 2009), whereas eosinophil-derived neurotoxin stimulates fibroblast proliferation. Eosinophil-derived cytokines TGF-B and fibroblast growth factor (FGF)-2 are well-known, potent pro-fibrogenic mediators (Puxeddu I et al., 2005a). With these means of promoting collagen deposition, the presence of eosinophils in WT mice may not allow for

a significant drug effect as seen in the previous OVA model (Doyle TM et al., 2011), but may allow for AMD3100 to attenuate collagen deposition in PHIL mice. Though this is suggested by our data (Figure 13), these results must be replicated.

4.4 Conclusion

In summary, our results show that HDM exposure induces EPC traffic and lung vascular changes comparable to the established OVA mouse model. Using this model in PHIL mice, HDM exposure stimulated lung accumulation of EPC and increase lung vascularity comparable to that observed in WT mice albeit at lower levels. Furthermore, use of AMD3100 concurrently with Ag attenuated lung accumulation of EPC and lung vascularity in PHIL mice which was comparable to the reductions seen in WT mice. A comparative analysis of the lung remodelling changes in PHIL versus WT mice suggests that eosinophils and eosinophil-derived products contribute to the worsening of pathological outcome measures of asthma such as bronchial vascularity via the release of pro-angiogenic factors.

In conclusion, our findings suggest that the lung accumulation of endothelial progenitor cells in HDM-induced asthma is independent of eosinophilic inflammation.

4.5 Limitations

4.5.1 Optimal concentration of drug

When compared with OVA, HDM exposure has previously been shown to produce differences in airway eosinophilia and maximal bronchoconstriction (DiGiovanni FA et al., 2009). Also, as the routes of entry are different between the two, the

allergenicity of HDM differs from OVA (Sjöbring U and Taylor JD, 2007) suggesting treatment protocols for HDM-induced lung dysfunction should be individualized for the allergen.

The concentration of AMD3100 used in this study was optimized for a brief OVA exposure model (Appendix B, Figure 21-23; Doyle T. MSc Thesis, 2009). Though the dosage was sufficient to detect drug induced changes in lung accumulation of EPC, bronchial vascularity and airway responsiveness, changes in airway inflammatory cells from collected BAL fluid were not easily identifiable. This suggests a greater concentration of drug may have been required to obtain optimal results. However, we were able to detect airway eosinophil changes in keeping with OVA/AMD by direct staining of left lung lobe sections with hematoxylin and eosin and thus can confirm AMD3100 was able to attenuate a factor of airway inflammation as well as speculate the other changes seen in Doyle *et al*. may have also subtly occurred (2011).

4.5.2 Drug specificity

AMD3100 is a CXCR4 antagonist used to inhibit the chemotactic activity of SDF-1α. Whilst SDF-1α is a chemoattractant of progenitor cells, it has also previously been shown to be chemotactic for neutrophils, monocytes, and lymphocytes in addition to eosinophils (Aiuti A et al., 1997; Bleul CC et al., 1996; Lukacs NW et al., 2002).

Our current study did not detect a reduction in inflammatory cells for WT HDM/AMD group (Figure 5), but did observe significantly lower macrophage and lymphocyte levels in PHIL HDM/AMD when compared with PHIL HDM (Figure 11). In the OVA-based study previously conducted by our lab, OVA/AMD group had

significantly lower levels of neutrophils, macrophages, and eosinophils when compared with OVA group (Doyle TM et al., 2011). With the knowledge that AMD3100 treatment attenuated macrophage, lymphocyte and neutrophil levels in the current, and previous study (Doyle TM et al., 2011), the potential role of these inflammatory cells in the morphometric changes observed in our study should be assessed in order to gain better insight into their contribution to our hypothesis.

4.5.3 Assessment of Airway Eosinophilia

Airway eosinophilia was assessed by enumerating eosinophils from BAL fluid as well as staining of lung sections with hematoxylin and eosin. Though this combination of analysis had previously been suggested to be sufficient (Walters EH, Ward C, and LI X, 1996), flow cytometric analysis may have provided additional benefits in comparing eosinophilia between groups. Eosinophil enumeration via flow cytometry ($CD45^{+}Gr\text{-}1^{low/int}SSC^{int/high}$) (Fattouh R et al., 2011) would have allowed for a percentage composition assessment of eosinophils from whole lung digests. If there is a difference between lung sizes of WT and PHIL mice, this would eliminate the size factor and look solely at lung content.

4.5.4 Collagen deposition

The significant effect of AMD3100 treatment on collagen deposition in a PHIL mouse model had been hindered in our study by a vehicle effect found in our control HDM/VEH group (Figure 13). As well, the optimal time to assess airway fibrosis is at 4 weeks post allergen challenge as cellular inflammation at earlier time points interferes with accurate staining for collagen deposition (Leigh R et al., 2002). However, the

suboptimal timing may not have a detrimental effect, as the 24hr post-challenge assessed WT HDM group was in keeping with the previously assessed 4 week post-challenge assessed OVA HDM group having a significantly higher collagen deposition (Doyle TM et al., 2011).

