DENDRITIC CELL SUBSETS IN ASTHMA

THE BIOLOGY OF DENDRITIC CELL SUBSETS IN ALLERGEN-INDUCED ASTHMA

By

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A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Doctor of Philosophy

DOCTOR OF PHILOSOPHY (2013)

Medical Sciences

McMaster University, Hamilton, Ontario

TITLE: The Biology of Dendritic Cell Subsets in Allergen-Induced Asthma

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NUMBER OF PAGES: cciv, 204

ABSTRACT

Asthma is an inflammatory disorder of the airways, and there has been growing insight into the cellular and molecular mechanisms underlying the inflammatory basis of this disease. Research into the inflammatory mechanisms of asthma has progressively shifted focus from downstream effectors, such as mast cells and eosinophils, up to Th2 lymphocytes and their proallergic cytokines. Even more upstream in the allergic cascade are dendritic cells (DCs), potent APCs that orchestrate immune responses. Evidence supporting a role of DCs in regulating airway allergic inflammation is derived mainly from animal studies. In animal models of asthma, myeloid DCs (mDCs) induce and maintain airway inflammation, while plasmacytoid DCs (pDCs) mediate tolerance and lung homeostasis. It remains uncertain, however, whether this concept of pro-allergic mDCs and anti-allergic pDCs translates from animal to human models. The overall objective of this thesis was to investigate the biology of DC subsets in allergen-induced asthma in asthmatic subjects. Initially, we demonstrate that both mDCs and pDCs increase in the airways of subjects with mild asthma after allergen inhalation. Next, we describe a distinct subpopulation of mDCs, called mDC2s, and demonstrate their association with allergy and asthma severity. Expanding on these findings, we show that mDC2s increase in the airways of mild asthmatics after allergen challenge. Lastly, we explore the potential of pharmacological therapies, anti-OX40L MAb and anti-TSLP MAb, to affect DCs in subjects with mild asthma, and demonstrate no effect of either drug on circulating DC subsets. The studies presented here provide evidence for multiple DC subtypes being involved in the regulation of allergen-induced inflammatory

responses, and support continued investigations into the biology of different DC subsets in allergen-induced asthma.

PREFACE

Each study described in this thesis is presented as an independent, but thematically-related, piece of work that required the contribution of many individuals.

Chapter 2:

Myeloid and plasmacytoid dendritic cells in induced sputum after allergen inhalation in subjects with asthma

Authors: Benny Dua, Richard Watson, Gail Gauvreau, Paul O'Byrne

This study was carried out during my work as a Master's student, and due to its relevance to this topic, it was agreed to include in this thesis. I, together with Dr. Gail Gauvreau and Dr. Paul O'Byrne, were responsible for the experimental design, interpretation of the results, and editing of this manuscript. Richard Watson conducted allergen challenges on asthmatic subjects.

Chapter 3:

Myeloid dendritic cells type 2 in allergic asthma

Authors: Benny Dua, Steven Smith, Takashi Kinoshita, Haruki Imaoka, Gail Gauvreau, Paul O'Byrne

This study was carried out during my work as a PhD student. I, together with Dr. Gail Gauvreau and Dr. Paul O'Byrne, were responsible for the experimental design, interpretation of the results, and editing of this manuscript. Dr. Steve Smith, Dr. Takashi Kinoshita and Dr. Haruki Imaoka assisted with the acquisition of the data.

Chapter 4:

Myeloid dendritic cells type 2 after allergen inhalation in asthmatic subjects

Authors: Benny Dua, Wei Tang, Richard Watson, Gail Gauvreau, Paul O'Byrne

This study was carried out during my work as a PhD student. I, together with Dr. Gail Gauvreau and Dr. Paul O'Byrne, were responsible for the experimental design, interpretation of the results, and editing of this manuscript. Dr. Wei Tang assisted with the acquisition of the data. Richard Watson conducted allergen challenges on asthmatic subjects.

Chapter 5:

The effects of anti-OX40L and anti-TSLP monoclonal antibodies on circulating dendritic cells

Authors: Benny Dua, Richard Watson, Gail Gauvreau, Paul O'Byrne

This study was carried out during my work as a PhD student. Dr. Gail Gauvreau and Dr. Paul O'Byrne were lead investigators in both these of large, multi-center clinical trials. I, together with Dr. Gail Gauvreau and Dr. Paul O'Byrne, were responsible for the interpretation of the results, and editing of this manuscript. Richard Watson conducted allergen challenges on asthmatic subjects. Other sites were responsible for coordinating their own study visits, and sent samples to McMaster University for measurements of mDC1s, mDC2s and pDCs.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Paul O'Byrne for giving me the opportunity to pursue my PhD. Not only are you an expert and true professional in your field, but you are a great mentor and role model. Your knowledge and drive for scientific discovery are inspiring, and I can only hope to emulate your example in my career as a clinician scientist. I am truly grateful for all the guidance, understanding and support throughout these years.

I would also like to extend my thanks to Dr. Gail Gauvreau. Although I wasn't your student on record, you treated me like one of your own. From the moment I stepped into the lab, you have always been there to help. Without your day-to-day guidance and support, this thesis would not have been possible.

To Dr. Kieran Killian, thank you for the knowledge you have imparted. You were always willinging to help, and our talks will not be forgotten. You have also mentored me throughout these years and it has been a pleasure and privilege to learn from you.

To the remaining members of my supervisory committee, Dr. Denis Snider and Dr. Mark Larche, I appreciate all your input and feedback throughout my PhD studies.

To the students and staff of the Cardiorespiratory Lab, thank you for all the ongoing support. From helping with studies, to having a few laughs, you made this experience a meanginful and enjoyable one.

Lastly, I want to thank my family and friends for your continued encouaragment, understanding and support. A special thank you goes to Bubloo, Hem, Mom and Dad.

My PhD would not have been possible without you. My debt to you is beyond measure and I am forever grateful for your unconditional love and support.

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LIST OF ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
ANOVA	analysis of variance
APCs	antigen presenting cells
BALF	bronchoalveolar lavage fluid
BDCA	blood dendritic cell antigen
CBC	complete blood count
CCL	chemokine ligand
CCR	chemokine receptor
c-KitL	c-Kit ligand
CLRs	C-type lectin-like receptors
DCCK	dendritic cell–derived chemokine
DCs	dendritic cells
DC-SIGN	DC-specific intercellular adhesion molecule-grabbing nonintegrin
Der p	Dermatophagoides pteronyssinus
DPBS	dulbecco's phosphate buffered saline
DTT	dithiothreitol
EAR	early asthmatic response
ECP	eosinophil cationic protein
ELC	EBI1-ligand chemokine
ELISA	enzyme-linked immunosorbant assay

EPO	eosinophil peroxidase
Fc	fragment, crystallisable
FcεRI	Fc epsilon receptor I
FEV_1	forced expiratory volume in one second
Flt-3L	FMS-related tyrosine kinase-3 ligand
FSC	forward scatter
GM-CSF	granulocyte macrophage-colony stimulating factor
HDM	house dust mite
HDM DP	house dust mite Dermatophagoides pteronyssinus
HIV	human immunodeficiency virus
ICAM	intercellular adhesion molecule
IDO	indoleamie 2,3-dioxygenase
IFN	interferon
IL	interleukin
LABA	long acting beta-2 agonists
LAR	late asthmatic response
LARC	liver activation regulated chemokine
LCs	langerhans cells
LFA	lymphocyte function-associated antigen
LPS	lipopolysaccharide
MAb	monoclonal antibody
MANOVA	multiple analysis of variance

MBP	major basic protein
MCh	methacholine
MDC	macrophage derived chemokine
mDCs	myeloid dendritic cells
mDC1s	myeloid dendritic cells type 1
mDC2s	myeloid dendritic cells type 2
MFI	median fluorescence intensity
MIP	macrophage inflammatory protein
MHC	major histocompatability complex
MIICs	MHC class II-rich compartments
MLNs	mediastinal lymph nodes
NLRs	NOD-like receptors
NSAIDS	nonsteroidal anti-inflammatiry drugs
OVA	ovalbumin
OX40L	OX40 ligand
PBS	phosphate buffered saline
PC_{20}	provocative concentration causing a 20% fall in FEV_1
pDCs	plasmacytoid dendritic cells
PDL-1	programmed death ligand 1
PPAR	peroxisome proliferator-activated receptor
PRRs	pattern-recognition receptors
RANTES	regulated upon activation, normal T-cell expressed and secreted

RGW	ragweed
SABA	short acting beta-2 agonists
SCF	stem cell factor
SCID	severe combined immunodeficiency
SLC	secondary lymphoid-tissue chemokine
SSC	side scatter
TARC	thymus and activation regulated chemokine
TCRs	T-cell receptors
TGF	tumour growth factor
Th	T-helper
TLRs	toll-like receptors
TNF	tumor necrosis factor
Treg	T-regulatory
TSLP	thymic stromal lymphopoietin
VC	vital capacity
WHO	World Health Organization

DECLARATION OF ACADEMIC ACHIEVEMENT

I have been the major contributor to the work presented in this thesis. At this moment, I would also like to acknowledge those who have helped contribute to the content of this thesis. Dr. Haruki Imaoka, Dr. Takashi Kinoshita and Dr. Wei Tang helped with subject enrollment and screening, and also assisted with performing diluent challenges. Richard Watson, Heather Campbell and George Obminski performed all the allergen challenges. Tara Strinich, Abbey Torek and Catie Obminski helped process sputum. The contribution of these individuals was important to the completion of this thesis.

CHAPTER 1

INTRODUCTION

Asthma is one of the most common chronic inflammatory diseases in the world. According to the World Health Organization (WHO), 300 million people suffer from asthma, and it is estimated that 1 in every 250 deaths worldwide is due to asthma (Bloemen et al., 2007; Masoli et al., 2004). Economic costs for asthma are significant, with direct and indirect medical expenditures expected to exceed the combined costs of HIV/AIDS and tuberculosis (Lambrecht, 2005). Within the next few decades, it is likely that an additional 100 million persons will be diagnosed with asthma (Masoli et al., 2004). To help reduce the rising prevalence of asthma, along with the substantial morbidity and mortality associated with this disease, a better understanding into the mechanisms that lead to both the development and expression of asthma is necessary.

1.1 Asthma

Asthma is a chronic inflammatory disease of the airways. The chronic inflammation is associated with physiological abnormalities of airway hyperresponsiveness to a wide variety inhaled physical and chemical stimuli, and variable airflow obstruction, that is often reversible spontaneously or with treatment (Batemen et al., 2008; O'Byrne et al., 2009). Asthmatics typically experience recurrent episodes of wheezing, breathlessness, chest tightness, and cough. Although symptoms are episodic, inflammation persists and is a constant histopathological feature within the airways of

asthmatics. The cellular pattern of inflammation commonly includes mast cells, eosinophils and T-helper (Th) 2 lymphocytes.

Multiple etiologies exist for the development and expression of asthma. Factors underlying asthma development can range from allergen sensitization and viral respiratory tract infections in infancy, to occupational exposures in adults (Global Strategy for Asthma Management and Prevention, 2012). Factors contributing to asthma exacerbations include allergen exposure in sensitized individuals, viral infections, and ingestions of non-steroidal anti-inflammatory agents (Global Strategy for Asthma Management and Prevention, 2012).

1.2 Asthma and Allergies

Environmental allergies are an important cause of asthma. Atopy describes a genetic predisposition to produce IgE antibodies in response to common airborne allergens, or foods. The majority of asthmatics are atopic, particularly those with childhood onset asthma, and significant asthma related morbidity and mortality are associated with exposure to allergens. Avoidance of allergens has been shown to improve asthma control and reduce the need for treatment (Platts-Mitts et al., 1982; Nelson, 2000).

1.2.1 Allergic Sensitization & T-helper cell Polarization

Allergy is an exaggerated immune response to an otherwise innocuous allergen. When an allergen enters the body, professional antigen presenting cells (APCs), such as

monocytes, B-cells and dendritic cells, capture the antigen and degrade it into short immunogenic peptides. Cleaved antigenic fragments are presented to naïve CD4+ Th cells on MHC class II molecules (Maurer et al., 1997). Naïve Th cells are subsequently polarized into distinct differentiated Th subsets, which depend on a multitude of factors, including antigen type and concentration, strength of peptide-bound MHC and T-cell receptor (TCR) interaction, co-stimulatory signals, and soluble mediators (Schipt et al., 2003). In allergic diseases like asthma, Th2 lymphocytes predominate, and secrete IL-4, IL-5, IL-9, and IL-13 which mediate allergic inflammation. Although Th2 cells are commonly associated with asthma, other Th-cell subsets have been linked to its pathogenesis, including Th17 and T-regulatory (Treg) cells (Hammad & Lambrecht, 2011).

1.2.2 Allergen Inhalation Challenge Model

Provoked models of asthma are extremely valuable in understanding the pathobiology of asthma. In subjects with mild stable asthma, inhalation challenge via aerosol delivery of specific allergens is an established and well characterized model to study the mechanisms of allergic airway inflammation, as well as the effectiveness of new drugs for asthma.

Subjects who inhale a sensitizing allergen experience acute bronchoconstriction within 10 minutes, which reaches a maximum bronchoconstrictor response within 30 minutes, and returns to baseline within 1 to 3 hours following inhalation (Gauvreau &

Evans, 2007). The acute onset of airway narrowing following inhaled allergen is termed the early asthmatic response. For the purposes of research, the early asthmatic response is defined as a minimum 20% fall in forced expiratory volume in one second (FEV_1) from baseline values. In approximately 50% of subjects who develop an early asthmatic response, airway narrowing will recur within 3 to 4 hours, will reach a maximum within 8 to 12 hours, and may be prolonged for up to 24 hours following allergen inhalation (O'Byrne et al., 1987). This delayed bronchoconstriction is termed the late asthmatic response, and is defined by a minimum 15% fall in FEV_1 from baseline values.

The induction of the early asthmatic response is mediated by circulating IgE antibodies. The administered allergen cross-links antigen-specific IgE that is bound to high-affinity IgE Fcepsilon receptors (FcεRI) on resident mast cells and circulating basophils. This leads to rapid degranulation and the release of a variety of preformed and newly formed mediators, including histamine, tryptase, cysteinyl leukotrienes, prostaglandins, platelet-activating factor, and various cytokines such as IL-2, IL-3, IL-4, IL-13, GM-CSF and TNF-α (Janeway, 2001; Oettegen & Geha, 2001). The release of these mediators leads to increased vascular permeability, enhanced mucus production and contraction of bronchial smooth muscle (Kay, 2001).

The late asthmatic response is mechanistically different from the early response, and is largely attributed to the recruitment of cellular inflammation. Allergen is presented to T-lymphocytes by APCs, leading to the release of a cascade of cytokines and chemokines that ultimately recruit various inflammatory cells, such as basophils,

lymphocytes, and eosinophils, to enter to the airways. Eosinophilic airway inflammation is a prominent feature of the late response (Gauvreau et al., 1999), and eosinophil activation releases pro-inflammatory mediators, including leukotrienes, eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and major basic protein (MBP), that constrict bronchial smooth muscle, damage bronchial structures and increase bronchial hyperresponsiveness (Gleich, 2000). The cellular inflammation observed during the late bronchoconstrictor response is associated with an increase in vascular permeability, mucus secretion, and airway hyperresponsiveness (Janway et al., 2001, O'Byrne et al., 1987).

1.3 Dendritic Cells

Dendritic cells (DCs) were first visualized in the basal layers of the skin epidermis by Paul Langerhans in 1868. These 'Langerhans cells' possessed long branched arms interdigitating between surrounding epithelial cells, which lead him to believe they were part of the nervous system. For more than a century, DCs remained uncharacterized until 1972, when Ralph Steinman discovered a unique population of cells in the mouse spleen, which have a prominent stellate shape (Banchereau & Steinman, 1998) (Figure 1) and continually retract and extend their dendrites to probe their environment (Steinman, 2007). For this discovery, Dr. Steinman was awarded the Nobel Prize in Medicine and Physiology in 2011. DCs were named for their 'tree-like' appearance, with their etymology deriving from the Greek word 'dendron' meaning tree.

DCs were subsequently described in all lymphoid organs, in the blood, bone marrow, and in several organs including the lung, gut and urogential tract. In the periphery, DCs act as sentinels to sample antigens, and then migrate to regional lymph nodes where they present these antigens to naïve T-cells. As such, DCs represent a unique class of white blood cell with a number of distinct features and functions.

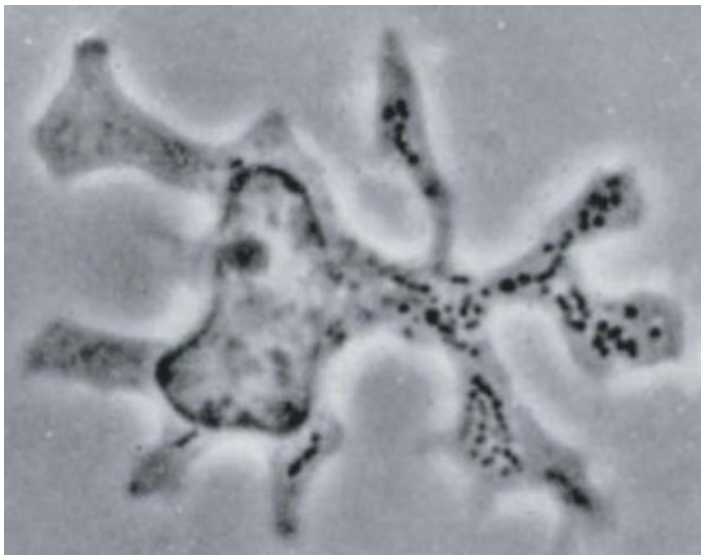

Figure 1: One of the first views of DCs in the spleen of a mouse (adapted from Steinman & Banchereau, 2007).

1.3.1 T-helper cell Interactions

DCs are crucial in regulating immune responses by bridging innate and adaptive immunity. DCs translate signals from antigens and the ensuing response from the innate immune system, to naïve T-cells, providing them with their first activation signals. These signals consist of a particular density of peptide-MHC II complexes (signal 1), costimulatory cell surface molecules (signal 2), and soluble cytokines and chemokines

(signal 3) (Figure 2). The signals at the interface between DCs and naive T-cells are likely the most critical factor in determining the polarization of CD4+ Th cells (Lipscomb and Masten, 2002). DCs are capable of promoting distinct Th cell subsets, including Th1 and Th2 lymphocytes.

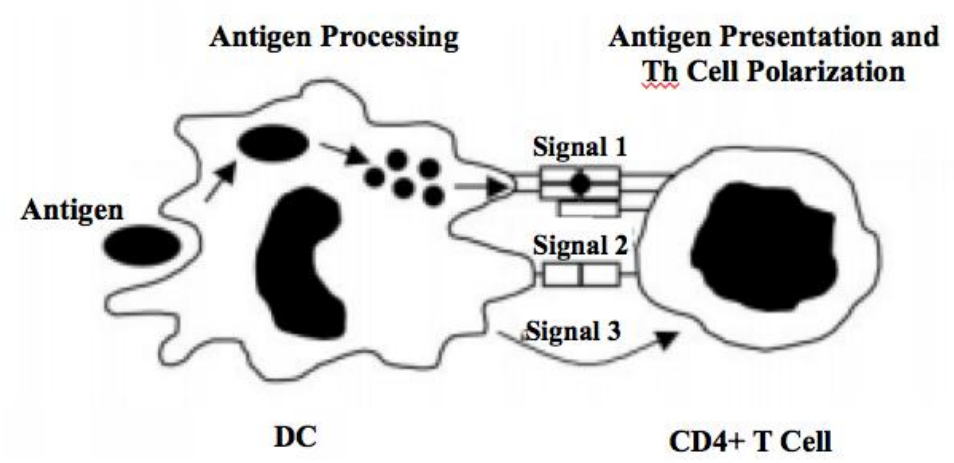

Figure 2: A model for DC-CD4+ Th cell interaction (adapted from Lipscomb and Masten, 2002).

A major influence in the differentiation of naïve CD4+ Th cells is the cytokine microenvironment. Instructive cytokines, including IL-4, IL-12, IL-10, IL-6 and TGF-β, are known to participate in Th cell differentiation, and most of these cytokines are produced by DCs themselves. A Th2 response is characterized by the production of IL-4, IL-5, IL-9 and IL-13, and typically occurs after parasitic infections or allergen exposures in sensitized individuals. DCs promote the polarization of Th2 cells through IL-6, which induces naïve T-cells to produce IL-4 - a Th2 polarizing cytokine. Although DCs cannot produce IL-4, DC secretion of IL-6 allows naïve IL-4 producing T-cells to differentiate

into Th2 effector cells, while inhibiting Th1 differentiation (Diehl et al., 2002). DCs can also produce IL-10, a Th1 inhibitory cytokine that may allow preferential expansion of Th2 cells under certain circumstances (Constant et al., 2002; Macatonia et al., 1993; Stumbles et al., 1998). Under steady state conditions, resting lung DCs fail to synthesize IL-12, and preferentially induce Th2 differentiation via IL-6 and IL-10 (Constant et al., 2002; Stumbles et al., 1998), supporting the concept that Th2 development occurs by default when IL-12 production is low. In contrast to Th2 cells, a Th1 response is characterized by IFN-γ and TNF production, and is the typical outcome after exposure of DCs to viruses or bacteria. Through pattern-recognition receptors (PRRs), including toll-like receptors (TLRs), C-type lectin-like receptors (CLRs), and NOD-like receptors (NLRs), DCs recognize microbial products and trigger the release of IL-12, a key Th1-promoting cytokine (Hilkens et al., 1997). In a mouse model of allergic asthma, over expression of IL-12 in DCs can reduce Th2-mediated airway inflammation, by skewing the immune response towards a Th1 profile (Kuipers et al., 2004). However, IL-12 is not necessary for Th1 development, as LPS-stimulated DCs can still reduce Th2-mediated inflammation and promote Th1 immunity, independent of IL-12 (Kuipers et al., 2003). DCs can also produce other Th1-inducing cytokines, including IL-18, IL-23 and IL-27 (Dreher et al., 2002; Kuipers & Lambrecht, 2004; Oppmann et al., 2000).

Costimulatory or accessory molecules on DCs are required to ensure T-cell division and differentiation into effector cells. Costimulatory molecules expressed on DCs include B7 proteins CD80, CD86 and ICOS-L, as well as TNF proteins CD40 and OX40L, and these bind to their respective receptors on T-cells. In animal models of

asthma, blocking the interaction of various costimulatory molecules of the B7 family (CD80,CD86 and ICOSL) or TNF family (OX40L) can reduce Th2-mediated airway inflammation (van Rijt & Lambrecht, 2005). Unlike the classification of cytokines, costimulatory molecules are generally not characterized with respect to their polarizing capacity or association with a Th subset, instead these molecules help provision the signal required by T-cells to differentiate and proliferate.

The processing and presentation of allergens by DCs results in a surface MHC-peptide signal that is recognized by TCRs on allergen-specific CD4+ Th cells. Several studies have indicated that low antigen doses or antigens with low TCR affinity tend to favour Th2 cell polarization, while high doses or high affinity antigens promote Th1 responses (Chaturvedi et al., 1996; Constant et al., 1995; Tao et al., 1997). The mechanism underlying the dose-dependent Th polarization is unclear, but may relate the strength of intracellular TCR signaling or other signals provided by DCs (Ruedl et al., 2000).

1.3.2 Ontogeny

Like other blood cells, DCs originate from hematopoietic stems cells within the bone marrow. DC progeny travel via the bloodstream to the tissues, where they colonize as immature non-dividing cells. Airway epithelial DCs turnover every 3 days (Holt et al., 1994; Nelson et al., 1995), and are maintained via continuous seeding of bone-marrow derived precursors from the blood (Fossum, 1989; Holt et al., 1994). Lung DC renewal

through proliferation of local progenitors is also a possibility, analogous to skin DC and macrophage populations (Coggle & Tarling, 1984; Merad et al., 2002). The rapid turnover of DCs is consistent with a sentinel role for these cells at sites of frequent antigen exposure. Several cytokines contribute to the growth and differentiation of DCs. SCF (c-KitL) and FLT-3L are cytokines produced by stromal cells that sustain DC progenitors (Banchereau & Steinman, 1998). GM-CSF, IL-4, TGF-β, TNF-α and IL-3 are cytokines released from many cell types that enhance DC differentiation (Banchereau & Steinman, 1998).

One of the earliest steps in the commitment of CD34+ hematopoietic stem cells is the development of either a common myeloid or lymphoid progenitor. In the bone marrow, these progenitors express FLT3 receptor (D'Amico & Wu, 2003; Karsunky et al., 2003; Karsunky et al., 2005) and will give rise to myeloid or plasmacytoid DCs respectively. CD34+ myeloid progenitor cells produce either CD14+ or CD14- CD11c+ DC precursors that differentiate into immature myeloid DCs in response to GM-CSF, TGF-β and IL-4 (Lipscomb & Masten, 2002). CD34+ lymphoid progenitor cells produce $CD11c^-$ IL3Rα DCs precursors that yield immature plasmacytoid DCs in response to IL-3 (Holt & Stumbles, 2000)

The dual DC differentiation model described above, highlights distinct developmental lineages for myeloid and plasmacytoid DCs. This model has been questioned by a series of careful studies, both on mouse precursors *in vivo* and human precursors in culture, that illustrated all DC subtypes could be generated from either

common myeloid or lymphoid progenitors (Chicha et al., 2004; Manz et al., 2001; Shigematsu et al., 2004; Traver et al., 2000; Wu et al., 2001). These findings underline considerable developmental plasticity in DC ontogeny, suggesting development to be more of an instructional and sequential multi-step process, rather than an all-or-nothing event. Moreover, others have suggested a dedicated circulating precursor for all DC lineages (del Hoyo et al., 2002), highlighting a common DC differentiation pathway.

1.3.3 Subsets

DCs do not represent a homogenous cell population. Although all DCs are capable of antigen uptake, processing and presentation, DC subtypes differ in location, migratory pathways and function. Subpopulations of DCs can be grossly divided into myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). Circulating mDCs share a common lineage with monocytes and macrophages, require GM-CSF for their survival, and express common myeloid surface markers including CD11c, CD33 and CD13 (Upham, 2003). Within circulation, mDCs represent between 0.5-1.0% of mononuclear cells (Upham & Stumbles, 2003), and can emerge in cultures from CD11c+ blood precursors in the presence of GM-CSF and either IL-4 or TNF-α (Caux et al., 1999; Romani et al., 1994; Sallusto & Lanzavecchia, 1994). mDCs can also originate from CD11c+ CD14+ monocytes in the absence of exogenous cytokines (Lyakh et al., 2002; Randolph et al., 1998; Randolph et al., 1999; Randolph et al., 2002), highlighting a physiological differentiation. Within epithelial tissues, Langerhans cells represent a

unique subtype of mDCs, expressing a distinct set of molecules, including E-cadherin, Langerin, CD1a and Birbeck granules (Liu, 2001). Commonly characterized in the skin epidermis, Langerhans cells can arise from both CD11c+ CD14+ and CD14- precursors, and require TGF-β for their differentiation. With respect to pDCs, these cells require IL-3 for their differentiation and express the IL-3Rα chain CD123, BDCA-2 and lymphoid development markers like CD4 (Rissoan et al., 1999). Unlike mDCs, pDCs have a low expression of CD11c and GM-CSF receptor, and lack myeloid markers CD14, CD13 and CD33 (Upham, 2003). pDCs are morphologically similar to plasma cells, represent less than 0.3% of circulating mononuclear cells, and are known for producing type I INFs (Upham & Stumbles, 2003).

In mice, DC subtypes have been well characterized. In the spleen, 3 CD11c+ mDC populations are found, based on their expression of CD8α and CD4 (CD8α+ CD4-, CD8α- CD4+ and CD8α- CD4-) (Vremec et al., 2000). The CD8α+ DCs are located in T-cell areas, while the CD8α- DCs are in the marginal zones of the spleen. In the lung, CD11c+ mDC subsets are characterized based on their expression of CD11b and CD103 (CD11b+ CD103- and CD11b- CD103+). CD11b mDCs reside beneath the basement membrane of the airways and lung parenchyma, while CD103 mDCs are associated with the respiratory epithelium, resembling Langerhans cells (Gill, 2012). Mouse pDCs are found in similar tissues as mDCs and although their exact anatomical location is not as clear, they can be found in large conducting airways and lung interstitium (Wikstorm & Stumbles, 2007).

In humans, the histological distribution of DC subtypes is not well characterized. Found in most lymphoid and non-lymphoid tissues, mDCs can be divided into type 1 mDCs expressing BDCA-1 and type 2 mDCs expressing BDCA-3, and pDCs expressing CD123 and BDCA-2 (Demedts et al., 2005).

1.3.4 Location

The localization of DCs reflects their function, as these APCs are found in areas of maximum antigen encounter like the skin, lungs and gut.

In the lungs, DCs are distributed throughout every compartment, including the conducting airways, lung parenchyma, alveolar space, interstitium, visceral pleura, and pulmonary vasculature (Gong et al., 1992; Holt et al., 1994; Holt et al., 1991; Pollard et al., 1990; Sertl et al., 1986; Suda et al., 1998). An extensive network of DCs exists within the mucosa and submucosa of the conducting airways, which represent the principal site for inhaled antigens. DCs are able to form long processes between the epithelium into the airway lumen, while maintaining the epithelial barrier intact via expression of tight junction proteins (Jahnsen et al., 2004; Sung et al., 2006). These dendriform cellular processes cover a substanial surface area for effective antigen sensing.

