THE INTERACTIONS OF TOLEROGENIC DENDRITIC CELLS, INDUCED REGULATORY T CELLS AND ANTIGEN-SPECIFIC IgG1-SECRETING PLASMA CELLS IN ASTHMA

A Thesis submitted to the College of

Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

In the Health Sciences Graduate Program

College of Medicine

University of Saskatchewan

Saskatoon, Saskatchewan, Canada

By

Yanna Ma

PERMISSION TO USE

In presenting this thesis in partial fulfilment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Program or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of materials in this thesis in whole or part should be addressed to:

The Health Sciences Graduate Program

College of Medicine

University of Saskatchewan

Saskatoon, Saskatchewan, S7N 5E5,

Canada

ABSTRACT

Allergic asthma is a chronic inflammatory airway disease that is dominated by Th2 immune responses, with accumulation of eosinophils, IgE and IgG1 production, and airway hyperresponsiveness. We reported previously that treatment of OVA-asthmatic mice with allergen-presenting IL-10-differentiated dendritic cells (DC) (DC10) leads to progressive and long-lasting full-spectrum asthma tolerance. However, little has been done in investigating a role for antigen-specific B cells in DC10-induced tolerance.

In this study, we characterized the surface markers of DC10 and found that these cells expressed lower levels of CD40, CD80, MHC II, PD-L1 and PD-L2 relative to immunostimulatory LPS-differentiated DCs (DCLPS). Co-culturing DC10 or DC10-induced regulatory T cells (iTreg) with $CD4^+$ Th2 effector T cells from asthmatic mice led to a marked suppression of DCLPS-induced T effector cell proliferation. Moreover, DC10 treatment of asthma phenotype mice down-regulated airway eosinophilic inflammation as determined 48 h after a recall allergen challenge, and reduced pulmonary parenchymal tissue OVA-specific IgG1-secreting (OVA-IgG1) plasma cell numbers. The number of lung OVA-specific IgG1 plasma cells decreased by 46.7% over a 2 week period in the absence of repeated allergen challenge, while the numbers of bone marrow OVA-specific IgG1 plasma cells stayed relatively stable over a 6 week period, as determined 48 h after a single allergen challenge of asthmatic mice. DC10 treatment had a significant impact on the serum of IgG1/IgE response.

To address the question of how DC10 influence OVA-IgG1 plasma cells responses, we co-cultured enzymatically-dispersed lung total cells from asthmatic mice with or without DC10, and found that the DC10 significantly suppressed OVA-IgG1 plasma cell antibody production. To determine whether DC10 required input from T cells to accomplish this, we co-cultured CD4 T cell-depleted, B cell-enriched populations from the lungs of asthmatic mice with or without DC10, and found that DC10 strongly (65.4+/-3.5%) suppressed OVA-IgG1 plasma cells in CD4 T cell-depleted lung cell cultures. To assess whether DC10-induced Treg also suppress IgG1-secretion, we co-cultured lung $CD4^+$ T cells from untreated or DC10-tolerized asthmatic mice with total lung cells from asthmatic donors, and found that the DC10-induced Tregs effectively (52.2+/-8.7%) suppressed OVA-IgG1 plasma cell responses. In summary, DC10 treatment strongly down-regulate OVA-specific IgG1 plasma cell responses of asthmatic mice,

both *in vivo* and *in vitro* by at least two mechanisms: directly via DC10 as well as indirectly through DC10-induced Tregs.

ACKNOWLEDGEMENTS

This thesis could not have been written without the support of many wonderful people. At the top of my list, as always, is my supervisor, Dr. John R. Gordon. Thanks for his patience, wisdom, guidance, enthusiasm, immense knowledge and financial support. I would like to thank the rest of my thesis committee: Dr. Barry Ziola, Dr. Volker Gerdts, Dr. Baljit Singh, Dr. Donald Cockcroft, Dr. Philip Griebel and coordinator Mrs. Angela Zoerb, for their critical comments, insightful questions and encouragement.

My sincere thanks also goes to our Research Associate Dr. Wojciech Dawicki for his enlightening teaching and help, as well as to our technician Mrs. Xiaobei Zhang for her thoughtful help, not only for the technical support in lab, but concern and kindness in personal life. Thanks to my fellow lab-mates Dr. Hui Huang, Chunyan Li, Nathan Wright, Shui Jiang, Sara A. Gordon, and Laura Churchman, for their care and sweet friendship, and to Mark Boyd, for his sincere help in my flow cytometry analysis. The more time I spend with them, the more thrilled I am to consider them not only friends, but family.

Last but not least, I would like to thank my dear and beloved family, my parents Xinrui Zhu, Jigeng Ma, my sister Yanru Ma and brother-in-law Guoshou Du, with their chubby newborn baby, Shengkang Du. My brother Xiaowei Ma, sister-in-law Xiaolei Niu and their cute and lovely children, Ziying Ma & Yingsong Ma. Without their love, I could not have devoted all the time and concentration required for me to realize my dream so far away from home. Thank my best and cherished friend Heng Wang in Tianjin, who is always with me by intellectual communication, vision and spiritual support in the past three years. I love them so much and miss them every single day in Saskatoon, Canada.

Dedicated to

My dear grandma

My beloved mum and dad

My sister and brother

My friend Heng Wang

For their unconditional love and support

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADAM: a disintegrin and metalloproteinase (cell surface marker)

AH: airway hyperresponsiveness

APC: antigen-presenting cells

ASM: airway smooth muscle

BAL: bronchoalveolar lavage

BALT: bronchus-associated lymphoid tissue

BCR: B cell receptor

Blimp-1: B lymphocyte-induced maturation protein-1

BMCMC: bone marrow-derived cultured mast cell

CCL: CC chemokine ligand

CCL11: CC chemokine ligand 11 (aka, eotaxin 1)

CCR: CC chemokine receptor

$CD8^+$ T cells: cytotoxic T cells

COPD: chronic obstructive pulmonary disease

CRTH2: chemoattractant receptor of Th2 cells, PTGDR2

CSR: class switch recombination

CT: computed tomography

CTLA-4: cytotoxic T-lymphocyte antigen 4

CTMC: connective tissue-type mast cell

DAMPs: damage-associated molecular patterns

DC: dendritic cell

DC10: IL-10-differentiated DC

DC-Dex: dexamethasone-induced tolerogenic DC

DCLPS: lipopolysaccharide-differentiated DC

DC-Rap: rapamycin differentiated mouse DC

DC-SIGN: dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

DC-VitD3: vitamin D3-induced tolerogenic DC

DC-VIP: vasoactive intestinal peptide-differentiated human DC

D region: diversity region

DTH: delayed-type hypersensitivity

EAE: experimental allergic encephalomyelitis

EBF1: early B cell factor 1

ECs: epithelial cells

E.coli: *Escherichia coli*

Eomes: eomesodermin

Eos: eosinophil

FDC: follicular DC

Flt3L: Flt3 ligand

FoxP3: forkhead box protein 3

GALT: gut-associated lymphoid tissue

GFP: green fluorescent protein

GILZ: glucocorticoid-induced leucine zipper

GM-CSF: granulocyte-macrophage colony-stimulating factor

HDM: house dust mite

hMoAB: humanized monoclonal antibody

HSCs: hematopoietic stem cells

iBALT: inducible bronchus-associated lymphoid tissue

IBD: inflammatory bowel disease

ICAM1: intercellular adhesion molecule 1

ICOS: inducible costimulator

ICS: inhaled corticosteroid

IDO: indoleamine 2, 3-dioxygenase

IFN-γ: interferon-γ

Ig: immunoglobulin

IL-2Rα: IL-2 receptor α-chain (also CD25)

i.p.: intraperitoneal

IPEX: Immune dysregulation, polyendocrinopathy, and enteropathy, X-linked syndrome

iTreg: induced Treg

i.v.: intravenous

J region: joining region

LFA1: lymphocyte function-associated antigen 1, integrin-αL

LPS: lipopolysaccharide

Lym.: lymphocytes

Mac.: macrophages

MBP: major basic protein

MHC: major histocompatibility complex

MLN: mesenteric lymph node

MLN: mediastinal lymph nodes

MMC: mucosal mast cells

MMP: matrix metalloproteinases

mTOR: mammalian target of rapamycin

MZ: marginal zone

NF- κ B: nuclear factor- κ B

NK: natural killer

NKT cells: natural killer T cells

NOD: non-obese diabetic (mouse strain)

nTreg: naturally-occurring Tregs

OVA: ovalbumin

PAMP: pathogen-associated molecular pattern

PAR-2: Proteinase activated receptor-2

Pax5: paired box protein 5

PD: programmed death

pDC: plasmacytoid DC

PDE: phosphodiesterase

PD-L1 or -L2: programmed death ligand-1 or -2

PGD2: prostaglandin D2

PGE2: prostaglandin E2

PRR: pattern recognition receptor

RBM: reticular basement membrane

ROR: retinoic acid receptor-related orphan nuclear receptor

s.c.: subcutaneous

SHM: somatic hypermutation

SLIT: sublingual immunotherapy

SLO: secondary lymph organ

SIT: allergen-specific immunotherapy

STAT: signalling transducer and activator of transcription

T_{cm}: central memory T cell

TCR: T cell receptor

TD: T-dependent

T_{em}: effector memory T cells

T_{FH}: T follicular helper cells

TLR: Toll-like receptor

TGF-β: transforming growth factor-β

Th cell: T helper cell

Tr1: IL-10-producing Treg

Treg: regulatory T cells

TSLP: thymic stromal lymphopoietin

t.t.: transtracheal

VIP: vasoactive intestinal peptide

V region: variable region

WAO: World Allergy Organization

CHAPTER 1: Literature review

1.1 Immunobiology of allergic asthma

Allergic diseases include asthma, atopic eczema, food allergy, allergic rhinitis, conjunctivitis, rinosinusitis and hypersensitivity to drugs or biological agents. According to the World Allergy Organization (WAO) White Book on allergy, there are about 300 million individuals of all ages with asthma worldwide, with 5% of all children affected. It is anticipated to increase to 400 million affected individuals by the year 2025, while 220-250 million people suffer from food allergies. Asthma causes 250,000 deaths every year, but it also brings a markedly reduced quality of life and very high direct or indirect costs in terms of socio-economic consequences. Asthma in children under the age of 5 is difficult to diagnosis, and especially so in infants, and thus is often untreated , but ≈5-10% of patients have severe asthma despite their condition being controlled by medications (Pawankar, Canonica et al. 2012).

Factors that influence the asthma phenotype

As asthma worsens, the airways become increasingly constricted and inflamed, filling with mucus. Patients experience classical symptoms including chest tightness, chronic cough, shortness of breath, and wheezing, especially at night or early in the morning. Both genetic and environmental factors can enhance the likelihood of developing asthma. Identifying these heritable candidate genes is useful in understanding disease mechanisms. For example, ADAM (a disintegrin and metalloproteinase) 33, initially identified in 2002, is expressed on smooth muscle cells and fibroblasts and is associated with long-standing airway inflammation. Subsequently, ORMDL3 (found at chromosome 17q21) was recognized from a genome-wide association study (GWAS) as a strong determinant of asthmatic inflammatory responses (Hedlin and van Hage 2012). The expression of number of genes analyzed by Bio-ontologic enrichment (e.g., IL8R1, IL1RL1, IL13, HLA-DOA, etc.) contributes critically to inflammatory responses and immune-regulation in asthma (Melén and Pershagen 2012), but there is insufficient evidence that the identified candidate genes can predict the likelihood of developing allergy disease with any degree of accuracy. The most common allergic diseases (e.g., asthma, rhinitis, and atopic dermatitis) are also strongly associated with exposure and sensitization to prevalent outdoor,

(e.g., birch or grass pollen, mould spores) or indoor (e.g., house dust mites, cockroach, cats, dogs or rodents, venom) allergens (Holt and Thomas 2005).