Confounding evidence was also suggested as the HDM chronic exposure study by Fattouh *et al*., had a significantly lower collagen deposition for PHIL HDM group when compared with WT HDM group (2011). This contradicts our finding suggesting HDM PHIL group had a significantly higher collagen deposition when compared with WT HDM group (Figures 13 & 7).

4.6 Future Directions

4.6.1 ΔdblGATA model

A pilot study was conducted using a second strain of eosinophil deficient mice referred to as ΔdblGATA. These mice are unable to produce eosinophils, but erythroid cells, megakaryocytes, mast cells and earlier hemopoietic precursors are not affected (Yu C et al., 2002).

A previous study using ΔdblGATA mice in an HDM chronic exposure model found HDM group had significantly increased total cell number and mononuclear cells, when compared with SAL group, in BAL fluid and these were significantly less in ΔdblGATA HDM group when compared with WT HDM (Fattouh R et al., 2011). Assessment of lung fibrosis also showed ΔdblGATA HDM group had significantly higher

collagen deposition when compared with ΔdblGATA SAL group and WT HDM group (Fattouh R et al., 2011).

Our current pilot study produced inconclusive results, showing only trends towards increases in most of the outcome indices measured. HDM group showed a trend towards increased EPC (Appendix A, Figure 15) and significant increases in MVD (Appendix A, Figure 16) and airway inflammation (Appendix A, Figure 17) when compared with SAL group. HDM group also had trends toward increased collagen deposition (Appendix A, Figure 18), and airway maximal resistance (Appendix A, Figure 19) compared to SAL group.

The ΔdblGATA pilot study was underpowered (SAL: n=5; HDM: n=6). Based on previous progenitor cell studies (Southam DS et al., 2004 and Doyle TM et al., 2011) we have used n=10 per group to see significant allergen-induced changes in lung-extracted EPC numbers and bronchial vascularity. Thus to support the findings from the PHIL mice that EPC migration and lung vascularity changes can occur independently of eosinophils, we will need to complete the ΔdblGATA study to n=10 per group.

4.6.2 Drug effects on Lymphocytes

As previously mentioned, the SDF-1α/CXCR4 mechanism is not specific to progenitor cells, and can also attract inflammatory cells such as lymphocytes (Luckas NW et al., 2002). Studies have shown that AMD3100 can promote a rapid and significant mobilization of all conventional T-lymphocyte populations as well as enhanced mobilization of Tregs and effector memory T-cells from the BM into PB (Kean LS et al., 2011). As the current thesis has found, eosinophils are not required in allergen-induced

increased EPC lung accumulation, bronchial vascularity, and development of AHR, it would also be beneficial to investigate the potential role/contribution lymphocytes play in these outcome measures.

Previous studies that have used PHIL mice and studied T-lymphocytes have found that eosinophils are required for T-cell recruitment into the lungs following allergen challenge (Jacobsen EA et al., 2008) and that T-cell mediated inflammation is not required for development of AHR (Leigh R et al., 2002). However, while Jacobsen et al. had found no significant difference between BAL lymphocyte numbers in PHIL saline and allergen groups (2008), a previous study by Fattouh et al. as well as the current thesis had found a significant increase in PHIL HDM group compared to PHIL SAL (2011). Thus, in an HDM chronic exposure model, a significant T-lymphocyte recruitment is not dependent on the presence of eosinophils, further supporting the need to assess their potential contribution to the morphological changes observed in our study.

CXCR7 (formally known as the orphan receptor RDC1), had been recently found as a secondary receptor for SDF-1α (Balabanian K et al., 2005). The levels of expression for CXCR7 on the cell surface of T-lymphocytes have been debated (Balabanian K et al., 2005; Hartmann TN et al., 2008). As well, CXCR7 has been proposed to function as a modulator of the SDF-1α/CXCR4 axis (Hartmann TN et al., 2008). However, T-lymphocytes from both studies mentioned above were taken from peripheral blood samples. Thus, the cell surface expression of CXCR7 and CXCR4 in lung-extracted T-lymphocytes as well as flow cytometric analysis of their relative levels following allergen

exposure and treatment with AMD3100 may further elucidate their mechanism of homing into the lungs.

4.6.3 Role of CXCR7 in EPC proliferation

A recent study by Costello CM et al. had found that while CXCR4 is essential for chemotactic cell migration in pulmonary hypertensive disease, CXCR7 is the receptor through which endothelial cell regeneration, repair and proliferation is mediated (2012). As a running hypothesis in our lab is, following allergen exposure, BM-derived EPC home into the lungs and differentiate into new vessels (Asahara T et al., 1997), contributing to increased bronchial vascularity, it may be beneficial to investigate the potential for an SDF-1α/CXCR7 axis modulating EPC proliferation within the lungs.