Original studies on respiratory tract DCs have identified DC-like cells as the most frequent APC within the lung (Holt et al., 1985). In the airway epithelium, DCs are found at densities between 500-1000 per square millimeter of epithelial surface (Holt et al., 1989; Schon-Hegrad et al., 1991; Sertl et al., 1986). The density of DCs in the

airways is related to the magnitude of antigen exposure, being greatest in the proximal airways, and diminishing towards the periphery. The density of DCs increases after exposure to pro-inflammatory stimuli, such as cigarette smoke (Soler et al., 1989), bacterial and viral infection (McWilliam et al., 1996; McWilliam et al., 1994), and inhaled allergen (McWilliam et al., 1996; McWilliam et al., 1994). In the airways, DCs turn over markedly faster than in other peripheral tissues or central lymphoid organs. While resting airway epithelial DCs turn over every 3 days (Holt et al., 1994; Nelson et al., 1995), Langerhans cells in the epidermis are renewed every 21 days (Fossum, 1989). The rapid turnover rates underline the importance of DCs in immune surveillance, particularly at major mucosal surfaces.

1.3.5 Antigen Handling

The capturing, processing and presentation of antigens by DCs is central to the initiation and propagation of immune responses. At mucosal tissues, DCs are in an immature state, adept for antigen uptake by 3 primary mechanisms. The first mechanism is by receptor-mediated endocytosis. Immature DCs express many specialized cell receptors for patterns associated with foreign antigens, immune complexes or opsonized particles. These include C-type lectin carbohydrate receptors (langerin, DC-SIGN, BDCA-2, macrophage mannose receptor) (Figdor et al., 2002; Jiang et al., 1995; Sallulsto et al., 1995), and Fcγ (CD32, CD64) and Fcε (FcεRI) receptors (Sallusto & Lanzavecchia, 1994). A second mechanism of antigen uptake is macropinocytosis. Engulfment of large amounts of fluid and solutes is taken up into pinocytic vesicles via

actin skeleton rearrangements, which then concentrate the soluble antigen in the endocyotic compartment (de Baey & Lanzavecchia, 2000). Thirdly, DCs are capable of phagocytosis. Particulate antigens, like latex beats, whole bacteria and viruses, and apoptotic and necrotic cell fragments, are readily phagocytosed by DCs (Banchereau et al., 2000; Inaba et al., 1993; Reis e Sousa et al., 1993; Svensson et al., 1997). Together, these mechanisms allow for a highly efficient system of antigen capture, allowing for DCs to detect antigen in picomolar and nanomolar concentrations (Sallulsto et al., 1995), often less than levels detected by other APCs.

Immature DCs also possess highly efficient mechanisms for processing and presenting antigens. Following antigen capture, exogenous antigens enter the endocytic pathway, whereby proteases initiate degradation. Unlike other APCs such as macrophages, DCs contain MHC class II-bearing compartments (MIIC), where peptide fragments associate with MHC II molecules (Cella et al., 1997; Pierre et al., 1997). MHC class II-peptide complexes rapidly form, and then transverse the cytoplasm for display on the cell surface. To maximize the number of MHC class II-peptide complexes presented, surface MHC class II molecules are continually recycled from the cell surface and acquire new antigens in the MIIC. During antigen-induced DC maturation however, the number and half-life of these surface MHC class II-peptide complexes are increased, allowing for maximal encounter with antigen-specific T-cells (Cella et al., 1997).

1.3.6 Maturation

In peripheral tissues, DCs are functionally immature, adept in capturing and processing antigens, but lack the requisite signals to stimulate T-cells. Following antigen recognition in the context of a pathogenic or inflammatory stimulus, immature DCs begin to mature, undergoing phenotypic and functional changes that result in the complete transition from antigen-capturing cells to professional APCs (Figure 3). While mature DCs lose the capacity to capture and process antigens, they acquire a strong capacity to activate T-cells. Maturing DCs upregulate their T-cell stimulating machinery and form an immunological synapse with T-cells, in which surface MHC class II-peptide complexes interact with TCRs, costimulatory molecules bind with corresponding T-cell co-receptors, and cytokines are released to polarize T-cell responses. DC maturation is intimately linked with their migration, being initiated in the periphery upon antigen encounter and completed during DC-T-cell interaction in the lymph nodes.

Mature DCs secrete chemokines to attract T-cells. DCs release MIP-3β and DCCK-1 to attract naïve T-cells, while TARC (CCL17) and MDC (CCL22) are released to attract activated T-cells (Cyster, 1999). Attraction of T-cells ensures rapid and efficient scanning of cells that will recognize antigen. T cells repeatedly engage and disengage contact with antigen-bearing DCs via adhesive interactions. Adhesion molecules on DCs, such as ICAM-1 and DC-SIGN, interact with their corresponding molecules LFA-1 and ICAM-2/3 on T-cells (Geijtenbeek et al., 2000; Gunzer et al.,

2000). These non-specific interactions allow for effective recognition of MHC class II-peptide complexes with their cognate TCRs (Lanzavecchia & Sallusto, 2000).

Following selection of antigen-specific T-cells, another signal is required between DCs and T-cells to initiate productive T-cell mediated immune responses. Costimulatory molecules expressed on mature DCs must interact with their receptors on T-cells. B7 molecules CD80 (B7-1) and CD86 (B7-2) are upregulated on mature DCs, and bind to their corresponding ligand CD28 on T-cells, allowing for their survival and proliferation (Caux et al., 1994). Other B7 family members include ICOS-L (B7RP-1) and PDL-1, and these may have a role in T-cell mediated inflammation (Dong et al., 2001; McAdam et al., 2001; Tafuri et al., 2001; Shin et al., 2003). TNF molecules CD40 and OX40L are also important costimulators on DCs. CD40-CD40L signaling leads to increased cytokine release by DCs, and enhanced expression of CD80 and CD86 (Bennett et al., 1998; Caux et al., 1994; Ridge et al., 1998; Schoenberger et al., 1998). OX40L is upregulated after stimulation with CD40L and epithelial-derived cytokines like IL-33 and TSLP (Liu, 2006; Ohshima et al., 1997), and is crucial for T-cell activation and survival (Chen et al., 1999; Gramaglia et al., 1998), as well as maintenance and reactivation of memory T-cells (Seshasayee et al., 2007; Wang & Liu, 2007).

As part of their maturation, DCs undergo changes in the expression of specific chemokine receptors. Maturing DCs express a different set of chemokine receptors to orchestrate their movements from peripheral tissues towards draining lymph nodes. Immature DCs express chemokine receptors CCR1, CCR2, CCR5 and CCR6, in order to

travel to sites of antigen exposure, where inflammatory chemokines are released. Following antigen capture, DCs begin to mature, whereby tissue homing receptors are downregulated (Lambrecht, 2001). Concurrently, lymph node homing receptors are upregulated, which direct the migration of maturing DCs to regional lymph nodes. Maturing DCs express chemokine receptors CCR7 and CXCR4, and chemoattract towards their ligands found within the T-cell areas of lymph nodes (Cyster, 1999; Dieu et al., 1998).

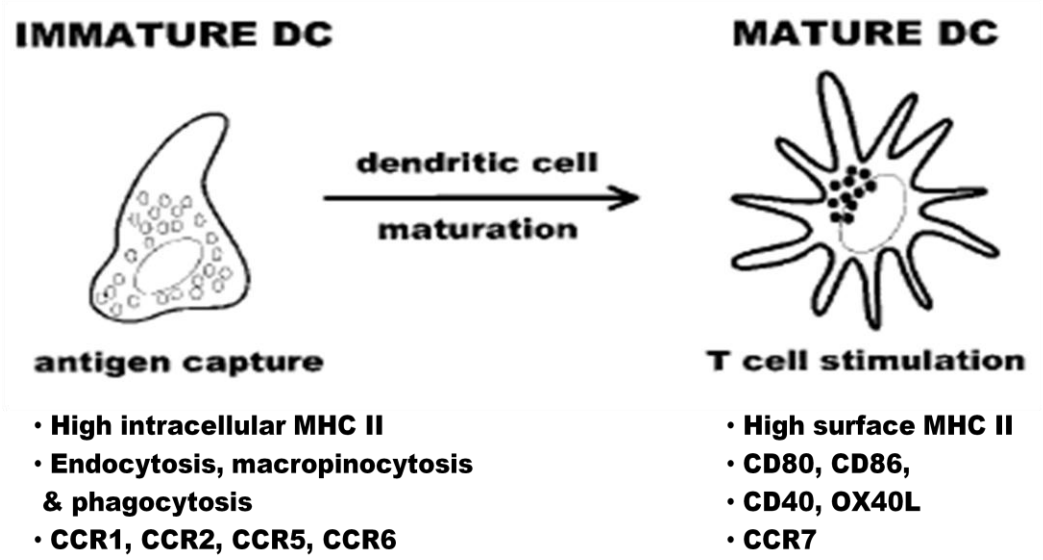

Figure 3: DC maturation (adapted from Steinman, 2001).

1.3.7 Migration

A critical feature of DCs at any stage of differentiation is their mobility. DCs are migratory cells, performing specialized functions as they traffic from one site to another.

Bone marrow-derived DCs migrate through the blood into tissues, where they become resident immature cells. Upon antigen recognition, DCs travel to regional lymph nodes to prime T-cells. The migration of DCs is regulated by their differential expression of chemokine receptors. Chemokine receptor expression is linked to DC maturation, as changes occur in receptor expression as DCs differentiate from an immature to a mature state. Chemokines help guide DCs to peripheral and lymphoid tissues via these chemokine receptors (Figure 4).

DCs migrate into peripheral tissues in response to antigen exposure (Jahnsen et al., 2001; Lambrecht et al., 1998). This increased migration represents the recruitment of circulating DC precursors in response to chemokines produced following local inflammation. As described above, immature DCs express a variety of chemokine receptors, including CCR1, CCR2, CCR5 and CCR6, and respond to a spectrum of chemokines, including RANTES, MIP-1α, MCP-1 and MIP-3α (Dieu et al., 1998; Power et al., 1997; Sozzani et al., 1997; Xu et al., 1996). Among these chemokines, MIP-3α (CCL20, LARC, Exodus-1) appears to be the most important chemokine guiding the *in vitro* migration of immature DCs (Dieu et al., 1998; Power et al., 1997), and it's primary chemokine receptor is CCR6 (Dieu et al., 1998; Dieu-Nosjean et al., 1999; Greaves et al., 1997). MIP-3α expression is restricted to the epithelium, while it is absent in lymphoid tissues (Dieu-Nosjean et al., 1999). *In vivo*, both mouse (Lundy et al., 2005, Osterholzer et al., 2005) and human (Farrell et al., 2007, Parameswaran et al., 2004) studies have supported a role for CCR6-mediated migration of DCs towards the lung.

Following antigen uptake in the periphery, DCs migrate towards regional lymph nodes (de Heer et al., 2004; Hammad et al., 2003a; Lambrecht et al., 2000b; Vermaelen et al., 2001). The migration of mature DCs also involves a coordinated action of chemokines and chemokine receptors. To travel towards lymphoid tissues, maturing DCs must first lose responsiveness to inflammatory chemokines that originally attracted them to the periphery. The response to inflammatory chemokines, like RANTES and MIP-3α, is rapidly lost through receptor downregulation or receptor desensitization (Dieu et al., 1998; Sallusto et al., 1998; Sozzani et al., 1999). Maturing DCs then upregulate the expression of CCR7 and CXCR4. CCR7 is exclusively expressed on mature DCs (Dieu et al., 1998; Soto et al., 1998; Sozzani et al., 1997), which accordingly acquire responsiveness to CCR7 ligands MIP-3β (CCL19, ELC) and 6C-kine (CCL21, SLC) (Chan et al., 1999; Dieu et al., 1998). 6C-kine is produced by lymphatic endothelial cells, while both 6C-kine and MIP-3β are produced by stromal cells and DCs in T-cell areas of lymph nodes (Dieu et al., 1998; Dieu-Nosjean et al., 1999; Gunn et al., 1998; Ngo et al., 1998; Yoshida et al., 1998). *In vivo*, mouse studies have supported a role for CCR7-mediated migration of DCs towards lymphoid tissues (Forster et al., 1999; Hammad et al., 2002).

Lipid mediators can also regulate DC migration. A leukotriene receptor antagonist, pranulukast, prevents the egress of circulating DCs towards the periphery (Parameswaran et al., 2004). Furthermore, both leukotriene C_4 and prostaglandin E_2 stimulate DC emigration towards draining lymph nodes (Kabashima et al., 2003; Robbiani et al., 2000; Scandella et al., 2003), and this may be through augmenting the

responsiveness of CCR7 to MIP-3β (Robbiani et al., 2000). On the other hand, prostaglandin D_2 inhibits DC emigration towards lymphoid tissues (Angeli et al., 2001; Hammad et al., 2003a), and this is also true for agonists of prostaglandin signaling, like PPAR-γ (Angeli et al., 2003; Hammad et al., 2004).

Spontaneous migration of DCs from peripheral tissues to draining lymph nodes can occur under non-inflammatory steady-state conditions (Vermaelen et al., 2001; Xia et al., 1995). The steady-state recruitment of DCs into peripheral tissues thus appears to be balanced by a continuous emigration of these cells into regional lymph nodes (Holt et al, 1994; Vermaelen et al., 2001). Under non-inflammatory conditions, it is believed that DCs migrate into draining lymph nodes to present innocuous or self-antigens in a tolerogenic form (Steinman & Nussenzweig, 2002). The immature phenotype retained by these spontaneously migrating DCs is likely critical for the development of immune tolerance (Upham, 2003).

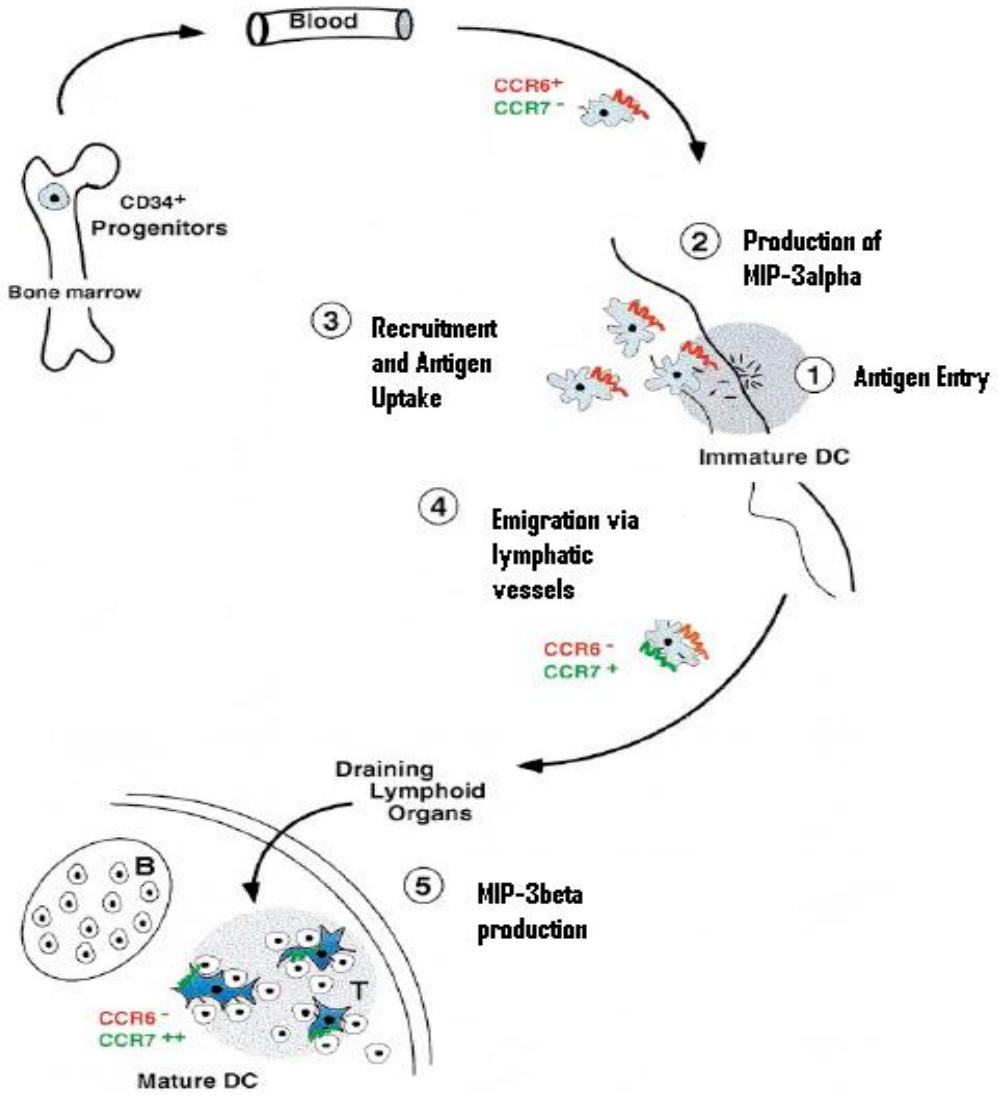

Figure 4: Model of DC mobilization upon inflammation in vivo (adapted from Dieu-Nosjean et al., 1999).

1.4 Dendritic Cells in Allergic Asthma

Allergic asthma is an inflammatory disorder of the airways, initiated by inhalation of environmental allergens. After allergen inhalation, the inflammatory reaction is orchestrated by activated Th2-cells releasing pro-allergic cytokines, typically IL-4, IL-5, IL-9 and IL-13. These cytokines promote eosinophil activation and chemotaxis, IgE production, and mucus hypersecretion. The paradigm that allergen specific Th2-cells control the salient features of the asthma includes the requirement for T-cells to be stimulated by APCs. DCs are the most potent APC in the lungs that initiate and regulate immune responses to inhaled allergen.

1.4.1 Animal Models

Like human DCs, rodent DCs originate from CD34+ bone marrow cells, are located in blood and mucosal tissues, possess antigen handling capabilities, express co-stimulatory molecules, mature and migrate in response to inflammatory signals, and are responsive to their microenvironment (Lipscomb & Masten, 2002). As such, research in rodents provides an appropriate model for the study of DCs in allergic asthma.

1.4.1.1 Myeloid DCs

An extensive network of bone-marrow derived DCs located above and beneath the basement membrane of the respiratory epithelium exists in rodents (Lambrecht et al.,

1998; Schon-Hegrad et al., 1991). In both mouse and rat models of asthma, significant increases in the numbers of airway DCs have been observed after exposure to allergen (Hammad et al., 2009; Lambrecht et al., 1998; van Rijt et al., 2002,). These cells are of myeloid lineage, and this increase is supported by an elevated production of myeloid progenitors in the bone marrow (Lambrecht et al., 1999; van Rijt et al., 2002). In the lung of rats, Holt and colleagues (1988, 1992) demonstrated that airway mDCs can capture inhaled ovalbumin (OVA) and process it into an immunogenic form on MHC class II molecules for presentation to T-cells. Following administration of large fluorescent-labeled antigens into the lungs of mice, lung derived mDCs can be traced in the T-cell areas of draining mediastinal lymph within 12 hours (Hammad et al., 2003a; Vermaelen et al., 2001). In regional lymph nodes, antigen-bearing mDCs from the airways can drive vigorous proliferation of T-cells *in vivo* (Hammad et al., 2003a; Lambrecht et al., 2000b). Collectively, these studies underline the importance of mDCs in controlling the immune response to inhaled allergen.

To establish a role for mDCs in the sensitization of antigen, Lambrecht and colleagues (2000a-c) injected bone marrow-derived allergen-pulsed mature mDCs into the trachea of naïve animals, and studied the occurrence of primary immune responses. In contrast to unpulsed mDCs, OVA-pulsed mDCs migrated into the draining lymph nodes and selected OVA-specific T-cells to proliferate. As early as 12 hours following intratracheal injection, mDCs started to interact with naïve T cells. Within 48 hours, antigen-specific T-cells were recruited from the blood into the lymph nodes, and began cellular division. To prove that OVA-pulsed DCs could induce the development of

effector T-cells with the ability to cause airway inflammation, mice were challenged with OVA aerosol 14 days after the primary immunization. Upon re-challenge, mice developed perivascular and peribronchial eosinophilic inflammation, and generalized goblet cell hyperplasia. These inflammatory changes were associated with Th cells producing IL-4 and IL-5. Together, these findings illustrated that mDCs were sufficient to cause sensitization to inhaled allergens.

Not only do DCs contribute to the primary immune response, they also have a crucial role mediating secondary responses to inhaled allergens. Although mDCs are observed to increase in the conducting airways of sensitized and challenged rodents after allergen (Huh et al., 2003; Lambrecht et al., 1999; van Rijt et al., 2002; Vermaelen & Pauwels, 2003), a functional role for mDCs in the secondary immune response is largely supported by knockout studies. Lambrecht and colleagues (1998) used transgenic mice to preferential express the suicide gene thymidine kinase in lineage-committed DCs. Treatment with the antiviral drug ganciclovir selectively depleted DCs from OVA-sensitized mice. In these mice, systemic removal of mDCs at the time of OVA challenge abrogated typical features of asthma, including airway inflammation, goblet cell hyperplasia and bronchial hyperresponsiveness. These findings were extended by van Rijt and co-workers (2005), who used CD11c-diptheria toxin receptor transgenic mice to conditionally deplete airway mDCs. Although systemic mDCs were not affected, local administration of diphtheria toxin to the airways also ablated other lung CD11c+ cells, including macrophages. During OVA challenge, selective removal of airway CD11c+ cells completely eliminated airway eosinophilia, goblet cell hyperplasia and bronchial

hyperresponsiveness, and this defect was restored by intratracheal injection of CD11c+ DCs, but not other APCs like macrophages. From these studies, it appears that mDCs are both necessary and sufficient for secondary immune responses to allergen. To support the idea that DCs could interact with T-cells locally in the airways, Huh and colleagues (2003) showed that antigen-bearing airway mDCs formed clusters with primed T-cells in the airway mucosa, leading to local maturation of DC function. Julia and co-workers (2000) also identified a subset of long-lived mDCs within the airways of mice, and these cells had a prolonged capacity for presenting allergen to Th2 cells *ex vivo*. In allergen challenged mice, mDCs are a prominent source of the chemokines TARC and MDC, which are involved in attracting memory CCR4+ Th2 cells to the airways (Kohl et al., 2006, Vermaelen et al., 2003)

In contrast to initiating and maintaining allergic inflammation, mDCs can also mediate tolerance to inhaled allergen. Although most inhaled antigens are transported to the lymph nodes by lung-derived mDCs, the usual outcome of inhaling harmless protein antigen is immunological tolerance. When OVA is introduced into the airways of naïve mice, it renders mice tolerant to subsequent immunizations with OVA in adjuvant, and effectively inhibits the development of allergic airway inflammation (Akbari et al., 2001; de Heer et al., 2004; Ostroukhova et al., 2004). Under non-inflammatory conditions, it is likely that harmless antigens cannot fully activate lung mDCs to induce an effective T-cell response (Reis, 2006; Sporri & Reis, 2005). Partially mature mDCs would then induce an abortive immune response, leading to the generation of unfit T-cells that fail to reach the threshold for survival and are deleted (Gett et al., 2003). Alternatively, partially

mature mDCs could stimulate the generation of IL-10 and/or TGF-β-producing Tregs, which have the potential of suppressing inflammatory responses by inhibiting effector T-cell function (Akbari et al., 2001; Ostroukhova et al., 2004). The exposure to lipid mediators, such as PPAR-γ agonists or prostaglandins, further supports the association between DC maturity and tolerance. These mediators keep mDCs in a persistently immature state; thus mDCs lose their capacity to prime for Th2-mediated airway inflammation, and induce protective immune responses in the lung (Hammad et al., 2003a; Hammad et al., 2004).

1.4.1.2 Plasmacytoid DCs

pDCs represent the other main subpopulation of DCs. pDCs are found in similar tissues as mDCs, like the lung, bone marrow and lymph nodes (Asselin-Paturel et al., 2003; Blasius et al., 2004); however, pDCs are phenotypically and functionally different than mDCs. pDCs have a lymphoid shape with a plasma cell morphology, express a different set of cell surface markers, and produce large amounts of type I INFs in response to most viruses. In contrast to mDCs, pDCs present exogenous antigens inefficiently and are poor at priming naïve T-cells (Colonna et al., 2004). The limited APC activity is likely attributed to the reduced ability of pDCs to capture, process, and load antigens onto MHC II molecules. Furthermore, pDCs have a lower expression of peptide-MHC class II complexes and costimulatory molecules compared to mDCs (Asselin-Paturel et al., 2001; Chehimi et al., 1989; Grouard et al., 1997).

A series of studies have suggested that pDCs mediate tolerance and homeostasis in the lung. After inhalation of allergen, antigen-bearing lung pDCs migrate to regional lymph nodes (de Heer et al., 2004; Vermaelen et al., 2001), but in contrast to mDCs, they do not present antigen to naïve T-cells in an immunogenic context (Boonstra et al., 2003; de Heer et al., 2004; Martin et al., 2002; Oriss et al., 2005). Functional evidence however for this immunoregulatory role stems from experiments in which pDCs were depleted using antibodies. De Heer and colleagues (2004) demonstrated that in mice depleted of pDCs, inhalation of inert OVA led to a break in tolerance, resulting in a Th2-mediated inflammatory response. Moreover, adoptive transfer of bone-marrow derived OVA-pulsed pDCs before sensitization completely suppressed the inflammatory response and restored tolerance. Kool and coworkers (2009) also demonstrated that selective removal of pDCs during allergen challenge augments airway inflammation, whereas transfer of cultured pDCs before challenge suppressed inflammation. Together, these findings highlight an immunoregulatory role for pDCs in the lung.

Although the mechanism by which depletion of pDCs results in sensitization is unknown; *in vitro* and *in vivo* data suggest that pDCs can directly suppress the potential of mDCs to generate effector T-cells (de Heer et al., 2004; Kohl et al., 2006; Kool et al., 2009). pDCs can also induce the formation of Tregs (de Heer et al., 2004; Martin et al., 2002; Ochando et al., 2006, Wakkach et al., 2003), possibly mediated by ICOS-L or PD-L1 (de Heer et al., 2004; Habicht et al., 2007). The immature phenotype of pDCs, low expression of peptide-MHC class II and costimulatory molecules may also be important for Treg formation (Hawiger et al., 2001; Jonuleit et al., 2001; Roncarolo et al., 2001).

Finally, pDCs can inhibit T-cells directly, through the production of the tryptophan catabolizing enzyme indoleamie 2,3-dioxygenase (IDO) (Fallarino et al., 2004) or the expression of PDL-1 (Lambrecht, 2005).

In contrast to immature pDCs, activated pDCs reorganize their morphology to display dendrites and augment the expression of MHC class II and costimulatory molecules; thus, enhancing their ability to prime naïve T-cells (de Heer et al., 2005, Reizis et al., 2011). Through exposure to various bacterial and viral products, particularly TLR7 and TLR9 agonists, pDCs become activated to produce type I IFNs and are capable of priming CD8+ T-cells and inducing efficient cytotoxic responses. (Cella et al., 2000; Dalod et al., 2003; Salio et al., 2004). In a mouse model of asthma, the tolerance to house-dust mite was abrogated when transferred pDCs were infected with respiratory syncytial virus (Tsuchida et al., 2012). As such, pDCs fine-tune the balance between immune response and tolerance.

1.4.2 Human Models

The characteristics and functional properties of lung DCs have been extensively studied in animal models of asthma. In these studies, DCs are known to be crucial for the initiation and regulation of the immune response to inhaled allergen. Research in humans has also provided some important insights on the function of DCs in asthma.

1.4.2.1 Myeloid DCs

Studies in humans have suggested that mDCs may be implicated in the sensitization to common inhaled allergen. Many *in vitro* studies have highlighted the differences in which mDCs from allergic subjects process and present allergens when compared to healthy individuals. When monocyte-derived mDCs from allergic subjects are pulsed with their relevant allergen, they preferentially induce Th2 cell responses from naïve autologous T-cells, compared to DCs from non-allergic individuals that induced Th1 responses (Bellinghausen et al., 2000; De Wit et al., 2000; Hammad et al., 2001). Furthermore, mDCs from allergic subjects have enhanced antigen uptake, increased T-cell stimulatory function, and express higher levels of HLA-DR, CD80, CD86 and IL-10, than non-allergic individuals (Bellinghausen et al., 2000, Hammad et al., 2001, McCarthy et al., 2007, van den Heuvel et al., 1998). mDCs may also contribute to secondary immune responses. *In vitro*, memory T-cells from allergic subjects preferentially induce Th2 responses when cultured with mDCs, compared to cultures from non-allergic individuals (Bellinghausen et al., 2000). Similarly, mDCs pulsed with allergen from allergic individuals selectively secrete Th2-cell specific chemokines TARC and MDC (Hammad et al., 2003b; Perros et al., 2009). In a humanized SCID mouse model, human monocyte-derived mDCs pulsed with Der p1 significantly enhanced peribronchial infiltrates of human CD45+ cells, human allergen specific IgE synthesis, and airway eosinophilia when administered to the lungs of sensitized mice (Hammad et al., 2002). Finally, isolation of bronchoalveolar lavage fluid (BALF) mDCs from allergic

asthmatics after allergen challenge induced strong T-cell proliferation *in vitro* (Schaumann et al., 2008).

Studies on human mDCs *in vivo* have also suggested a role for these cells in ongoing allergic inflammation. In humans, mDCs are increased in the airways of allergic asthmatics compared to healthy subjects (Koh et al., 2005; McCarthy et al., 2007; Moller et al., 1996; Novak et al., 2003; Tunon-De-Lara et al., 1996). These airway mDCs are known to express higher levels of FcεRI (Novak et al., 2003; Tunon-De-Lara et al., 1996), HLA-DR (Moller et al., 1996), and correlate with the number of activated eosinophils in the airways (Koh et al., 2005).

Following allergen challenge, mDCs egress from the circulation, and migrate into the airways. In the peripheral blood of allergic asthmatics, both Upham et al. (2002) and Parameswaran et al. (2004) observed baseline falls in the levels of mDCs between 3hrs and 24hrs after allergen inhalation. Farrell and colleagues (2007) also reported similar falls in circulating mDCs, but to account for any diurnal changes, they administered a diluent challenge and found circulating mDCs to be significantly lower at 6hrs and 24hrs after allergen compared with diluent. In the lungs, Jahnsen and coworkers (2001) demonstrated a baseline increase in the number of mDCs in the airway mucosa 4hrs to 5hrs after allergen challenge. Similarly, Bratke and colleagues (2007) showed that mDCs increase significantly in the BALF of subjects with allergic asthma 24hrs after segmental allergen challenge compared with saline challenge. A concomitant decrease in circulating mDCs was also observed after allergen challenge (Bratke et al., 20007).