Immunobiology of asthma

The molecular and cellular mechanisms underlying allergic asthma are very complex. Antigen-presenting cells (APC) including dendritic cells (DCs) and macrophages, are richly represented in tissues associated with body surfaces (i.e. skin, lung, nose, and intestine), such that they are in a position to detect perturbations within our environment. DCs are considered the pre-eminent APCs and are a critical link between innate and adaptive immunity (de Heer, Hammad et al. 2005). They respond quickly to environmental changes and can differentiate extensively to become immunogenic accessory cells (Steinman 2012). After antigen inhalation, local DCs accumulate rapidly at the site of antigen deposition on the bronchial epithelium, while circulating DC precursors are also recruited by chemokines upon local inflammation (McWilliam, Nelson et al. 1994). DC maturation takes place as the DCs migrate from their tissue niche to the lung-draining lymph nodes via the lymphatics, during which the chemokine receptor CCR7 is upregulated, guiding the DC to home to draining mediastinal lymph nodes (MLN) (Sánchez-Sánchez, Riol-Blanco et al. 2006). DCs phagocytose and process antigens, then present the processed antigen peptides on their cell surface as major histocompatibility complex (MHC) II-peptide complexes, and thereby provide antigen-specific signals to cognate CD4 T cells that recognize the allergen peptides being presented. At the same time, DC also elevate the level of surface costimulatory and adhesion molecules, such as CD80 and CD86, which bind both CD28 and CTLA-4 on T cells (Guermonprez, Valladeau et al. 2002).

As they mature, DC loses their ability to digest and present new antigens (Romani, Koide et al. 1989). Fully-mature DC in the draining (mediastinal) lymph nodes facilitate naive T cell proliferation and differentiation into effector T helper cells (Förster, Schubel et al. 1999). DCs attract T and B lymphocytes by releasing chemokines (e.g., a C-C chemokine expressed on human DCs, DC-CK1) (Adema, Hartgers et al. 1997), maintain the viability of recirculating T cells (Banchereau and Steinman 1998), and enhance the T cell's stimulatory capacity and cytokine production. IL-12 production by DCs, although transient, gives rise to myeloid DC priming of either Th1 or Th2 responses (Langenkamp, Messi et al. 2000). There are at least four

subsets of $CD4^+$ T cells, including T-helper (Th) 1, Th2, Th17 and regulatory T cells (Tregs). Asthma is dominated by Th2 immune responses that lead to the accumulation of eosinophils, as well as IgE and IgG1 production (with sensitization of tissue mast cells and circulating basophils), and airway hyperresponsiveness (AHR). The airway obstruction and inflammation development is very common (Pawankar, Canonica et al. 2011). Th2 cytokines foster the classic allergic responses, such as IL-4-driven B cell class-switching from IgG to IgE, IL-5-mediated eosinophilic infiltration, and IL-13-dependent airway hyperresponsiveness and goblet cell hyperplasia (Holgate 2012). Mast cell-bound or circulating IgE antibody can be detected by skin prick testing or *in vitro* assays, respectively. By binding to FcεRI (IgE receptor)-bearing cells, antigen-specific IgE secreted by plasma cells is able to sensitize mast cells and basophils for subsequent activation on exposure to cognate allergen (Kay 2001). Tregs are essential for the maintenance of self-tolerance and the modulation of immune responses to infections or allergens (Yssel, Lecart et al. 2001). Severe asthma can be characterized as neutrophilic or mixed eosinophilic/neutrophilic in phenotype, with the latter mediated by Th17 cells that secrete the IL-17A found at elevated levels in bronchoalveolar lavage (BAL) fluids and lung biopsies (Molet, Hamid et al. 2001). Similarly, cytokines produced by Th17 cells promote mucus over-production (e.g., IL-17), activate airway epithelial cell secretion of CXCL8, which is a chemoattractant for neutrophils (e.g., IL-17), and foster airway remodelling with its smooth muscle cell hyperplasia and collagen deposition (e.g., IL-17, IL-22) (Aujla and Alcorn 2011).

1.1.1 Dendritic cells (DCs)

Initially, scientists questioned the mechanisms behind cell-mediated immune responses, how antigens were introduced to the immune system, and how that translated into the induction of clonal T cell responses. In 1970s, Steinman found a novel cell in the spleen, which was different from macrophages in that these cells had few lysosomes, a distinct morphology, expressed high levels of MHC molecules, and were highly potent immune stimulators (Steinman and Cohn 1974). Now we know that these dendritic cells (DC), so named because of their distinctive dendritic processes, are professional APC, and seemingly more potent than B cells or macrophages (Lechler and Batchelor 1982).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is broadly recognized as an important factor in DC development and maturation since it was found to be a critical factor for the maturation of Langerhans cells (Witmer-Pack, Olivier et al. 1987). GM-CSF and IL-4, or IL-13, with or without additional maturation stimuli (e.g., lipopolysaccharide; LPS) can also induce DC differentiation from precursors (Sallusto, Cella et al. 1995). Moreover, Flt3 ligand (Flt3L) dramatically increases the numbers of mature DC and their expression of CD86, MHC II, CD11c, and DEC205 (Maraskovsky, Brasel et al. 1996). Inflammatory stimuli, e.g., TNF-α, IL-1 or lipopolysaccharide (LPS), can facilitate DC maturation by upregulating their adhesion, costimulatory signals, MHCII synthesis and decreasing endocytosis (Cella, Engering et al. 1997).

DC are located at the interfaces of the body with the environment, such as the skin, respiratory tract, and mucosal sites, and include distinct types of cells (e.g., $CD8^+$, $CD8^-$, plasmacytoid, and monocyte-derived DC). Exogenous antigens can be presented by DCs in a MHC II-dependent fashion and thereby induce specific T cell responses. There are four basic stages of DC development that have been identified, including bone marrow progenitors, circulating precursor DC, and tissue-resident immature or mature DCs. Precursor DCs patrol and circulate in the blood before populating peripheral tissues (e.g., the lungs) and lymphoid tissues. The respiratory tract dendritic cells serve in surveillance for inhaled antigens. At least 85% airway DCs are renewed every 24-36 h (Holt, Haining et al. 1994); mature DC complete their life cycle by migrating to tissue-draining lymph nodes (Holt, Haining et al. 1994). Tissue-resident immature DCs have high endocytic and phagocytic abilities and therefore readily capture antigens, but on cellular maturation DC lose their capacity to capture antigen, upregulate their expression of costimulatory signals for antigen presentation and migrate to their resident tissue draining lymphoid organs (Banchereau, Briere et al. 2000).

1.1.1.1 Subsets of dendritic cells in mouse and human

Dendritic cells differ in localization, phenotype and function. The mouse spleen contains three subsets of DCs-$CD4^-CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^-$ DCs (Vremec, Pooley et al. 2000). Lymph nodes contain two extra DC subsets which cannot be found in the spleen and apparently migrate from the lymphatics to the lymph nodes; one is $CD4^-CD8^-CD11b^+$ DCs which express modest levels of CD205, while the other one, found in skin-draining lymph nodes, expresses high levels

of langerin (Henri, Vremec et al. 2001). The circulating tissue DCs precursors have not been all identified yet, but we know that blood-borne precursors continue to replace splenic and lymph node DCs in murine models. For instance, splenic $CD8\alpha^+$ DCs continuously gain access to the spleen via the vasculature (Randolph, Ochando et al. 2008). The gut-associated lymphoid DCs include those in mesenteric lymph nodes (MLNs), intestinal lamina propria, and isolated lymphoid follicles (Steinman and Banchereau 2007). $CD11c^{hi}CD103^+CD11b^+CX3CR1^-$ cells and $CD11c^{int}CD103^-CD11b^-CX3CR1^+$ cells are two $CD11c^+$ mononuclear populations in the lamina propria (Farache, Koren et al. 2013). CD103, the αE integrin subunit, is expressed on almost all lamina propria DCs and on a subset of MLN DCs (Johansson-Lindbom, Svensson et al. 2005). In the lungs, $CD11c^{hi}$ myeloid DC are found in conducting airway and in the interstitium, while $CD11c^-$ cells have been identified in the airway mucosa (von Garnier, Filgueira et al. 2005). $CD11c^+$, MHC II^+, and $CD11b^+$, but not $CD8\alpha^-$ (de Heer, Hammad et al. 2005), are expressed on airway DCs in mice, which also express CD103 and tight junction proteins (Del Rio, Bernhardt et al. 2010; Vroman, van den Blink et al. 2014). $CD11c^+CD1c^+$, $CD11c^+$BDCA-3^+ myeloid cells, and $CD11c^-$BDCA-2^+ plasmacytoid DCs are three populations of DCs identified in human bronchoalveolar lavage and lung digest studies (Demedts, Brusselle et al. 2005), and they correspond to $CD11b^+CD103^-$, $CD11b^-CD103^+$langerin$^+$ conventional and plasmacytoid DCs subsets in mice (Lambrecht and Hammad 2012). As in the intestinal tract and lungs, skin DC are well-positioned and quickly respond to cutaneous insults with induction of Teff responses or tolerance. Skin-derived DCs have at least three subsets, self-renewing epidermal langerin$^+CD103^-$ Langerhans cells (Chorro and Geissmann 2010), langerin$^-CD103^-$ (Igyártó, Haley et al. 2011), and langerin$^+CD103^+$ (Merad, Ginhoux et al. 2008) dermal cells. In the steady-state, hepatic myeloid DCs produce high levels of IL-10 (Bamboat, Ocuin et al. 2010), IL-27 (Chen, Jiang et al. 2009), retinoic acid and phosphodiesterase 2 (PDE2) to induce tolerance (Xia, Guo et al. 2008). On the other hand, hepatic plasmacytoid DCs secrete high levels of IL-10 and express an immature phenotype, whereby they induce tolerogenic T cell responses (Dubois, Joubert et al. 2009). BDCA-1^+ DCs in the human liver contribute to induction of $CD25^+Foxp3^+$ Tregs (Bamboat, Stableford et al. 2009).

In contrast to mouse DCs, there have been relatively few studies on mature human DCs isolated from fresh tissues. Human blood DCs express a variety range of markers which reflect

their differentiation or maturation stage rather than being markers of separate subsets (Hart 1997). Two subsets of DCs have been identified in human peripheral blood, $MHCII^{+}CD11c^{+}CD123^{lo}$ myeloid dendritic cells and $MHCII^{+}CD1c^{-}CD123^{hi}$ plasmacytoid DCs (Bamboat, Stableford et al. 2009; Ueno, Klechevsky et al. 2011). Human Langerhans DCs have been identified as a separate DC subtype with distinct cell markers, including CD1a and langerin (Shortman and Liu 2002). Similar to mouse splenic DCs, human splenic and tonsillar DCs express CD4, CD11b, and CD11c (McIlroy, Autran et al. 1995). The majority of human thymic DCs express $CD11c^{+}CD11b^{-}CD45RO^{lo}$ and lack myeloid markers, similar to mouse thymic $CD8^{+}$ DCs, while a minority are $CD11c^{hi}CD11b^{+}CD45RO^{hi}$ with many myeloid markers, also as with mouse thymic $CD8^{-}$ DCs (Shortman and Liu 2002).

1.1.1.2 Dendritic cells and T/B cells interaction

All immune reactions are initiated in lymphoid organs. The 'decision' to be made between the DCs and T cells during their interaction is whether that interaction leads to effector or tolerogenic responses. Dendritic cells are generated from bone marrow progenitors, circulate in blood as precursors, and reside in peripheral tissues as immature cells. The various sub-populations of DCs phagocytose and process antigens to their constituent peptides and respond to environmental triggers by up-regulating their antigen presentation-associated surface molecules and receptors (e.g., MHC II, CD40/80/86, IL-1R) and lymph node-homing chemokines (i.e. CCR7), but they also downregulate their phagocytic activities and ability to process new antigens (Romani, Koide et al. 1989). Numerous factors influence and regulate DC maturation, including pathogen-associated molecules (i.e. LPS) and proinflammatory signals in the local environment (i.e. TNF, IL-1, IL-6) (Banchereau, Briere et al. 2000).

DCs are important in the initiation and regulation of immune response and tolerance, as determined by their surface phenotype, localization, cytokines and antigen presentation. They migrate to tissue-draining lymphoid organs and present MHC-antigen peptide complexes to naive T cells, with the support of costimulatory signals and cytokines (e.g., chemokines, TNF, IL-1, IL-12) (Heufler, Koch et al. 1996; Gordon, Ma et al. 2014). According to the allergen sources and participation of costimulatory signals, DCs induce the polarization and differentiation of distinct types of T cell responses that align well with different classes of

pathogens (e.g., Th1, Th2, Th17, or regulatory T cells [Tregs]). DC-T cell interactions are also fostered by adhesion molecules, such as integrins β1 and β2, and immunoglobulin superfamily members (CD2, CD50, CD54, CD58) (Hart 1997). DCs can provide other signals to direct the trafficking of T cells. For example, in skin-draining lymph nodes, DCs release vitamin D metabolites to induce T cells expression of CCR10, so that the T cells become responsive to the skin-homing chemokine CCL127 (Sigmundsdottir, Pan et al. 2007). Likewise, in the gut, DCs produce retinoic acid (RA) and transforming growth factor (TGF)-β, and thereby induce T cells to express the gut-homing receptors α4β7 and CCR9 (Weiner, da Cunha et al. 2011). These activated T cells leave the lymph nodes via the lymphatics, circulate in the blood and finally arrive in their designated inflamed gut tissues. On one hand, mature DCs are capable of inducing immunogenic reactions leading to allergic inflammatory immune responses while, on the other, the maturation stage of the DCs, the allergen challenge dose, the distribution of the various DC subsets and many other unpredictable factors can modulate the DCs ability to induce tolerogenic responses (tolerogenic DCs). Indeed, such tolerogenic DC have become an immunotherapy candidate approach in immune-mediated diseases (Rutella and Lemoli 2004; Steinman 2012).