4.6.4 Drug effects in lung fibrosis

Concurrent treatment with AMD3100 was found to produce a trend towards decreased collagen deposition when compared to HDM group in WT mice and did significantly attenuate airway fibrosis to SAL levels in PHIL mice. As these outcome measures were 24 hours post final allergen challenge and a delay period of 4 weeks is normally required to assess collagen deposition, this may suggest that, if given the optimal wait period (after which eosinophil levels would have declined and not contributed to fibrosis (Kay AB et al., 2004; Leigh R et al., 2002)), WT mice would have identical results to PHIL. A previous study by Song et al. showed that treatment with AMD3100 inhibits fibrocyte mobilization to the lung and reduces airway fibrosis (2010). Thus, a more detailed assessment of the role AMD3100 plays in airway fibrosis,

primarily focusing on fibrocyte homing to the lungs, may be useful in further understanding this drug's effects.

4.6.5 Clinical AMD3100 experiments in lung

AMD3100 has recently approved by the FDA for clinical trials for auto hemopoietic stem cell transplants, where it is used for stem cell mobilization from the bone marrow (Kean MP et al., 2011). We have shown that AMD3100 is also capable of preventing overall lung dysfunction and development of AHR, when administered concurrently, from the current thesis and previous studies in mouse models (Doyle TM et al., 2011; Lukacs NW et al., 2002). Though this drug has been most effective in a preventative treatment regimen, in a reversal treatment regimen, AMD3100 significantly attenuated primitive progenitor cell numbers and bronchial vascularity, as well as showed a trend towards decreased maximal airway resistance to incremental doses of MCh (Doyle TM et al., 2011). As AMD3100 was able to attenuate bronchial vascularity and the development of AHR after the asthmatic phenotype was established, these effects would greatly benefit patients. A plausible next step would be to optimize a human airway treatment protocol for AMD3100 and assess the effects of this drug in clinical trials.

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APPENDIX A- Pilot Study: HDM Chronic Exposure Model in eosinophil deficient ΔdblGATA Mice

To further confirm the results of the PHIL data, a second strain of eosinophil deficient mice ΔdblGATA underwent the HDM chronic exposure protocol and we assessed for the remodelling factors as mentioned previously (Figure 2). Two groups of mice were utilized, SAL control (n=5) and HDM (n=6).

Appendix A.1 Lung-extracted progenitor cell numbers

A trend towards decreased lung-extracted PC (Sca-1$^+$c-kit$^+$) was observed in HDM exposed mice compared to SAL (HDM: 4 169±2 411 vs. SAL: 19 705±4 220 cells, p= 0.57). A trend towards increased EPC (Sca-1$^+$c-kit$^+$VEGFR-2$^+$) was observed in the HDM group compared to SAL (HDM: 2 222±1 302vs. SAL: 4 653±884 cells, p= 0.83) (Appendix A, Figure 15).