Together, these studies suggest that mDCs are recruited directly from the bloodstream into the airways, and support a role for these cells in mediating allergen-induced airway inflammation.

1.4.2.2 Plasmacytoid DCs

Like their counterparts in rodents, human pDCs have been associated with the induction of immunological tolerance. *In vitro*, peripheral blood-derived immature pDCs induce an anergic state in human antigen-specific CD4+ T-cell lines, which fail to proliferate when optimally restimulated (Kuwana et al., 2001). pDCs isolated from the BALF of asthmatics after allergen challenge also could not induce T-cell proliferation *in vitro* (Schaumann et al., 2008). The reduced ability to prime T-cells may be explained by their lower expression of MHC class II and costimulatory molecules (Demedts et al., 2005; Masten et al., 2006; Schaumann et al., 2008). Moreover, many studies have demonstrated that human pDCs have the capability to induce functional Tregs that can suppress effector T-cells *in vitro* (Ito et al., 2007; Moseman et al., 2004; Palomares et al., 2012).

Studies on human pDCs *in vivo* have also suggested a role for these cells in regulating allergen-induced inflammation. In a cohort study of children, Upham and colleagues (2009) found that the number of circulating pDCs in infancy inversely correlates with asthma development during the first 5 years of life. Conversely, Matsuda and coworkers (2002) found higher numbers of circulating pDCs in adult patients with

allergic asthma compared to healthy individuals. Following allergen inhalation, both Parameswaran et al. (2004) and Farrell et al (2007) reported falls in circulating pDCs numbers. In contrast to human mDCs, the migration of pDCs towards the lung is not well established. Initial reports suggested that short-term allergen challenge was insufficient for the recruitment of pDCs to the airways (Jahnsen et al., 2001); however, a more recent study found pDCs do accumulate in the BALF of allergic asthmatics 24 hours after challenge (Bratke et al., 2007). Collectively, these studies implicate pDCs in the regulation of the immune response to inhaled allergen; however, pDCs need to be studied more carefully before conclusions about their functional role in asthma are drawn.

1.4.2.3 DCs as Therapeutic Targets

As evidence supports a role for DCs in mounting immune responses during ongoing inflammation, interfering with their function could constitute a novel form of treatment for allergic diseases. Steroids are currently the cornerstone of anti-inflammatory treatment in asthma. Inhaled steroids reduce the number of DCs in the airways of patients with allergic asthma (Bocchino et al., 1997; Hoogsteden et al., 1999; Moller et al., 1996). Steroids directly affect DCs, inhibiting their differentiation and maturation (van den Heuvel et al., 1999; Woltman et al., 2000), as well as inducing their apoptosis (Brokaw et al., 1998). Alternate or adjunct asthma therapies have also affected DCs, including pranlukast (Parameswaran et al., 2004) and omalizumab (Chanez et al.,

2012). Recently, several new molecules able to alter DC function in allergic inflammation have been identified, including epithelial-derived cytokines and costimulatory ligands, and these might represent new therapeutic targets for the treatment of allergic asthma.

1.5 Summary

DCs are professional APCs that mediate the immune response to inhaled allergens. Different subtypes of DCs perform different functions, not only during sensitization but also during established inflammation. In animal models of asthma, the induction and maintenance of airway inflammation is principally a function of mDCs, whereas the tolerance to inhaled allergens is largely a function of pDCs. It remains uncertain however, whether this concept of pro-allergic mDCs and anti-allergic pDCs translates from animal to human models. Continued research on the biology of DCs will be fundamental to understanding the pathogenesis of allergic asthma.

1.6 Research Studies

The overall objective of this thesis was to investigate the biology of DC subsets in allergen-induced asthma. We postulated that different subtypes of DCs have different roles in the regulation of immune responses to inhaled allergen in mild allergic asthmatic subjects.

1.6.1 Myeloid and plasmacytoid dendritic cells in induced sputum after allergen inhalation in subjects with asthma (Chapter 2)

Hypothesis: mDCs are recruited into the airways during the allergen-induced inflammatory response, whereas pDCs migrate into the airways during the resolution of these responses.

Aim: To examine changes in sputum mDCs and pDCs after allergen inhalation in subjects with asthma.

1.6.2 Myeloid dendritic cells type 2 in allergic asthma (Chapter 3)

Hypothesis: mDC2s are increased in the blood of subjects with allergy and asthma.

Aim: To examine circulating mDC2s among moderate/severe atopic asthmatics, mild atopic asthmatics, atopic non-asthmatics and healthy subjects.

1.6.3 Myeloid dendritic cells type 2 after allergen inhalation in asthmatic subjects (Chapter 4)

Hypothesis: mDC2s are recruited into the airways during the allergen-induced inflammatory response.

Aim: To examine changes in circulating and sputum mDC2s after allergen inhalation in subjects with asthma.

1.6.4 The effects of anti-OX40L and anti-TSLP monoclonal antibodies on circulating dendritic cells (Chapter 5)

Hypothesis: Circulating mDC1s and mDC2s are reduced in subjects treated with human monoclonal antibodies (MAbs) against OX40L and TSLP. Circulating pDCs will remain unchanged.

Aim: To examine circulating mDC1s, mDC2s and pDCs in subjects with asthma treated with the human MAbs anti-OX40L and anti-TSLP.

CHAPTER 2

Myeloid and plasmacytoid dendritic cells in induced sputum after allergen inhalation in subjects with asthma

This manuscript appeared in the Journal of Allergy and Clinical Immunology [2010;126(1):133-139], and has been granted permission by Elsevier to include in this thesis.

Different subtypes of DCs perform different functions. Evidence from animal models of asthma has demonstrated that mDCs induce and maintain airway inflammation [9-11], whereas pDCs mediate tolerance and homeostasis in the lung [12]. Since DCs are migratory cells and studies have showed that DCs efflux from the blood in response to allergen [13-15], we investigated changes in mDCs and pDCs in the sputum of subjects with mild asthma after allergen inhalation. We demonstrate that both mDCs and pDCs increase in the airways of mild asthmatics after allergen challenge.

MYELOID AND PLASMACYTOID DENDRITIC CELLS IN INDUCED SPUTUM AFTER ALLERGEN INHALATION IN ASTHMATIC SUBJECTS

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Funding: Canadian Institutes for Health Research Grant #9362

Running head: Sputum dendritic cells after allergen challenge

Word Count: 3070

ABSTRACT

Background: Dendritic cells are professional antigen presenting cells that mediate the response to inhaled allergens. In animal models, the induction and maintenance of allergic airway inflammation is primarily a function of myeloid dendritic cells, while the tolerization to inhaled allergens is likely a function of plasmacytoid dendritic cells.

Objective: To investigate changes in sputum myeloid and plasmacytoid dendritic cells after allergen inhalation in asthmatic subjects. Also, the number of myeloid and plasmacytoid dendritic cells expressing both chemokine receptor (CCR) 6 and 7, and their chemokine ligands macrophage inflammatory protein (MIP)-3α and 3β were measured in sputum supernatants.

Methods: Sputum was induced from 12 dual-responder allergic asthmatics before and 7hrs, 24hrs and 72hrs after inhalation of diluent and allergen. Dendritic cells were enumerated via flow cytometry and the chemokines using enzyme-linked immunosorbent assays.

Results: The number of sputum myeloid dendritic cells was significantly higher 24h following allergen challenge when compared to diluent. Similarly, sputum plasmacytoid dendritic cells also increased significantly at 24h after allergen challenge. Also, a significant increase in CCR6+ myeloid dendritic cells numbers occurred 72h following allergen challenge. In contrast, CCR7+ myeloid dendritic cells, as well as the number of CCR6+ and CCR7+ plasmacytoid dendritic cells, were not different between challenges.

Finally, allergen challenge increased sputum levels of MIP-3α, but not MIP-3β, when compared to baseline.

Conclusions: Both myeloid and plasmacytoid dendritic cells increase in the sputum of asthmatic subjects following allergen challenge, suggesting that both subsets are involved in the pathogenesis of allergen responses in asthma.

KEY MESSAGES

- This study is the first to demonstrate that both myeloid dendritic cells and plasmacytoid dendritic cells increase in the sputum of allergic asthmatic subjects following allergen inhalation, suggesting that both subsets are involved in the responses to inhaled allergens.
- Continued insight into dendritic cell biology, including its migration and function, will be fundamental in improving our understanding of the mechanisms of allergic asthma.

CAPSULE SUMMARY

Both subsets of dendritic cells increase in induced sputum from allergic asthmatics following allergen inhalation. Research into the migration and function of dendritic cell subsets will improve our understanding of allergic asthma and ultimately allow for therapeutic intervention based upon dendritic cell biology.

KEY WORDS

Allergic asthma, sputum, antigen presenting cells, myeloid dendritic cells, plasmacytoid dendritic cells, CCR6, CCR7

ABBREVIATIONS

APCs	Antigen presenting cells
CCR	Chemokine receptor
DCs	Dendritic cells
ELISA	Enzyme-linked immunosorbant assay
FEV_1	Forced expired volume in 1 second
MANOVA	Multiple analysis of variance
MIP	Macrophage inflammatory protein
mDCs	Myeloid dendritic cells
pDCs	plasmacytoid dendritic cells
Th2	T-helper 2

INTRODUCTION

Allergic asthma is an inflammatory disease of the airways that is characterized by eosinophilic inflammation, mucus hypersecretion, airway hyperresponsiveness and airway remodeling, triggered by inhalation of environmental allergens [1]. Activated T-cells producing T-helper (Th2) cytokines are believed to orchestrate the inflammatory reaction observed in allergic asthma. T-cells however are unable to respond to allergen independently of antigen presenting cells (APCs). Since dendritic cells (DCs) are the most potent APC within the airways, DCs have garnered much interest in their potential role in the regulation of immune responses to inhaled allergens.

In humans, two major subpopulations of DCs exist: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). Circulating mDCs share a common lineage with monocytes and macrophages, and represent between 0.5-1.0% of mononuclear cells within circulation [2]. In contrast, pDCs express lymphoid development markers, bear a morphological resemblance to plasma cells and represent less than 0.3% of circulating mononuclear cells [2].

A critical feature of DCs at any stage of differentiation is their mobility. Dendritic cells are migratory cells, performing specialized functions as they traffic from one site to another. The migration of DCs is regulated by their differential expression of chemokine receptors and the response to chemokine gradients guide DCs to peripheral and lymphoid tissues. The chemokine macrophage inflammatory protein (MIP)-3α (CCL20, LARC, Exodus-1) is produced by epithelial cells [3,4] and is believed to mediate the recruitment

of chemokine receptor (CCR)6+ immature DCs to the airways following allergen deposition in the lung. Antigen capture induces immature DCs to undergo maturation, whereby DCs traffic towards regional lymph nodes in order to present antigens to naïve T-cells. Maturing DCs lose responsiveness to inflammatory chemokines, through receptor down-regulation or desensitization [3,5,6], and concomitantly up-regulate the expression of CCR7. The expression of CCR7 has been exclusively documented on mature DCs [3,6,7], which then acquire responsiveness to its ligand MIP-3β (CCL19, ELC, Exodus-3), which is expressed in T-cell rich areas of the lymph nodes [3,8]. Although these expression of these chemokine receptors are believed to important for DC migration, CCR6 and CCR7 are usually expressed by only a fraction of DCs.

In animal models of allergic asthma, the induction and maintenance of airway inflammation is primarily a function of mDCs [9-11]. In contrast, tolerization to inhaled allergens is likely a function of pDCs [12]. However, it is still uncertain whether the concept of pro-allergic mDCs and anti-allergic pDCs translates from animal to humans. Previous research in peripheral blood has suggested that DCs migrate from circulation during the allergen-induced asthmatic response [13-15]; however, a comprehensive study investigating DCs in the airways following allergen inhalation has yet to be conducted. Airway DCs have been identified in asthmatic airway biopsies [16] and in a small number of allergic asthmatic subjects in induced sputum [17]; however, the temporal association of allergen-induced airway inflammation and airway mDCs and pDCs has not been examined. Accordingly, we examined the kinetics of DCs in the sputum of allergic asthmatic subjects following inhalation of allergen. Sputum mDCs and pDCs, as well as

the number of mDCs and pDCs expressing both CCR6 and CCR7, were enumerated following allergen and diluent challenges. Furthermore, the levels of CCR6 ligand MIP-3α and CCR7 ligand MIP-3β were also enumerated in sputum supernatant following both challenges.

METHODS

Subjects

Twelve mild allergic asthmatic subjects, aged between 18 and 55 years, were enrolled (Table 1). All were atopic, based on one or more skin wheals responses to common aeroallergens, had a forced expired volume in 1 second (FEV_1) greater or equal to 70% of predicted, and all had previously documented dual airway responses to inhaled allergen, as determined by a fall in FEV_1 at least 20% within the first 2h, followed by second fall in FEV_1 of at least 15% 3h to 7h following allergen inhalation. All subjects were treated only with intermittent (not daily) β_2-agonists. Subjects were excluded if they were pregnant, current smokers, or ex-smoker with more than 10 pack-years. All subjects gave written informed consent, and this study was approved by the Hamilton Health Science Research Ethics Board.

Study Design

A randomized, diluent-controlled allergen inhalation study was performed to examine changes in sputum DCs in subjects developing a late asthmatic response. All subjects were screened on Visit 1 with a methacholine inhalation challenge to assess airway hyperresponsiveness, skin prick testing to confirm atopy, and sputum induction. On Visit 2, subjects were randomized to receive either a diluent or allergen inhalation challenge and sputum was induced 7h following challenge. Subjects returned for Visits 3 and 4 to undergo a sputum induction, both at 24h and 72h post-challenge respectively. A washout period of 2-4 weeks was enforced between challenges.

Allergen Inhalation

Allergen inhalation was performed as previously described [15]. The allergen producing the largest diameter skin wheel was diluted in saline for inhalation. The concentration of allergen required to achieve a 20% decrease in FEV_1 (the allergen PC_{20}), was predicted using the methacholine PC_{20} and the titration of allergen determined from the skin prick test [18]. The early bronchoconstrictor response was recorded as the greatest fall in FEV_1 between 0 to 2h following allergen inhalation, whereas the greatest drop in FEV_1 between 3h to 7h was recorded as the late bronchoconstrictor response. For diluent challenges, the same procedure was followed as per inhalation of allergen; however, 0.9% normal saline was used for 3 inhalations only.

Sputum Induction and Processing

Sputum was induced before, 7h, 24h and 72h following challenge using 7 minute inhalations of 3%, 4% and 5% hypertonic saline as described elsewhere [19]. Sputum was separated from saliva, treated with 0.1% dithiothreitol and processed as described elsewhere [20].

Immunofluorescent Staining

The following monoclonal antibodies were used: PACIFIC BLUE-CD45 (eBioscience), FITC-Lin1, APCH7-HLA-DR, APC-CD11c, PECy5-CD123, PE-CCR6, PECy7-CCR7 (Becton-Dickinson Biosciences), as well as the isotype controls for CD11c, CD123, CCR6 and CCR7. Before these monoclonal antibodies were added, the DPBS-suspended

sputum cells were centrifuged at 1500 rpm for 10 minutes at room temperature. The supernatant was discarded and 200 uL of mouse block (Sigma Aldrich, St Louis, Missouri, USA) were mixed with the cell pellet. The cells were divided into separate tubes with isotype control and test antibodies. Following staining of sputum cells with appropriate antibodies, the samples were vortexed gently and incubated in the dark at 4°C for 30 minutes. Next, 2 mL of FACS buffer (0.1% sodium azide and 0.5% BSA in PBS) were added to each tube and the samples were then centrifuged at 1500rpm for 10 minutes at 4°C. The supernatant was aspirated and the cells were fixed with 300 μL of 1% paraformaldehyde. Stained cells were then stored in the dark at 4^{o}C until flow cytometric acquisition within 48h following staining.

Due to the paucity of cells in sputum, blood was substituted for sputum to make compensatory controls. During the collection of baseline, 7h and 24h sputum samples, one set of compensation tubes were used as controls, while another set of compensation tubes were used for the 72h sputum sample. Along with an unstained control, each color was compensated for. Following staining of peripheral blood with individual compensatory colors, the compensation controls were vortexed gently and incubated in the dark at 4^{o}C for 25 minutes. Next, compensation tubes were treated with FACS Lysing Solution (Becton Dickinson Biosciences), vortexed and incubated in the dark at 4^{o}C for 5 minutes. Following the lysis of red blood cells, 2 mL of FACS buffer were added to each tube and the compensation controls were then centrifuged at 1500 rpm for 10 minutes at 4°C. The supernatant was aspirated and another washing with FACs buffer

was repeated. After centrifuging and removing the supernatant, the blood was fixed with 300 μL of 1% paraformaldehyde.

Flow Cytometry Acquisition

Cells were acquired with a 15-colour LSR II flow cytometer equipped with 3 lasers (Becton Dickinson Instrument Systems; Becton-Dickinson) using the FACSDiva software program (Becton-Dickinson Biosciences, Mississauga, Canada). Nine parameters were acquired: linear forward angle light scatter (FSC), linear side angle light scatter (SSC), log PACIFIC BLUE, log FITC, log APCH7, log APC, log PECy5, log PE and log PeCy7 fluorescence. According to previous experiments with DCs in blood [13-15], approximately 100 to 200,000 mononuclear cells were collected upon flow cytometric acquisition in order to obtain a sufficient amount of CCR6+ and CCR7+ DCs.

Enumeration and Analysis of DCs

DCs were enumerated as previously described for in blood [15]. A mononuclear gate was set up to enumerate the number of mononuclear cells, while excluding as much debris as possible, on the CD45 vs. SSC plot. Offline analyses of flow cytometric acquisition were performed using FlowJo software (Tree Star Inc., Ashland, Oregon, USA). Gating strategies for enumerating DCs were followed according to previous flow cytometric analysis conducted on peripheral blood DCs (see Figure 1) [15].

Sputum DC Counts

Following analysis, total sputum DC numbers were calculated. From the flow cytometric acquisition, mDCs, pDCs, CCR6 mDCs and pDCs and CCR7 mDCs and pDCs were expressed as a percentage of the mononuclear cells collected. Next, this fraction was multiplied by the number of sputum mononuclear cells which were calculated using the total and differential cell counts of the sputum sample. The result is the total sputum DC counts expressed as DCs per gram of sputum.

ELISA of Sputum Supernatant

DuoSet ELISA Development kits (R&D Systems Inc, Minneapolis, Minnesota, USA) were used to measure natural and recombinant human MIP-3α and MIP-3β. Assays were run according to DuoSet ELISA kit instructions.

Statistical Analysis

All statistics are expressed as the mean ± SEM. Statistical analyses were performed using Statistica version 8. Post-hoc Fischer tests were used to compare allergen versus diluent values at various time points using multiple analysis of variance (MANOVA). Significance was accepted at $p<0.05$.

RESULTS

Airway Physiology

All subjects demonstrated a dual response to allergen inhalation, with a mean maximum baseline fall in FEV_1 of 38.4% ± 2.2% during the early response ($p<0.05$), followed by a 24.0% ± 3.5% fall during the late response ($p<0.05$). No significant change in FEV_1 occurred during the diluent challenge.

Sputum Inflammatory Cells

Allergen challenge significantly increased the number of sputum eosinophils, both at 7h and 24h ($p<0.05$), as well as the number of neutrophils at 24h ($p<0.05$), compared to the diluent challenge (Table 2). In contrast, the number of mononuclear cells (monocytes and lymphocytes) was not significantly different between allergen and diluent at any time points (Table 2).

Allergen-induced Changes in Sputum Dendritic Cells

There was a significant increase in the number of sputum mDCs at 24h after allergen, when compared to diluent ($3.28 \pm 1.31 \times 10^4$/g post diluent vs. $8.91 \pm 2.70 \times 10^4$/g post allergen, $p<0.05$)(Figure 2A). Furthermore, a significant increase was observed in the number of sputum CCR6+ mDCs at 72h after allergen ($0.33 \pm 0.14 \times 10^3$/g post diluent vs. $4.43 \pm 2.61 \times 10^3$/g post allergen, $p<0.05$) (Table 3). Numbers of CCR7+ mDCs were not significantly different between allergen and diluent at any time point (Table 3).

Furthermore, the expression of CCR6 and CCR7 on mDCs was not different from its isotype expression on mDCs (Figure 3A).

There was a significant increase in the number of sputum pDCs at 24h after allergen when compared to diluent ($1.60 \pm 0.87 \times 10^4$/g post diluent vs. $3.99 \pm 1.22 \times 10^4$/g post allergen, $p<0.05$)(Figure 2B). However, the numbers of sputum CCR6+ and CCR7+ pDCs were not significantly different between diluent and allergen at any time point (Table 3). The expression of CCR6 and CCR7 on pDCs was also not different from its isotype expression on pDCs (Figure 3B). There were no significant correlations between the changes in sputum DC numbers and the magnitude of the allergen-induced late responses or sputum eosinophil or neutrophil numbers.

Allergen-induced Changes in Sputum Supernatant Chemokine Levels

Allergen challenged increased sputum levels of MIP-3α when compared to baseline (Figure 4A); however, MIP-3α levels were not significantly different between allergen and diluent at any time point examined. The levels of MIP-3β were not significantly different between diluent and allergen (Figure 4B).

DISCUSSION

This study has demonstrated that both sputum mDC and pDC numbers increase following allergen inhalation in allergic asthmatic subjects. Both subsets were significantly increased at 24h following allergen challenge when compared to diluent challenge. In addition, the number of immature mDCs, as measured by the expression of CCR6+ on their surface, was increased at 72h after allergen challenge. The increase in DCs was associated with a significant increase in MIP-3α, the chemokine for immature DCs, when compared to baseline measurements.

Previous studies on peripheral blood have established a decrease in circulating mDCs during the asthmatic response in allergic asthmatic subjects [13-15]. In particular, a study by Farrell et al [15] demonstrated that circulating mDCs were significantly lower at 6h and 24h following allergen challenge, when compared to diluent. Following their disappearance from circulation, it has been speculated that these cells migrate into the airways. Studies in both the airway mucosa [16] and the airway lumen [17,21] have illustrated an increase in airway mDCs following allergen challenge. The current study is the first to provide a comprehensive evaluation of airway mDCs following allergen challenge by measuring the number of mDCs over multiple time points, as well as accounting for any diurnal variation in their numbers, by comparison to a diluent challenge. The study identified an increase in the number of sputum mDCs, thereby supporting the hypothesis that following allergen inhalation, mDCs disappear from circulation, and migrate into the airway tissue. However, this study does not provide

direct evidence that this trafficking occurs. Taken together, these findings support a role for mDCs in mediating the late asthmatic response and allergen-induced airway inflammation.

Research on pDCs has received less attention when compared to mDCs. Although baseline falls in circulating pDCs have been observed following allergen challenge [14,15], numbers of pDCs were not different when compared to diluent in allergic asthmatic subjects [15]. In the airways, Jahnsen et al [16] failed to demonstrate an accumulation of pDCs in the bronchial mucosa following short-term allergen challenge; however, pDCs do increase in the nasal mucosa following several days of repeated allergen exposure in allergic rhinitis subjects [22]. These initial studies in blood and airway tissue suggested that pDCs follow a different kinetic pattern than mDCs. The alternate migration profile was thought to be associated with a distinct functional role for pDCs in the allergic process, to resolve inflammation and restore homeostasis. In the current study, the number of pDCs increased in the airway lumen at the same time as mDCs, which casts doubt on earlier hypotheses that pDCs follow a different, more delayed recruitment towards the airways. Furthermore, these results suggest a more complex, yet active role for pDCs during the allergic response than previously ascribed.

To characterize further both immature and mature mDCs and pDCs, we examined the expression of specific chemokine receptors, CCR6 and CCR7, on both these subpopulations of DCs. Under inflammatory conditions, epithelial cells release MIP-3α [3,4] and therefore, it is believed that immature CCR6+ DCs migrate from the bone marrow, through the blood, and into the airways. Furthermore, in mice models of allergic

asthma, experiments in CCR6 knockout mice have demonstrated a reduced inflammatory response, partially attributed to a decrease in lung DC numbers [23]. In the study by Farrell et al [15], a decrease in circulating levels of CCR6+ mDCs was found following allergen challenge when compared to diluent, while in the current study, the number of CCR6+ mDCs was increased at 72h following allergen challenge. When comparing the increase in mDCs to the change in CCR6+ mDCs at 24hrs post allergen challenge, it is apparent that a majority of mDCs do not express CCR6. It is possible that these DCs are undergoing maturation, whereby they possess a profile between an immature and mature phenotype. The proposed model of DC mobilization has yet to be established within a human framework and therefore, more research *in vivo* is necessary to examine the role this receptor plays in the migration of DCs towards the lung.

Circulating levels of CCR7+ mDCs are extremely low both before and after allergen challenge [15]. The expression of MIP-3β is restricted to T-cell rich areas of regional lymph nodes [3,24,25] and it is believed that mature antigen-bearing CCR7+ DCs migrate from the airways into the draining lymph nodes to interact with naïve antigen-specific T-cells. In our study, we did not find a difference between allergen and diluent, further highlighting the belief that CCR7+ mDCs migrate towards the lymph nodes. Although studies *in vivo* have been limited, our findings do support the proposed model of DC mobilization following antigen capture.

The numbers of sputum CCR6+ and CCR7+ pDCs was also enumerated. A significant efflux of circulating CCR6+ pDCs following allergen inhalation has been previously described [15]; however, in the present study, we did not find a difference in the number

of sputum CCR6+ pDCs or CCR7+ pDCs between allergen and diluent challenges. In animal studies, antigen-bearing pDCs have been shown to migrate towards the draining lymph nodes in murine models of allergic asthma [26-28], but the migration profile of pDCs in allergic humans still remains poorly defined.

CCR6 is the major functional receptor for MIP-3α on immature DCs, as illustrated by a strong correlation between migration profiles and receptor expressions [3,24,29]. The production of MIP-3α is restricted to epithelial cells, as observed in the human lung and liver, and is induced in the crypts of inflamed tonsils [3] and by human airway epithelial cells stimulated with pro-inflammatory stimuli [4]. Parameswaran et al [14] reported a significant increase in sputum levels of MIP-3α at 7hrs following allergen challenge when compared to baseline levels. By contrast, another study could demonstrate no significant differences in serum MIP-3α expression between allergen and diluents [15]. We have shown an increase in sputum MIP-3α levels, when compared to baseline, both at 7h and at 24h post allergen challenge; however, these changes were not significantly different when compared to diluent challenge. CCR7 is the major functional receptor for MIP-3β on mature DCs [3]. Although MIP-3β levels did not change, the increase in CCR7+ mDCs might represent those DCs that have captured allergen and matured, but not migrated back through the epithelium from the airway lumen. In the present study, MIP-3β levels were not significantly different between diluent and allergen, which is consistent with the findings of Farrell et al [15], in which serum levels of MIP-3β were not different between allergen and diluent.

In summary, both mDCs and pDCs increase in the sputum of allergic asthmatic subjects following allergen inhalation. These results are consistent with the hypothesized role for mDCs in allergen presentation and the induction of allergen-induced airway inflammation. The appearance of pDCs is also consistent with a possible role in the resolution of allergen-induced airway inflammation, but appear in the airways earlier that would be expected from the time-course of their transit from the circulation.

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Table 1. Subject characteristics.

Sex	Age (years)	% Predicted FEV_1	$MchPC_{20}$ (mg/ml)	Ag Inhaled	Final [Ag] Inhaled	% Fall in FEV_1 (EAR & LAR)	
M	56	92	1.09	RGW	1:128	44.3	15.8
F	22	102	1.89	HDM DP	1:512	42.2	15.6
F	23	113	5.43	HDM DP	1:128	46.5	35.2
M	20	71	0.85	HDM DP	1:512	32.6	56.5
F	22	94	3.48	RGW	1:256	27.3	19.1
F	27	97	14.93	HDM DP	1:512	30.9	16.0
M	36	78	2.5	GRASS	1:256	45.1	32.4
F	23	92	7.67	HDM DP	1:32	24.2	22.6
F	22	98	1.28	HDM DP	1:512	36.5	17.3
M	23	91	1.52	CAT	1:64	43.3	16.7
F	23	85	0.14	CAT	1:128	45.5	15.2
M	44	73	13.45	HDM DP	1:256	42.4	26.1
#M:F	**Mean:**	**Mean:**				**Mean:**	**Mean:**
5:7	**28.4 ± 3.2**	**90.5 ± 3.5**				**38.4 ± 2.2**	**24.0 ± 3.5**

FEV_1 Forced expired volume in 1 second

Mch Methacholine

PC_{20} Provocative concentration causing a 20% fall in FEV_1

Ag Allergen

RGW Ragweed

HDM House dust mite

EAR Early asthmatic response

LAR Late asthmatic response

Data are presented as mean ± SEM.

Table 2. Inflammatory cells in sputum.

	Eosinophils (10^6 cells/g sputum)		**Neutrophils** (10^6 cells/g sputum)		**Mononuclear Cells** (10^6 cells/g sputum)	
Time (hrs)	**Diluent**	**Allergen**	**Diluent**	**Allergen**	**Diluent**	**Allergen**
0	0.08 ± 0.03	0.10 ± 0.04	0.87 ± 0.31	0.63 ± 0.24	1.66 ± 0.38	1.24 ± 0.22
7	0.32 ± 0.11	1.40 ± 0.44*	1.09 ± 0.32	2.40 ± 0.60	1.13 ± 0.33	0.92 ± 0.15
24	0.39 ± 0.12	1.71 ± 0.59*	1.14 ± 0.50	2.61 ± 0.49*	1.71 ± 0.33	1.97 ± 0.35
72	0.51 ± 0.36	1.33 ± 0.45	0.55 ± 0.19	1.03 ± 0.28	1.18 ± 0.26	1.41 ± 0.34

* Difference between diluent and allergen at $p<0.05$

Data are presented as mean ± SEM.