DC subsets may determine different classes of immune response by providing a variety of cytokines. For instance, in mice, splenic $CD8\alpha^+$ lymphoid DC and $CD8\alpha^-$ myeloid DC subsets prime naive CD4 T cells to make Th1 and Th2 cytokines, respectively (Maldonado-López, De Smedt et al. 1999; Pulendran, Smith et al. 1999). In humans, the monocyte-derived $CD11c^+$ and $CD11c^-$ DC subsets polarize naive T cells toward Th1 and Th2 profiles, respectively (Rissoan, Soumelis et al. 1999). It was shown that in the presence of free antigen, DCs prime T helper cells, which further interact with B cells, resulting in antigen-specific antibody production (Sornasse, Flamand et al. 1992). In addition to stimulating naive T cells, DCs were reported to directly regulate naive and memory B cells. For example, DCs can induce surface IgA expression mediated by TGF- β , instead of IL-10 or IL-12, on CD40-activated naive B cells, although IL-10 is critical for further differentiation of IgA-secreting B cells (Fayette, Dubois et al. 1997). In rats, DCs can capture intact antigens and transfer these to naive B cells to initiate antigen specific Th2-related antibody responses (Wykes, Pombo et al. 1998).

1.1.2 Allergen-specific antibodies

1.1.2.1 B cell development and subsets

B lymphocytes represent a critical part in of the adaptive immune responses. B cell precursors are generated from hematopoietic stem cells (HSCs) in the bone marrow. Depending on exposure to environmental stimuli, HSC transcription factors drive HSCs development, such that these cells can give rise to all blood cell types. B cells undergo development from pre-pro B cells, pro-B cells (early and late), and pre-B cells (large and small) to immature B cells in the bone marrow. These early committed B cell precursors are characterized by various surface markers (such as B220, CD19, CD24, IL-7R, etc.), transcription factors (e.g. EBF1 [early B cell factor 1], Pax5 [paired box protein 5] etc.), and Ig gene rearrangement (Table 1.1). Newly-formed IgM^+ bone marrow B cells are immature B cells, although they successfully express BCR. By expressing CCR2 chemokine receptors, immature B cells emigrate from bone marrow and traffic into the spleen through further steps of maturation (Flaishon, Becker-Herman et al. 2004). Similarly, transitional B cells (T1 and T2) in the periphery are characterized by their surface immunoglobulin receptors and membrane markers (Fig. 1.1) (Samitas, Lötvall et al. 2010). T1 and T2 both have the capacity to differentiate into mature B cells in follicles or the marginal zone of the spleen where they undergo positive or negative selection, after which they recirculate as full mature B lymphocytes (i.e., follicular (B-2) B cells, B-1 B cells and marginal zone B cells) (Allman and Pillai 2008).

Once they obtain the ability to recirculate, the mature follicular B cells migrate to B cell areas of lymph nodes, Peyer's patches, and the spleen through the blood and lymphatics. Follicular B cells mainly participate in T cell-dependent (TD) immune responses to protein antigens in the B cell follicles of lymph nodes, located adjacent to T cell zones (Genestier, Taillardet et al. 2007). In the follicular niche, naive follicular B cells can present antigen to naive T cells when they receive synergistic signals via the B cell receptor (BCR), CD40 and TLRs (Allman and Pillai 2008). However, if follicular B cells are stimulated only via their TLRs, they do not acquire the capacity to differentiate into antibody-secreting cells (Genestier, Taillardet et al. 2007). In early T cell-dependent responses, follicular B cells may differentiate into short-lived plasma cells, but they don't migrate to distinct sites. In contrast, germinal center B cells can eventually differentiate into memory B cells or plasma cells, which exit germinal center, re-enter the

circulation and migrate to the bone marrow, residing there as long-lived plasma cells (Fig. 1.2, Table 1.2) (Batista and Harwood 2009). The main populations of B-1 B cells, which are subdivided into B-1a and B-1b B cells, are not normally found in lymph node or bone marrow, but reside in the pleural and peritoneal cavities, as well as a small population in the spleen. B-1a and B-1b B cells contribute to innate-like and adaptive immune responses, respectively (Allman and Pillai 2008). Natural immunoglobulin M (IgM) and IgA are produced by B-1 B cells to T-independent (TI) antigens for early protection against bacterial infection and mucosal pathogens, respectively (Fagarasan and Honjo 2003). B-1 B cells can migrate from the peritoneum to the mesenteric lymph nodes, and home to the intestinal lamina propria. TLR signalling can downregulate their expression of integrins and CD9, facilitating egress of B-1 cells from the peritoneum. Some cytokines, like IL-5 and IL-10, can also induce B-1 B cells activation and differentiation (Allman and Pillai 2008).

Marginal zone B cells, the first line of defense against blood-borne particulate T cell-independent pathogens, are located between the marginal sinus and the red pulp (Liu, Oldfield et al. 1988). In rodents, LPS and other pathogen-driven responses may decrease adhesive integrins, leading these activated B cells to migrate out of the marginal zone into the splenic red pulp, where they differentiate into short-lived plasma cells (Martin and Kearney 2002). Marginal zone B cells may also foster T cell-dependent responses to proteins by direct activation of T cells or delivery of those antigens to follicular B cells (Allman and Pillai 2008).

Table 1.1 Differentiation diagram of Pre-pro B cells to immature B cells in the bone marrow, depicting the expression of cell surface markers, transcription factors, status of Ig genes, and surface Ig receptor expression.

Cell Stage	Pre-Pro B cells	Early Pro-B cells	Late Pro-B cells	Large Pre-B cells	Small Pre-B cells	Immature B cells
Lineage Surface Markers	$B220^+$ (CD45R) $CD19^-$ c-Kit^- $Flt3^+$ $CD93^+$ $CD43^+$ $IL\text{-}7R^{low}$ BP-1^- $CD24^{low/-}$ $CD25^-$	$B220^+$ $CD19^+$ c-Kit^{low} $Flt3^-$ $IL\text{-}7R^{low/+}$ $CD93^+$ $CD24^+$ $CD25^-$ $CD43^+$ BP-1^-	$B220^+$ $CD19^+$ c-Kit^{low} $Flt3^-$ $IL\text{-}7R^+$ $CD93^+$ $CD24^+$ $CD25^{+/-}$ $CD43^+$ BP-1^+	$B220^+$ $CD19^+$ c-Kit^- $Flt3^-$ $CD93^+$ $CD24^{++}$ $CD25^+$ $IL\text{-}7R^+$ $CD43^{+/-}$ BP-1^+	$B220^+$ $CD19^+$ c-Kit^- $Flt3^-$ $CD93^+$ $CD24^{++}$ $CD25^+$ $IL\text{-}7R^+$ $CD43^-$ BP-$1^{+/-}$ IgM^-/IgD^-	$B220^+$ $CD19^+$ $CD25^-$ c-Kit^- $Flt3^-$ $IL\text{-}7R^-$ $CD23^-$ $CD93^+$ $CD24^{++}$ $CD43^-$ BP-1^-
Transcription Factor	$PU.1^{low}$, EBF-1^+, $E2A^+$, $TdT^{+/-}$, RAG^{low}	$PU.1^{low}$, EBF-1^+, $E2A^+$, TdT^+, $Pax5^{+/-}$, RAG^+	$PU.1^{low}$, EBF-1^+, $E2A^+$, TdT^+, $Pax5^+$, RAG^+	$PU.1^{low}$, EBF-1^+, $E2A^+$, TdT^-, $Pax5^+$, RAG^-	$PU.1^{low}$, EBF-1^+, $E2A^+$, TdT^-, $Pax5^+$, RAG^+	$PU.1^{low}$, EBF-1^+, $E2A^+$, TdT^-, $Pax5^+$, RAG^{low}
Status of Ig Genes	Preparing for the first step of Ig gene recombination	D_HJ_H	$V_HD_HJ_H$	$V_HD_HJ_H$	$V_HD_HJ_HV_LJ_L$ rearrangement begins	$V_HD_HJ_HV_LJ_L$
Surface Ig Receptor Expression	None	None	None	Pre-BCR	Pre-BCR	IgM

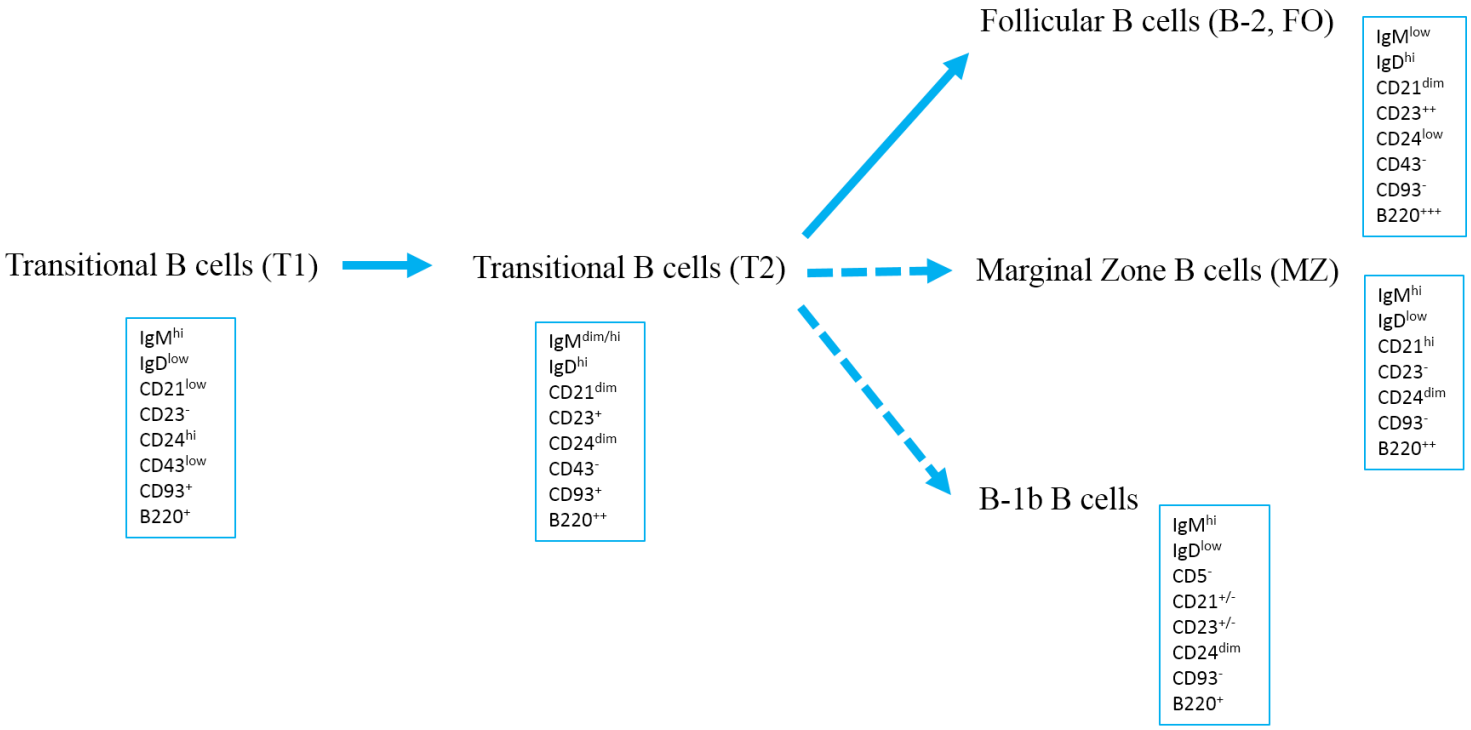


Figure 1.1 Differentiation and maturation process of B cells in spleen. Immature B cells migrate from bone marrow to spleen to mature. Peripheral B cells are characterized by surface molecules and transcription factors. Transitional B cells (T1) differentiate into T2 cells which give rise to follicular B cells. T2 B cells may also generate marginal zone B cells and B-1 B cells. Table 1.1 and Fig. 1.1 adopted from Fig. 1 & 2 in reference (Samitas, Lötvall et al. 2010).