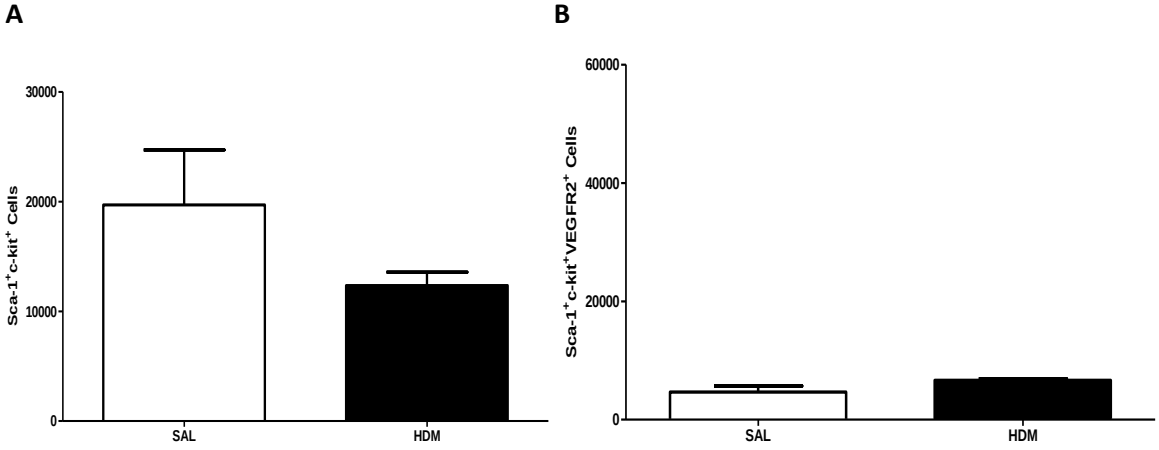


Figure 15: ΔdblGATA mice (SAL: n=5, HDM: n=6) lung-extracted (A) Total primitive progenitor (Sca-1$^+$c-kit$^+$) and (B) Total endothelial progenitor (EPC) (Sca-1$^+$c-kit$^+$VEGFR-2$^+$) cell populations in lung samples 24hrs post allergen challenge enumerated by flow cytometry. There was a trend towards higher primitive progenitor cells in the SAL group compared to HDM exposed mice. EPC numbers were similar in both SAL and HDM groups. (*p<0.05 compared to saline and #p<0.05 compared to HDM) Data expressed as mean± standard error of the mean.

Appendix A.2 Bronchial vascularity

MVD was significantly increased in the HDM group compared to SAL mice (HDM: 139.81±31.67 vs. SAL: 43.16±26.50; $p<0.005$) (Appendix A, Figure 16).

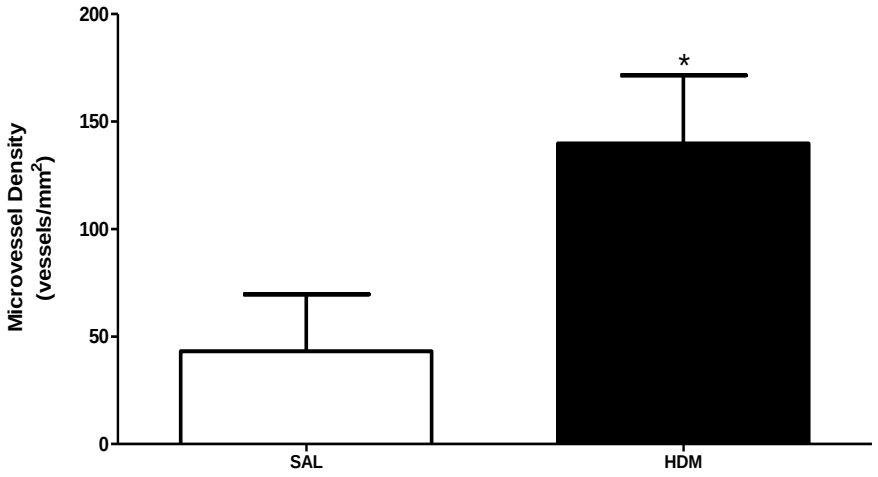


Figure 16: ΔdblGATA mice (SAL: n=5, HDM: n=6) (A) Microvessel Density (MVD) as measured by von Willebrand factor staining (vWF) - images from 24hrs post-final allergen challenge at 40X magnification. (B) Vessels counted were 10µm or less (arrows) in a 50µm bandwidth area around the main airway. MVD was significantly higher in HDM groups compared to SAL. (*p<0.05 compared to saline and #p<0.05 compared to HDM) Data expressed as mean± standard error of the mean.

Appendix A.3 Airway inflammation via BAL

Compared to SAL groups, HDM treated mice had significantly elevated total cell counts (SAL: 4.23±0.65 vs. HDM: 8.69± 0.70 ($x10^4$) cells, p=0.005), neutrophils (SAL: 0.17±0.05 vs. HDM: 2.63± 0.33 ($x10^4$) cells, p<0.0001), and lymphocytes (SAL: 0.45±0.21 vs. HDM: 3.21± 0.61 ($x10^4$) cells, p<0.0001). As this strain is eosinophil deficient, no eosinophils were detected in BAL fluid (Appendix A, Figure 17).