Table 3. Allergen-induced changes in CCR6 and CCR7 mDCs and pDCs.

	CCR6 mDCs (10^3 cells/g sputum)		**CCR7 mDCs** (10^3 cells/g sputum)		**CCR6 pDCs** (10^3 cells/g sputum)		**CCR7 pDCs** (10^3 cells/g sputum)	
Time (hrs)	**Diluent**	**Allergen**	**Diluent**	**Allergen**	**Diluent**	**Allergen**	**Diluent**	**Allergen**
0	2.24 ± 1.32	1.79 ± 0.78	5.13 ± 2.64	3.05 ± 1.12	3.63 ± 1.85	3.66 ± 1.12	1.54 ± 0.50	4.00 ± 1.63
7	0.49 ± 0.19	2.23 ± 0.90	1.73 ± 0.90	2.78 ± 0.94	1.27 ± 0.31	2.12 ± 1.24	1.64 ± 0.36	2.21 ± 1.10
24	3.52 ± 2.20	5.04 ± 1.28	5.45 ± 2.62	10.16 ± 3.81	5.74 ± 3.61	3.63 ± 1.37	5.27 ± 2.78	8.05 ± 2.79
72	0.33 ± 0.14	4.43 ± 2.61*	7.57 ± 3.80	10.24 ± 6.12	1.08 ± 0.27	3.72 ± 2.21	1.72 ± 0.54	3.62 ± 1.33

mDCs = myeloid dendritic cells; pDCs = plasmacytoid dendritic cells

* Difference between diluent and allergen at $p<0.05$

Data are presented as mean ± SEM.

FIGURE LEGENDS

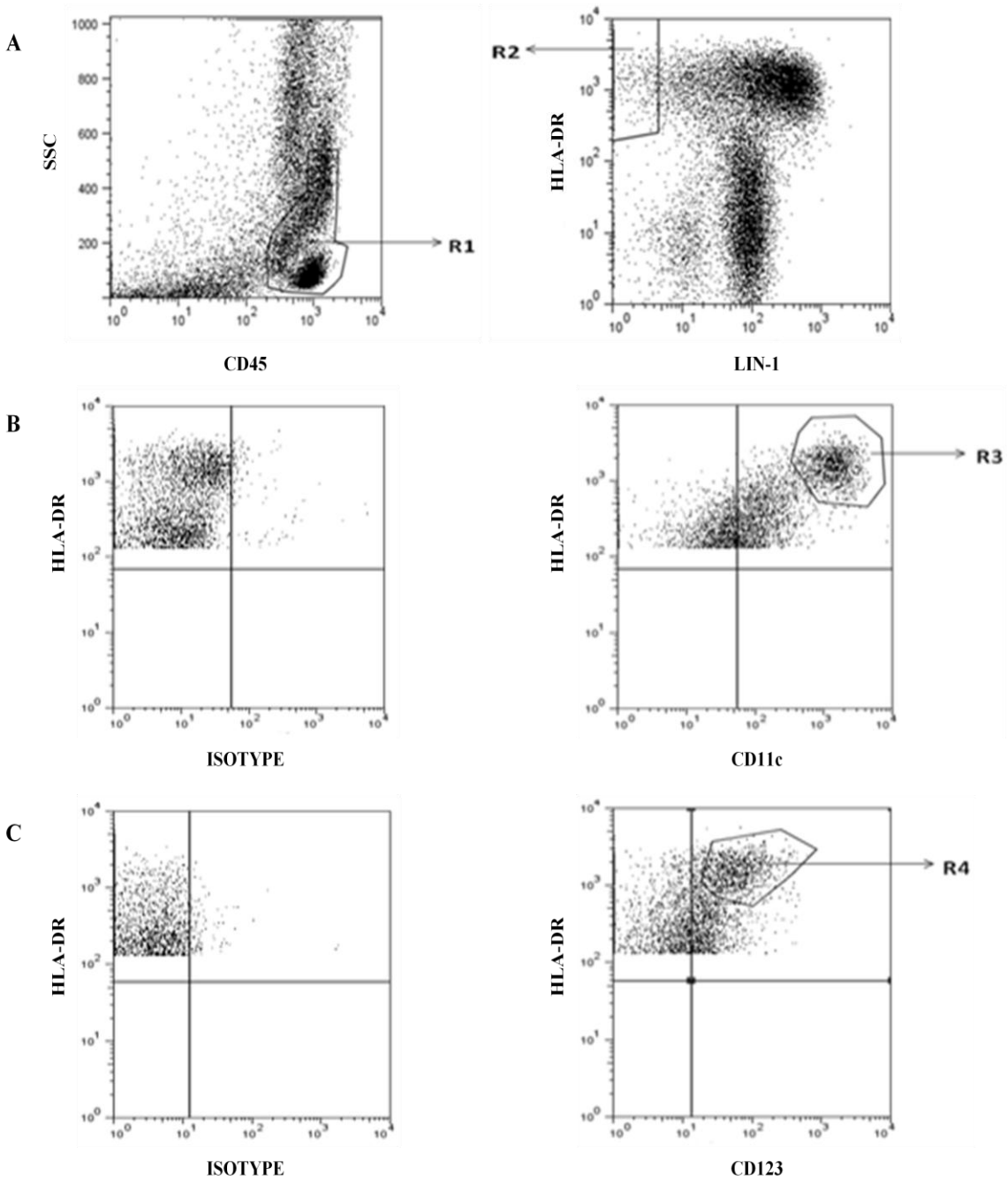
Figure 1. Gating strategy for enumeration of DCs. **A**, Monounuclear gate R1 is set up around the mononuclear cell population on the SSC vs. CD45 plot and then transferred to a Lin1 vs HLA-DR plot to identify DC populations (R2). B, Gating around the mDC population (R3). **C**, Gating around the pDCs population (R4).

Figure 2. Kinetics of sputum mDCs **(A)** and pDCs **(B)** following inhalation of diluent versus allergen. * Difference between baseline and 24hrs post allergen measurements at $p<0.05$ for; ** Difference between diluent and allergen measurements at $p<0.05$. Data are presented as mean ± SEM.

Figure 3. Expression of CCR6 and CCR7 on mDCs **(A)** and pDCs **(B)** compared to its isotype expression.

Figure 4. Kinetics of sputum supernatant levels of MIP-3α **(A)** and MIP-3β **(B)** following inhalation of diluent versus allergen. * Difference between baseline and 24hrs post allergen measurements at $p<0.05$. Data are presented as mean ± SEM.

Figure 1.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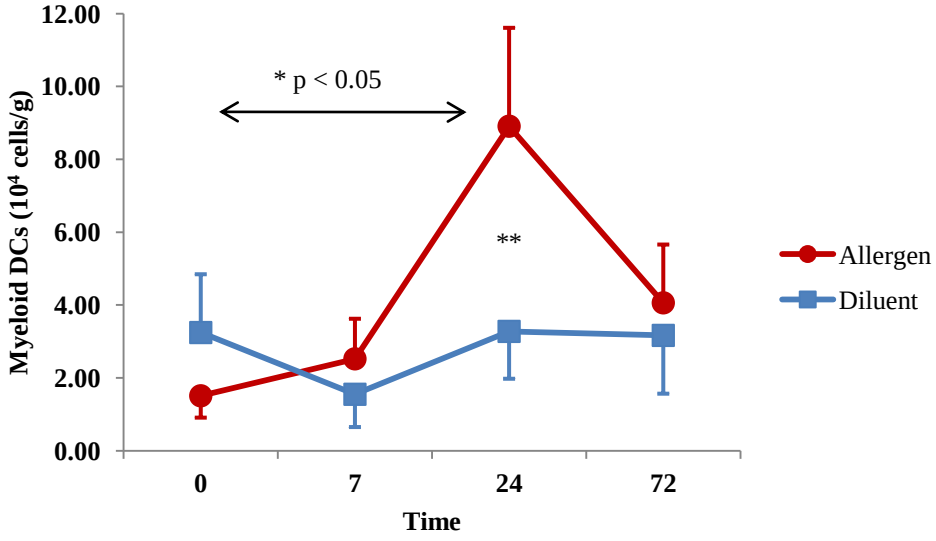
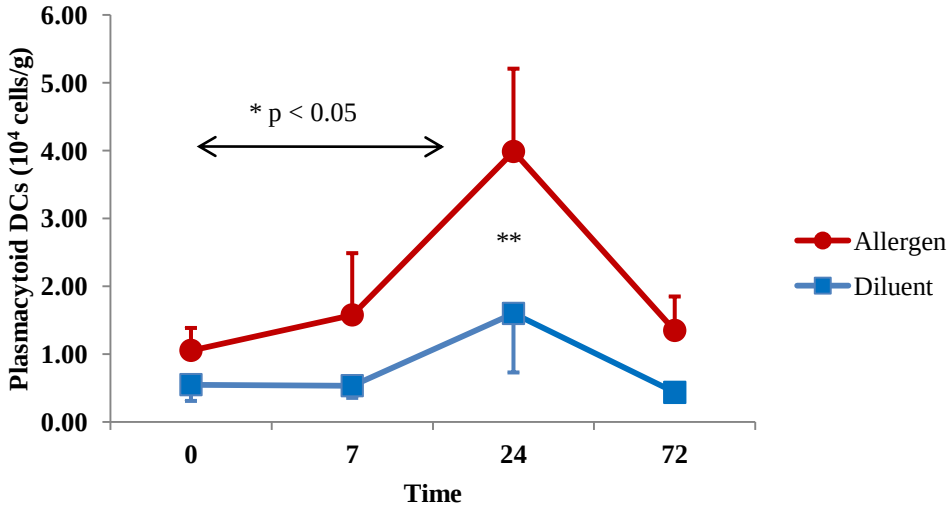
SSC
CD45
R1
HLA-DR
LIN-1
R2
B
HLA-DR
ISOTYPE
HLA-DR
CD11c
R3
C
HLA-DR
ISOTYPE
HLA-DR
CD123
R4

Figure 2.

A


12.00
10.00
8.00
6.00
4.00
2.00
0.00
Myeloid DCs (10⁴ cells/g)
* p < 0.05
**
0
7
24
72
Time
Allergen
Diluent

B


6.00
5.00
4.00
3.00
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1.00
0.00
Plasmacytoid DCs (10⁴ cells/g)
* p < 0.05
**
0
7
24
72
Time
Allergen
Diluent

Figure 3.

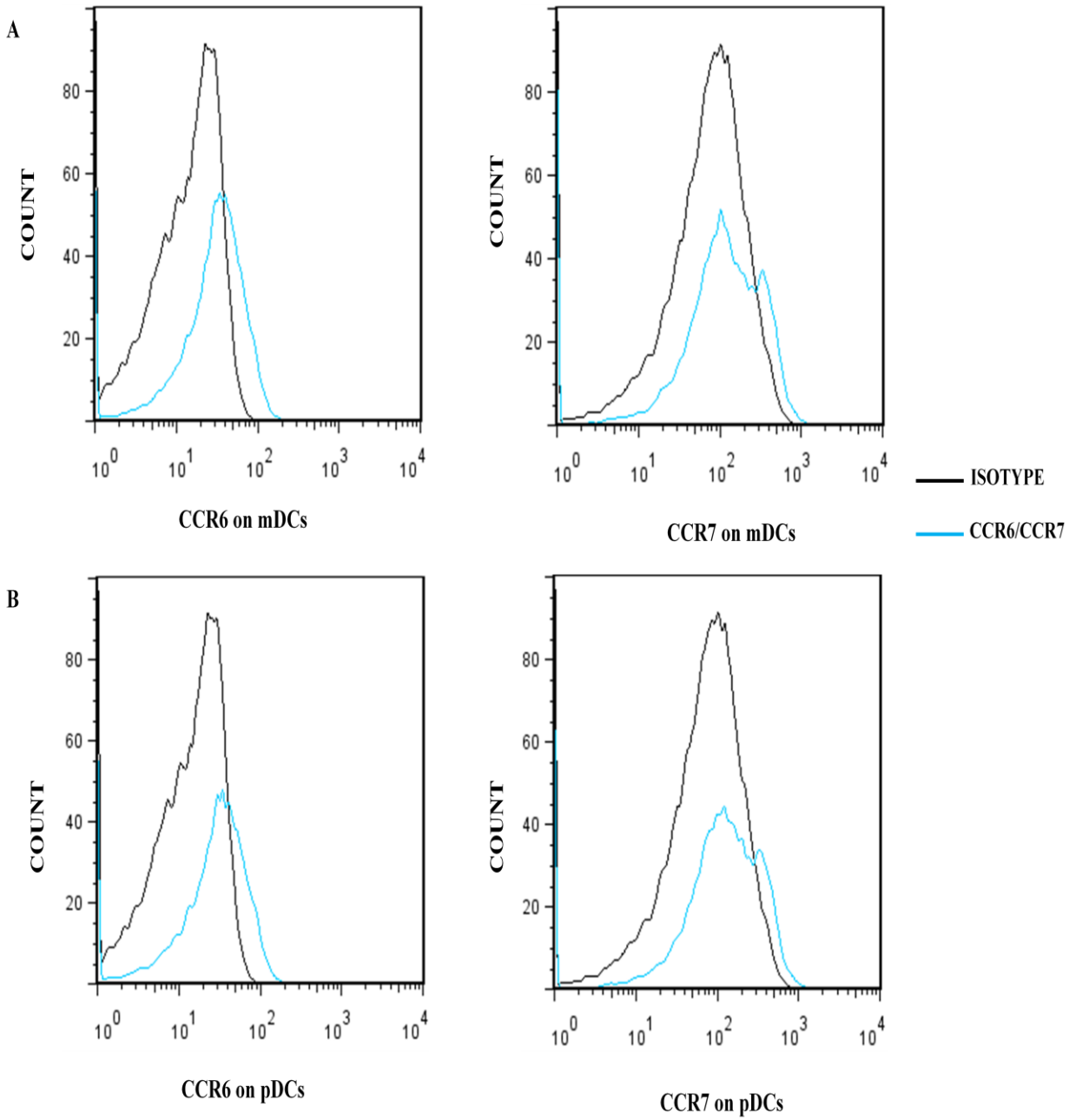
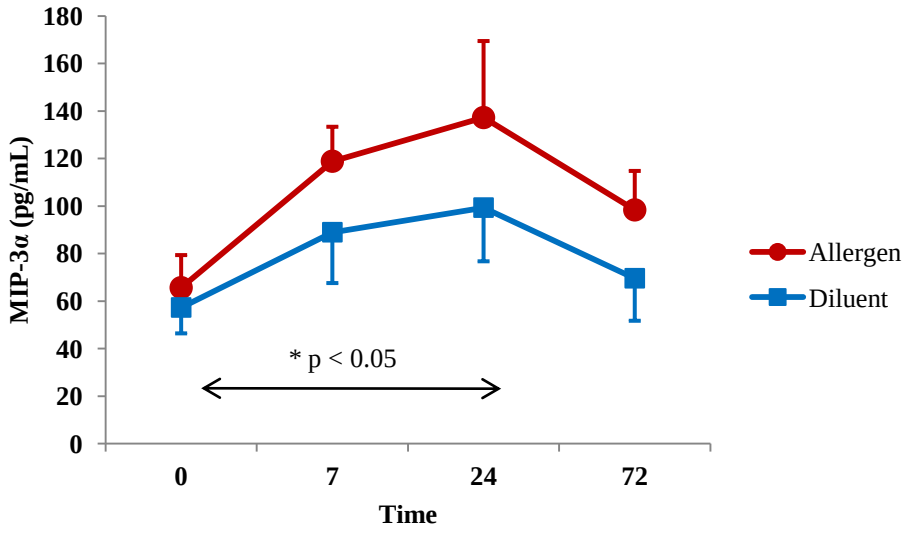
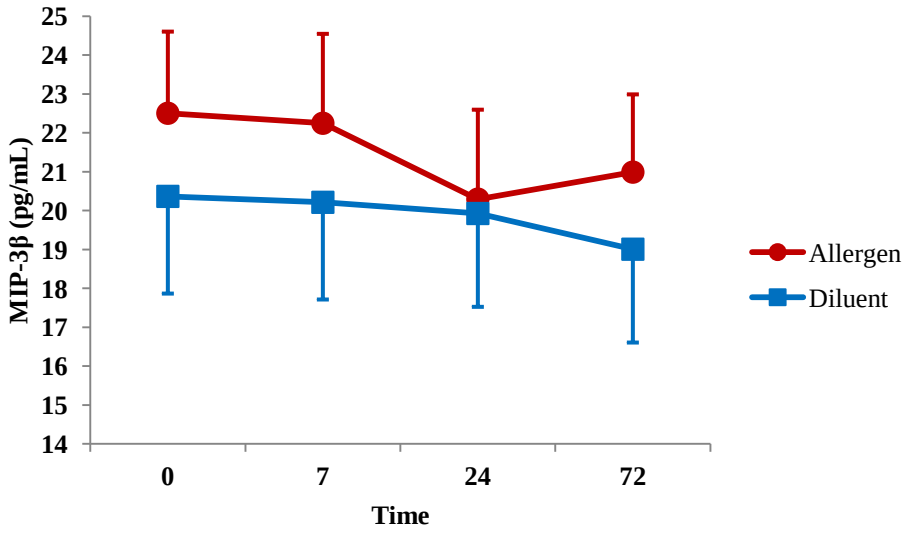

A
B
COUNT
CCR6 on mDCs
CCR7 on mDCs
CCR6 on pDCs
CCR7 on pDCs
ISOTYPE
CCR6/CCR7

Figure 4.

A


180
160
140
120
100
80
60
40
20
0
MIP-3α (pg/mL)
* p < 0.05
0
7
24
72
Time
Allergen
Diluent

B


25
24
23
22
21
20
19
18
17
16
15
14
MIP-3β (pg/mL)
0
7
24
72
Time
Allergen
Diluent

CHAPTER 3

Myeloid dendritic cells type 2 in allergic asthma

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mDCs do not represent a homogenous cell population. Within the mDC lineage, two distinct populations have been identified, which are mDC1s and mDC2s [5,6]. An unexpected role for mDC2s was recently suggested in allergic disease when comparing subjects with allergy and asthma to controls [8]. As such, we enumerated circulating mDC2s, as well as mDC1s and pDCs, in healthy, atopic non-asthmatic, mild atopic asthmatic and moderate/severe atopic asthmatic subjects. We find that circulating mDC2s are lower in atopic subjects compared to healthy controls and in asthmatic subjects compared to non-asthmatic subjects.

MYELOID DENDRITIC CELLS TYPE 2 IN ALLERGIC ASTHMA

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Word count: 2077

ABSTRACT

Background: Dendritic cells (DCs) are professional antigen presenting cells that mediate the responses to inhaled allergens. DCs are not a homogenous cell population in humans, with myelod dendritic cells (mDCs) and plasmacytoid dendritic (pDCs) being the 2 major subpopulations. A new subtype of myeloid DC, termed myeloid dendritic cells type 2 (mDC2s), has been identified in both the circulation and the lung, and has been suggested to have a role in allergic asthma.

Methods: Circulating mDC2s, as well as mDC1s and pDCs, were enumerated in 19 healthy, 18 atopic non-asthmatic, 18 mild atopic asthmatic and 16 moderate/severe atopic asthmatic subjects using flow cytometry.

Results: The number of circulating mDC2s was significantly lower in atopic subjects compared to healthy controls and in asthmatic subjects compared to non-asthmatic subjects. There was a trend towards lower levels of circulating mDC2s with increasing allergy and asthma severity. The largest differences were seen in moderate/severe atopic asthmatics being 430.78 ± 48.91 / mL compared to healthy controls being 767.05 ± 101.64 / mL ($p<0.05$). Moderate/severe atopic asthmatics also had a significantly lower number of circulating pDCs. Circulating numbers or percentages of mDC1s were not different among any of the subject groups.

Conclusions: Circulating mDC2s are lower in atopic and asthmatic subjects, which suggests that these cells efflux from the blood into the airways in patients with allergic disease.

INTRODUCTION

Allergic asthma is an inflammatory disease of the airways, initiated by inhaled environmental allergens. The inflammatory reaction is controlled by activated Th2 cells releasing pro-inflammatory cytokines, causing eosinophil activation and chemotaxis, IgE production and mucus hypersecretion. Without antigen presenting cells (APCs), T cells are unable to respond to allergen. Dendritic cells (DCs) are the most potent APC in the lungs that orchestrate immune responses to inhaled allergen.

DCs do not represent a homogenous cell population and two main subpopulations of DCs exist: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). Both subsets are recognized by distinct phenotypic characteristics, and have different functions in the regulation of responses to inhaled allergens. From animal models of allergic asthma, mDCs induce and maintain airway inflammation [1-3], while pDCs induce tolerance to inhaled allergens [4].

Two distinct populations have been identified within the myeloid DC lineage – mDCs type 1 (mDC1s) and mDCs type 2 (mDC2s) [5,6]. Specific blood dendritic cell antigens (BDCA) are used to distinguish among DC populations. Expression of BDCA-2 (CD303) and BDCA-4 (CD304, Neuropilin-1) is confined to pDCs, while BDCA-1 (CD1c) and BDCA-3 (CD141, thrombomodulin) are expressed on mDC1s and mDC2s respectively. DCs represent about 1% of all peripheral blood mononuclear cells in healty individuals, of which 0.60% are mDC1s, 0.37% are pDCs, and 0.03% are mDC2s. mDC2s share many characteristics with mDC1s, including phenotype, morphology,

endocytic capacity, and maturation [5]; however, mDC2s do not express CD32, CD64 and FcεRI [5], and stimulate T-cell proliferation less efficiently than mDC1s, albeit better than pDCs [7].

Yerkovich and colleagues [8] have suggested a role for mDC2s in allergic disease. After *in vitro* stimulation with house dust mite (HDM), BDCA-3 expression on blood DCs was higher in atopic individuals compared to non-atopic subjects, and these mDC2s induced a strong Th2 polarized response compared to DCs lacking BDCA-3 [8]. Circulating mDC2s were also more frequent in subjects with allergy and asthma compared to healthy individuals [8, 9]. In the lung, mDC2s have been found in the airway tissue [10] and lumen [11], and studies have demonstrated mDC2s to be higher in atopic asthmatics compared to healthy individuals [12,13].

There is much less known about the role of mDC2s in allergic disease, when compared to mDC1s or pDCs. To help understand the possible role of mDC2s in allergic asthma, we examined the number of circulating mDC2s across different population groups with allergy and asthma. As such, mDC2s were enumerated in peripheral blood and compared between healthy subjects and atopic non-asthmatic, mild atopic asthmatic and moderate/severe atopic asthmatic subjects. We hypothesized that, consistent with the results of Yerkovich and coworkers [8], circulating mDC2s are increased in subjects with allergy and asthma compared to healthy individuals.

METHODS

Subjects

Nineteen healthy subjects, 18 atopic non-asthmatic subjects, 18 mild atopic asthmatic subjects and 16 atopic moderate/severe asthmatic subjects were enrolled (Table 1). All subjects underwent spirometry, skin prick testing and blood sampling. Except for the moderate/severe atopic asthmatics, all subjects underwent a methacholine inhalation challenge. Healthy subjects were classified on the basis of a negative skin test to common aeroallergens, a PC_{20} > 16 mg/ml and a negative history for any respiratory disease. Atopic non-asthmatic subjects were classified as having 1 or more skin wheal responses to common aeroallergens, a PC_{20} > 16 mg/ml and a negative history for asthma. Mild atopic asthmatics were classified as having a positive skin test, a PC_{20} < 16 mg/ml, a $FEV_1 \geq 80\%$ predicted, and a history for mild asthma, with no need for inhaled corticosteroid (ICS) maintenance treatment. Moderate/severe atopic asthmatic subjects were classified as having a positive skin test, a $FEV_1 \leq 80\%$ predicted, and a history of moderate/ severe asthma, including the need for regular use of an ICS with a long-acting β_2-agonist (LABA) as maintenance treatment. Subjects were excluded if they were pregnant, experienced an asthma exacerbation or a respiratory infection within the previous 6 weeks, were current smokers, or ex-smokers with more than 10 pack-years. All subjects gave written informed consent, and this study was approved by the Hamilton Health Science Research Ethics Board (REB # 09-317).

Immunofluorescent Staining

A commercial blood DC enumeration kit was used to phenotype circulating mDC2s (Miltenyi Biotech, Auburn, CA, USA). A cocktail of monoclonal antibodies including APC-BDCA-3, PE-BDCA-1, FITC-BDCA-2, and PE-Cy5-CD14-CD19, as well as a corresponding cocktail of isotype controls, were used to identify mDC2s, mDC1s and pDCs. Staining was performed according to kit instructions.

Flow Cytometry Acquisition

Cells were acquired with a 15-color LSR II flow cytometer equipped with 3 lasers (BD Instrument Systems, Mississauga, ON, Canada) using the FACSDiva software program (BD Biosciences, Mississauga, ON, Canada). Six parameters were acquired: linear forward angle light scatter, linear side angle light scatter, log PE, log FITC, log APC, and log PECy5. Approximately 1 million cells were collected on flow-cytometric acquisition to obtain a sufficient number of mDC2s.

Enumeration and Analysis of DCs

Dendritic cells were enumerated as per kit instructions using FlowJo software (Tree Star Inc, Ashland, OR, USA). An initial gate was set up to capture leukocytes, excluding debris and platelets on the FSC vs SSC plot. Next, a subsequent gate was made to exclude B-cells, monocytes, granulocytes and dead cells on the SSC vs CD14-CD19 plot. In this plot, DCs are known to be found in the bottom left region, with a SSC value between lymphocytes and granulocytes. Finally, individual gates were created to

enumerate mDC1s (BDCA-1+), pDCs (BDCA-2+) and mDC2s (BDCA-3+) (Figure 1). Since BDCA-3 is also expressed at much lower levels on other cell types, only cells with high expression were included.

Circulating DC Counts

From the flow cytometric acquisition, mDC1s, pDCs and mDC2s were expressed as a percentage of total white blood cells collected. This fraction was then multiplied by the absolute number of white blood cells per mL in the blood sample. The result is the total number of circulating DCs expressed per mL of blood.

Statistical Analysis

All statistics are expressed as means ± SEMs. Statistical analyses were performed by using Prism Software (GraphPad Software Inc., La Jolla, CA, USA). Dunn's post tests were performed to compare individual groups using a 1-way ANOVA. Significance was accepted at $p < 0.05$.

RESULTS

The moderate/severe atopic asthmatics were significantly older and, as expected from the enrollment criteria, had a lower resting absolute FEV_1 and FEV_1 % predicted compared to each of the other 3 groups. Also, as expected, serum levels of IgE were significantly lower in healthy subjects, when compared to the atopic non-asthmatics, mild atopic asthmatics and moderate/severe atopic asthmatics ($p<0.05$) (Table 1).

Healthy subjects had the greatest number of circulating mDC2s, followed by atopic non-asthmatics, mild atopic asthmatics and finally moderate/severe atopic asthmatics (Figure 2). When subjects were divided based on atopy and asthma, circulating mDC2s were lower in the atopic (524.66 ± 43.05 / mL) and asthma (476.91 ± 44.53 / mL) groups, compared to the non-atopic (767.05 ± 101.64 / mL) and non-asthmatic (693.00 ± 68.30 / mL) groups (Figure 3) ($p=0.03$, $p=0.02$ respectively). There was a trend towards lower mDC2 levels with increasing allergy and asthma severity ($p=0.066$). In addition, there was a significant difference in the number of circulating mDC2s between healthy subjects (767.05 ± 101.64 / mL) compared to moderate/severe atopic asthmatics (430.78 ± 48.91 / mL) (Figure 2) ($p<0.05$). This was also true for the percentage of mDC2s (data not shown). There was also a trend towards a positive correlation between FEV_1 % predicted and the number of mDC2s among all subjects ($r=0.23$, $p=0.053$).

Circulating numbers or percentages of mDC1s were not different among any of the subject groups (Figure 4). In contrast, moderate/severe atopic asthmatics had a significantly lower number of circulating pDCs (4687.29 ± 592.67 / mL), when compared

to both mild atopic asthmatics (7320.39 ± 840.14 / mL) ($p<0.05$) and atopic non-asthmatics (8961.10 ± 733.40 / mL) ($p<0.05$) (Figure 4). This was also true for the percentage of pDCs (data not shown).

DISCUSSION

This study has demonstrated that circulating numbers of mDC2s are significantly lower in atopic and asthmatic subjects. The greatest differences were observed between moderate/severe atopic asthmatics and healthy subjects, and there was a trend towards lowering circulating mDC2 levels with increasing allergy and asthma severity. In addition, there was a trend towards a significant, but weak, positive relationship between FEV_1 % predicted and the number of mDC2s.

Unexpectedly, our results were different to those of Yerkovich and colleagues [8]. Using the same commercial kit, these investigators measured the percentage of circulating mDC2s among healthy, atopic non-asthmatic and atopic asthmatic subjects, but did not include a moderate/severe atopic asthmatic group. These subjects were adolescents, and a minority of the atopic asthmatics were taking inhaled steroids. They demonstrated circulating mDC2s were higher in atopic asthmatics compared to healthy subjects [8]. When subjects were further divided into atopic and asthmatic groups, circulating mDC2s were higher in these groups compared to non-atopic and non-asthmatic subjects respectively [8]. The authors illustrated a relationship between the HDM allergen and mDC2s, demonstrating *in vitro* HDM exposure up-regulates BDCA-3 on DCs – the marker for mDC2s [8]. It is plausible that the increase in circulating mDC2s is a result of the perennial exposure to HDM in their study, as supported by the fact that all atopic subjects were HDM positive. A minority of subjects in our current study were HDM sensitized; therefore, it is possible that our results may more accurately reflect the relative

number of circulating mDC2s in atopic asthmatics, without concomitant allergen exposure.