Table 1.2 Cell surface markers of transcription factor Blimp1^{int} and Blimp1^{hi} plasma cells

Surface marker	Plasma cells	
	Blimp1^{int}	**Blimp1^{hi}**
IgM	-	-
IgD	-	-
B220	+	-
CD138	+	+
CD21	-	-
CD23	-	-
CD5	-	-
CD9	++	++
CD43	+	-
CD11b/Mac-1	-	-
CD19	+	-
MHC II	+	-
FcγRII	+++	+++
FcRH3	-	-
CXCR5	-	-
CXCR4	+	+

Referenced from table 1 in (Fairfax, Kallies et al. 2008). B lymphocyte-induced maturation protein-1 (Blimp-1) is critical for development of antibody secreting cells and maintenance of long lived plasma cells. High and intermediate levels expression of Blimp-1 represent different maturational stages of plasma cells. Blimp-1^{hi} is more mature compare to Blimp-1^{int}. Thus, Blimp-1^{hi} retain lower levels of B220, CD19 and MHC II (Fairfax, Kallies et al. 2008). There are some other transcription factors that are also relevant to plasma cell differentiation, like XBP-1, Pax-5^{low} and Bcl-6^{low}.

1.1.2.2 Antigen recognition and B cell differentiation

B cells can recognize both soluble and membrane-bound antigen. Membrane-associated antigens are recognized as more important for B cell activation *in vivo*, because of their associations with immunological synapses, which foster B cell activation and the antigen recognition process more than does soluble antigen (Depoil, Fleire et al. 2007). In addition, marginal zone macrophages, follicular DCs (FDCs) and other DCs can 'hold' intact antigens with which they activate naive B cells primary responses before antigen-specific antibody is produced (Koppel, Wieland et al. 2005). Adhesion molecules such as ICAM-1, VCAM-1, which are expressed on FDCs and DCs, are important participants in B cell responses (Koopman, Parmentier et al. 1991; Kushnir, Liu et al. 1998).

B cell receptor (BCR) mediates B cell-induced T cell activation through a cascade of tyrosine phosphorylation events (Carrasco and Batista 2006). MHC II is also engaged, in as much as MHC II-knock-out μMT mice (i.e., MHC II-deficient, B cell-deficient mice; MHC $II^{B-/-}$) have impairments in T cell responses after antigen challenge, suggesting that B cells contribute as APCs to T cell activation (Crawford, MacLeod et al. 2006). However, compared to DCs, B cells are thought of as inefficient 'professional' APCs. After antigen encounter, B cells up-regulate their lymph node-homing receptors (CCR7), surface molecules (e.g., CD86), migrate towards and aggregate at the follicle-T cell zone border as a large population of $IgM^{med}IgD^{hi}CD21^{med}CD31^{hi}$ cells (Klaus, Humphrey et al. 1980; Mandels, Phippsi et al. 1980). These B cells can process antigens to their constituent peptides, present these peptides on MHC II molecules to $CD4^+$ T cells, which reciprocally provide help for B cell proliferation and differentiation (Rock, Benacerraf et al. 1984).

Extrafollicular plasmablasts, formed from activated B cells, produce antibody as part of the early immune response in primary follicle. In addition, differentiated B cells can also enter the germinal center environment, a specialized structure within the follicles of secondary lymphoid organs (secondary follicle). These cells then become long-lived memory B cells that are regulated by T follicular helper cells (T_{FH}) and have a long-lasting ability to respond to secondary allergy challenge. These B cell-T_{FH} cell interactions involve signalling via the T cell receptor (TCR), membrane protein CD40L (Liu, Joshua et al. 1989), and the cytokines IL-4, IL-10 and IL-21 (Bryant, Ma et al. 2007; Linterman, Beaton et al. 2010; Vinuesa and Chang

2013). Post-germinal centre memory B cells retain their high affinity BCR, do not secret antibody, but have the intrinsic ability to response more quickly than naive B cells (Tangye, Avery et al. 2003). Alternately, differentiated B cells undergo immunoglobulin gene somatic hypermutation (SHM) leading to higher affinity BCRs on antibody-secreting plasma cells in the germinal centre (Goodnow, Vinuesa et al. 2010). Depending on expression of CXCR4 (the receptor for bone marrow stroma-produced CXCL12), most of GC-derived plasma cells migrate to the bone marrow or local mucosa-associated lymphoid tissues (Hauser, Debes et al. 2002). The bone marrow provides a final niche for survival of those plasma cells.

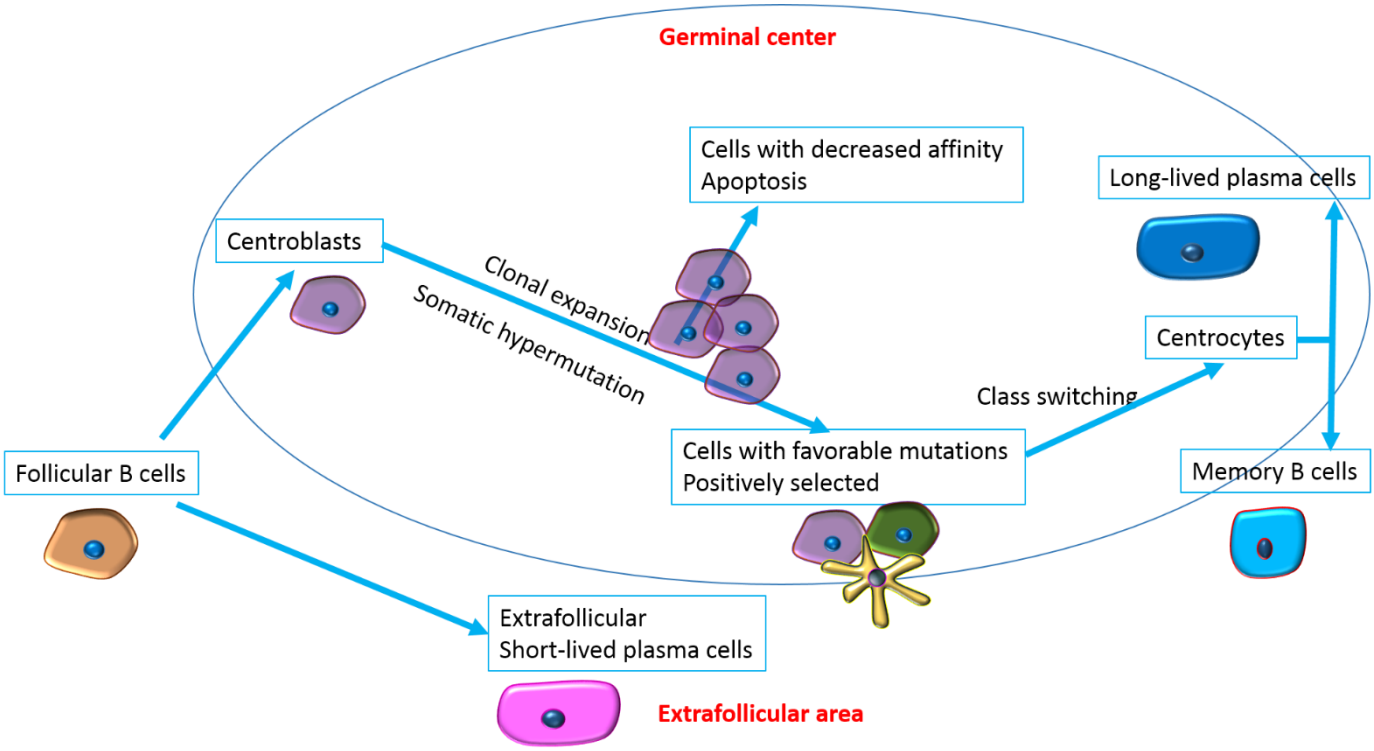


Figure 1.2 Activation and differentiation of follicular B cells in germinal center. Follicular B cells encounter the antigen and are activated by local follicular dendritic cells. Follicular B cells are either differentiated into extrafollicular short-lived plasma cells (primary follicle), or migrate to germinal center to become to centroblasts (secondary follicle). Undergo clonal expansion and somatic hypermutation, some decreased affinity centroblasts are negatively selected and die by apoptosis, while others are positively selected and finally, differentiated into long-lived plasma cells and memory B cells. Adopted from Fig. 3 in reference (Samitas, Lötvall et al. 2010).

1.1.2.3 Allergen-specific antibodies (IgA, IgG, IgE)

Variable (V), diversity (D), and joining (J) gene segments of the immunoglobulin genes randomly connect together to induce diversity of antibody repertoire and also produce unique heavy- and light-chain IgV regions (Hesslein and Schatz 2001), with the V regions determining antigen-specificity to the antibodies (Fig. 1.3). Initially, activated B cells produce IgM/IgD antibodies, but these B cells subsequently undergo Ig heavy-chain class switch recombination (CSR) (Zhang, Alt et al. 1995), and light-chain somatic hypermutation (SHM), to generate high-affinity IgA, IgG and IgE antibodies, each of which carries out distinct functions (Jacobs and Bross 2001; Manis, Tian et al. 2002). There are nine antibody isotypes (within 5 classes) in humans: IgM, IgD, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, and IgE (Spiegelberg 1989). Antibodies that are expressed as secreted (Ig) or membrane-bound (mIg) forms arise from alternate splicing of a common mRNA (Alt, Bothwell et al. 1980).

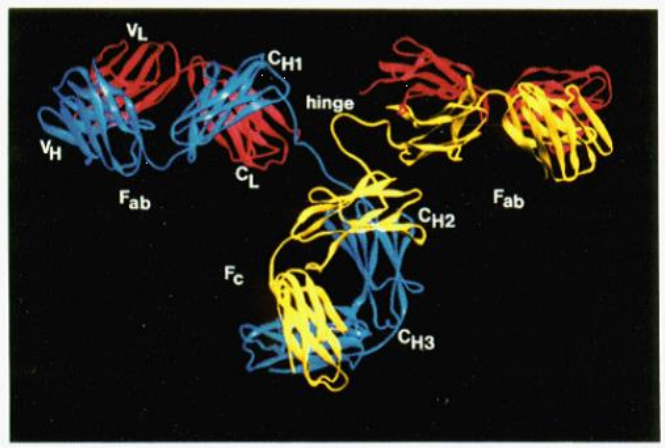


Figure 1.3 Intact monoclonal antibody structure for canine lymphoma. Antibody has a "Y" structure with two identical Fab fragments and one Fc fragment joined by a flexible hinge region. The two Fab fragments form the antigen binding sites. Each antibody molecule has two identical heavy (H) and light (L) chains. In the N-terminal variable (V) domain, VH and VL are in the heavy and light chain, respectively. Constant (C, CL, CH1, CH2, CH3, CH4) domain will give different classes. Adopted from Fig. 1 in (Davies and Chacko 1993). Copyright 1992 Nature (Harris, Larson et al. 1992).

IgA has been mainly defined as a first-line of mucosal immune response, part of the innate mucosal defences (Pilette, Durham et al. 2004). It has the highest rate of synthesis (40–60 $mg \cdot kg^{-1} \cdot day^{-1}$) among the immunoglobulins (Conley and Delacroix 1987), while IgG antibody-producing cells represent about 5%-20% of antibody-secreting cells in the bronchi (Crabbe, Carbonara et al. 1965). Mucosal tissue-secreted IgA is mostly in a dimeric form, while bone marrow plasma cell-derived (serum) IgA is mainly monomeric. Resident cells of the airway wall, like bronchial epithelial cells (Salvi and Holgate 1999), secrete cytokines such as IL-10, IL-2, IL-5, and IL-6 to promote clonal proliferation of antigen-specific, IgA-committed B cells (Fayette, Dubois et al. 1997), although TGF-β also fosters class-switching to IgA (Coffman, Lebman et al. 1989). On the other hand, mast cells and infiltrating Th2 lymphocytes provide IL-4 and/or IL-13 cytokines to support B cell IgE-switching in allergic reactions (Phillips, Everson et al. 1990). IgA triggers phagocytosis, release of oxidants and proinflammatory mediators in the airways (Polat, Laufer et al. 1993), and improves the viscoelastic properties of the airway secretions (Puchelle, Jacqot et al. 1986). Eosinophil activation results in secretion of IL-4, IL-5, among other cytokines (Woerly, Roger et al. 1999). It has been demonstrated that IgA and IgG subclass (IgG3-IgG1) deficiencies are associated with a high risk of chronic obstructive pulmonary disease (COPD) (Oxelius, Hanson et al. 1985). IgG antibody-mediated hypersensitivity activity is displayed in animal models, but it is not evident in humans (Gould, Sutton et al. 2003). It was shown that IgG and IgA are much more efficient in inducing inflammatory mediators release from monocytes than IgE (Spiegelberg 1989).