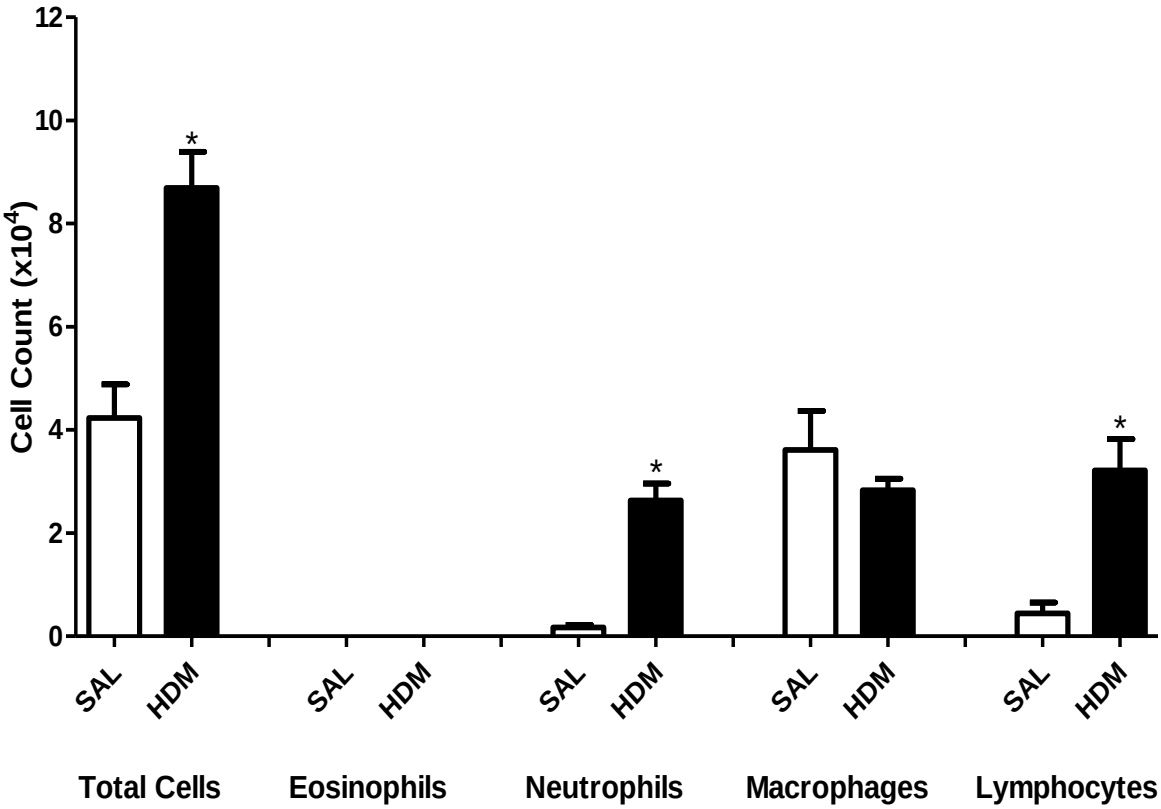


Figure 17: Airway inflammation as measured by bronchial alveolar lavage (BAL) for ΔdblGATA mice (SAL: n=5, HDM: n=6). HDM exposed mice had a significantly increased number of inflammatory cells (excluding macrophages) compared to SAL. (*p<0.05 compared to saline and #p<0.05 compared to HDM) Data expressed as mean± standard error of the mean.

Appendix A.4 Lung eosinophilia

To confirm ΔdblGATA mice are devoid of eosinophils, an H&E stain was performed on lung sections. Negligible numbers of eosinophils (1-2 eosinophils/group) were observed in SAL and HDM groups (SAL: 0.00± 0.00, HDM: 0.00±0.00 eosinophils/mm^2) (Figure 18).

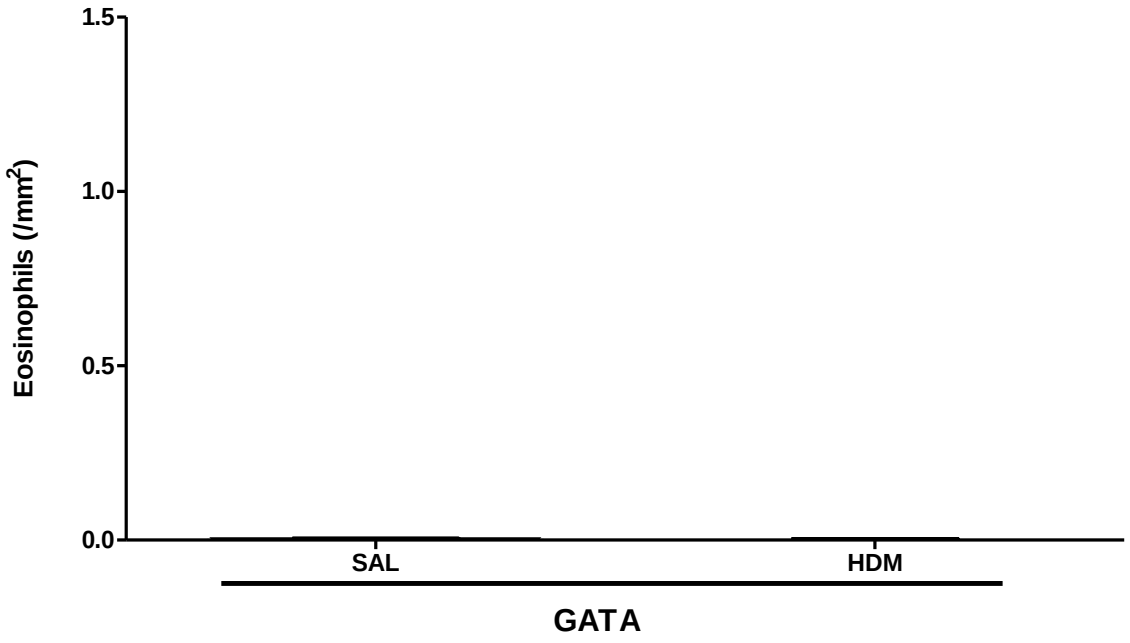


Figure 18: ΔdblGATA (n=5-6/group) (A) Airway eosinophils as measured by hematoxylin and eosin (H&E) stain- images from 24hrs post-final allergen challenge at 40x magnification. (B) Negligible numbers of eosinophils were observed in SAL, HDM and HDM/AMD groups. (*p<0.05 compared to saline and #p<0.05 compared to HDM) Data expressed as mean± standard error of the mean.