The populations studied included a group of moderate/severe allergic asthmatics, who required regular treatment with ICS to maintain asthma control. None of the other groups were using regular therapy for allergy or asthma at the time of the study. Thus, the differences observed in mDC2 numbers in the moderate/severe population may be because of the maintenance asthma treatment. However, the decline in mDC2 numbers with increasing asthma and allergy, along with a lack of difference in mDC1s between moderate/severe subjects and other groups, argues against an effect of ICS treatment lowering DC numbers. It is also possible that age may have influenced mDC2 numbers, as the moderate/severe asthmatics were older than the other groups. However, there was no correlation between age and mDC2 levels, and when a subgroup of moderate/severe asthmatics was compared with a subgroup of healthy subjects whose ages were similar, the mDC2 numbers between these groups remained significantly different. As such, we believe neither ICS treatment, nor age, had an effect on the differences observed in mDC2 numbers.

Circulating mDC1 numbers were not different among groups, and this was consistent with previous results from Yerkovich and colleagues, who demonstrated no differences in mDC1s among healthy, atopic non-asthmatic and atopic asthmatic subjects [8]. pDCs have been shown to be increased in subjects with asthma [14], and in consideration of their ability to mediate immune tolerance, this may be a compensatory response to

regulate allergic inflammation. Of interest, the circulating pDCs were significantly lower in the moderate/severe asthmatics when compared to the atopic and mild asthmatic groups. Other studies have suggested that it is the relative balance between mDCs and pDCs which determines the magnitude of allergen-induced responses [14].

Previous studies have suggested that, in contrast to the circulation, mDC2 numbers are at comparable or even higher levels than mDC1s and pDCs in the lung [10,11], suggesting a preferential localization to this compartment. Furthermore, mDC2s were found to be increased in the BALF and sputum of atopic asthmatics compared to healthy subjects [12,13]. The relevance of the increased localization and presence of mDC2s in the lung is uncertain, but may account for the decreased number of circulating mDC2s we observed in our study. We have previously shown that both mDCs and pDCs increase in the sputum of asthmatic subjects following allergen challenge [15]. Sputum mDC2s were not quantified in this study; however, it is possible that mDC2s are more frequent in the airways of subjects with allergy and asthma.

In summary, this study demonstrates, for the first time, that circulating mDC2s are lower in atopic and allergic asthmatics compared to healthy subjects. The relatively lower numbers of circulating mDC2s in atopic asthmatic subjects might help explain their increased presence in the lung. The role of mDC2s in allergic asthma is not clear, and future studies will aim to characterize mDC2s in the airways, and examine the effects of allergen inhalation on these cells.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. John Upham, University of Brisbane, for his help in the interpretation of these results.

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Table 1. Subject characteristics.

	Healthy	Atopic Non-Asthmatic	Mild Atopic Asthmatic	Moderate/ Severe Atopic Asthmatic
Sample Size (n)	19	18	18	16
Sex, male/female	6/13	7/11	6/12	3/13
Age	24.32 ± 2.31*	26.50 ± 1.76*	33.11 ± 3.59*	49.19 ± 3.84
FEV_1 % predicted	94.96 ± 1.73*	93.14 ± 3.10*	95.43 ± 2.65*	62.10 ± 3.23
Methacholine PC_{20} (mg/ml)	>16	>16	2.38 ± 0.30	_
ICS/LABA Use	0	0	0	16
HDM DP Allergy	0	11	8	6
Total serum IgE (KU/L)	23.05 ± 5.89	139.83 ± 24.99†	299.94 ± 94.25†	663.75 ± 487.71†

FEV_1 Forced expiratory volume in 1s

PC_{20} Provocative concentration causing a 20% fall in FEV_1

ICS Inhaled corticosteroid steroid

LABA Long-acting β_2-agonist

Data are presented as means ± SEMs (PC_{20} is presented as geometric mean ± geometric SEM)

* Difference to moderate/severe atopic asthmatic group. $p < 0.05$

† Difference to healthy group. $p < 0.05$

FIGURE LEGENDS

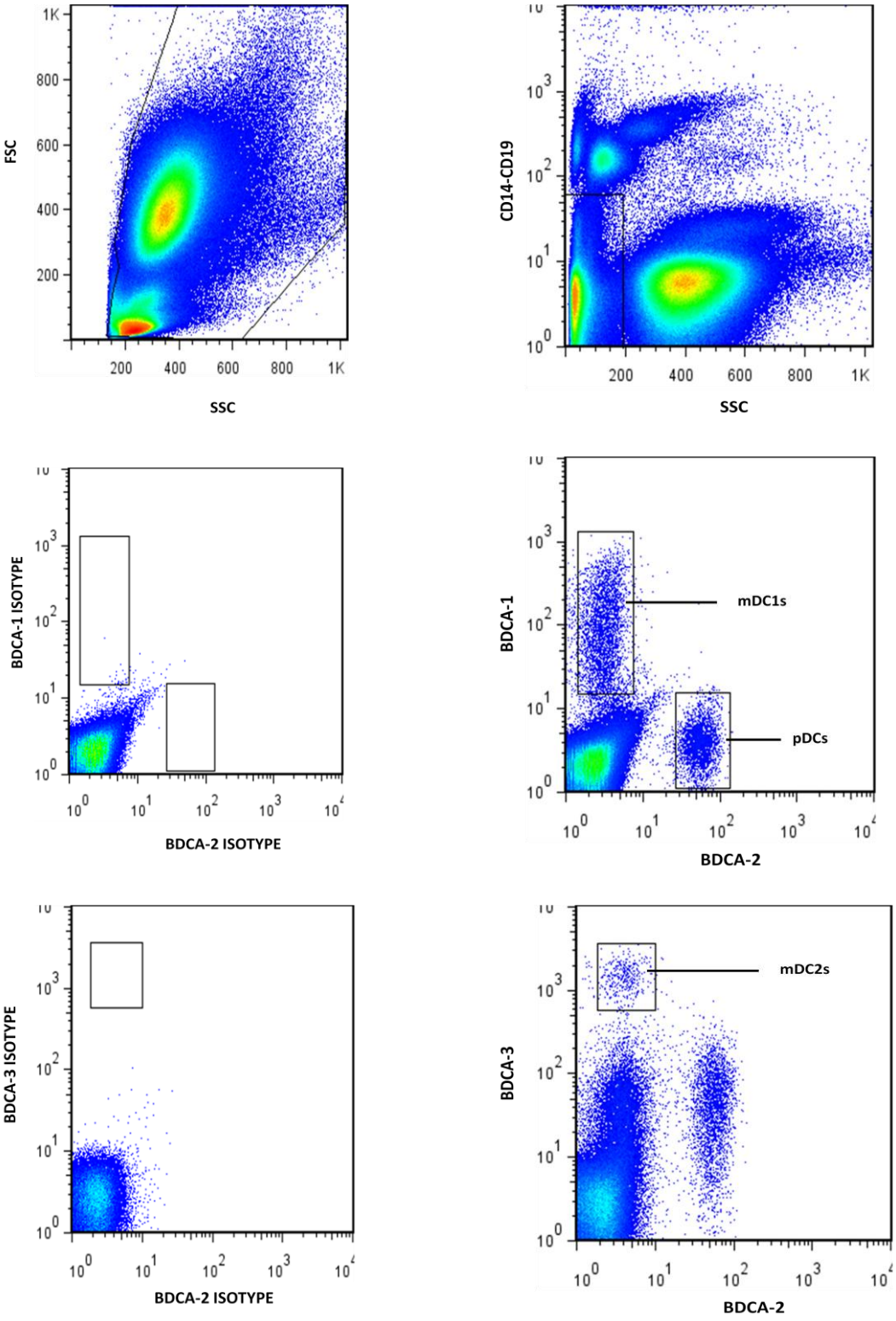
Figure 1. Flow cytometric gating strategy for phenotyping of circulating DCs. An initial leukocyte gate was set up around all leukocytes and then transferred to a SSC vs CD14-CD19 plot to exclude various cell populations. Individual gates were then created to identify mDC2s, mDC1s and pDCs.

Figure 2. Comparison of mDC2 numbers between healthy, atopic non-asthmatic, mild atopic asthmatic and moderate/severe atopic asthmatic subjects. Data are presented as means ± SEMs.

Figure 3. Comparison of mDC2 numbers between non-atopic and atopic groups (**A**), and non-asthmatic and asthmatic groups (**B**). Data are presented as means ± SEMs.

Figure 4. Comparison of mDC1 (**A**) and pDC (**B**) numbers between healthy, atopic non-asthmatic, mild atopic asthmatic and moderate/severe atopic asthmatic subjects. Data are presented as means ± SEMs.

Figure 1.


FSC
SSC
CD14-CD19
SSC
BDCA-1 ISOTYPE
BDCA-2 ISOTYPE
BDCA-1
mDC1s
pDCs
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BDCA-2 ISOTYPE
BDCA-3
mDC2s
BDCA-2

Figure 2.

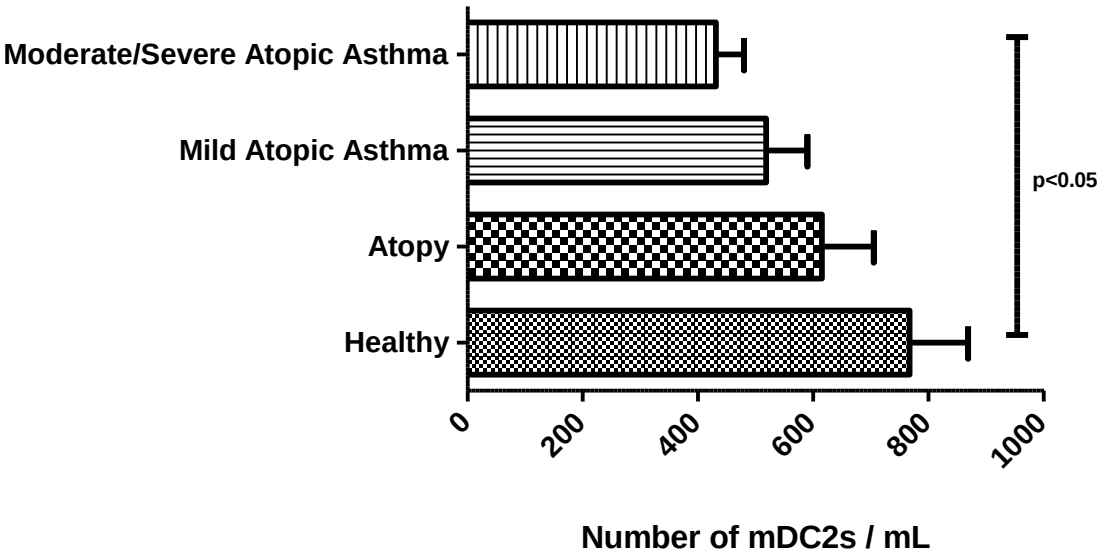
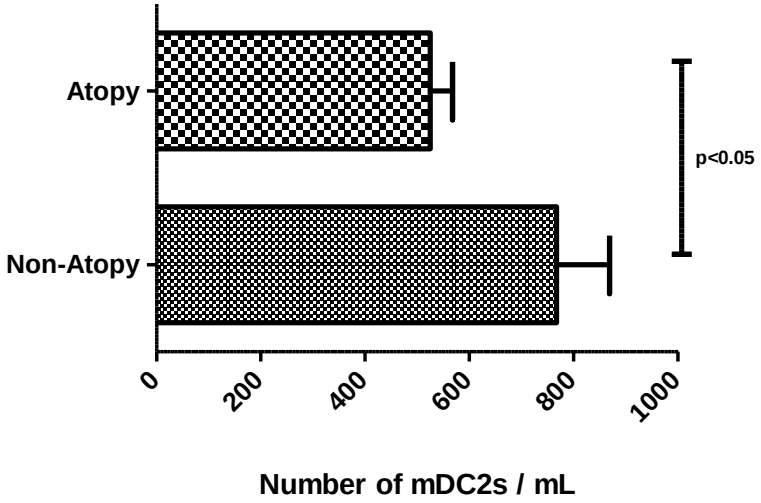

Moderate/Severe Atopic Asthma
Mild Atopic Asthma
Atopy
Healthy
p<0.05
0
200
400
600
800
1000
Number of mDC2s / mL

Figure 3.

A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Non-Atopy
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0
200
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600
800
1000
Number of mDC2s / mL

B

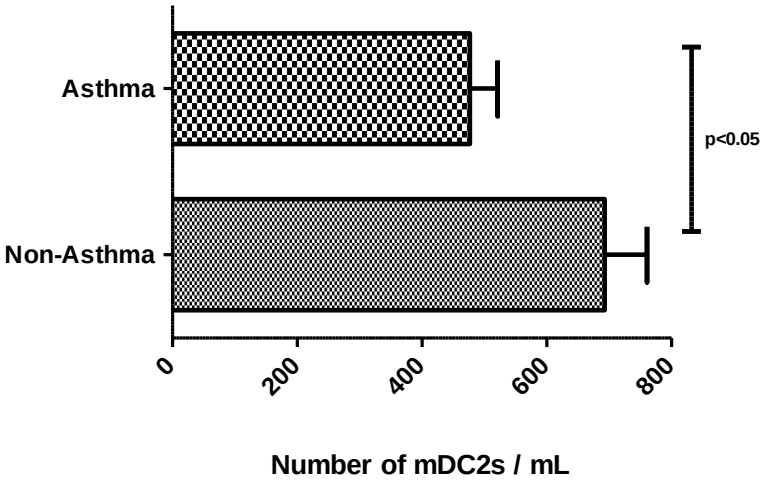
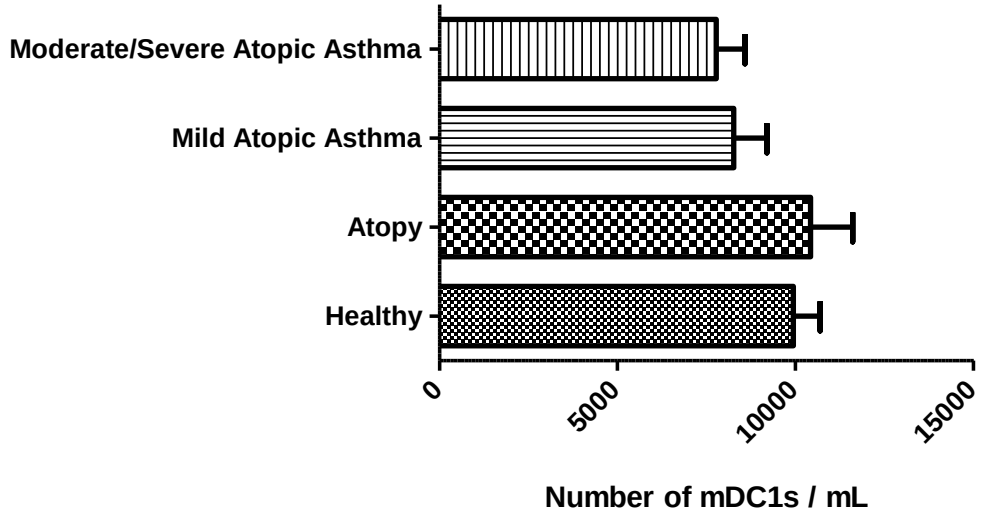
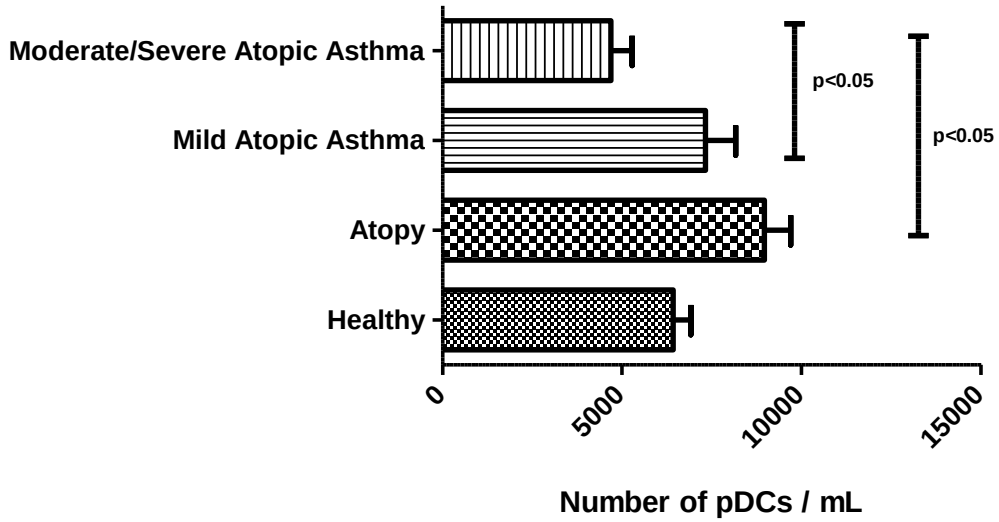

Asthma
Non-Asthma
p<0.05
0
200
400
600
800
Number of mDC2s / mL

Figure 4.

A


Moderate/Severe Atopic Asthma
Mild Atopic Asthma
Atopy
Healthy
0
5000
10000
15000
Number of mDC1s / mL

B


Moderate/Severe Atopic Asthma
Mild Atopic Asthma
Atopy
Healthy
p<0.05
p<0.05
0
5000
10000
15000
Number of pDCs / mL

CHAPTER 4

Myeloid dendritic cells type 2 after allergen inhalation in asthmatic subjects

This manuscript has been submitted to the Journal of Allergy and Clinical Immunology.

In the previous chapter, we studied mDC2s, a minor subset of mDCs in peripheral blood, which are distinct from the more prevalent mDC1 subset. Our findings support the notion that mDC2s preferentially locate to the lung [12,13]. To elaborate on these findings, we examined the effect of *in vivo* allergen exposure on mDC2s. As such, we investigated changes in circulating and sputum mDC2s after allergen inhalation in subjects with asthma. We demonstrate that sputum mDC2s increase in the airways of mild asthmatics after allergen challenge.

MYELOID DENDRITIC CELLS TYPE 2 AFTER ALLERGEN INHALATION IN ASTHMATIC SUBJECTS

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Funding: Canadian Institutes of Health Research Grant # 150887

Running head: Myeloid dendritic cells type 2 after allergen challenge

Word count: 2641

ABSTRACT

Background: Dendritic cells (DCs) are professional antigen presenting cells that mediate the response to inhaled allergen. A major division in DC ontogeny exists between myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). A subtype of mDC expressing thrombomodulin, termed myeloid DCs type 2 (mDC2s), has been identified in both the circulation and lung, and has recently been suggested to have a role in allergic asthma.

Objective: To investigate changes in circulating and sputum mDC2s after allergen inhalation in subjects with asthma.

Methods: Peripheral blood and induced sputum was obtained before and 3 hours, 7 hours, and 24 hours after inhalation of diluent and allergen from allergic asthmatic subjects who develop both allergen-induced early and late phase responses. mDC2s were measured by flow cytometry. Soluble BDCA-3 (thrombomodulin) was measured in sputum by ELISA.

Results: The number of sputum mDC2s significantly increased 24 hours after allergen challenge compared with diluent. The expression of BDCA-3 on sputum mDCs also increased, albeit non-significantly, at 7 hours and 24 hours after allergen. Soluble BDCA-3 in sputum and the number of circulating mDC2s were not different between allergen and diluent.

Conclusions: mDC2s increase in the sputum of subjects with asthma after allergen challenge, suggesting this subtype of mDC is involved in the regulation of allergen responses in the lung.

KEY MESSAGES

- This study is the first to explore the *in vivo* effects of allergen inhalation on circulating and airway mDC2s. We have demonstrated that mDC2s increase in the sputum of allergic asthmatic subjects following allergen inhalation, suggesting that this subpopulation of DC is involved in the immune response to inhaled allergen.
- Continued research exploring DC heterogeneity, including the migration and function of various DC subsets, will be important in improving our understanding of the mechanisms of allergic asthma.

CAPSULE SUMMARY

mDC2s increase in induced sputum from allergic asthmatics following allergen inhalation. Research into the various DC subtypes, including their migration and function, will improve our understanding of allergic asthma and ultimately allow for therapeutic intervention based upon dendritic cell biology.

KEY WORDS

allergic asthma, antigen presenting cells, BDCA-3, myeloid dendritic cells type 2 (mDC2s), sputum, thrombomodulin

ABBREVIATIONS

APCs	antigen presenting cells
BDCA	blood dendritic cell antigen
DCs	dendritic cells
FEV_1	forced expired volume in 1 second
HDM	house dust mite
mDC1s	myeloid dendritic cells type 1
mDC2s	myeloid dendritic cells type 2
pDCs	plasmacytoid dendritic cells

INTRODUCTION

Allergic asthma is an inflammatory disease of the airways, initiated by inhalation of environmental allergens. After allergen inhalation, the inflammatory reaction is orchestrated by activated Th2 cells releasing pro-inflammatory cytokines, causing eosinophil activation and chemotaxis, IgE production and mucus hypersecretion. T cells however, are unable to respond to allergen independently of antigen presenting cells (APCs). Dendritic cells (DCs) are the most potent APC in the lungs that initiate and regulate immune responses to inhaled allergen.

DCs do not represent a homogenous cell population. In humans, a major division in DC ontogeny exists between myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). Circulating mDCs share a common lineage with monocytes and macrophages, whereas pDCs express lymphoid development markers and bear a morphological resemblance to plasma cells [1]. Both subsets are recognized by distinct phenotypic characteristics, and have different functions in the regulation of responses to inhaled allergens. From animal models of allergic asthma, mDCs have a role in the induction and maintenance of airway inflammation [2-4] while pDCs have a role in the tolerance to inhaled allergens [5].

Within the myeloid DC lineage, two distinct populations have been identified, which are mDCs type 1 (mDC1s) and mDCs type 2 (mDC2s) [6,7]. To discriminate among DC populations, specific blood dendritic cell antigens (BDCA) are used. Expression of BDCA-2 is restricted to pDCs, while BDCA-1 (CD1c) and BDCA-3 (CD141, thrombomodulin) are expressed on mDC1s and mDC2s respectively [6]. In healthy

individuals, DCs represent about 1% of all peripheral blood mononuclear cells, of which 0.60% are mDC1s, 0.37% are pDCs, and 0.03% are mDC2s. This small population of mDC2s shares many characteristics with mDC1s, including phenotype, morphology, endocytic capacity, and maturation [6]; however, mDC2s do not express CD32, CD64 or FcεRI [6], and stimulate T-cell proliferation less efficiently than mDC1s, albeit better than pDCs [8].

A role for mDC2s in allergic disease was recently suggested by Yerkovich and colleagues [9]. After *in vitro* stimulation with house dust mite (HDM), BDCA-3 expression on blood DCs was higher in atopic individuals compared to non-atopic subjects, and these mDC2s induced a strong Th2 polarized response compared to DCs lacking BDCA-3 [9]. Furthermore, mDC2s were found to be more prevalent in the peripheral blood of subjects with allergy and asthma compared to healthy individuals [9-11]. In the lung, mDC2s have been identified in the airway tissue [12] and lumen [13], and studies have demonstrated mDC2s to be higher in the airways of atopic asthmatics compared to healthy individuals [14, 15].

The migration of DCs towards antigen in the periphery, followed by the trafficking towards lymphoid tissues, is fundamental to their function as APCs. To date, no study has examined the *in vivo* effects of allergen on mDC2 migration. As such, we examined the kinetics of mDC2s in subjects with allergic asthma after inhalation of allergen. mDC2s in both peripheral blood and sputum were enumerated after allergen and diluent challenges. The levels of BDCA-3 (thrombomodulin) were also enumerated in sputum

after both challenges.

METHODS

Subjects

Twenty mild allergic asthmatic subjects, between 18 and 65 years, were enrolled in the study (Table 1). All subjects were atopic on the basis of 1 or more skin wheals responses to common aeroallergens, had an FEV_1 greater or equal to 70% of predicted, and had previously documented dual airway responses to inhaled allergen, as determined by a fall in FEV_1 of at least 20% within the first 2 hours, followed by second fall in FEV_1 of at least 15% 3 hours to 7 hours after allergen inhalation. All subjects were treated with only intermittent (not daily) β_2-agonists. Subjects were excluded if they were pregnant, current smokers, or ex-smokers with more than 10 pack-years. All subjects gave written informed consent, and this study was approved by the Hamilton Health Science Research Ethics Board.

Study Design

All subjects were screened on day 1 with a methacholine inhalation challenge to assess airway hyperresponsiveness and a skin prick test to confirm atopy. On day 2, subjects were randomized to receive either a diluent or allergen inhalation challenge. Subjects returned on day 3 to undergo another methacholine inhalation challenge. In twelve subjects, blood samples were collected before and at 3 hours, 7 hours, and 24 hours following both allergen and diluent. In a different eight subjects, sputum was induced before and at 7 hours and 24 hours following each challenge. A washout period of 2 to 4 weeks was enforced between challenges.

Allergen inhalation

Allergen inhalation was performed as previously described [16]. The allergen producing the largest diameter skin wheel was diluted in saline for inhalation. The concentration of allergen required to achieve a 20% decrease in FEV_1 (the allergen PC_{20}) was predicted using the methacholine PC_{20} and the titration of allergen determined from the skin prick test [17]. The early bronchoconstrictor response was recorded as the greatest fall in FEV_1 between 0 and 2 hours after allergen inhalation, whereas the greatest drop in FEV_1 between 3 hours and 7 hours was recorded as the late bronchoconstrictor response. For diluent challenges, the same procedure was followed as per inhalation of allergen; however, 0.9% normal saline was used for 3 inhalations only.

Blood collection

Venous blood was withdrawn into vacuum blood collection tubes containing either lithium heparin or EDTA. Blood was collected before and 3 hours, 7 hours, and 24 hours after challenge. Each time, 4 mL of blood was collected for immunofluorescent staining of mDC2s, while another 2 mL was collected for routine blood count.

Sputum induction and processing

Sputum was induced before and 7 hours and 24 hours after challenge by using 7-minute inhalations of 3%, 4%, and 5% hypertonic saline as described elsewhere [18]. Sputum was separated from saliva, treated with 0.1% dithiothreitol, and processed as described elsewhere [19].

Immunofluorescent staining

A commercial blood DC enumeration kit was used to phenotype circulating mDC2s (Miltenyi Biotec, Auburn, CA, USA). A cocktail of monoclonal antibodies including APC-BDCA-3, PE-BDCA-1, FITC-BDCA-2, and PE-Cy5-CD14-CD19, as well as a corresponding cocktail of isotype controls, were used to identify mDC2s, mDC1s and pDCs. Staining was performed according to kit instructions. To phenotype sputum mDC2s, the following monoclonal antibodies were used: EFLUORO450-CD45, FITC-Lin1, APC-h7-HLADR, APC-CD11c and PE-BDCA-3, as well as an isotype control for BDCA-3. Staining was performed according to a previous method we developed for sputum DCs [20].

Flow cytometry acquisition

Cells were acquired with a 15-color LSR II flow cytometer equipped with 3 lasers (BD Instrument Systems, Mississauga, ON, Canada) using the FACSDiva software program (BD Biosciences, Mississauga, ON, Canada). For circulating mDC2s, mDC1s and pDCs, 6 parameters were acquired: linear forward angle light scatter, linear side angle light scatter, log APC, log PE, log FITC and log PE-Cy5. Approximately 1 million cells were collected on flow cytometric acquisition to obtain a sufficient number of circulating mDC2s. For sputum mDC2s, 7 parameters were acquired: linear forward angle light scatter, linear side angle light scatter, log EFLUORO450, log FITC, log APC-h7, log APC and log PE. According to previous experiments with DCs in sputum [20], approximately 1×10^4 to 2×10^4 mononuclear cells were collected on flow cytometric acquisition to obtain a sufficient amount of sputum mDC2s.

Enumeration and analysis of DCs

Circulating mDC2s were enumerated according to kit instructions using FlowJo software (Tree Star Inc, Ashland, OR, USA). An initial gate was set up to capture leukocytes, excluding debris and platelets on the FSC vs SSC plot. Next, a subsequent gate was made to exclude B-cells, monocytes, granulocytes and dead cells on the SSC vs CD14-CD19 plot. In this plot, DCs are known to be found in the bottom left region, with a SSC value between lymphocytes and granulocytes. Finally, individual gates were created to enumerate mDC2s (BDCA-3+), as well as mDC1s (BDCA-1+) and pDCs (BDCA-2+) (Figure 1). Sputum mDC2s were enumerated based upon previous flow cytometric analysis on sputum DCs [20] also using FlowJo software. An initial mononuclear gate was set up to capture mononuclear cells and exclude debris on the CD45 vs. SSC plot. Another gate was then made on the Lin1 vs. HLADR plot to exclude a variety of white blood cells, including macrophages, B-lymphocytes and T-lymphocytes. These cells were then transferred to a CD11c vs. HLADR plot to identify mDCs. Finally, mDC2s were identified on the BDCA-3 vs. HLADR plot (Figure 2).

Circulating and sputum mDC2 counts

After analysis, total mDC2 numbers were calculated. From the flow cytometric acquisition, mDC2s were expressed as a percentage of the parent population gated. For circulating mDC2s, this fraction was multiplied by the white blood cell count, yielding the total number of circulating mDC2s per mL of blood. For sputum mDC2s, this fraction was multiplied by the number of sputum mononuclear cells, which was

calculated by using the absolute and differential cell counts of the sputum sample. The result is the total number of sputum mDC2s per gram of sputum.

ELISA of sputum supernatant

Quantikine ELISA kits (R&D Systems Inc., Minneapolis, MN, USA) were used to measure human BDCA-3 (thrombomodulin). Assays were run neat, according to kit instructions.

Statistical analysis

All statistics are expressed as means ± SEMs (with the exception of the PC_{20} data, which is expressed as geometric means ± geometric SEMs). Statistical analyses were performed by using Prism Software (GraphPad Software Inc., La Jolla, CA, USA). Using a 2-way repeated measures ANOVA, *post hoc* tests compared allergen versus diluent values at various time points. Significance was accepted at $p < 0.05$.