IgE can be secreted by B cells in the lymph nodes, gut- or airway-associated lymphoid tissues of individuals with atopic or non-atopic asthma (Takhar, Corrigan et al. 2007), food allergy (Coeffier, Lorentz et al. 2005), or allergic rhinitis (KleinJan, Vinke et al. 2000) or any other form of atopy. The IgE-secreting B cells require heavy-chain class switching of germline segment genes from IgM and IgG class to IgE through somatic recombination. Therefore, IgE switching is associated with greater numbers of cell cycle divisions than switching to IgG; in this process, cells can drop out at any differentiation stage to become antibody-secreting plasma cells or to undergo apoptosis-the B cells of allergic individuals preferentially switch to IgE secretion (Gould, Sutton et al. 2003; Stone, Prussin et al. 2010). Compared to a serum half-life of 20 days for IgG, IgE immunoglobulins have a serum half-life of 2 or 3 days. Having said that, IgE that is

bound to the FcεRI on mast cells or basophils is said to be exceptionally long-lived, such that mast cell reactivity may not be reflected by the serum levels of these antibodies. The normal serum concentration of IgE is ~150 ng/ml, compared to 10 mg/ml of IgG in normal individuals (Gould, Sutton et al. 2003). In atopic individuals, the circulating IgE levels may reach over 10-times higher than normal levels, indicating high risk of developing allergies. The high affinity receptor for IgE, FcεRI, is expressed at high levels on mast cell and basophil membranes (200,000 molecules/cell), but much less so on Langerhans cells and other dendritic cells, platelets, monocytes and eosinophils (Gould, Sutton et al. 2003). It is said that cross-linking by allergen of as few as 200 FcεRI-bound IgE molecules on a mast cell is sufficient to fully activate these cells (Galli and Tsai 2012). IgE also binds to low-affinity FcεRII receptors on lymphocytes, monocytes, macrophages (Anderson and Spiegelberg 1981), basophils (MacGlashan, Bochner et al. 1997) and eosinophils (Spiegelberg 1989).

1.1.3 T cells

Naive T cells arise from the thymus and recirculate via the vasculature through the paracortical regions of secondary lymph organs (SLOs) until they detect cognate antigen peptides being presented by antigen-presenting cells. T cells enter lymph nodes from the blood via specialized post-capillary venules (high endothelial venules, HEVs) which express homing molecules such as L-selectin (CD62L), CC-chemokine receptor 7 (CCR7), CC-chemokine ligand 21 (CCL21), lymphocyte function-associated antigen 1 (LFA1; also known as integrin-αL) and intercellular adhesion molecule 1 (ICAM1). HEVs support high levels of lymphocyte traffic under steady-state conditions, with these homing molecules constitutively participating in T cell rolling, activation and arrest (Masopust and Schenkel 2013). In secondary lymph organs, naive T cells scan APCs for cognate antigen peptides being presented on MHC II and, if unsuccessful in this search, they exit the lymph nodes through efferent lymphatics and pass through the thoracic duct into the blood (Gowans 1959). Once naive T cells detect cognate antigen in the lymph nodes, they undergo a pronounced clonal expansion and generate antigen-specific effector T cells that migrate out to the periphery to engage their cognate targets locally. After having served their effector functions, the majority of the activated T cells go through programmed cell death, leaving small numbers of T cells that differentiate into relatively stable memory T cells. Memory

T cells respond very efficiently on subsequent antigen exposure, proliferating and differentiating rapidly once again into effector cells (Kallies 2008). There are also cytotoxic T cells ($CD8^+$ T) and natural killer T cells (NKT), which can destroy virally-infected cells by binding to MHC I molecules and through recognition of glycolipid antigen presented on CD1d, respectively. γδ T cells express a $CD4^-CD8^-$ phenotype and reside as intraepithelial lymphocytes (IEL) in the intestine and genitourinary tract. γδ T cells have potential roles in antimicrobial and antitumor immunosurveillance and immune regulation in human disease(Girardi 2006). In this review, we mainly focus on αβ $CD4^+$ T cells.

1.1.3.1 T cell subsets

Short-lived $CD4^+$ T cells play central roles in adaptive immune responses in a variety of settings, including autoimmunity, asthma, tumour immunity and allergic responses. Following antigen activation and costimulatory signals, $CD4^+$ T cells differentiate into one of several distinct subsets of T helper (Th) cells, including Th1, Th2, Th17, T_{FH}, Th22, or Treg, as characterized by their cytokine profiles and functions. Other subsets, including IL-10-producing Tr1 cells, TGF-β-producing Th3 cells, and IL-9 producing Th9 cells were identified to suppress Th1, Th2, Th17 immune responses and induce tolerance (Zhu, Yamane et al. 2010). Overlapping cytokine secretion and master transcription factor profiles became important additional lineage-defining identifiers for differentiated Th cells subsets (Baumjohann and Ansel 2013). Memory $CD8^+$ and $CD4^+$ T cells can be defined as $CD44^+CD62L^+CCR7^+$ central memory T cells (T_{CM}) and $CD44^+CD62L^-CCR7^-$ effector memory T cells (T_{EM}) based on their distinct chemokine receptors and homing molecules (Sallusto, Lenig et al. 1999).

1.1.3.2 Cytokines produced by T cell subsets

Cytokines play critical roles in the differentiation and effector functions of $CD4^+$ T cells. Upon TCR activation via cognate antigen presentation, naive $CD4^+$ T cells differentiate into distinct Th lineages with unique cytokine receptors, such that they can respond to cytokines produced by accessory cells (Table 1.3). Th1 and Th2 clones were mainly define by their cytokines and surface molecules. Th1 cells produce interferon (IFN)-γ, IL-2, TNF-α and lymphotoxin, which

are required for clearance of intracellular pathogens. IL-12, produced by dendritic cells, is a critical in promoting T-bet and IFN-γ production, as well as Th1 cell proliferation and cellular survival. Th2 cells produce IL-4, IL-5, IL-9, IL-13 and TNF-α, and mediate immune responses against parasites and allergens. Th2 cells also produce modest amount of IL-2, which is important in autocrine regulation of $CD4^+$ T cell metabolism and proliferation. Th17 cells produce IL-17A, IL-17F, IL-6, TNF-α and IL-22, which are thought to be signature cytokines in immunity against extracellular bacteria and fungi. IL-17 is important for recruitment of neutrophils, eosinophil infiltration, and survival of macrophages (Pulendran, Tang et al. 2010). Although IL-21 is produced by Th17 cells, other Th cells can also secret IL-21 (Korn, Bettelli et al. 2007). IL-23 is critical in development and/or maintenance of Th17 cells (Bettelli, Carrier et al. 2006). In the presence of TGF-β and IL-2, $CD4^+$ T cells can differentiate into Treg, with these cytokines being critical for cellular development, function and homeostasis (Akkoc, Akdis et al. 2011). As noted above, a major function of CD4 T cells is to provide help for B cell antibody production, class switch recombination and somatic hypermutation in germinal centres. Those CD4 T cells are T_{FH} cells, which can be IL-4-secreting cells which provide help for B cells in follicles and germinal centers (Reinhardt, Liang et al. 2009). Others have reported that either IL-4 or IFN-γ is secreted by T_{FH} that interact with B cells in the germinal center, depending on how they were primed (King, Tangye et al. 2008). Th22 cells are characterized by secretion of IL-22 and TNF-α (Eyerich, Eyerich et al. 2009). Both $CD4^+$ and $CD8^+$ T_{EM} can differentially produce IFN-γ, IL-4 and IL-5 following antigen stimulation. While T_{CM} produce IL-2, but after stimulation T_{CM} efficiently differentiate into effector cells which produce elevated levels of IFN-γ or IL-4 (Sallusto, Geginat et al. 2004). IL-7 and IL-15 were defined as cytokines important to the maintenance, survival and homeostasis of memory T cells (Sprent, Cho et al. 2008).

1.1.3.3 Transcription factors for each T cell lineage

Master transcription factors and the signalling transducer and activator of transcription (STAT) proteins regulate cytokine secretion and Th cell fate determination (Table 1.3). T-bet is the main transcription factor for Th1 cell differentiation and IFN-γ production (Szabo, Kim et al. 2000). Over-expression of T-bet in Th2 cells results in these cells acquiring competence to produce IFN-γ, with a loss of their ability to produce IL-4. $Tbx21^{-/-}$ (T-bet-knockout) mice spontaneously

develop airway hypersensitivity, while T-bet-expressing T cells are significantly reduced in human asthmatic airways (Finotto, Neurath et al. 2002). IFN-γ-induced STAT1 activation is very important for T-bet induction of Th1 differentiation *in vitro* (Lighvani, Frucht et al. 2001; Afkarian, Sedy et al. 2002). STAT2 can combine with STAT1 to form a heterodimer in response to type I IFNs (Park, Li et al. 2000). Activation of STAT4 can directly induce IFN-γ production and T-bet during Th1 differentiation (Usui, Preiss et al. 2006).

GATA3 was the first identified master regulator of Th2 cells (Zheng and Flavell 1997). It promotes Th2 commitment, suppressing Th1 cell differentiation and thereby selectively stimulating Th2 cell growth (Zhu, Yamane et al. 2006). During Th2 or Th1 differentiation, GATA3 expression is up- or down-regulated, respectively (Zhang, Cohn et al. 1997), but it also has roles in developing CD4 T cells and naive CD4 T cells (Ho, Tai et al. 2009). Fully differentiated Th2 cells in which GATA3 expression has been reduced express very little IL-4, but display completely blocked IL-5 and IL-13 production, suggesting that GATA3 regulates each of these cytokines in Th2 cells (Zhu, Min et al. 2004). STAT5 is also important to Th2 cells; low levels of STAT5 are sufficient for cell survival and differentiation, while strong STAT5 signalling is required for Th2 cell division (Zhu, Cote-Sierra et al. 2003). STAT6 is both a major inducer of Th2 cell expansion and differentiation (Kaplan, Schindler et al. 1996), and is necessary and sufficient to induce high levels of GATA-3 in Th2 cells (Kurata, Lee et al. 1999).

Th17 cells express high levels of the transcription factor retinoic acid receptor-related orphan nuclear receptor gamma t (RORγt), the Th17 cell master regulator (Ivanov, McKenzie et al. 2006). Over 50% of activated Th17 cells expressing RORγt produce IL-17, although RORα, a related nuclear receptor which is also upregulated on Th17 cells, promotes IL-17 production by RORγt-deficient cells (Yang, Pappu et al. 2008). The cytokines IL-6, IL-21 and IL-23, which are involved in Th17 cell differentiation, expansion and maintenance, activate STAT3 (Zhou, Ivanov et al. 2007). IL-2 is also required for the expansion of differentiated Th17 cells (Amadi-Obi, Yu et al. 2007).

Bcl-6 is necessary and sufficient to induce T_{FH}-associated molecules, including CXCR5 (a critical B cell follicle homing receptor) (Breitfeld, Ohl et al. 2000), programmed death (PD)-1, IL-6R, and IL-21R (Nurieva, Chung et al. 2009) but, reciprocally, it also suppresses Th1, Th2, and Th17 cytokine production. STAT5 can also regulate the activity of Bcl-6 in B cells, and therefore may be important for T_{FH} cell differentiation (Scheeren, Naspetti et al. 2005).

Foxp3 is a master transcription factor for at least some populations of Treg and it is required for those cells to maintain their suppressive activity (Williams and Rudensky 2007). Moreover, conventional T cells acquire a Treg phenotype, including an inability to produce Th cytokines (anergy) and a Th suppressor activity, by enforced expression of Foxp3 (Fontenot, Gavin et al. 2003). Repeated TCR signalling in $Foxp3^-$ naive CD4 T cells combined with simultaneous exposure to TGF-β converts these cells into (induced) $Foxp3^+$ CD4 T cells, also known as iTregs (Chen, Jin et al. 2003). STAT3 activation by IL-6 can downregulate Foxp3 expression in Tregs (Korn, Bettelli et al. 2007), whereas STAT5 signalling contributes to Foxp3 induction (Burchill, Yang et al. 2007).