Appendix A.5 Collagen deposition

HDM exposed mice had a trend towards a higher percentage of stain compared to SAL (HDM: 11.42±1.07 vs. SAL: 10.07±1.62%, p=0.61) (Appendix A, Figure 19).

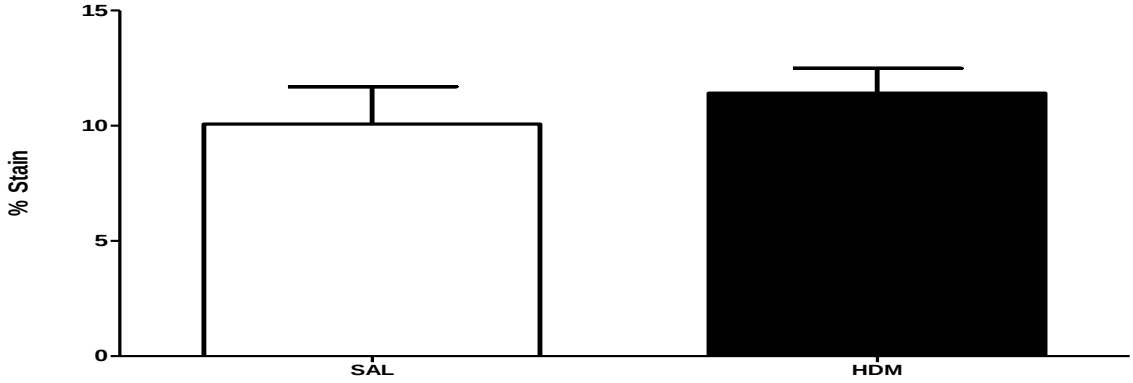


Figure 19: ΔdblGATA (SAL: n=5, HDM: n=6) (A) Collagen deposition as measured by picrosirius red (PSR) stain- images from 24hrs post-final allergen challenge at 20x magnification. (B) HDM exposed mice had a trend toward increased collagen deposition compared to SAL (*p<0.05 compared to saline and #p<0.05 compared to HDM) Data expressed as mean± standard error of the mean.

Appendix A.6 Airway Responsiveness

In the dose-response curve, HDM exposed mice had a trend towards a greater response to MCh compared to SAL (Appendix A, Figure 20A).

There was a trend towards an increase in maximal airway resistance to MCh for the HDM group compared to SAL (HDM: 1.85±0.62 vs. SAL: 1.92±1.07 $cmH_2O/ml/s$, p=0.51) (Appendix A, Figure 20B).