RESULTS

Airway physiology

All subjects demonstrated a dual response to allergen inhalation. Subjects had a mean maximum baseline fall in FEV_1 of 39.2% ± 1.4% during the early response ($p<0.05$), followed by a 23.6% ± 1.5% fall during the late response ($p<0.05$) (Table 1). No significant change in FEV_1 occurred during the diluent challenge.

Sputum inflammatory cells

Allergen challenge significantly increased the number of sputum eosinophils at 7 hours post challenge compared with diluent ($p<0.05$) (Table 2). In contrast, the number of mononuclear cells (monocytes and lymphocytes) and neutrophils were not significantly different between allergen and diluent at any time point (Table 2).

Allergen-induced changes in circulating and sputum mDC2s

Allergen inhalation significantly increased the number of sputum mDC2s at 24 hours after allergen inhalation. When compared to diluent at 24hrs, sputum mDC2s were significantly increased following allergen to 0.036 ± 0.01 $x10^6$ cells/g sputum post allergen compared to 0.013 ± 0.005 $x10^6$ cells/g sputum post diluent ($p<0.05$) (Figure 3A). This was also true for the percentage of sputum mDC2s (data not shown). Allergen challenge also increased the expression of BDCA-3 on mDCs at 7 hours and 24 hours; however, these levels were not significantly from diluent (Figure 4A). With respect to peripheral blood, allergen challenge did not affect the number or percentage of circulating mDC2s, with no differences observed between allergen and diluent at any

time point (Figure 3B). This was also true for the numbers and percentages of circulating mDC1s and pDCs (data not shown).

Allergen-induced changes in sputum BDCA-3 (thrombomodulin) levels

Sputum levels of BDCA-3 were not significantly different between allergen and diluent at any time point (Figure 4B).

DISCUSSION

This study has demonstrated that sputum mDC2s increase after allergen inhalation in subjects with allergic asthma. mDC2s were significantly increased at 24 hours after allergen challenge compared with diluent challenge. The expression levels of BDCA-3 on sputum mDCs were also increased at 7 hours and 24 hours after allergen challenge; however, these levels were not significantly different from diluent. Circulating mDC2s were not different between allergen and diluent.

Previous studies in humans have demonstrated that mDCs increase in the airways in subjects with allergic asthma [14, 20-22]. Work done by our group showed that mDCs significantly increase in the sputum of allergic asthmatics at 24 hours after allergen challenge compared with diluent [20]. In the current study, we found a similar result with respect to mDC2s. Assuming that mDCs are comprised of mDC1s and mDC2s, when comparing the number of mDC2s at 24 hours post allergen in this study, with the corresponding number of mDCs in our previous study [20], it is evident that mDC2s represent a significant proportion of mDCs that migrate into the airways, and mDC2s are found in relatively comparable numbers with mDC1s in the airways after challenge. Although mDC2s represent a small minority of DCs in circulation, this preferential localization towards the lung is supported by other studies comparing mDC1s, mDC2s and pDCs in human tissue and BALF [12,13].

The expression of BDCA-3 on mDCs was increased, albeit non-significantly, after allergen challenge. The only other study to evaluate the *in vivo* effects of allergen

exposure on mDC2s also found a similar increase in BDCA-3 on airway DCs [22]; however, neither the numbers nor the percentages of mDC2s were reported in that study. BDCA-3 can be released from the cell surface and measured in biological fluids. Sputum levels of BDCA-3 increased 24 hours after allergen challenge, but the variability in BDCA-3 levels precluded any statistically significant finding. Taken together, these findings suggest mDC2s to be an important subpopulation of mDCs that mediates the late asthmatic response and allergen-induced airway inflammation.

There was no change in circulating mDC2s after allergen challenge. This is in contrast to our previous work that demonstrated an efflux of circulating mDCs from the blood during the asthmatic response [23-25]. Farrell and colleagues [25] demonstrated that circulating mDCs were significantly lower at 6 hours and 24 hours after allergen compared with diluent. It is possible that circulating mDC2s follow a different kinetic pattern in response to allergen exposure when compared to mDC1s; however, the increase in sputum mDC2s we observed does argue against this altered migration. Furthermore, mDC2s represent such a small population of DCs in the blood, that it may difficult to detect subtle changes in their frequency.

A relationship between allergy to HDM and mDC2s was suggested by Yerkovich and colleagues [9], who demonstrated *in vitro* HDM exposure up-regulates BDCA-3 on DCs. This group has shown that mDC2s were more prevalent in the peripheral blood of subjects with allergy and asthma compared to healthy individuals [9]. By contrast, we have recently demonstrated that circulating mDC2s are lower in atopic and asthmatic

subjects compared to control subjects [26]. It is plausible that the reported increase in circulating mDC2s is a result of the perennial exposure to HDM, which is a protease allergen, and known to induce differential changes in DCs, and condition DCs to induce a Th2 polarized response [27,28]. Yerkovich and coworkers suggested that the protease activity of HDM allergens may up regulate BDCA-3 [9], as it is a natural receptor for thrombin, a serine protease. In the current study, *in vivo* allergen exposure increased the number of sputum mDC2, and the expression of BDCA-3 on mDC2s. The majority of these subjects were challenged with allergens other than HDM (Table 1B) and therefore, the responsiveness of mDC2s is not restricted to HDM allergen alone.

Traditionally, BDCA-3 (thrombomodulin) is associated with the anticoagulation cascade. Initially found on endothelial cells, it binds with thrombin to regulate hemostasis. With respect to immune function, the role of BDCA-3 is less well understood. Although mDC1s and mDC2s share many characteristics, mDC2s do have a unique ability to cross-present necrotic viral cell antigens to CD8 T-cells [29]. In mice, BDCA-3 appears to have an anti-inflammatory role [30]. In humans however, mDC2s are associated with Th2 responses [9], and are likely to have a similar role in regulating airway inflammation as mDC1s after allergen challenge. It is evident that much more research on mDC2s will be necessary to understand its exact role in humans, particularly in the context of allergic disease.

In summary, this study demonstrates, for the first time, that mDC2s are increased in the sputum of subjects with asthma after allergen inhalation. The increase in mDC2s into the

airways during the asthmatic response suggests these cells are important in the induction and regulation of allergen-induced airway inflammation.

ACKNOWLEDGEMENTS

The authors would like to thank Heather Campbell and George Obminski for their help with the allergen and methacholine challenges.

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Table 1. Subject characteristics.

1A. Circulating mDC2s.

Sex	**Age (years)**	**% Predicted FEV_1**	**$MchPC_{20}$ (mg/ml)**	**Ag Inhaled**	**% Fall in FEV_1 (EAR & LAR)**	
F	22	98.29	1.04	Cat	40	17
F	20	105.97	4.63	Ragweed	35	29
F	24	91.59	9.19	HDM DP	54	25
M	20	93.11	12.06	Grass	44	22
F	51	88.15	1.28	HDM DP	43	33
M	41	73.49	1.18	Grass	40	23
F	20	95.08	0.14	Cat	39	18
M	61	88.51	12.48	Cat	33	27
M	26	92.67	14.28	HDM DP	38	15
M	51	92.16	7.75	HDM DP	33	35
F	41	98.82	0.83	Ragweed	42	16
M	19	70.85	0.11	Cat	28	27
#M:F	**Mean:**	**Mean:**	**Mean:**		**Mean:**	**Mean:**
6:6	**33.0 ± 4.4**	**90.72 ± 2.87**	**1.32 ± 0.51**		**39.1 ± 1.9**	**23.9 ± 1.9**

1B. Sputum mDC2s.

Sex	**Age (years)**	**% Predicted FEV_1**	**MchPC_{20} (mg/ml)**	**Ag Inhaled**	**% Fall in FEV_1 (EAR & LAR)**	
F	20	102.11	9.05	Horse	49	28
M	19	80.65	0.31	Cat	35	30
F	24	90.03	5.24	Cat	43	16
M	51	92.16	7.75	HDM DP	33	35
F	19	80.25	5.8	Ragweed	29	23
M	41	98.82	0.83	Ragweed	42	16
M	27	98.51	2.71	Ragweed	42	16
M	44	79.16	32	Grass	41	22
#M:F	**Mean:**	**Mean:**	**Mean:**		**Mean:**	**Mean:**
4:4	**30.6 ± 4.5**	**90.21 ± 3.28**	**3.77 ± 0.55**		**39.2 ± 2.3**	**23.2 ± 2.6**

FEV_1 Forced expired volume in 1 second

Mch Methacholine

PC_{20} Provocative concentration causing a 20% fall in FEV_1

Ag Allergen

HDM House dust mite

EAR Early asthmatic response

LAR Late asthmatic response

Data are presented as means ± SEMs. (PC_{20} is expressed as geometric means ± geometric SEMs).

Table 2. Inflammatory cells in sputum.

	Eosinophils (10^6 cells/g sputum)		**Neutrophils** (10^6 cells/g sputum)		**Mononuclear Cells** (10^6 cells/g sputum)	
Time (hours)	**Diluent**	**Allergen**	**Diluent**	**Allergen**	**Diluent**	**Allergen**
0	0.09 ± 0.05	0.16 ± 0.10	1.63 ± 0.58	0.65 ± 0.23	2.87 ± 0.60	2.49 ± 0.60
7	0.10 ± 0.05	1.82 ± 1.01*	3.22 ± 1.27	4.07 ± 1.88	1.92 ± 0.45	2.23 ± 0.39
24	0.12 ± 0.04	0.49 ± 0.07	4.94 ± 1.04	3.65 ± 1.41	1.56 ± 0.36	2.07 ± 0.73

*Difference between diluent and allergen measurements at $p < 0.05$.

Data are presented as means ± SEMs.

FIGURE LEGENDS

Figure 1. Flow cytometric gating strategy for phenotyping of circulating DCs. An initial leukocyte gate was set up around all leukocytes and then transferred to a SSC vs CD14-CD19 plot to exclude various cell populations. Individual gates were then created to identify mDC2s, mDC1s and pDCs.

Figure 2. Flow cytometric gating strategy for phenotyping of sputum mDC2s. A monoculear gate was initially set up to capture mononuclear cells (R1), and then transferred to a Lin1 vs HLADR plot to exclude various cell populations (R2). Gates were then set to identify mDCs (R3) and mDC2s (R4).

Figure 3. (A) Kinetics of sputum mDC2s after inhalation of diluent versus allergen. (**B)** Kinetics of circulating mDC2s after inhalation of diluent versus allergen. *Difference between baseline and 24 hours post allergen measurements at $p < 0.05$. **Difference between diluent and allergen measurements at 24hrs at $p < 0.05$. Data are presented as means ± SEMs.

Figure 4. (A) Kinetics of BDCA-3 expression on sputum mDCs after inhalation of diluent versus allergen. (**B)** Kinetics of sputum supernatant levels of BDCA-3 (thrombomodulin) after inhalation of diluent versus allergen. Data are presented as means ± SEMs.

Figure 1.

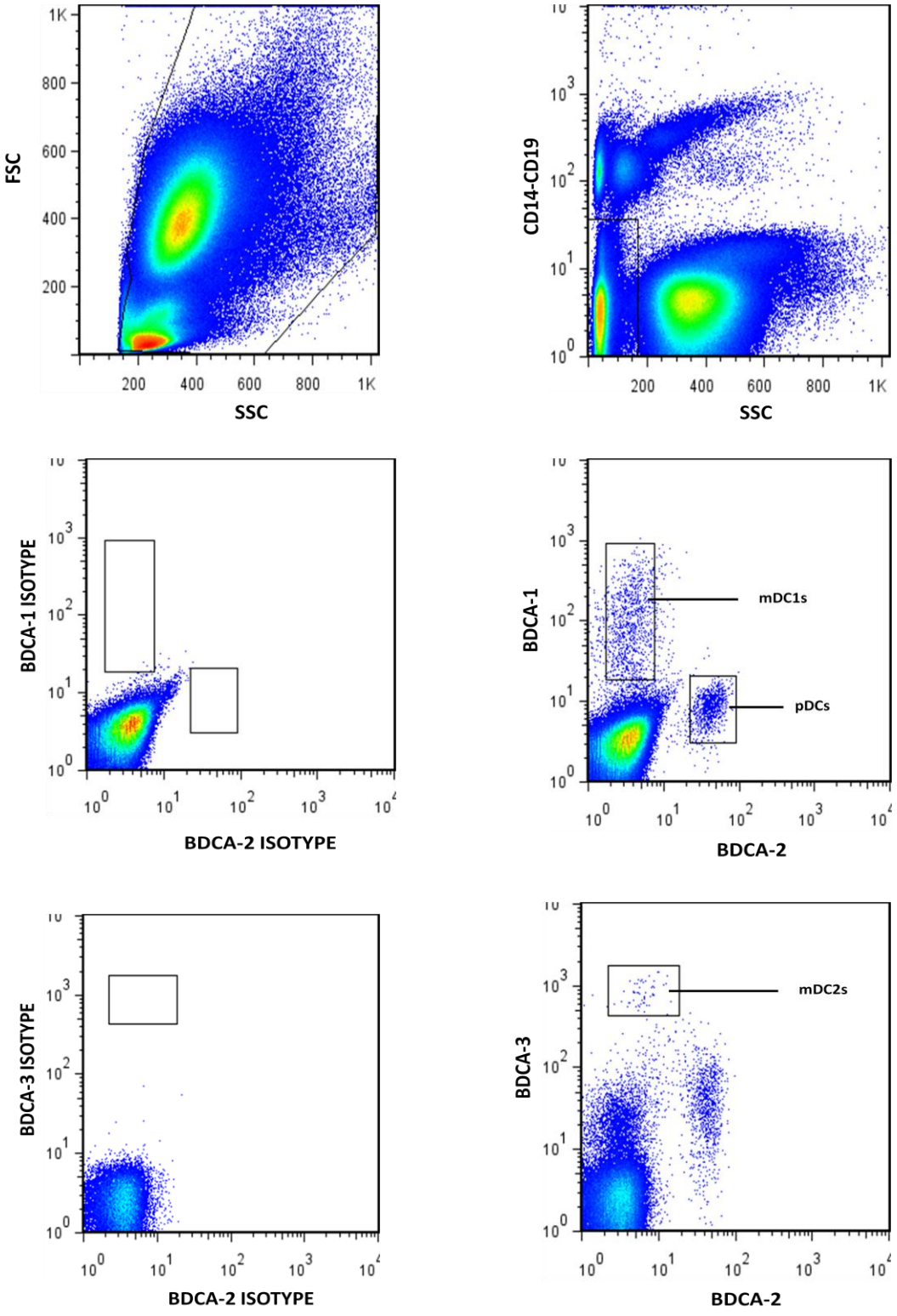

FSC
SSC
CD14-CD19
BDCA-1 ISOTYPE
BDCA-2 ISOTYPE
BDCA-1
mDC1s
pDCs
BDCA-2
BDCA-3 ISOTYPE
BDCA-2 ISOTYPE
BDCA-3
mDC2s
BDCA-2

Figure 2.

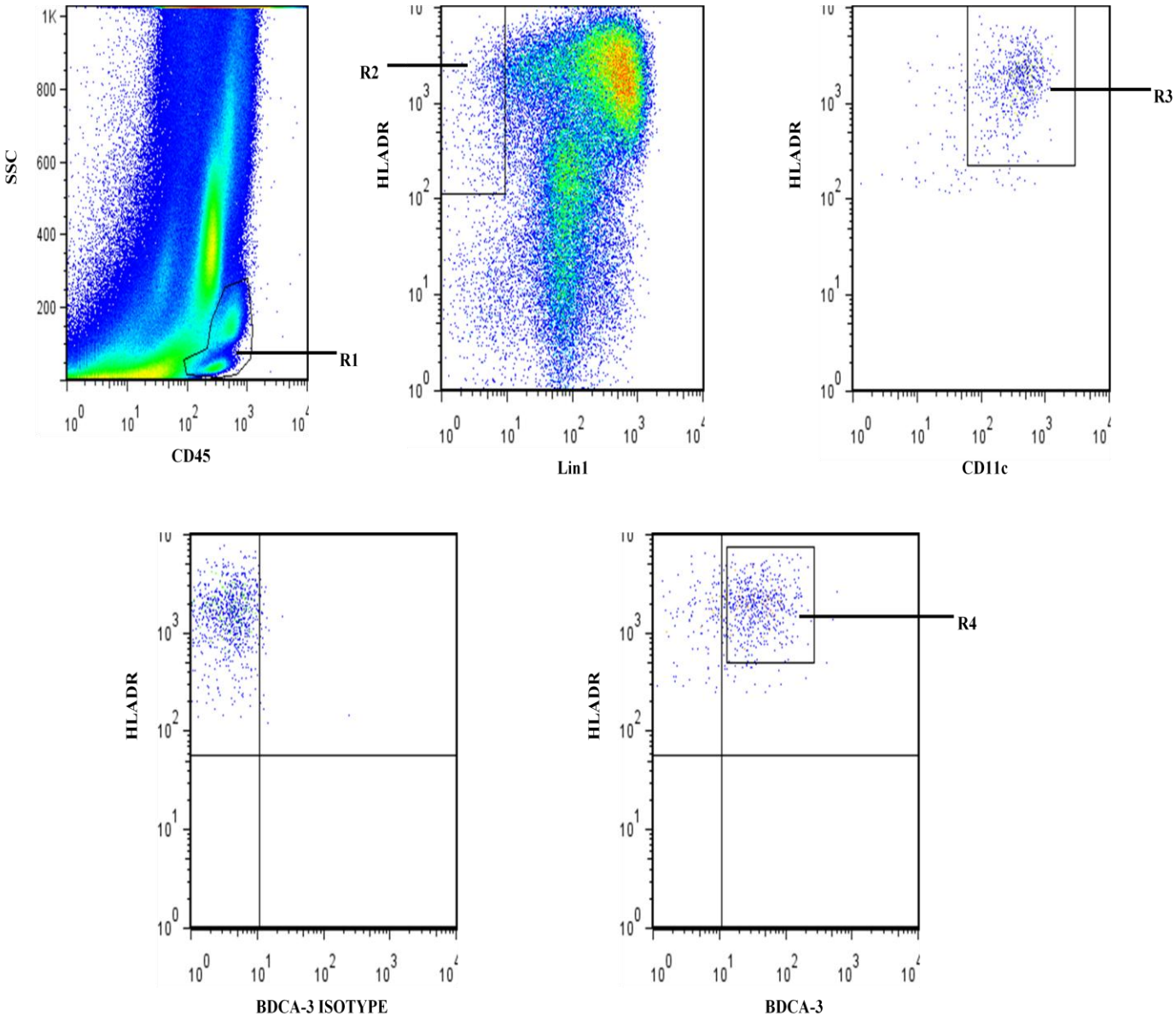
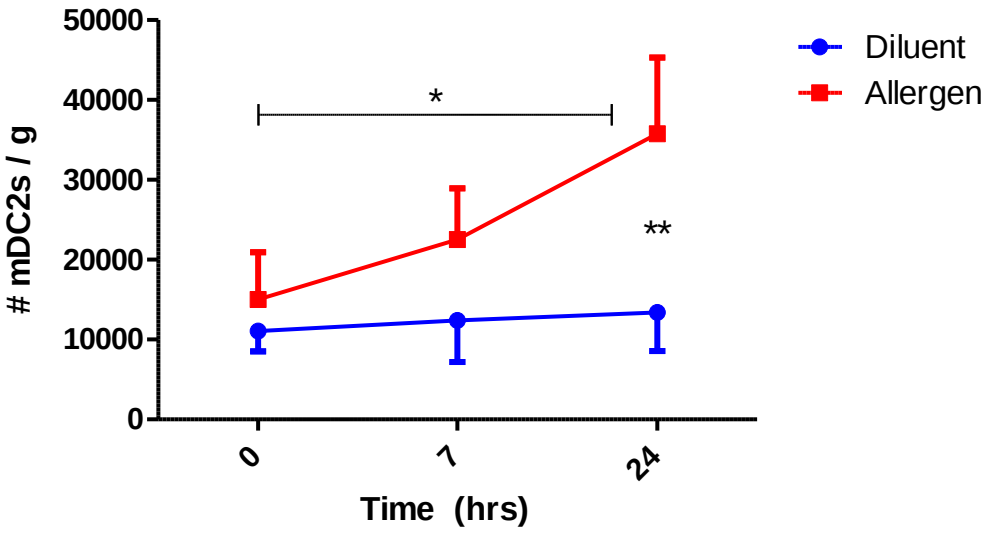

SSC
CD45
R1
HLADR
Lin1
R2
HLADR
CD11c
R3
HLADR
BDCA-3 ISOTYPE
HLADR
BDCA-3
R4

Figure 3.

A


50000
40000
30000
20000
10000
0
mDC2s / g
*
**
0
7
24
Time (hrs)
Diluent
Allergen

B

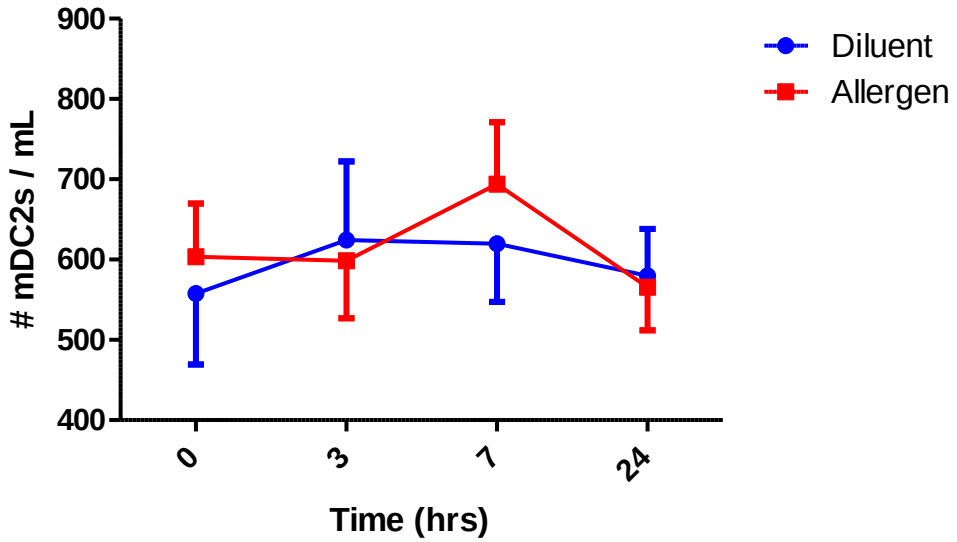
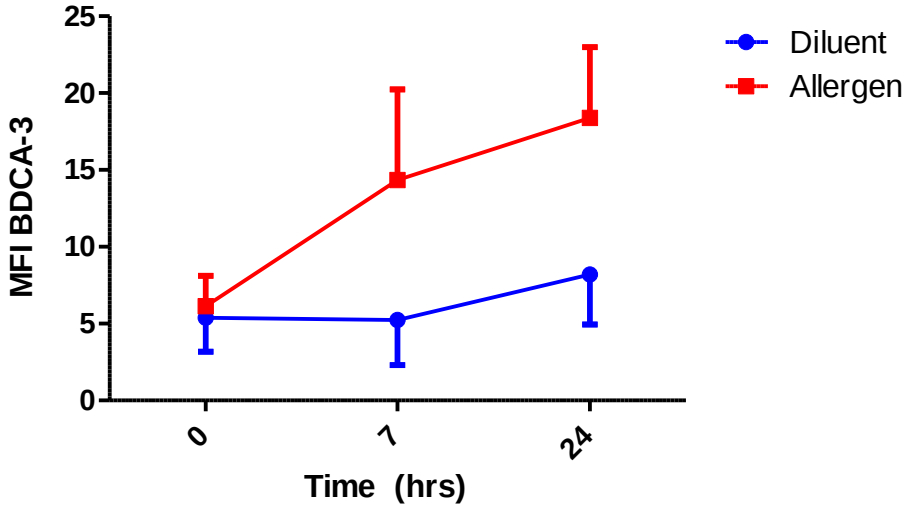
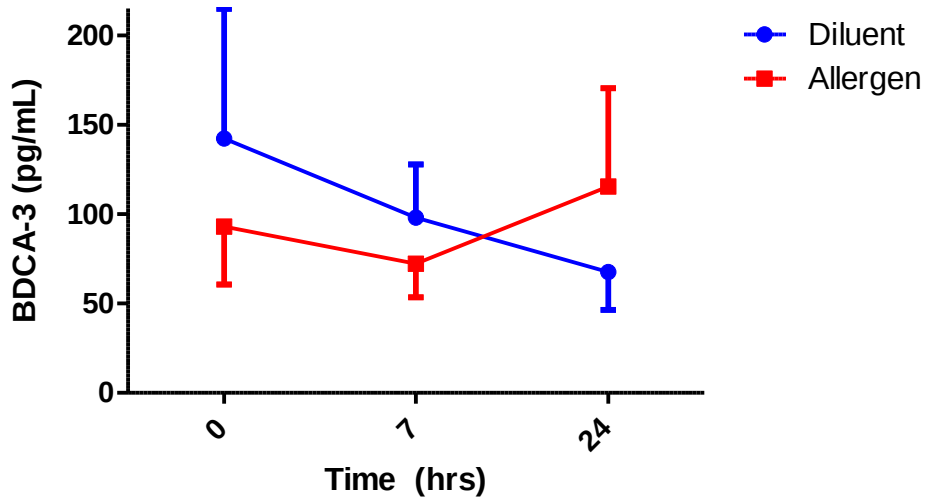

900
800
700
600
500
400
mDC2s / mL
0
3
7
24
Time (hrs)
Diluent
Allergen

Figure 4.

A


25
20
15
10
5
0
MFI BDCA-3
0
7
24
Time (hrs)
Diluent
Allergen

B


200
150
100
50
0
BDCA-3 (pg/mL)
0
7
24
Time (hrs)
Diluent
Allergen

CHAPTER 5

The effects of anti-OX40L and anti-TSLP monoclonal antibodies on circulating dendritic cells

This manuscript has not yet been submitted.

Since DCs are important in the initiation and promotion of immune responses to inhaled allergen, molecules that interfere with DC function have been developed as possible therapeutic treatments for allergic asthma. Animal models of asthma have established the therapeutic potential of drugs that disrupt TSLP and OX40L signaling. In mice treated with antibodies that block either TSLP or OX40L signaling, the anti-inflammatory effects of these drugs were partly mediated through their antagonizing effects on DCs (Seshasayee et al., 2007; Shi et al., 2008). As part of two large clinical trials, we explored the potential of pharmacological therapies, anti-OX40L MAb and anti-TSLP MAb, to affect circulating mDC1s, mDC2s and pDCs in subjects with mild asthma. We find that treatment with anti-OX40L MAb or anti-TSLP MAb had no effect on circulating DC subsets.

THE EFFECTS OF ANTI-OX40L AND ANTI-TSLP MONOCLONAL ANTIBODIES ON CIRCULATING DENDRITIC CELLS

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Running head: Anti-OX40L and anti-TSLP MAbs on DCs

Word count: 2274

ABSTRACT

Background: Dendritic cells (DCs) initiate and maintain immune responses to inhaled allergen. Several new molecules capable of regulating DC function have been identified, and these might represent new therapeutic targets for the treatment of allergic asthma.

Objective: To investigate changes in circulating DC subsets after treatment with anti-OX40L and anti-TSLP monoclonal antibodies (MAbs) in subjects with asthma.

Methods: Two separate double-blind, randomized, placebo-controlled, parallel-group, clinical trials were conducted to evaluate the efficacy of humanized anti-OX40L and anti-TSLP MAbs in subjects with allergic asthma. Allergen challenges were carried out 56 and 113 days after the first dose of anti-OX40L MAb, and 42 and 84 days after the first dose of anti-TSLP MAb. Myeloid DCs type 1 (mDC1s), myeloid DCs type 2 (mDC2s), and plasmacytoid DCs (pDCs) were measured in blood before and after allergen challenge during the treatment period.

Results: Compared to placebo, treatment with anti-OX40L MAb did not attenuate the early or late phase asthmatic responses at Days 56 or 113 compared to placebo, or affect the circulating numbers of mDC1s, mDC2s or pDCs. Treatment with anti-TSLP MAb reduced both allergen-induced early and late asthmatic responses when compared to placebo. Anti-TSLP MAb did not affect circulating numbers of mDC1s, mDC2s or pDCs compared to placebo.

Conclusions: Although anti-OX40L and anti-TSLP MAbs exhibited pharmacological activity, there was no effect on circulating DCs. It is possible that measuring DCs in the

blood was insufficient to detect any significant effects of anti-OX40L MAb and anti-TSLP MAb on these cells.

KEY WORDS

allergic asthma, antigen presenting cells, DCs, OX40L, TSLP

ABBREVIATIONS

APCs	antigen presenting cells
BDCA	blood dendritic cell antigen
DCs	dendritic cells
huMAb	human monoclonal antibody
MAb	monoclonal antibody
MDC	macrophage derived chemokine
mDC1s	myeloid dendritic cells type 1
mDC2s	myeloid dendritic cells type 2
MLN	mediastinal lymph nodes
OX40L	OX40 ligand
pDCs	plasmacytoid dendritic cells
TARC	thymus and activation regulated chemokine
Th	T-helper
TLR	toll-like receptor
TSLP	thymic stromal lymphopoietin

INTRODUCTION

Dendritic cells (DCs) are crucial in mounting and maintaining immune responses to inhaled allergen, and interfering with their function could represent a novel form of treatment for allergic asthma. Inhaled steroids are currently the cornerstone of anti-inflammatory treatment in asthma, and in spite of their efficacy, may cause local adverse reactions and have the potential for systemic side effects. Recently, several new molecules capable of regulating DC function have been identified, and these might represent new therapeutic targets for the treatment of allergic asthma.

Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine that is produced primarily by epithelial cells in response to inflammation. The TSLP receptor complex is heterodimeric, consisting of a TSLP-binding chain, referred to as TSLPR, and the IL-7Rα chain (Pandey et al., 2000; Park et al., 2000; Reche et al., 2001). Human mDCs express both TSLPR and IL-7Rα, and are found to express the highest levels of both transcript and protein TSLPR among various hematopoietic cell lineages (Wang & Liu, 2009). In response to TSLP, mDCs undergo maturation by upregulating the expression of HLA-DR, CD80, CD83 and CD86 (Reche et al., 2001). Unlike other common DC-activation signals, TSLP does not stimulate the production of Th1-polarizing cytokines; mDCs produce eotaxin-2, IL-8, and the Th2 cell-attracting chemokines TARC and MDC (Liu, 2006). Furthermore, TLSP-treated mDCs preferentially prime naive CD4+ T-cells to differentiate into Th2 cells, capable of secreting IL-4, IL-5 and IL-13 (Soumelis et al., 2002). TSLP-treated mDCs also induce the expansion and further polarization of Th2

memory cells (Wang et al., 2006), and TSLP can directly impair Treg function (Nguyen et al., 2010).

OX40 (CD134) is a co-stimulatory receptor transiently expressed on activated T cells (Croft, 2003; Kaur & Brightling, 2012); its cognate ligand, OX40L, is mainly expressed by APCs, including activated DCs (Chen et al., 1999; Ohshima et al., 1998). OX40L is induced on DCs following stimulation with TSLP, CD40L and TLR ligands (Sugamura et al., 2004). OX40-OX40L interaction is crucial for T-cell activation and survival (Chen et al., 1999; Gramaglia et al., 1998), and is involved in the maintenance and reactivation of memory T-cells (Seshasayee et al., 2007; Wang & Liu, 2007). OX40-OX40L signalling can also suppress Treg formation and function (Ito et al., 2006).

In an attempt to identify the molecular mechanism by which TSLP-activated mDCs induce and maintain Th2 responses, OX40L was identified as a key molecule expressed by these DCs (Ito et al., 2005). *In vivo* blockade of OX40L significantly inhibits TSLP-driven Th2 allergic inflammation (Seshasayee et al., 2007), and OX40L expressed by TSLP-activated DCs is important for the expansion of Th2 memory cells (Wang et al., 2006). These studies suggest that OX40L functions to induce Th2 cells and maintain Th2 memory cells during TSLP-DC-mediated allergic responses.

In mouse models of allergic asthma, OX40L and TSLP are important molecules in the development of inflammatory responses. In response to allergen, mice lacking OX40 or OX40L exhibited significantly decreased inflammatory responses in the lung, including reduced eosinophilia, airway hyperreactivity, mucus secretion, serum IgE and Th2

cytokine levels (Arestides et al., 2002; Hoshino et al., 2003; Jember et al., 2001; Salek-Ardakani et al., 2003). Anti-OX40L antibodies were also shown to inhibit Th2 inflammation (Seshasayee et al., 2007). Furthermore, over-expression of TSLP in the lungs of mice induced spontaneous Th2 inflammatory responses in the airways (Zhou et al., 2005), while mice lacking TSLPR failed to develop airway inflammation after allergen challenge (Al-Shami et al., 2005; Zhou et al., 2005). Blockade of TSLP was also shown to alleviate allergic airway inflammation (Al-Shami et al., 2005; Shi et al., 2008). In the current study, we describe the effects of 2 human monoclonal antibodies (MAbs) on circulating DCs; an anti-OX40L antibody that blocks OX40-0X40L signaling and an anti-TSLP antibody that blocks TSLP signaling through its TSLP receptor complex. The pharmacological activities of these antibodies were evaluated in two separate clinical trials in subjects with mild allergic asthma using a model of allergen inhalation. We hypothesized that circulating mDC1s and mDC2s are reduced in subjects treated with human MAbs against OX40L and TSLP.

METHODS

Anti-OX40L huMAb trial

Study design

The study design for this trial is described by Gauvreau and colleagues (2013). Briefly, 28 mild allergic subjects with asthma were recruited for a phase II, multi-center, double-blind, placebo-controlled, randomized, parallel-group trial to evaluate the efficacy of intravenous anti-OX40L huMAb (Table 1). Subjects received either study drug (n=14) or placebo equivalent (n=14) every 4 weeks, for a total of 4 doses. Subjects received a loading dose of 8 mg/kg intravenously on Day 1, followed by monthly 4 mg/kg doses on Days 29, 57 and 85. Allergen challenges were conducted during screening, and on Days 56 and 113 (2 and 4 months after the first dose, respectively) (Figure 1). The primary outcome was the allergen-induced late asthmatic response. Secondary outcomes included the allergen-induced early asthmatic response, airway hyperresponsiveness, serum IgE levels, and blood and sputum eosinophils.

Circulating DCs

Peripheral blood DCs were measured 24 hours before and 24 hours after allergen challenge, during both screening (Days -13 and -15) and treatment periods (Days 55, 57, 112, 114). Each time, approximately 20mL of blood was collected for exploratory cell measurements, which included measuring DCs. Blood samples collected from McMaster University were processed immediatlely after collection. Samples collected from other

sites were kept cold and shipped to McMaster University. These samples were processed within 24 hours of collection.

Anti-TSLP huMAb trial

Study design

Briefly, 30 mild allergic subjects with asthma were recruited for a phase I, multi-center, double-blind, placebo-controlled, randomized, parallel-group trial to evaluate the efficacy of intravenous anti-TSLP huMAb (Table 1). Subjects received either study drug (n=15) or placebo equivalent (n=15) every 4 weeks, for a total of 3 doses. Subjects received 700 mg intravenously on Day 1, followed by monthly infusions on Days 29 and 57. Allergen challenges were conducted during screening, and on Days 42 and 84 (Figure 2). The primary outcome was the allergen-induced late asthmatic response, as well as the safety and tolerability of anti-TSLP huMAb. Secondary outcomes included the allergen-induced early asthmatic response and the pharmacokinetic profile of anti-TSLP huMAb.

Circulating DCs

Peripheral blood DCs were measured on Day 1 pre-dosing, and 24 hours before and 24 hours after allergen challenge during the treatment period (Days 41, 43, 83, 85). Each time, approximately 50mL of blood was collected for exploratory cell measurements, which included measuring DCs. Blood samples collected from McMaster University were processed immediatlely after collection. Samples collected from other sites were

kept cold and shipped to McMaster University. These samples were processed within 24 hours of collection.

Immunofluorescent Staining of DCs

A commercial blood enumeration kit was used to phenotype circulating DCs (Miltenyi Biotech, Auburn, CA, USA). A cocktail of monoclonal antibodies including APC-BDCA-3, PE-BDCA-1, FITC-BDCA-2, and PE-Cy5-CD14-CD19, as well as a corresponding cocktail of isotype controls, were used to identify mDC1s, mDC2s and pDCs. Staining was performed according to kit instructions.

Flow Cytometry Acquisition

Cells were acquired with a 15-color LSR II flow cytometer equipped with 3 lasers (BD Instrument Systems, Mississauga, ON, Canada) using the FACSDiva software program (BD Biosciences, Mississauga, ON, Canada). Six parameters were acquired: linear forward angle light scatter, linear side angle light scatter, log PE, log FITC, log APC, and log PECy5. Approximately 1 million cells were collected on flow-cytometric acquisition to obtain a sufficient number of mDC2s.

Enumeration and Analysis of DCs

DCs were enumerated as per kit instructions using FlowJo software (Tree Star Inc, Ashland, OR, USA). An initial gate was set up to capture leukocytes, excluding debris and platelets on the FSC vs SSC plot. Next, a subsequent gate was made to exclude B-cells, monocytes, granulocytes and dead cells on the SSC vs CD14-CD19 plot. In this

plot, DCs are known to be found in the bottom left region, with a SSC value between lymphocytes and granulocytes. Finally, individual gates were created to enumerate mDC1s (BDCA-1+), mDC2s (BDCA-3+) and pDCs (BDCA-2+) (Figure 3). Since BDCA-3 is also expressed at much lower levels on other cell types, only cells with high expression were included.

Circulating DC Counts

From the flow cytometric acquisition, mDC1s, mDC2s and pDCs were expressed as a percentage of total white blood cells collected. This fraction was then multiplied by the absolute number of white blood cells per mL in the blood sample. The result is the total number of circulating DCs expressed per mL of blood.

Statistical Analysis

All statistics are expressed as means ± SEMs (unless otherwise indicted). Statistical analyses were performed by using Prism Software (GraphPad Software Inc., La Jolla, CA, USA). Using a 2-way ANOVA, *post hoc* tests compared drug versus placebo values at various time points. Significance was accepted at $p < 0.05$.

RESULTS

Anti-OX40L huMAb trial

Treatment with anti-OX40L MAb did not attenuate the allergen-induced early or late asthmatic responses on Days 56 or 113 compared to placebo (Figure 4). Total serum IgE levels on Day 113 and sputum eosinophils on Day 112 were significantly reduced in the anti-OX40L MAb group compared to the placebo group. There was no effect of anti-OX40L MAb on airway hyperresponsiveness or circulating eosinophils.

Effect of anti-OX40L MAb on circulating DCs

Circulating numbers of mDC1s, mDC2s and pDCs were similar between placebo and anti-OX40L MAb groups during screening. No significant changes were observed in circulating mDC1s, mDC2s or pDCs between placebo and anti-OX40L MAb groups during the treatment period (Figure 5).

Anti-TSLP huMAb trial

Treatment with anti-TSLP MAb reduced both allergen-induced early and late asthmatic responses when compared to placebo (Figure 6).

Effect of anti-TSLP MAb on circulating DCs

Circulating numbers of mDC1s, mDC2s and pDCs were similar between placebo and anti-TSLP MAb groups on Day 1. No significant changes were observed in circulating mDC1s, mDC2s or pDCs between placebo and anti-TSLP MAb groups during the treatment period (Figure 7).

DISCUSSION

This study evaluated the effects of 2 human MAbs on circulating DCs in 2 separate clinical trials. Treatment with anti-OX40L MAb or anti-TSLP MAb did not affect circulating numbers of mDC1s, mDC2s or pDCs compared to placebo in subjects with mild asthma.

Since DCs are crucial to the initiation and progression of immune responses to inhaled allergen, molecules that interfere with DC function have been developed as possible therapeutic treatments for allergic asthma. Animal models of allergic asthma have been instrumental in establishing the therapeutic potential of drugs that disrupt OX40L and TSLP signaling. In mouse and non-human primate models of TSLP-driven allergic inflammation, treatment with anti-OX40L MAb resulted in a significant reduction in Th2 cytokine and antigen-specific IgE levels, along with a significant attenuation of airway eosinophils, and effector and memory T-cells (Seshasayee et al., 2007). Similarly, blockade of TSLP using a TSLPR-Fc fusion protein or a TSLPR-blocking antibody in mice challenged with allergen significantly reduced the allergic inflammatory response, including airway eosinophilia, goblet cell hyperplasia and Th2 cytokine production (Al-Shami et al., 2005; Shi et al., 2008). Studies in humans have also suggested an association between asthma and both OX40L and TSLP. OX40L expression was found to be increased in the bronchial submucosa of mild asthmatics compared to normal subjects, and correlated with IL-4+ cells and eosinophils in the airway tissue (Siddiqui et al., 2010). Increased numbers of TSLP-expressing cells were also found in the epithelium and submucosa of asthmatic patients compared to normal subjects, and

correlated with disease severity and the expression of Th2-attracting chemokines (Ying et al., 2005). Moreover, genetic polymorphisms in the promoter region of the TSLP gene were associated with bronchial asthma (Harada et al., 2011). Taken together, these studies suggest a role for OX40L and TSLP in asthma.

In this study, anti-OX40L MAb did not affect circulating numbers of DCs in subjects with mild asthma. In mice treated with anti-OX40L MAb, these antibodies not only interfered with OX40-OX40L signaling but also depleted OX40L-expressing cells, including lung and MLN DCs, through antibody-dependent cell-mediated cytotoxicity (Seshasayee et al., 2007) The depletion of DCs limited DC-derived inflammatory signals, including cellular costimulation for T-cells and the production of Th2-attracting chemokines (Ito et al., 2005; Soumelis et al., 2002). The exposures levels of anti-OX40L MAb in our study were lower than that observed in animal studies (Gauvreau et al., 2013), and this may account for not only the limited effect of anti-OX40L MAb on airway responses, but also the limited effect on circulating DCs. Measurements of airway DCs and assays of DC function were not examined in this study; therefore, it is possible that anti-OX40L MAb may still modulate DC number and function.

Anti-TSLP MAb also did not affect circulating numbers of DCs in subjects with mild asthma. In mice treated with TSLPR antibodies, the anti-inflammatory effects of TSLPR blocking were achieved by inhibiting the maturation and migration of airway DCs, as well as their ability to initiate CD4+ T-cell responses (Shi et al., 2008). Disrupting TSLP signaling prevented Th2-mediated inflammation, at least in part by regulating the function of DCs (Shi et al., 2008). These effects are consistent with the effects of TSLP

on human DCs *in vitro* (Ito et al., 2005; Wang et al., 2006). Although anti-TSLP MAb had an effect on allergen-induced airway responses, it did not change circulating DC subsets. It is tempting to speculate that measuring DCs locally in the airways, including their number and maturation status, as well as examining their chemokine production and T-cell stimulatory capabilities *ex vivo*, would reveal significant effects of anti-TSLP MAb on DCs.

In summary, treatment with anti-OX40L MAb or anti-TSLP MAb had no effect on circulating DC subsets in subjects with mild asthma. Despite limitations in the collection and functional analysis of DCs, our study is the first to describe the *in vivo* effects of anti-OX40L MAb and anti-TSLP MAb on circulating DCs in humans. Continued research on the effects of anti-OX40L MAb and anti-TSLP MAb on both blood and airway DCs will be critical in understanding their therapeutic potential as novel treatments for allergic asthma.

ACKNOWLEDGEMENTS

The authors would like to thank Genetech and Amgen for supplying the MAbs, and the study coordinators at each site for sending us samples.

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Table 1. Subject characteristics.

	Anti-OX40L huMAb trial		**Anti-TSLP huMAb trial**	
Group	Placebo	Anti-OX40L MAb	Placebo	Anti-TSLP MAb
Sample Size (n)	14	14	15	15
Sex, male/female	7/7	8/6	4/11	5/10
Age	33.9 (12.0)	33.4 (13.3)	31.6 (11.5)	31.5 (11.2)
FEV_1 % predicted	84.9 (14.7)	91.7 (11.4)	108.0 (17.0)	103.3 (16.1)
Methacholine PC_{20} (mg/ml)	0.79 (0.05-13.5)	1.62 (0.3-11.6)	0.60 (0.37-29.60)	0.30 (0.02-19.60)

MAb Monoclonal antibody

FEV_1 Forced expiratory volume in 1s

PC_{20} Provocative concentration causing a 20% fall in FEV_1

OX40L OX40 ligand

TSLP thymic stromal lymphopoietin

Data are presented as means (standard deviations)

FIGURE LEGENDS

Figure 1. Study schematic for the anti-OX40L huMAb trial. (Adapted from Gauvreau et al., 2013).

Figure 2. Study schematic for the anti-TSLP huMAb trial.

Figure 3. Flow cytometric gating strategy for phenotyping of circulating DCs. An initial leukocyte gate was set up around all leukocytes and then transferred to a SSC vs CD14-CD19 plot to exclude various cell populations. Individual gates were then created to identify mDC1s, mDC2s, and pDCs.

Figure 4. Allergen-induced changes in FEV_1 during the screening period, and 56 and 113 days post-dosing with placebo and OX40L MAb. Data are presented as means ± SEMs. (Adapted from Gauvreau et al., 2013).

Figure 5. Circulating numbers of mDC1s (top panel), mDC2s (middle panel) and pDCs (bottom panel) following treatment with placebo (open bars) and anti-OX40L MAb (solid bars) measured during the treatment period before (Days 55, 112) and 24 hours after (Days 57, 114) allergen challenge. Data are presented as means ± SEMs.

Figure 6. Allergen-induced changes in FEV_1 during the screening period, and 42 and 84 days post-dosing with placebo and TSLP MAb. Data are presented as means with 95% confidence intervals.

Figure 7. Circulating numbers of mDC1s (top panel), mDC2s (middle panel) and pDCs (bottom panel) following treatment with placebo (open bars) and anti-TSLP MAb (solid bars) measured during the treatment period before (Days 41, 83) and 24 hours after (Days 43, 85) allergen challenge. Data are presented as means ± SEMs.

Figure 1.

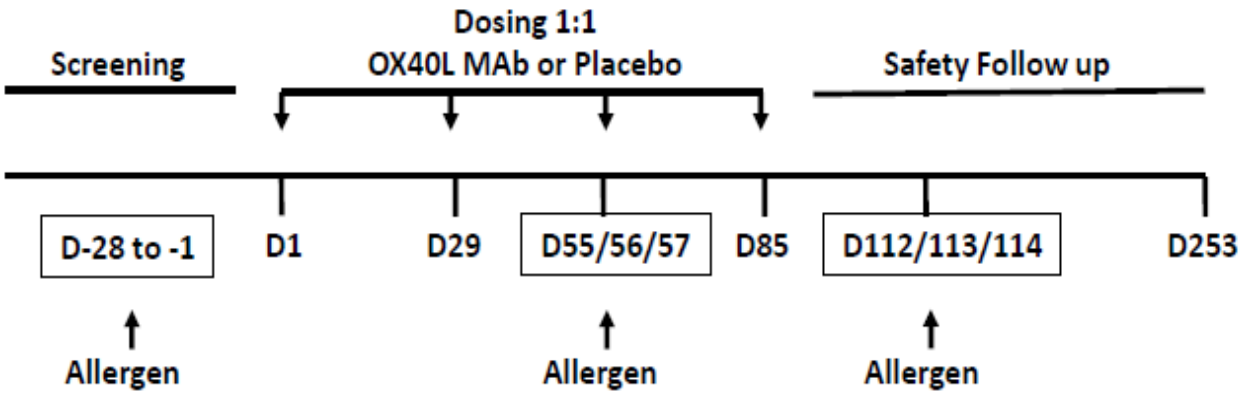

Dosing 1:1
OX40L MAb or Placebo
Screening
Safety Follow up
D-28 to -1
D1
D29
D55/56/57
D85
D112/113/114
D253
Allergen
Allergen
Allergen

Figure 2.

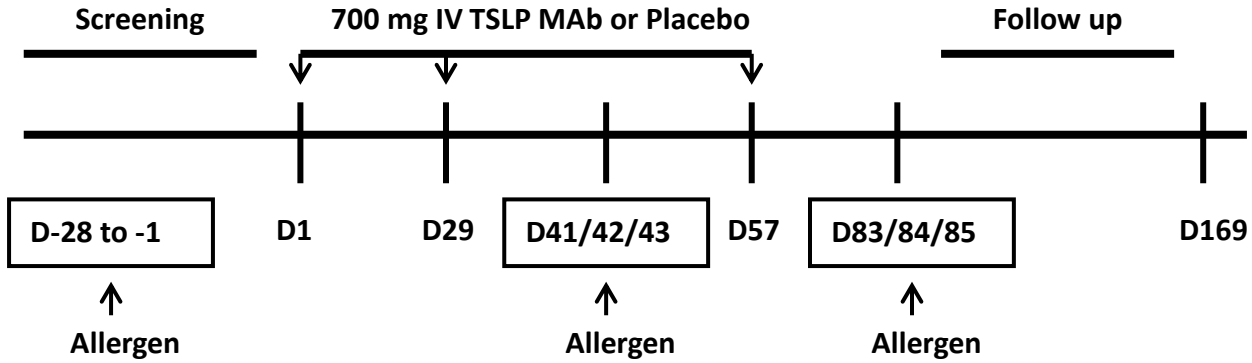

Screening
700 mg IV TSLP MAb or Placebo
Follow up
D-28 to -1
D1
D29
D41/42/43
D57
D83/84/85
D169
Allergen
Allergen
Allergen

Figure 3.

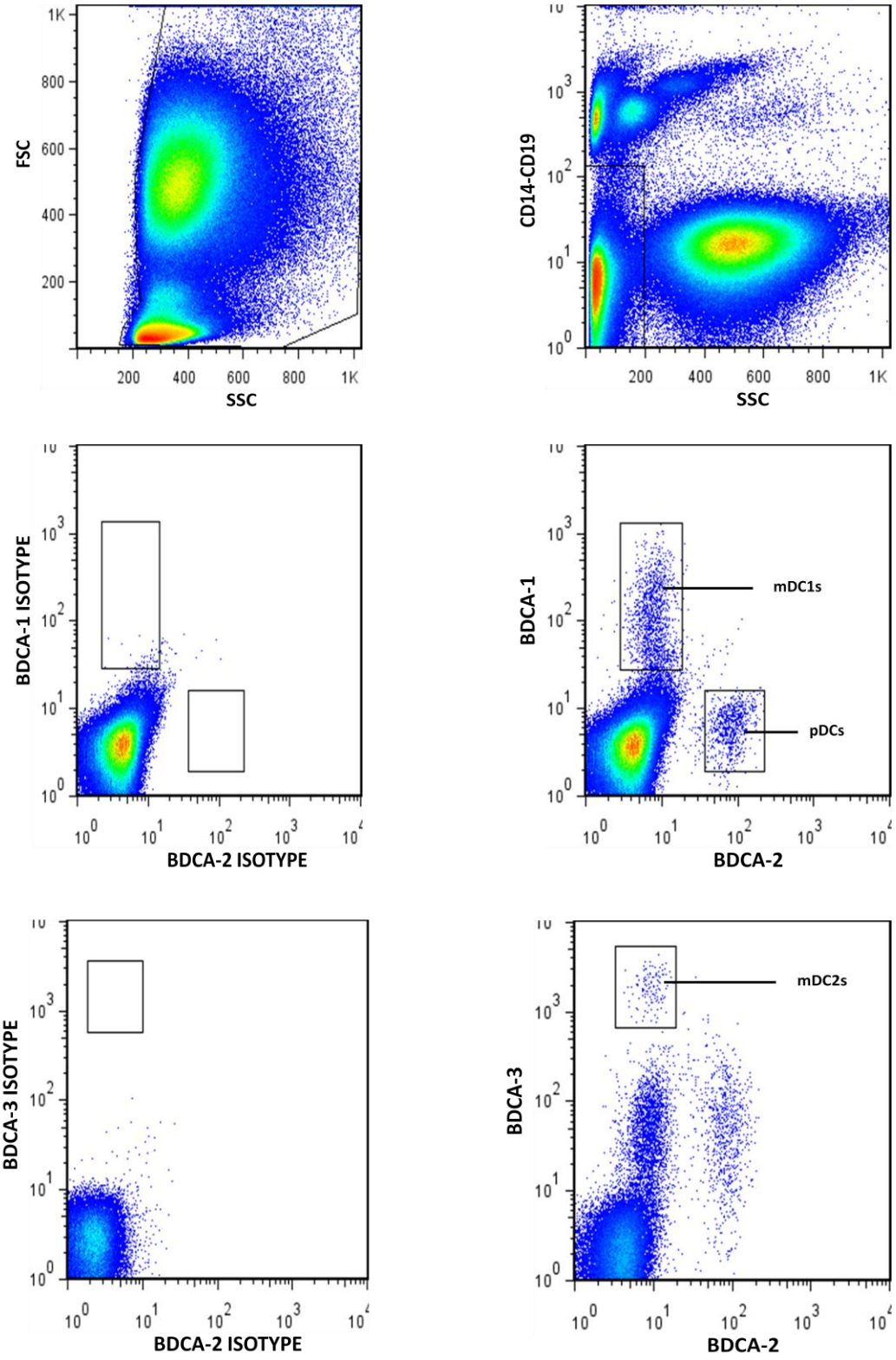

FSC
SSC
CD14-CD19
SSC
BDCA-1 ISOTYPE
BDCA-2 ISOTYPE
BDCA-1
mDC1s
pDCs
BDCA-2
BDCA-3 ISOTYPE
BDCA-2 ISOTYPE
BDCA-3
mDC2s
BDCA-2

Figure 4.

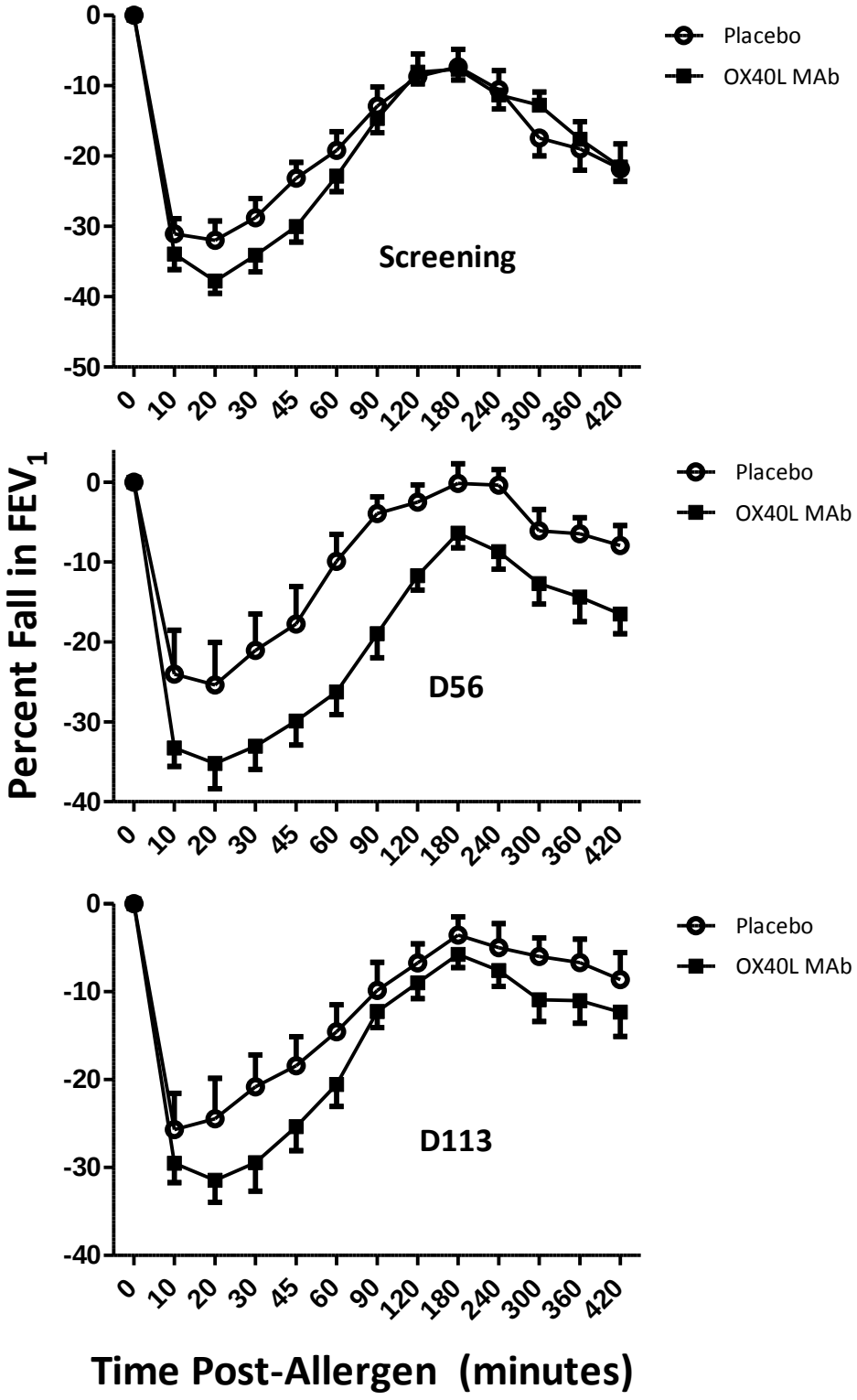

Placebo
OX40L MAb
Screening
D56
D113
Percent Fall in FEV1
Time Post-Allergen (minutes)
0
-10
-20
-30
-40
-50
0 10 20 30 45 60 90 120 180 240 300 360 420

Figure 5.

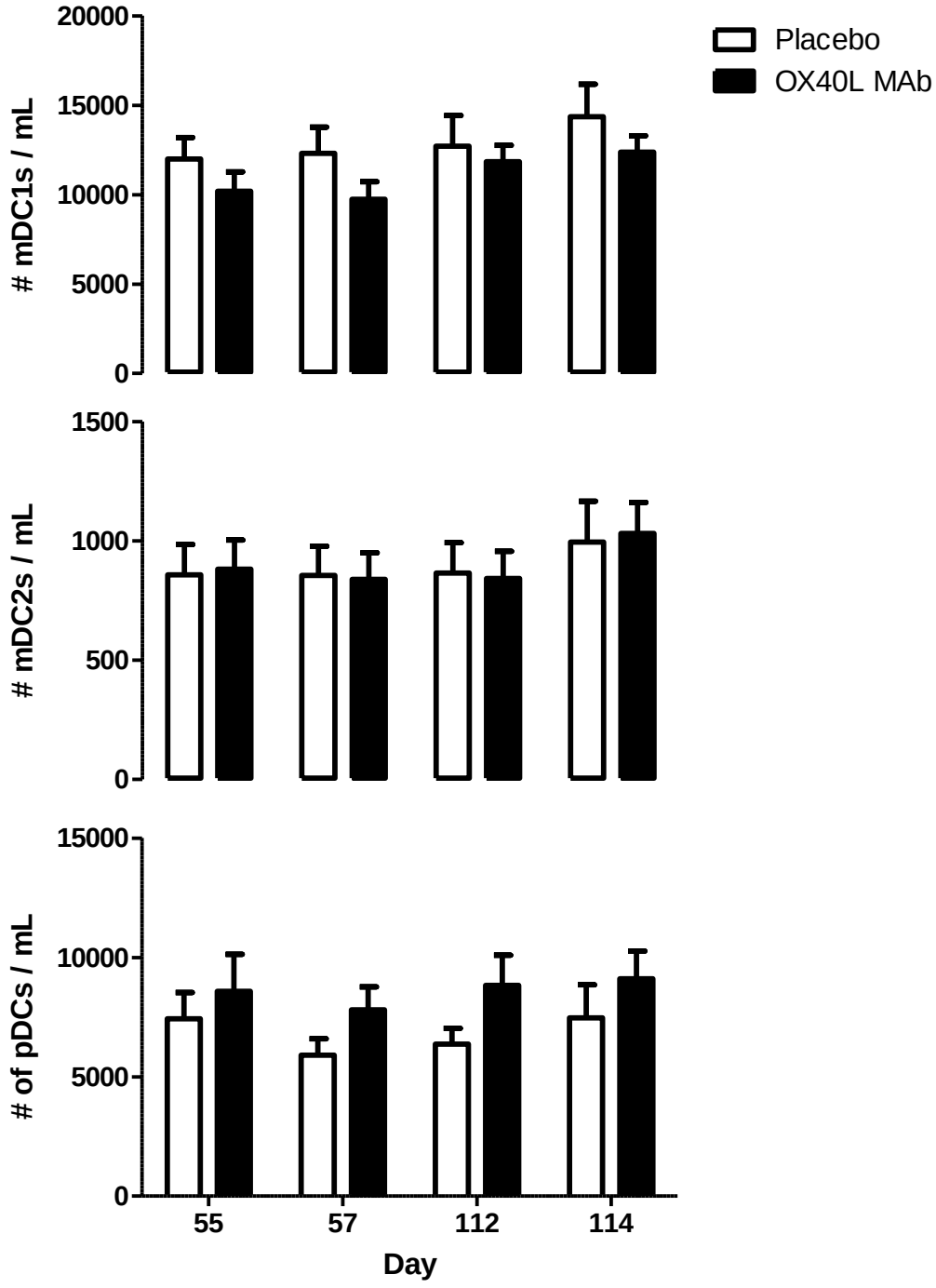

Placebo
OX40L MAb
mDC1s / mL
20000
15000
10000
5000
0
mDC2s / mL
1500
1000
500
0
# of pDCs / mL
15000
10000
5000
0
55
57
112
114
Day

Figure 6.