A number of transcription factors have been implicated in memory T cells, including T-bet, eomesodermin (Eomes), Bcl-6 and Blimp-1. T-bet and Eomes belong to the T-box family of transcription factors which cooperate to promote $CD8^+$ memory T cell survival and generation, while Blimp-1, induced by IL-2, has come to light as a critical transcription factor in guiding effector/memory T cell homeostasis, such that Blimp-1-deficient mice show severely impaired T cell-mediated immune responses (Kallies, Hawkins et al. 2006). Bcl-6 is an essential transcription factor for survival and maintenance of germinal centre B cells, where it is proposed to suppress Blimp-1 expression. However, the role of Bcl-6 in activation of $CD8^+$ T cells may not be identical to its role in germinal centre B cells. For instance, germinal centre B cells are not detected in Bcl-$6^{-/-}$ mice, while modest numbers of memory $CD8^+$ T cells can be found in these mice (Kallies 2008).

Table 1.3 $CD4^+$ T cell subsets, cytokines and transcription factors

$CD4^+$ T cell subsets	Cytokines	Transcription Factors
Th1 cell	IFN-γ, IL-2, TNF-α, lymphotoxin	T-bet, STAT1, STAT2, STAT4
Th2 cell	IL-4, IL-5, IL-9, IL-13, TNF-α, IL-2	GATA3, STAT5, STAT6
Th17 cell	IL-17A, IL-17F, IL-6, TNF-α, IL-22	RORγt, STAT3
TFH cell	IL-4, IFN-γ	Bcl-6, STAT5
Th22 cell	IL-22, TNF-α	undetermined
Treg	IL-10	Foxp3, STAT3, STAT5
Tm	undetermined	T-bet, Eomesodermin (Eomes), Bcl-6, Blimp-1
Th3 cell	TGF-β	undetermined
Th9 cell	IL-9	undetermined
Tr1	IL-10	c-Maf

1.1.4 Mast cells

1.1.4.1 Development and proliferation

Mast cells are derived from bone marrow hematopoietic stem cells and circulate as immature progenitor cells. They differentiate and mature *in situ* in vascularized tissues and serosal cavities in which they reside. They are widely distributed throughout vascularized tissues, especially close to blood vessels, smooth muscle cells, nerves, etc., and particularly near surfaces exposed to the environment (e.g., skin, airways and gastrointestinal tract). Thus, they are one of the first cells to recognize allergens, but only if the cells are armed with antigen-specific IgE molecules. The main factors that influence mast cell proliferation and phenotype are the stem cell factor (SCF) ligand for c-Kit, IL-3, the Th2-associated cytokines IL-4 and IL-9, as well as some other growth factors, cytokines and chemokines (Kawakami and Galli 2002). It has been shown that c-Kit-deficient mice or (WB×C57BL/6) F1-W/W^{v} (WBB6F1-W/W^{v}) are selectively deficient in mast cells, but can be reconstituted by adoptive transfer of genetically-compatible mast cells generated *in vitro* (Tsai, Tam et al. 2002). The phenotype of such adoptively transferred mast cells is strikingly affected by local microenvironment factors. For instance, mast cells in the peritoneal cavity, skin, spleen and the muscularis propria of the glandular stomach exhibit connective tissue-type mast cell features (CTMC). By contrast, the mast cells within the mucosa of the glandular stomach are identified as mucosal mast cells (MMC) (Nakano, Sonoda et al. 1985).

1.1.4.2 Activation and secretion of mediators

Mast cells can be activated by a variety of stimuli to release a diverse array of biologic products, which mediate potent inflammatory or immunoregulatory effects. Depending on the type and strength of the stimulus, mast cells can release distinct kinds of mediators, such as performed mediators (i.e. histamine), lipid-derived mediators (i.e. PGE2, LTB4, etc.), cytokines (i.e. IL-1, IFN-γ, TNF, etc.), chemokines (i.e. CCL3, CCL20, etc.), free radicals (i.e. nitric oxide, etc.), and so on (Gonzalez-Espinosa, Odom et al. 2003; Galli, Kalesnikoff et al. 2005). In asthma, those mediators induce contraction of the airway smooth muscles, vasodilation, increased vascular permeability and mucus overproduction, but also recruit circulating inflammatory cells

(e.g. leukocytes, predominantly neutrophils) (Wershil, Wang et al. 1991). If the response is systemic, it can lead to anaphylaxis, a potentially catastrophic immune response resulting in death in individuals without access to immediate medical attention (i.e., epinephrine injection).

There are high- and low-affinity IgE receptors, which are critical for IgE binding. CD23 (FcεRII, the low-affinity IgE receptor) is a C-type lectin which is present in a membrane-bound form on epithelial cells, B cells and myeloid cells, mediates robust and diverse IgE responses to antigens (Gould and Sutton 2008). As noted above, cross-linking of the high affinity IgE receptors on mast cells (FcεRI) drives allergen-dependant cellular activation (Kawakami and Galli 2002) and thereby the pathology of allergic disorders like anaphylaxis and asthma (Williams and Galli 2000). In principle, at least two FcεRI-bound IgE molecules must be cross-linked or 'aggregated' on the surface of mast cells (or basophils) to initiate anaphylactic cell degranulation, resulting in hypersensitivity responses within minutes of antigen exposure (Galli and Tsai 2012). Antigen-IgE complexes can also activate mast cells, basophils, DCs and macrophages, thus promoting allergic inflammation. Once activated, mediators released by these cells (e.g., histamine, TNF, PDG2) can in turn promote the recruitment and maturation of DCs to support further allergen-specific responses (Galli and Tsai 2012).

Mast cells are also key effector cells in Th2 and IgE-associated immune responses. IFN-γ-stimulated human mast cells express the FcγRI, but not the FcγRIII, suggesting that mast cells are involved in certain IgG-dependant responses in humans as well (Woolhiser, Brockow et al. 2004). In addition, another important function of mast cells is to initiate innate immunity against microbial invasion through Toll-like receptor (TLRs), complement receptor, endogenous peptide, cytokine, etc., signalling. (Marshall 2004). It was shown that, by presenting bacterial antigens on MHC I, mouse bone marrow-derived cultured mast cells (BMCMCs) can promote antigen-specific T cell activation (Malaviya, Twesten et al. 1996).

1.1.4.3 Influence of mast cells on DCs, T cells or B cells

Many observations suggested that mast cell products, including TNF, IL-1 (Cumberbatch, Dearman et al. 2000), IL-16 (Kaser, Dunzendorfer et al. 1999), CCL19 (Robbiani, Finch et al. 2000), and prostaglandin E1 (Kabashima, Sakata et al. 2003), can foster DC migration, maturation and function. Histamine and prostaglandins E2 and D2 (PGE2 and PGD2) (Bochenek,

Nagraba et al. 2001) can inhibit IL-2 production by DCs, leading to the polarization of Th2 cells. Thymic stromal lymphopoietin (TSLP) from mast cells can promote DC modulation and the induction of CCL17 (also called TARC), a chemokine that induces Th2 cell migration (Soumelis, Reche et al. 2002). It has also been shown that IgE and antigen-stimulated skin mast cells secrete histamine, which promotes H2 receptor-dependent Langerhans cell migration to lymph nodes (Jawdat, Albert et al. 2004).

T cell recruitment to local sites of inflammation is affected by local expression of adhesion molecules and chemokines. Mast cells can directly promote T cells migration via their chemotactic factors, including IL-16, CCL2, CCL20, CXCL10 (Nakajima, Inagaki et al. 2002; Sayama, Diehn et al. 2002; Lin, Maher et al. 2003), or indirectly by up-regulation of cell surface molecules such as E-selection or vascular cell adhesion molecules I (VCAM1) on endothelial cells (Marshall 2004). Many mediators released by mast cells also contribute to the polarization and phenotype of T cells. For instance, histamine facilitates Th1 cell activation by H1 receptors, while H2 receptor signalling inhibits Th1 and Th2 cell activation (Jutel, Watanabe et al. 2001).

The interactions of CD40 expressed on B cells with CD40L (also called CD154) on T cells is very important for antibody class-switch and germinal center formation in secondary lymphoid organs (Kawabe, Naka et al. 1994). Similarly, certain tissue mast cells express CD40L, and can thereby interact with B cells leading to IgE-switching in the presence of IL-4 or adenosine, but without help from T cells (Galli, Nakae et al. 2005).

1.1.5 Basophils

1.1.5.1 Development and differentiation

Basophils are rare circulating granulocytes that develop from $CD34^+$ hematopoietic progenitors but, unlike mast cells, they complete their maturation in the bone marrow and circulate in the periphery as fully mature cells. Basophils comprise about less than 1% of peripheral blood leukocytes and have a half-life of approximately a few days (Falcone, Haas et al. 2000). IL-3 is a key differentiation factor for basophils, and is sufficient to drive stem cell differentiation into basophils (Stone, Prussin et al. 2010). Phenotypically, unlike mast cells and eosinophils, basophils lack expression of the surface marker c-Kit (CD117) and the chemokine receptor CCR3. They are rarely found in normal tissues (Marone, Triggiani et al. 2005), but are present at

considerably increased numbers at sites of allergic inflammation in asthmatic individuals or atopic dermatitic patients (Gauvreau, Lee et al. 2000; Macfarlane, Kon et al. 2000). Basophils share many features with mast cells, including secretion of Th2 cytokines (IL-4, IL-13), histamine, and expression of the high-affinity IgE receptor (FcεRI) and lipid mediators, indicating that they are involved in immune responses (Stone, Prussin et al. 2010).

1.1.5.2 Activation, mediators and function

Basophils express FcεRI, the expression levels of which correlate with circulating IgE levels. Cross-linking of FcεRI-bound IgE leads to basophil activation, granule exocytosis and mediator release. Stimulation via several chemokines or cytokines (e.g., IL-3, IL-5, IL-33 [a member of the IL-1 superfamily]) augments basophil degranulation and IL-4/IL-13 secretion following FcεRI activation (Pecaric-Petkovic, Didichenko et al. 2009), but the anaphylatoxin C5a can activate basophils by itself (Stone, Prussin et al. 2010). Innate immune response signalling in basopils has been investigated; TLR2 ligands stimulation of basophils leads to NF-κB nuclear localization and cytokine production (Bieneman, Chichester et al. 2005).

Histamine is the major preformed mediator secreted by basophils, while heparin is expressed at lower levels in basophils relative to mast cells, although both express similar levels of tryptase. After activation, basophils rapidly produce LTC4, LTD4 and LTE4 (but not PGD2), which can increase vascular permeability and bronchoconstriction. IL-4, IL-13, and CD40L expression by basophils have been reported to drive B cell IgE class-switching, such that neutralizing IL-4 or CD40L can abrogate IgE synthesis. Activated basophils upregulate CD40L, which engages CD40 expressed on B cells to foster IgE production (Yanagihara, Kajiwara et al. 1998).

Basophils facilitate the development of $CD4^+$ T cell, eosinophil, neutrophil and B cell responses through the many cytokines they secrete. Basophil secretion of IL-4 can induce naïve T cells to develop into Th2 cells - little Th2 differentiation is observed in an IL-4-deficient basophil coculture system (Denzel, Maus et al. 2008). After IgE cross-linking, human basophils secrete IL-25 (IL-17E), which is critical in allergic inflammation (Wang, Angkasekwinai et al. 2007), indicating that basophils may augment the activation of Th2 memory T cells via such IL-25 expression. Studies using IL-4 promoter-enhanced green fluorescent protein (GFP) transgenic mice demonstrated that basophils rapidly secrete IL-4, IL-13 after activation (Hu-Li,

Pannetier et al. 2001) but, as also occurs with mast cells and eosinophils, IL-4 or IL-13 are not secreted without such stimuli (Gessner, Mohrs et al. 2005). In IgE-mediated chronic allergic dermatitis, eosinopils and neutrophils infiltration is critically dependent on the presence of basophils (Obata, Mukai et al. 2007). Chronic allergic inflammation is certainly supported by basophils through antigen, IgE and FcεRI signaling, and that is independent of any direct involvement by T cells or mast cells (Mukai, Matsuoka et al. 2005). Depletion of basophils ameliorates IgG-mediated passive anaphylaxis in mouse models, indicating a distinct pathway of allergen-induced systemic anaphylaxis mediated by basophils, IgG and platelet-activating factor (PAF) (Tsujimura, Obata et al. 2008). Basophils may also contribute to enhance memory B cell responses through their capture of large amounts of intact antigens on their surface and secretion of IL-4 and IL-6 after restimulation of soluble antigen (Denzel, Maus et al. 2008). IL-4, histamine, leukotriene C4, generated by basophils, are thought to be primary drivers of acute and chronic allergic inflammation symptoms (Marone, Triggiani et al. 2005).