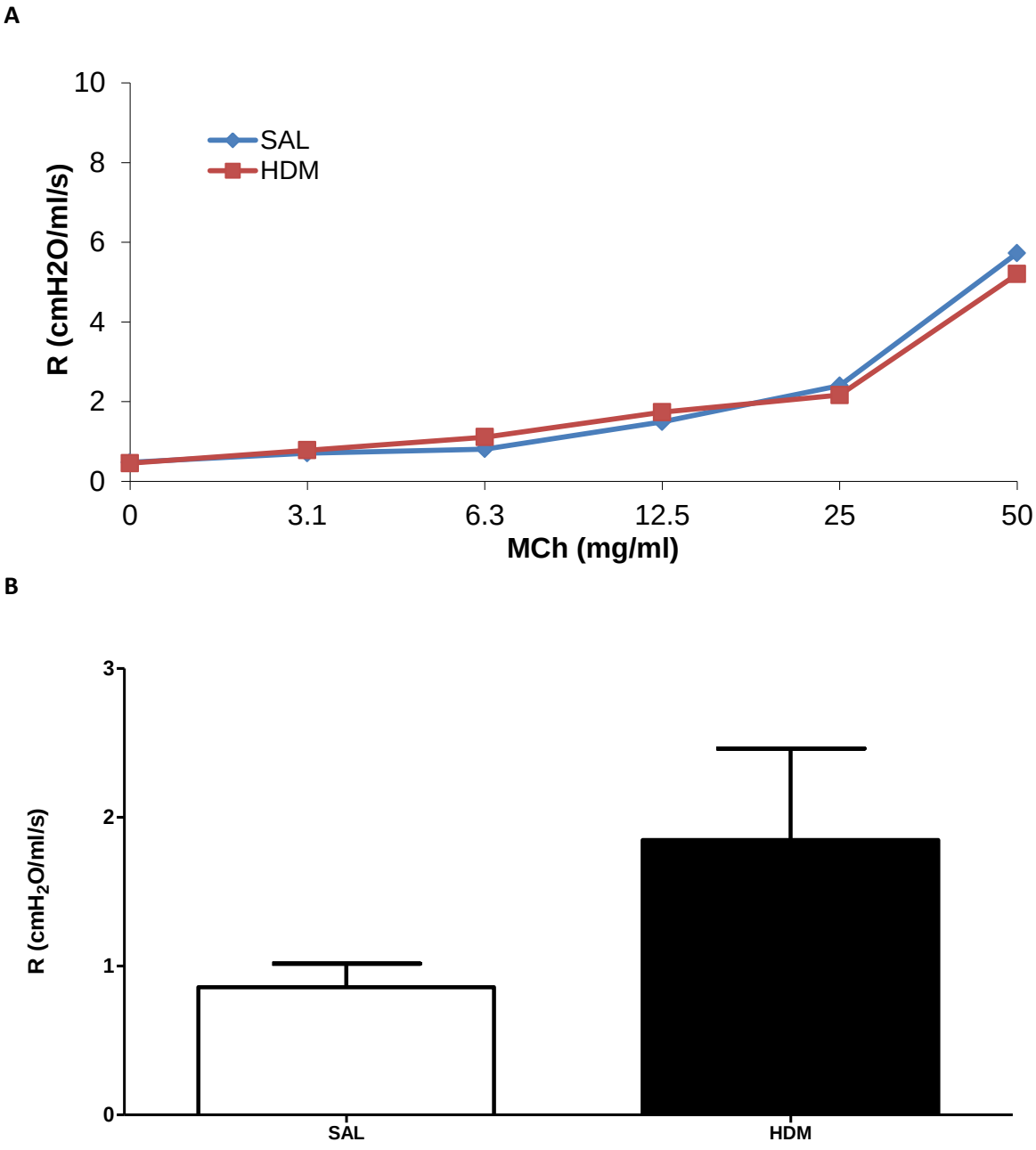
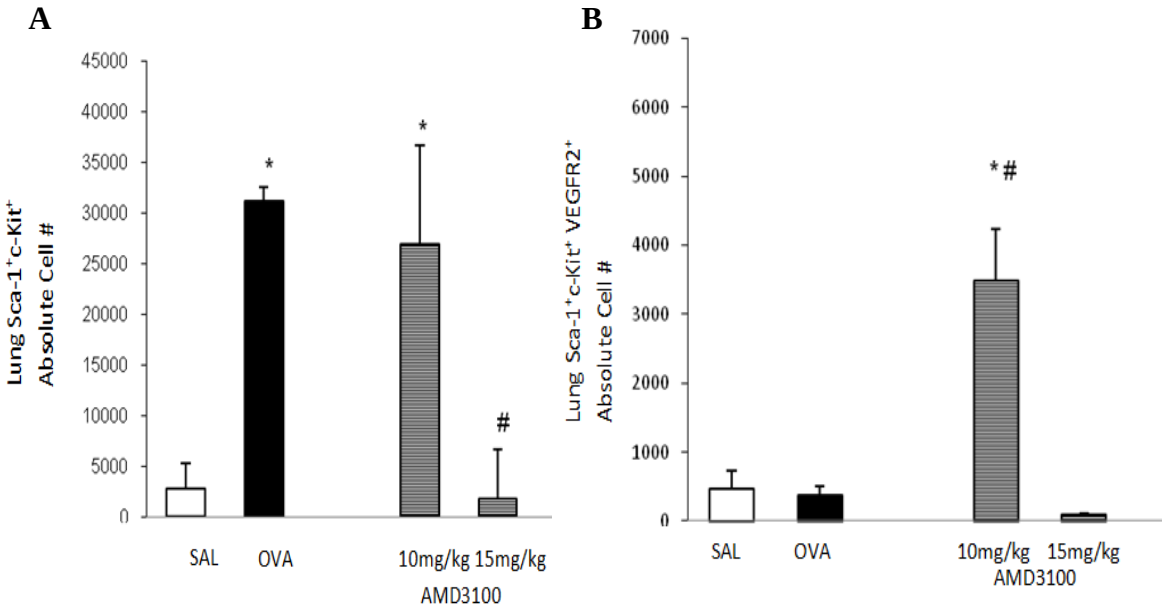
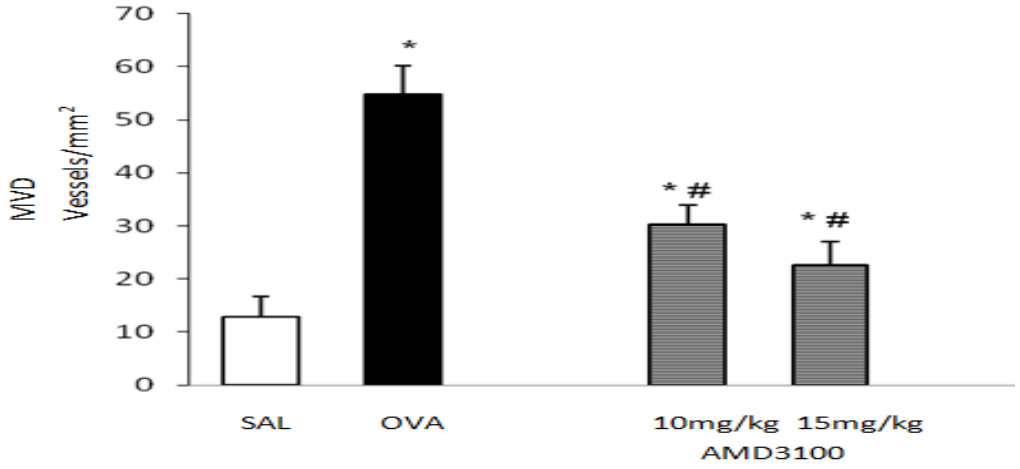