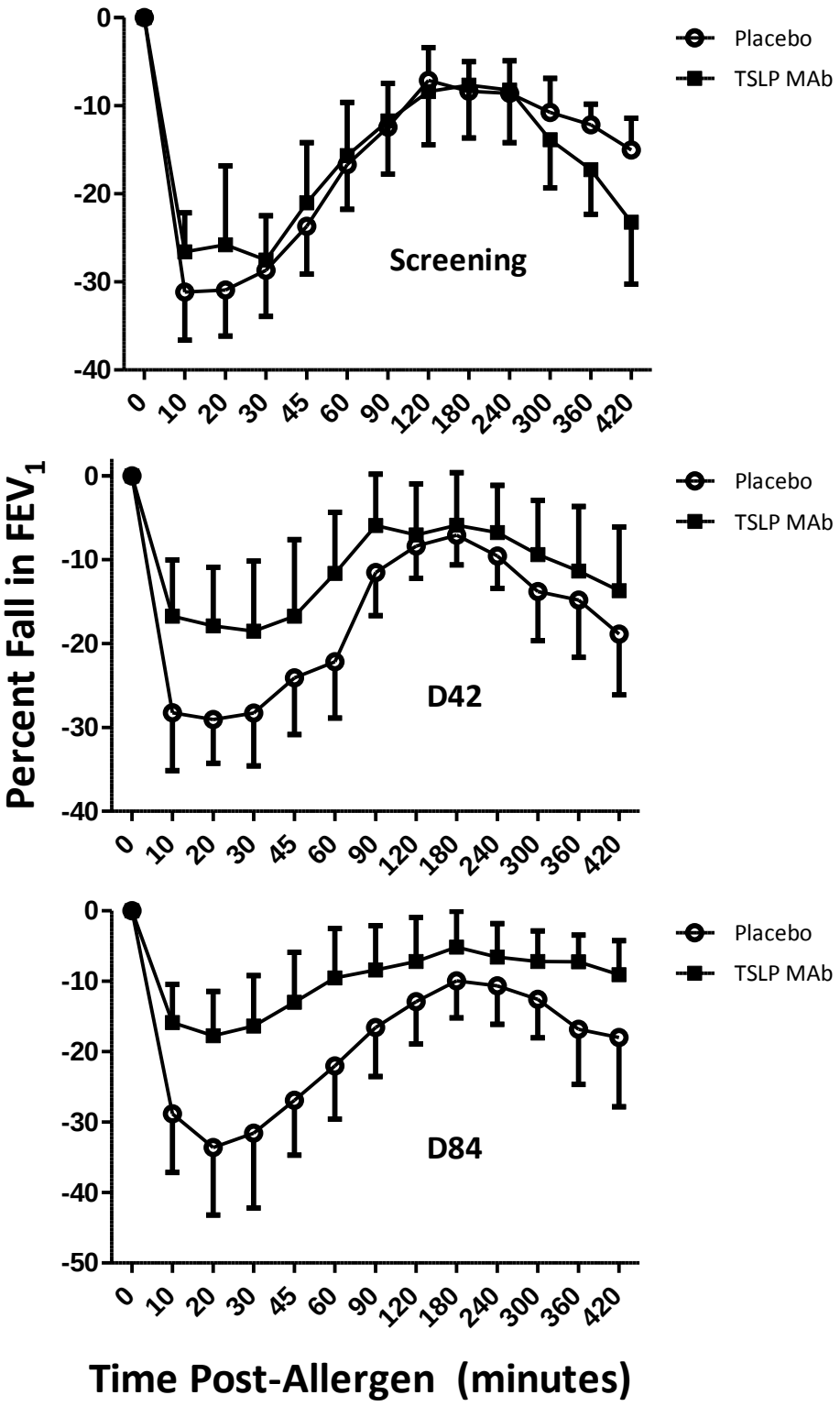
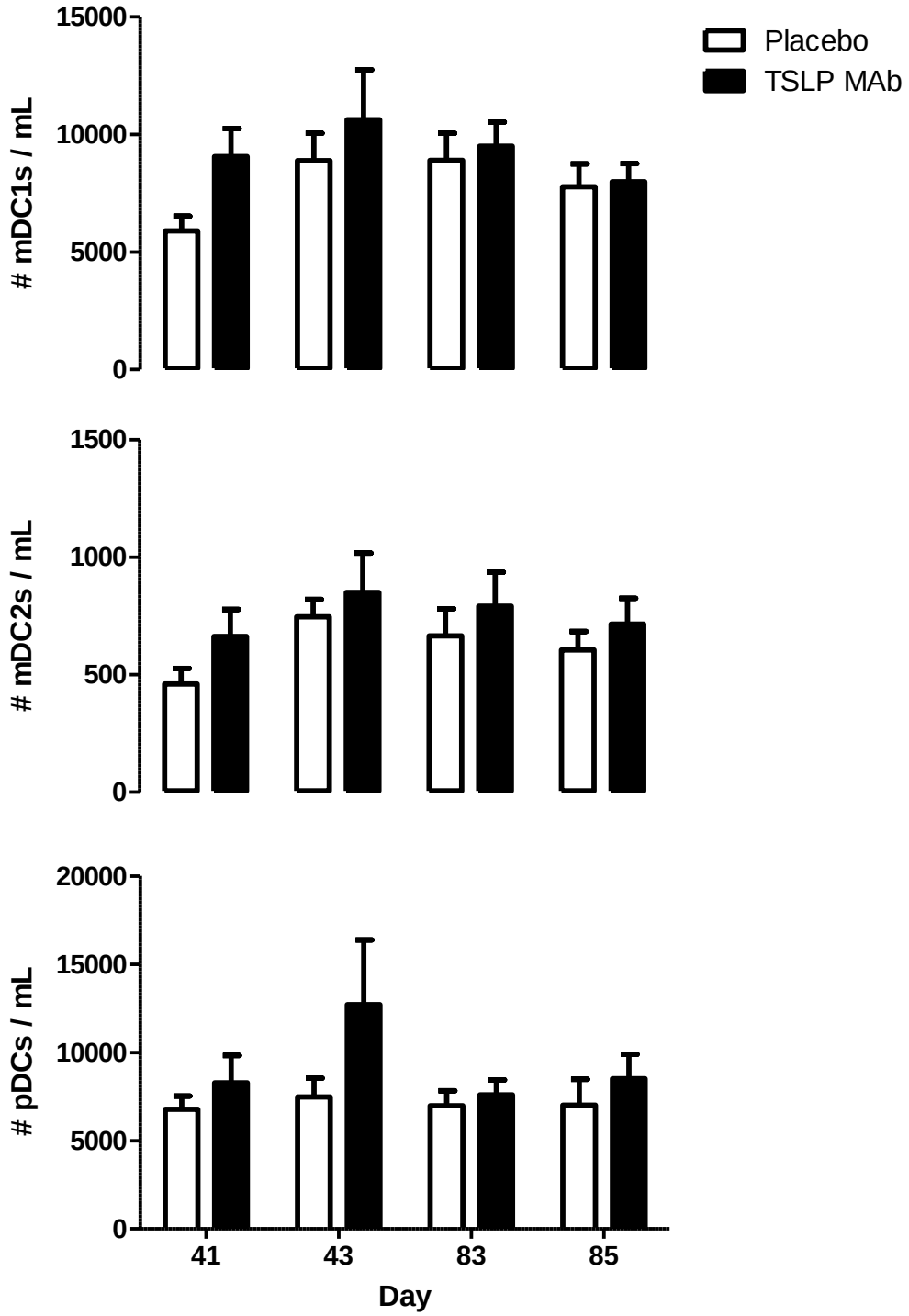

Placebo
TSLP MAb
Screening
D42
D84
Percent Fall in FEV1
Time Post-Allergen (minutes)
0
-10
-20
-30
-40
-50
0 10 20 30 45 60 90 120 180 240 300 360 420

Figure 7.


Placebo
TSLP MAb
mDC1s / mL
15000
10000
5000
0
mDC2s / mL
1500
1000
500
0
pDCs / mL
20000
15000
10000
5000
0
41
43
83
85
Day

CHAPTER 6

DISCUSSION

Asthma is an inflammatory disorder of the airways, and there has been growing insight into the cellular and molecular mechanisms underlying the inflammatory basis of this disease. Research into the inflammatory mechanisms of asthma has progressively shifted focus from downstream effectors, such as mast cells, IgE and eosinophils, up to Th2 lymphocytes and their proallergic cytokines. Even more upstream in the allergic cascade are DCs, potent APCs that orchestrate immune responses. Evidence supporting a role of DCs in regulating allergic inflammation is derived mainly from animal studies. In animal models of asthma, the induction and maintenance of airway inflammation is principally a function of mDCs, whereas the tolerance to inhaled allergens is largely a function of pDCs. It remains uncertain, however, whether this concept of pro-allergic mDCs and anti-allergic pDCs translates from animal to human models. The overall objective of this thesis was to investigate the biology of DC subsets in allergen-induced asthma. We postulated that different subtypes of DCs have different roles in the regulation of immune responses to inhaled allergen in mild allergic asthmatic subjects.

The studies presented in this thesis provide evidence for multiple DC subtypes being involved in the regulation of allergen-induced inflammatory responses. In chapter 2, we demonstrate that both mDCs and pDCs increase in the airways of subjects with mild asthma after allergen inhalation. In chapter 3, we describe a distinct subpopulation of mDCs, called mDC2s, and demonstrate their association with allergy and asthma

severity. We show in Chapter 4 that mDC2s also increase in the airways of mild asthmatics after allergen challenge. Lastly, in chapter 5, we explore the potential of pharmacological therapies, anti-OX40L MAb and anti-TSLP MAb, to affect DCs in subjects with mild asthma.

6.1 mDCs vs pDCs

Subpopulations of DCs in humans can be grossly divided into mDCs and pDCs. Although all DCs are capable of antigen uptake, processing and presentation, mDCs and pDCs differ in phenotype, localization, and function.

6.1.1 Phenotype

In humans, mDCs express markers shared with monocytes and macrophages, including CD11c and CD33 (Upham, 2003), whereas pDCs express CD123 and lymphoid development markers like CD4 (Rissoan et al., 1999). Expression of CD11c and CD123, or the expression of a group of BDCA antibodies, are commonly used to differentiate between human mDC and pDC subsets. In our studies, we have used both sets of markers to phenotype DCs. The striking differences in phenotype between mDCs and pDCs suggested these cells belong to distinct developmental lineages. However, the identification of E2-2, a specific transcriptional regulator for pDCs, challenged the idea that mDCs and pDCs are developmentally unrelated, as a deletion in the E2-2 gene

causes pDCs to spontaneously convert into mDCs-like cells (Cisse et al., 2008; Ghosh et al., 2010).

6.1.2 Location

The division of labour among DC subsets is particularly obvious in different compartments in the mouse lung. Associated with the respiratory epithelium, CD103+ mDCs project their dendrites between epithelial cells, enabling them to sample the airway lumen (Sung et al., 2006). These cells express tight junction proteins, which allow them to be firmly situated within the epithelial cell layer (Sung et al., 2006). Allergens with enzymatic activity, like Der p 1 of HDM, cause cleavage of epithelial tight junctions (Wan et al., 1999), allowing for these DCs to be activated and migrate towards lymph nodes. Immediately below the epithelium, CD11b+ mDCs reside in the lamina propria of the conducting airways. These cells are suited for priming and restimulating effector CD4+ T-cells (del Rio et al., 2007; van Rijt et al., 2005) , and are a rich source of the Th2 attracting chemokines TARC and MDC (Sung et al., 2006; van Rijt et al., 2005).

The anatomical location of mouse pDCs is not as clear. pDCs are found in large conducting airways and the interstitium of the lung (Wikstorm & Stumbles., 2007). Compared to mouse DCs, little is known regarding the anatomical localization of DC subtypes in the human lung. In our studies, we have demonstrated that both mDCs and pDCs can be found in the blood and airways of subjects with asthma.

6.1.3 Function

Along with distinct phenotypic and anatomical characteristics, mDCs are functionally different than pDCs. In humans, lung mDCs and pDC express different TLRs and therefore, are activated by different pathogenic stimuli. Through high TLR2 and TLR4 expression, mDCs produce inflammatory cytokines in response to stimuli from gram-positive and gram-negative bacteria (Demedts et al., 2006). In contrast, pDCs predominately express TLR7 and TLR9, and release antiviral and proinflammatory cytokines in response to stimuli from specific viruses and bacteria (Demedts et al., 2006). mDCs and pDCs are also different with respect to their ability to stimulate T-cells. Lung mDCs are strong induces of T-cell proliferation, while pDCs hardly induce any T-cell division (Demedts et al., 2006). The low T-cell stimulatory capacity of lung pDCs may be explained by their lower expression of MHC class II and costimulatory molecules compared to lung mDCs (Demedts et al., 2005; Masten et al., 2006; Schaumann et al., 2008). Furthermore, human mDCs and pDCs chemoattract to different chemokines, and produce different chemokines in response to various bacterial, viral and T-cell derived stimuli. Compared to blood pDCs, mDCs migrate in response to inflammatory chemokines and selectively produce the Th2-attracting chemokines TARC and MDC (Penna et al., 2001; Penna et al., 2002)

6.1.4 Animal and human models of asthma

Evidence supporting distinct functions for DC subtypes in asthma is derived mainly from animal studies. A role for mDCs in promoting allergic inflammation is demonstrated by the fact that their depletion at the time of allergen challenge abrogated typical features of asthma (Lambrecht et al., 1998; van Rijt et al., 2005), while their reinjection restored these features (van Rijt et al., 2005). In contrast, when pDCs are deleted from the lungs, inhaled tolerance is abolished and airway inflammation is augmented (de Heer et al., 2004; Kool et al., 2009), whereas transfer of pDCs suppresses inflammation and restores tolerance (de Heer et al., 2004; Kool et al., 2009).

Studies in humans have also suggested distinct roles for these cells in regulating allergen-induced inflammation. In subjects with mild asthma, both mDCs and pDCs are decreased in the blood following allergen inhalation (Farrell et al., 2007; Parameswaran et al., 2004); however, only circulating mDCs after inhaled allergen are different from numbers measured after inhaled diluent, suggesting significant diurnal changes in circulating pDCs (Farrell et al., 2007). Following their egress from the blood, initial reports demonstrated that mDCs, but not pDCs, accumulate in the lungs of mild asthmatics following short-term allergen challenge (Jahnsen et al., 2001). In our study, we were the first to examine the temporal association between allergen-induced airway inflammation and airway DCs, and found that both mDCs and pDCs increase in the sputum of mild asthmatics following allergen challenge (Dua et al., 2010). Moreover, airway mDCs, but not pDCs, from allergic asthmatics after allergen challenge could

induce strong T-cell proliferation *ex vivo* (Schaumann et al., 2008). Taken together, these results are consistent with a role for mDCs in the induction of allergen-induced airway inflammation, whereas the appearance of pDCs suggests a more active role for these cells in the resolution of the allergic response.

6.2 mDC2s

There are two distinct populations of mDCs, mDC1s and mDC2s (Dzionek et al., 2000; Ito et al., 1999). mDC2s express high levels of BDCA-3 and this marker is used exclusively to distinguish these cells from mDC1s. Compared to mDC1s, mDC2s do not express CD32, CD64 or FcεRI (Dzionek et al., 2000). Like other DC subsets, mDC2s are found in lymph nodes, bone marrow, blood and the lungs. In our studies, we have identified mDC2s in the bloodstream and airways of human subjects. Functionally, lung mDC2s and mDC1s express similar TLRs, including TLR2 and TLR4, and produce similar proinflammatory cytokines in response these TLR ligands (Demedts et al., 2006). With respect to T-cell proliferation, lung mDC2s have an intermediate T-cell stimulatory capacity, stimulating T-cells less efficiently than mDC1s, but better than pDCs (Demedts et al., 2006). The expression of MHC class II and costimulatory molecules on lung mDC2s, compared with mDC1s and pDCs, is consistent with this moderate capacity to stimulate T-cells (Demedts et al., 2005). Finally, unlike mDC1s, circulating mDC2s express higher levels of TLR3, and have a unique ability to cross-present necrotic viral cell antigens to CD8 T-cells (Jongbloed et al., 2010).

BDCA-3 (thrombomodulin) is traditonally associated with the anticoagulation cascade, binding with thrombin to regulate hemostasis. An additional and unexpected role for BDCA-3 expressed on DCs is emerging in asthma. Upregulation of BDCA-3 after *in vitro* HDM exposure distinguishes DCs from allergic versus non-allergic subjects, and these mDC2s induce a stronger Th2 response compared to DCs lacking BDCA-3 (Yerkovich et al., 2009). In our study, we were the first to demonstrate that circulating mDC2s are lower in allergic and asthmatic subjects compared to control subjects (Dua et al., 2013a). This is contrary to previous studies (Hayashi et al., 2013; Spears et al., 2011; Yerkovich et al., 2009) and we speculate that our results may more accurately reflect the number of circulating mDC2s in allergic subjects without concomitant allergen exposure. The lower numbers of circulating mDC2s in allergic asthmatics might help explain their increased presence in the lung (Demedts et al., 2005; Tsoumakidou et al., 2006). In contrast to mDC2s, we did not find circulating mDC1s to be different among our study groups (Dua et al., 2013a).

Studies on the *in vivo* effects of allergen on mDC2s have been limited. Our study was the first to evaluate the effects of allergen inhalation on circulating and airway mDC2s in subjects with mild asthma. We showed that mDC2s increase in the sputum of allergic asthmatics following allergen challenge (Dua et al., 2013b). In the circulation, mDC2s represent a minor subset of mDCs, comprising less than one-tenth the numbers of mDC1s (Dua et al., 2013a). In the airways however, we found that mDC2s are in relatively comparable numbers with mDC1s, and that an equally significant proportion of mDC2s migrate into the lungs after challenge (Dua et al., 2013b). The preferential

localization of mDC2s towards the lung is supported by other studies comparing mDC subsets in the lung (Demedts et al., 2005; Tsoumakidou et al., 2006). The increase in mDC2s into the airways during the asthmatic response may suggest these cells are important in the induction and regulation of allergen-induced airway inflammation.

With respect to animal models of asthma, transfer of antigen-pulsed mDC2s in mice reduces airway hyperresponsiveness and inhibits lung inflammation, including the number of eosinophils and the levels of IL-5 and IgE, compared to DCs lacking BDCA-3 (Takagi et al., 2011). Moreover, these mDC2s have reduced phagocytosis, lower expression of maturation markers, secrete fewer pro-inflammatory but more anti-inflammatory cytokines, stimulate T-cells to proliferate less, and induce a weaker Th2 response (Takagi et al., 2011). The tolerogenic role suggested for mDC2s here contrasts to the immunogenic role found for mDCs in mouse (Lambrecht et al., 1998; van Rijt et al., 2005), and for mDC2s in humans (Yerkovich et al., 2009).

6.3 Therapeutic regulation of DC subsets

Targeting the function of DCs may constitute a novel form of treatment for allergic asthma. Therapeutic strategies however, must not run the risk of excessive immune suppression, or even raise the susceptibility to respiratory infections. Exploiting the function of different DC subsets will be important in the development of effective therapeutic interventions.

TSLP and OX40L are important molecules in the development of inflammatory responses. TSLP is produced primarily by epithelial cells in response to inflammation and promotes Th2 inflammatory responses through its activation of DCs. OX40L is expressed on DCs following stimulation with TSLP, and is important in primary and secondary Th2 responses. Animal models of asthma have established the therapeutic potential of drugs that disrupt TSLP and OX40L signaling. In mice treated with antibodies that block either TSLP or OX40L signaling, the anti-inflammatory effects of these drugs were partly mediated through their antagonizing effects on mDCs (Seshasayee et al., 2007; Shi et al., 2008). Furthermore, the importance of the TSLP-OX40L axis in inducing and maintaining Th2 responses in humans is mediated primarily through mDCs (Ito et al., 2005; Wang et al., 2006). mDCs have a role in the promotion of allergic inflammation (Lambrecht et al., 1998; van Rijt et al., 2005), and it follows that both TSLP and OX40L would exert their pro-allergic actions via mDCs. In our study, we described the effects of human MAbs against OX40L and TSLP on circulating mDC1s, mDC2s and pDCs in subjects with asthma. Although treatment with anti-OX40L MAb or anti-TSLP MAb did not affect any circulating DC subset, we believe that measuring mDCs locally in the airways, or examining their function *ex vivo*, would reveal significant anti-inflammatory effects of these drugs.

Another way of exploiting the function of DCs is to reprogram the Th2 response. pDCs have an important role in inhalation tolerance and homeostasis in the lung, and therefore, triggering this particular subset of DC might be a useful therapeutic strategy. *In vivo* expansion of pDCs by Flt3L, a hematopoietic growth factor that selectively

enhances specific populations of immune cells, suppresses features of asthma in mice when given during secondary challenge (Kool et al., 2009). The anti-inflammatory effects of this cytokine are mediated by shifting the balance between mDCs and pDCs, favouring the accumulation of pDCs in the lung (Kool et al., 2009). Furthermore, the anaphylatoxin C5a protects from allergic sensitization in the lungs of mice by setting the balance in favour of lung pDCs over mDCs (Kohl et al., 2006). The cytokine osteopontin also works through regulation of DC subsets. Osteopontin is produced in higher amounts in the airways of asthmatics, and neutralization of osteopontin during primary sensitization in mice decreases Th2 responses through increasing pDCs in draining lymph nodes (Xanthou et al., 2007). The role for pDCs presented here is consistent with the finding that a relative deficiency of circulating pDCs in infancy is a risk factor for the development of asthma (Upham et al., 2009).

6.4 Limitations

Despite the number of techniques available, DCs remain difficult to study. Firstly, DCs are rare cells *in vivo*, as they represent at most a few percent of the total cell population in a given organ. Secondly, DCs are exquisitely sensitive to stress signals from the environment and therefore, isolating DCs from tissues often induces activation artifacts. Finally, in contrast to other leukocytes, DCs cannot be immunophenotyped using one single marker. Human DCs are identified by a panel of cell surface markers, typically by the expression of MHC class II and by the absence of common lineage

markers for other leukocytes, including those for T-cells, B-cells, NK cells, monocytes and granulocytes.

In light of the above considerations, we acknowledge certain limitations in our studies. In chapter 2, we examined mDCs and pDCs in induced sputum of subjects with asthma. Although tests of DC function, like T-cell proliferation, would have strengthened our findings, technical difficulties in isolating DCs in sputum, combined with the limited and variable numbers of DCs collected in sputum, prevented us from doing so. In chapter 3, we enumerated mDC2s in the peripheral blood of healthy, allergic non-asthmatic, mild allergic asthmatic and moderate/severe allergic asthmatic subjects. mDC2s were lower in our moderate/severe asthmatic group and in spite of our desire to measure DCs locally in the airways, inducing sputum from subjects with severely reduced lung function was not feasible. In chapter 4, we examined mDC2s in the blood and induced sputum of subjects with asthma. Although *ex vivo* tests on DCs in blood are routinely conducted and relatively easy, these assays require a substantial volume of blood. Subjects enrolled in our study were also taking part in other larger clinical trials, and therefore, we were unable to perform these assays due to the maximum allowable blood draw limits for each subject. Lastly in chapter 5, as part of two large clinical trials, we described the effects of human MAbs against OX40L and TSLP on circulating DCs in subjects with asthma. In both trials, circulating DCs were studied as exploratory cells and consequently, there was not enough blood available to study their function *ex vivo*, or enough sputum cells to measure DCs locally in the airways.

6.5 Summary

The overall objective of this thesis was to investigate the biology of DC subsets in allergen-induced asthma. Initially, we demonstrated that both mDCs and pDCs increase in the airways of mild asthmatic subjects after allergen inhalation. This study was the first to not only identify DCs in sputum, but also examine the temporal association between allergen-induced airway inflammation and airway DCs in asthmatic subjects. These findings were consistent with a role for mDCs in the induction of allergen-induced airway inflammation, whereas the appearance of pDCs suggested a more active role for these cells in the resolution of the allergic response. Next, we studied a distinct subpopulation of mDCs, called mDC2s, in the peripheral blood of healthy, atopic non-asthmatic, mild atopic asthmatic and moderate/severe atopic asthmatic subjects. Here, we demonstrated for the first time that circulating mDC2s are lower in subjects with allergy and asthma. It is possible that circulating mDC2s are being recruited into the airways, which may explain their increased presence in the lungs of subjects with allergic disease. Following this result, we examined the trafficking of circulating and airway mDC2s after allergen challenge in subjects with mild allergic asthma. No study to date has examined the *in vivo* effects of allergen on mDC2 migration. We found that mDC2s also increase in the airways of mild asthmatics after allergen challenge, suggesting these cells may be an important subpopulation of mDCs that regulates the late asthmatic response and allergen-induced airway inflammation. Finally, we explored the potential of pharmacological therapies, anti-OX40L MAb and anti-TSLP MAb, to affect DCs in subjects with mild asthma. Although treatment with anti-OX40L MAb or anti-TSLP

MAb did not affect circulating mDC1s, mDC2s or pDCs, this study was the first to describe the *in vivo* effects of anti-OX40L MAb and anti-TSLP MAb on circulating DCs in humans. We believe measuring DCs locally in the airways, or examining their function *ex vivo*, may have revealed significant effects of these drugs.

Taken together, the studies presented here provide evidence for multiple DC subtypes being involved in the regulation of allergen-induced inflammatory responses, and support continued investigations into the biology of different DC subsets in allergen-induced asthma.

6.6 Future Directions

6.6.1 Migration

The migration of DCs is central to their function as APCs. As the number and activation of lung DCs during secondary challenge regulates allergic inflammation, it will be important to study the factors that control the recruitment, survival or egress of DCs from the lung. It is widely assumed that CCR6 is the predominant chemokine receptor directing immature DCs towards the airways (Dieu et al., 1998; Dieu-Nosjean et al., 1999; Greaves et al., 1997; Yang et al., 1999). In our study, the majority of airway DCs did not express CCR6, and we did not find a significant increase in the number of CCR6 mDCs or pDCs during the allergic response (Dua et al., 2010). Using mixed bone-marrow chimaeras in mice, CCR2 but not CCR5 or CCR6, was the predominant receptor

for attracting DCs to the lungs following allergen challenge (Robays et al., 2007). Moreover, mDCs and pDCs differ in the capacity to migrate to chemotactic stimuli because of functional differences in chemokine receptors (Penna et al., 2001). As such, continued research on the expression of different chemokine receptors on different DC subsets will be critical in understanding the mechanisms involved in DC trafficking, and this may reveal potential therapeutic targets.

6.6.2 mDC2s

Multiple subtypes of DCs can be found in the lung. mDC2s have more recently emerged as a minor subset of mDCs in human peripheral blood, distinct from the more prevalent mDC1 subset. We and others have shown that mDC2s preferentially localize to the lung, where they are comparable in number to lung mDC1s (Demedts et al., 2005; Dua et al., 2013b; Tsoumakidou et al., 2006). The relevance of this increased localization is uncertain, but some authors have suggested mDC1s and mDC2s to represent maturational stages of the same cell type (Lindstedt et al., 2007). BDCA-3 is expressed on other cell types and can be induced on other DCs following *in vitro* culture (Dzionek et al., 2000); therefore, it will be important to correctly phenotype mDC2s, and distinguish them from mDC1s and pDCs.

Elegant experiments in animal models of asthma have demonstrated a role for mDCs in the induction and maintenance of allergic inflammation. Although it is reasonable to assume that mDC1s and mDC2s may both be pro-allergic, some authors

have suggested otherwise (Takagi et al., 2011), and therefore it will be necessary to conduct similar experiments in animals with both subtypes of mDCs. Similarly in humans, investigations into mDCs must account for both mDC1s and mDC2s. Although we, and others, have suggested a possible role for mDC2s in allergen-induced asthma (Dua et al., 2013b; Yerkovich et al., 2009), the function of mDC2s in the initiation or persistence of allergic asthma remains uncertain.

Future research of DC biology must appreciate the concept that different subtypes of DCs perform different functions. From this, pharmacological strategies may emerge that have therapeutic value in the treatment of allergic asthma.

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