1.1.6 Eosinophils

1.1.6.1 Development and circulation

Blood and tissue eosinophilia is a hallmark of many disorders, including allergies, asthma, helminth infections, eosinophilic gastrointestinal disorders, hypereosinophilic syndrome, etc. Increased accumulation of eosinophil progenitors is an important checkpoint in disease-associated eosinophilia in allergic asthma (Sehmi, Dorman et al. 2003; Mori, Iwasaki et al. 2009). After being formed in the bone marrow, eosinophils are released into the circulation, where they normally comprise less than 1-5% of peripheral blood leukocytes (Fulkerson and Rothenberg 2013). Committed eosinophil progenitors develop from $CD34^+$ hematopoietic stem cells with the help of IL-3, GM-CSF, and in particular IL-5, which promotes eosinophil development, expansion, and recruitment. The eosinophil lineage is identified by cellular expression of CD34, CCR3 and the IL-5 receptor. During allergic responses or helminth inflammation, IL-5 secreted at the site of inflammation causes eosinophil release from bone marrow, although the CCR3 receptor ligand CCL11/eotaxin-1, which is induced following allergen challenge, can also cause eosinophil release (Stone, Prussin et al. 2010). Once released, IL-5-stimulated eosinophils enter the circulation and are attracted to the sites of allergic

inflammation, where they have a half-life of 8-18 hours. IL-4 and IL-13 expressed at gastrointestinal mucosal surfaces or at Th2-dominated sites of inflammation fosters local eosinophil recruitment through upregulation of eotaxin (CCL11 and CCL26) or vascular adhesion molecule-1 expression. In addition, PAF, LTD2, C5a and CCL5 are potential eosinophil chemotactic factors (Stone, Prussin et al. 2010).

1.1.6.2 Activation mediators and function

Many mediators, such as IL-3, IL-5, CC chemokines, PAF and GM-CSF, can promote eosinophil activation. Similar to mast cells and basophils, cross-linking of IgG or IgA Fc receptors can activate eosinophils. CCR3, a chemokine receptor abundantly expressed on eosinophils, promotes their accumulation, while eotaxin-induced CCR3 activation induces eosinophil degranulation and upregulation of adhesion molecules (Kampen, Stafford et al. 2000). The α4β1 integrin is an adhesion molecule that is also important for eosinophil accumulation in the lung in response to allergen (Nakajima, Sano et al. 1994). The CCR3-CCL11/eotaxin-1 axis (Ahrens, Waddell et al. 2008) and CRTH2 (chemoattractant receptor of Th2 cells, also known as PTGDR2) (Hirai, Tanaka et al. 2001) are important for eosinophil recruitment in experimental asthma models and eosinophilic gastrointestinal disorders. PGD2 activates eosinophils through the CRTH2 (Heinemann, Schuligoi et al. 2003), resulting in eosinophil recruitment, activation and mobilization from the bone marrow.

Once activated, eosinophils release proinflammatory mediators, granule-stored cationic proteins, cytokines and newly-synthesized eicosanoids. Mature human eosinophils contain considerable numbers of crystalloid secondary granules, composed of highly-charged cationic protein, neurotoxin, peroxidase and two eosinophil granule major basic proteins (MBPs) (Hogan, Rosenberg et al. 2008). Those granules contribute to tissue damage, organ dysfunction and tissue remodeling in asthma and anti-viral responses (Domachowske, Dyer et al. 1998). In addition to these cationic proteins, eosinophils release lipid-derived mediators, including LTC4, PGE2, PAF and thomboxane, but also low levels of cytokines, chemokines, enzymes, or growth factors, contributing to a diverse array of biological activities in infection and inflammation (Muniz, Weller et al. 2012). Those cytokines are stored within the cellular granules and are released on degranulation (Stone, Prussin et al. 2010). IL-4 has the ability to stimulate adaptive immunity

upon eosinophils activation. Clinical studies and animal models of asthma have indicated that eosinophils play causal roles in airway remodeling, hyperresponsiveness and mucus overproduction (Blanchard and Rothenberg 2009). That is, eosinophil lineage-deficient mice are protected against asthma phenotype pathology (Lee, Dimina et al. 2004). IL-5 strongly induces signal transduction relating to eosinophil maturation, survival (Takatsu 2011) and peripheral and tissue eosinophilia (Haldar, Brightling et al. 2009). IL-13 is a key cytokine in recruitment of eosinophils to inflammatory sites in animal models (Pope, Fulkerson et al. 2005), as well as in humans in response to airway allergen challenge (Prieto, Lensmar et al. 2000). IL-25 and IL-33, secreted as Th2-like cytokines, primarily activate basophils and eosinopils and promote allergic phenotype (Pecaric-Petkovic, Didichenko et al. 2009).

1.1.7 Lung structural cells

1.1.7.1 Epithelial cells are the front-line of allergen sensitization

Airway epithelial cells (ECs) and their tight junctions constitute an important physical barrier and the first line of defence in response to lung challenge by microorganisms, allergens and viruses, being positioned at the interface between the environment and the host (Xiao, Puddicombe et al. 2011). Airway remodeling, or structural changes associated with a progressive decline of lung function in asthmatics, includes smooth muscle hypertrophy around the airway wall, thickening of the basement membrane caused by deposition of extracellular matrix components, a breach in epithelial integrity, and mucus-producing goblet cell hyperplasia (Lambrecht and Hammad 2012). The airway epithelium is critical for immune responses to allergens, viruses and environmental changes, and contributes to asthma pathogenesis. By expression of many pattern recognition receptors (PRRs) such as the TLRs, NOD-like receptors, C-type lectins, and protease-activated receptors (PAR), epithelial cells detect environmental stimuli and translate those signals to the DCs that link innate and adaptive immunity. PRRs can rapidly recognize and respond to pathogen-associated molecular patterns (PAMPs) of pathogens or damage-associated molecular patterns (DAMPs) associated with dying cells and tissue damage, leading to release of cytokines, chemokines and antimicrobial peptides (Hertz, Wu et al. 2003; Finkelman, Lempitski et al. 2006; Uehara, Fujimoto et al. 2007; Page, Ledford et al. 2010). Recently, EC activation was recognized as a key trigger in responses to inhaled allergens,

activating local DCs (Lambrecht and Hammad 2009), which subsequently migrate to draining lymph nodes and present antigen to T cells (Lambrecht and Hammad 2012). Exposure to PAMPs and DAMPs promotes activation of ECs and thereby can trigger both innate and adaptive immune responses. The activation of ECs by PRRs is regulated by down-stream signalling intermediates such as NF-κB, which controls expression of an array of inflammatory cytokine genes (Poynter, Irvin et al. 2002).

Influence of ECs on immune cells

There have been many studies directed at understanding the mechanisms by which ECs act on DC. House dust mite (HDM)-triggered activation of TLR4 on ECs induces production of TSLP, IL-25, IL-33 and GM-CSF(Chustz, Nagarkar et al. 2011). Proteinase-activated receptor-2 (PAR-2) is a critical element in regulation of matrix metalloproteinase-9 (MMP-9) release by airway ECs (Vliagoftis, Schwingshackl et al. 2000). IL-33-activated DC exacerbate OVA-induced Th2 immune responses in allergic lung inflammation (Besnard, Togbe et al. 2011). TSLP can stimulate bronchial EC proliferation and IL-13 production (Semlali, Jacques et al. 2010), while over-expression of TSLP and GM-CSF in the lungs of mice induces spontaneous Th2 sensitization to the otherwise innocuous protein ovalbumin (OVA) (Stämpfli, Wiley et al. 1998; Zhou, Comeau et al. 2005). CC chemokine ligand 2 (CCL2) and CCL20 expression by airway ECs attracts monocytes and immature DCs to migrate into the lung during HDM inhalation (Hammad, Chieppa et al. 2009; Nathan, Peterson et al. 2009), while it is known that homodimers of IL-12p40 released by bronchial ECs are chemotactic for monocytes and DCs (Walter, Kajiwara et al. 2001).

Cytokines secreted by ECs also activate other immune cells, including basophils, eosinophils, innate lymphoid cells and mast cells (Fort, Cheung et al. 2001; Schneider, Petit-Bertron et al. 2009; Neill, Wong et al. 2010; Saenz, Siracusa et al. 2010). Bone marrow basophils can differentiate under the influence of, and be activated by, TSLP (Siracusa, Saenz et al. 2011), while IL-4 secreted by basophils can further promote Th2 cell responses (Hammad, Plantinga et al. 2010). GM-CSF and the chemokines IL-8, CCL11, and CCL17 produced by ECs can attract neutrophils, eosinophils, and CD4 Th2 cells, respectively, in response to the Th2 cytokines IL-4 and IL-13 (Matsukura, Stellato et al. 2001; Lordan, Bucchieri et al. 2002).

1.1.7.2 Smooth muscle cells

As noted, airway remodeling is a key feature of asthma, and part of that is increases in the numbers of airway smooth muscle (ASM) surrounding the airway wall, leading to airway narrowing. It was found that ASM cells from asthmatic patients proliferate faster in culture than those obtained from non-asthmatic individuals, suggesting that they are in an activated state (Johnson, Roth et al. 2001). Mediators in bronchoalveolar lavage (BAL) fluids from asthmatic individuals are mitogenic for ASM cells, and display enhanced mitogenic activity after allergen challenge (Naureckas, Maurice Ndukwu et al. 1999). Through passive sensitization of human ASM, the IgE found in sensitizing serum increases ASM cell proliferation and cytokine production (e.g. TNF-α) (Black and Johnson 2000), indicating that ASM cells are not simply a physical barrier in asthma.

1.1.7.3 Macrophages

Macrophages are the most abundant leukocytes found in the lungs, which harbour three subsets of these cells: bronchial macrophages, alveolar macrophages and interstitial macrophages. Alveolar macrophages (AMs) are prominent immune cells found in the airways. AMs are derived from circulating blood monocytes. Monocyte chemoattractant protein-1 (MCP-1) is critical in recruitment of monocytes into inflamed tissue. AMs can fight against pathogens by multiple immunological pathways, or manifest anti-inflammatory response to tissue damage. For instance, adoptive transfer of AMs from unsensitized mice significantly ameliorated airway hyperresponsiveness and inflammation in asthma. Intravenously administered antigen-pulsed macrophages migrate to the spleen where they induced a long-term immunosuppressive response through induction of Tregs. Moreover, AMs express lower levels expression of co-stimulatory molecules, (CD80 and CD86), and elevated levels of IL-10 in non-asthmatic people than asthmatics. However, a lot of literature indicates that AMs have pro-inflammatory roles in asthma. Activated neutrophils are recruited to alveolar space by AMs and undergo programmed cell death. Apoptotic neutrophils are recognized and phagocytosed by AMs, resulting in relieved pulmonary inflammation. Thus, macrophages in allergic asthma participate in the maintenance of homeostasis through their anti-inflammatory responses, but also at times through their abilities to promote inflammation (Balhara and Gounni 2012).

1.2 Asthma therapy

1.2.1 Inhaled corticosteroids (ICSs)

All levels of persistent asthma require daily anti-inflammatory treatment. Once diagnosed, according to the frequency of symptoms or degree of impairment in pulmonary function, an asthma severity classification can be determined, classifying the patient as having mild intermittent, mild persistent, moderate persistent, or severe persistent disease. Based on the severity, daily inhaled corticosteroid (ICS) treatments may be recommended. Currently, ICS therapy is the most effective and safest treatment for persistent asthma; it is also the first line therapy for reducing the need for systemic corticosteroids in acute asthmatic patients. Precisely how ICSs suppress the inflammatory response is not fully clear, but they do affect a variety of inflammatory pathways. By binding to glucocorticoid receptor on respiratory epithelial cells, ICS molecules enter the nucleus of those cells, where they induce transcription of genes encoding proteins (i.e. IL-10) that decrease inflammation and inhibit transcription of Th2-fostering genes (IL-4, IL-5, IL13, TNF-α) (Fong and Levin 2007).

ICSs also decrease cytokine production by other immune cells, such as mast cells, T lymphocytes and eosinophils, but they may upregulate β-adrenergic receptors on ASM cells, modulating their secretion of chemokines and cytokines that drive ASM proliferation and function (Fernandes, Mitchell et al. 2003; Mauad, Bel et al. 2007). If properly administered, there are almost no side effects of ICSs, but potential complications include the development of oral candidiasis, which is preventable, or bronchospasms, which can be managed by β2-adrenergic agonist pretreatment (Fong and Levin 2007). Due to the shallow dose-response curve for ICSs, patients usually do better with delivery of add-on medications rather than increases in the doses of ICSs. The most effective add-on medications are the long-acting inhaled β-agonists (Pawankar, Canonica et al. 2011), which improve asthma control and reduce exacerbations, unlike short-acting inhaled β2-agonists (McFadden Jr 1995). Low doses of ICSs can decrease the risk of death from asthma (Lipworth 1999).