Figure 20: ΔdblGATA (SAL: n=5, HDM: n=6) (A) Airway resistance curve and (B) Maximal airway resistance 24hrs post allergen challenge. HDM exposed mice had a trend towards higher resistance compared to the SAL group. (*p<0.05 compared to saline and #p<0.05 compared to HDM) Data expressed as mean± standard error of the mean. Note: values up to 25mg/ml were used in the analysis.

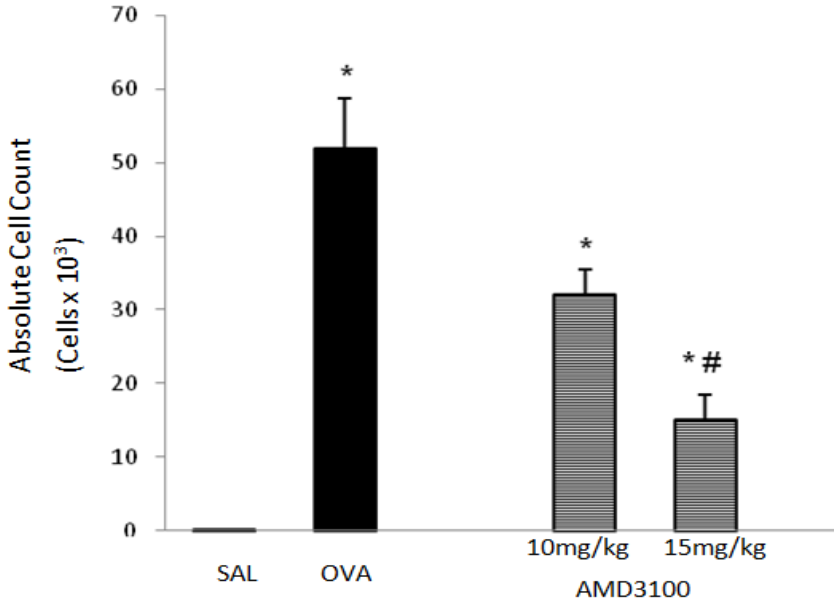
APPENDIX B- OVA Pilot Study: Optimizing AMD3100 Dosage



(A) Primitive progenitor cell (Sca-1+c-Kit+) and (B) Total vascular endothelial progenitor cell (Sca-1+c-Kit+VEGF2R+) populations in lung samples (n=5) 24hrs post allergen challenge. 15mg/kg of AMD 3100 maintained lung progenitors at saline levels. (*p< 0.05 compared to saline and #p<0.05 compared to OVA). Data expressed as mean ± standard error of the mean. (Doyle TM, MSc Thesis, 2009).



Microvessel Density (MVD) as measured by von Willebrand factor staining (vWF) (n=5) 24hrs post allergen challenge where AMD3100 in all dosages effectively attenuated MVD (*p< 0.05 compared to saline and # p<0.05 compared to OVA). Data expressed as mean ± standard error of the mean. (Doyle TM, MSc Thesis, 2009).



Airway eosinophilia (n=5) 24hrs post allergen challenge. All dosage groups of AMD 3100 effectively attenuating eosinophilia (*p< 0.05 compared to saline and # p<0.05 compared to OVA). Data expressed as mean ± standard error of the mean. (Doyle TM, MSc Thesis, 2009).