For severe corticosteroid-dependent asthma patients, etanercept and the anti-TNF-α antibody infliximab are two powerful TNF-α neutralization approaches (Howarth, Babu et al. 2005). The

levels of TNF-α, its receptor, and converting enzyme are upregulated in BAL and blood monocytes of asthma patients. Etanercept, a monoclonal antibody specific for TNF-α that prevents free TNF from binding to its receptors, decreases airway hyperresponsiveness and asthma symptoms and increases lung function in asthmatic patients (Howarth, Babu et al. 2005; Berry, Hargadon et al. 2006). In murine models of acute airway inflammation, the inhibitors of TNF-α-converting enzyme significantly decrease lung inflammation (Trifilieff, Walker et al. 2002). By binding and neutralizing the soluble TNF-α homotrimer and its membrane-bound precursor, Infliximab decreases moderate exacerbations and reduces sputum inflammatory cytokine levels in moderate asthma patients, but has no effect on lung function (Erin, Leaker et al. 2006).

1.2.2 Allergen Specific Immunotherapy-SIT

Allergen specific immunotherapy (SIT) is a clinically proven therapy for some allergic immune disorders, whereby functional tolerance to allergic symptoms can be achieved. SIT involves repeated (e.g., weekly) subcutaneous injection of continuously escalating sub-immunogenic doses of allergenic extract over up to years, which minimizes unintentional allergic reactions, especially anaphylaxis. The mucosal route for administration of allergen has been used with grass pollen extracts in allergic individuals (Akkoc, Akdis et al. 2011).

In house dust mite allergic asthma patients, sublingual SIT improves lung function and bronchial hypersensitivity (Bousquet, Scheinmann et al. 1999). Pollen immunotherapy effectively decreases IgE-mediated seasonal allergic rhinitis symptoms (Durham, Walker et al. 1999), while a standardized cat dander extract can be effective for cat allergen-related rhinitis (Varney, Edwards et al. 1997). However, some studies have shown that local bronchial SIT fails in reducing symptom scores, although there was reduced early bronchial sensitivity and drug consumption after immunotherapy (Crimi, Voltolini et al. 1991).

After successful SIT immunization, both naturally-occurring and inducible antigen-specific regulatory T cells (Tregs) are generated, and these secrete elevated levels of IL-10 and TGF-β and suppress Th1, Th2, and Th17 responses. Moreover, antigen-specific B cell undergo class-switching to secrete IgG4, rather than IgE antibodies. IgG4 can engage allergens,

preventing them from cross-linking of IgE on the FcεRI of mast cells, eosinophils and basophils (Akkoc, Akdis et al. 2011), inhibiting release of mediators and thereby the Th2 cell homing which contributes to type I hypersensitivity. IL-10-producing Tregs (i.e., Tr1 cells) can be induced by SIT therapy in humans. For instance, mucosal immunotherapy induces Tr1 cells by inducing IL-10 and TGF-β responses to HDM, birch pollen or food allergens (Jutel, Akdis et al. 2003). Even though sublingual immunotherapy (SLIT) is less effective than subcutaneous immunotherapy (SCIT), SLIT seems relatively safer compared to SCIT in allergic disease (Gidaro, Marcucci et al. 2005). In SLIT therapy, local oral mucosal epidermal dendritic cells (i.e., Langerhans cells) take up allergen via their FcεRI (Allam, Novak et al. 2003), leading to production of IL-10 and induction of Tregs *in vitro* (Allam, Peng et al. 2008). Although peripheral T cell tolerance can be induced by SIT, it is still not clear whether B cell tolerance occurs or not, particularly as SIT tolerance seems dependent on continued expression of IgG4.

1.2.3 Anti-IgE antibody-omalizumab

It is well known that IgE antibody plays a critical role in chronic allergic airway inflammation, as it is associated with triggering of multiple types of immune cells, including eosinophils, mast cells, and basophils, via the high (FcεRI) or low (FcεRII) affinity IgE receptors. Elevated serum allergen-specific IgE levels are a key element in the pathogenesis of asthma. Omalizumab is a biologically-engineered, humanized recombinant anti-IgE monoclonal antibody for treatment of allergic bronchial asthma. Because cross-linking IgE triggers degranulation and synthesis of generated mediators from IgE-sensitized cells, omalizumab was engineered in such a way that it is able to bind to the same IgE Fc sites used by the FcεRI and FcεRII on those cells, thereby preventing IgE binding and subsequent cross-linking. Consequently, the high affinity receptors on immune cells are blocked and IgE effector functions are inhibited. As such, eosinophils and mast cells are not activated via their IgE receptors, reducing the occurrence of asthma symptoms (D'Amato, Stanziola et al. 2014). The advantage of this therapy is that it neutralizes unbound serum IgE, without affecting other antibody classes. Computed Tomography (CT) has been used to measure the effect of omalizumab on airway wall thickening. In one study, thirty patients were selected with (n=14) or without (n=16) omalizumab treatment for 16 weeks, after which it was confirmed that omalizumab had reduced airway wall thickness, with significant reductions in

eosinophils, and increases in pulmonary function (Hoshino and Ohtawa 2012). Another study evaluated the long term (one year) efficacy of treatment with omalizumab and showed that it had reduced the thickening of the reticular basement membrane (RBM) and eosinophil infiltration in bronchial biopsies (Riccio, Dal Negro et al. 2011). Several large randomized trials have been established and shown that omalizumab can be beneficial as an add-on therapy in severe persistent asthma patients (Corren, Casale et al. 2009; Busse, Morgan et al. 2011; Braunstahl, Chen et al. 2013).

1.2.4 Metalloproteinase and phosphodiesterase (PDE) inhibitors

Matrix metalloproteinases (MMP) are a family of enzymes that can degrade extracellular matrix components as a part of fetal and post-natal cell migration, tissue modeling, and lung angiogenesis. Although their expression, for the most part, is down-regulated after fetal alveolar development, the expression of many MMP family members is upregulated during acute and chronic phases of lung disease (Greenlee, Werb et al. 2007). Upregulated levels of MMP-1, MMP-2, MMP-3, MMP-8, and MMP-9 are found in sputum and BAL fluids from asthmatic patients (Demedts, Brusselle et al. 2005), suggesting that targeting MMPs could be a potential treatment in asthma. Marimastat, a synthetic MMP inhibitor, was initially used in a clinical cancer trial before being tested in mild asthma patients. Even though there were no significant changes in sputum inflammatory cells, asthma symptoms or exhaled nitric oxide, marimastat reduced airway hyperresponsiveness in these asthmatic individuals (Bruce and Thomas 2005). Phosphodiesterases (PDEs) regulate signalling responses to intracellular gradients of cAMP and cGMP. The PDE4 inhibitor roflumilast has been shown to decrease airway epithelial thickness, TGF-β accumulation, and airway inflammation in an animal model of allergic asthma (Kumar, Herbert et al. 2003).

Some other new drugs that target CCR3 (Wegmann, Goggel et al. 2007), TGF-β (Leung, Niimi et al. 2006), tryptase (Oh, Pae et al. 2002), or TLR-7/8 (Camateros, Tamaoka et al. 2007), or that induce immunomodulation (Fanucchi, Schelegle et al. 2004; Lee, Cho et al. 2006) have been developed and tested in animal models, and shown to decrease eosinophilia, inflammatory

cytokines production, mucus secretion and airway remodeling, although they have not been tested in human as yet.

1.2.5 T cell epitope immunotherapy

Allergen-specific peptides, or altered peptide ligands, which contain relevant allergen epitopes, have been used to induce antigen-specific T cell anergy and thereby provide an alternative therapy. After treatment of T cell epitope peptides of the major honeybee venom allergen, phospholipase A2 (PLA), patients displayed reduced peripheral blood mononuclear cell specific proliferative responses (Müller, Akdis et al. 1998). Recombinant DNA technology was used to clone allergens and such synthetic peptides, and particularly ones that bind with great efficacy to MHC II (Li and Boussiotis 2008). Cat- and grass pollen- (Jutel, Jaeger et al. 2005) allergic patients have been successfully treated in clinical trials by using mixed recombinant allergen-specific peptides (Oldfield, Larche et al. 2002). The treated patients expressed high levels of allergen-specific IgG1 and IgG4 antibody responses, with some level of peripheral T cell tolerance, as determined by elevated IL-10 production and reduced production of cytokines such IL-4, IL-5, IL-13, and IFN-γ (Larché and Wraith 2005).

1.2.6 Selective B cell depletion

Given the importance of IgE in allergic disease, allergen-specific IgE-producing B cells might be a good target to eliminate potential harmful consequences of IgE production (Firer 2014). Some antibodies, which target a unique segment of the IgE membrane-binding region, were developed but clinical trial results are not available as yet (Brightbill, Jeet et al. 2010; Chowdhury, Chen et al. 2012).

1.2.7 Eosinophils

Blockade of eosinophil migration from the bloodstream to tissues has been investigated as a potential therapy for asthma. Lebrikizumab, an IL-13-specific neutralizing antibody, was shown to be effective for patients with high periostin and blood eosinophil levels (Corren, Lemanske Jr

et al. 2011), but as IL-5 is important for eosinophil proliferation and maturation, humanized IL-5-specific antibodies (mepolizumab and reslizumab) were also tested (Haldar, Brightling et al. 2009; Castro, Mathur et al. 2011) and shown to decrease tissue eosinophilia and oesophageal eosinophil infiltration (Straumann, Conus et al. 2009). Another approach to blockade of eosinophils is to deliver IL-5R-specific cytotoxic antibody, which would bind to and kill IL-5R-positive cells. Benralizumab, a humanized monoclonal antibody, binds to IL-5R to block IL-5 activity through antibody-dependent cell-mediated cytotoxicity (Kolbeck, Kozhich et al. 2010). Alternately, to inhibit IL-5-mediated effects on eosinophils, one could downregulate the other subunit of IL-5R, the common β-chain (βc). The inhaled drug TPI ASM8, which contains two phosphorothioate-modified antisense oligonucleotides, can degrade mRNA encoding human CCR3 and βc (Imaoka, Campbell et al. 2011). Sialic acid-binding immunoglobulin-like lectins (SIGLECs) are cell surface proteins, with SIGLEC-F being expressed exclusively on murine eosinophils (Bochner 2009). The administration of SIGLEC-F-targeting antibodies to mice with hypereosinophilia results in rapidly reduced blood and tissue eosinophilia, as well as eosinophil apoptosis (Zimmermann, McBride et al. 2008). Omalizumab, a recombinant anti-IgE monoclonal antibody, strongly reduces blood eosinophils in eosinophilic gastrointestinal disorder patients (Foroughi, Foster et al. 2007). Thymic stromal lymphopoietin (TSLP) provides survival signals and modulates cell surface adhesion molecule expression in human eosinophils (Wong, Hu et al. 2010). Some studies focused on the therapeutic target of TSLP, such as a fully human monoclonal antibody (AMG 157) which interrupts the interaction of TSLP and its receptor (Fulkerson and Rothenberg 2013), resulted in reduced allergen-induced bronchoconstriction and indexes of airway inflammation in allergic asthma patients (Gauvreau, O'Byrne et al. 2014).

1.3 Tolerance in allergic disease

1.3.1 Tolerogenic dendritic cells

1.3.1.1 Steady-state immature DCs and induced tolerogenic DCs

In the steady-state, lymphoid organ and tissue DCs express an immature, or tolerogenic phenotype. For instance, steady-state splenic $CD8\alpha^+$ DCs that have been pulsed with antigen induce partial tolerance in a mouse model of asthma, dampening airway eosinophilia, Th2

cytokine responses, and AHR, through augmentation of IL-10, TGF-β and indoleamine 2,3-dioxygenase (IDO) production (Gordon, Ma et al. 2014). Signalling via such DCs leads to increased apoptosis and decreased IL-2 expression by T cells through FasL-dependent mechanisms (Süss and Shortman 1996). Tissue resident DCs remain tolerogenic after acquiring innocuous antigens - these steady-state mucosal DCs migrate to draining lymph nodes and present these antigens to T cells in a tolerogenic fashion (Holt and Upham 2004). Unlike immunostimulatory DC, tolerogenic DCs often express low levels of MHC II and costimulatory signals (Jonuleit, Schmitt et al. 2000; Hubo, Trinschek et al. 2013), as well as an array of cytokines (e.g., TNF, Flt3L), chemokines (e.g., MIP2, RANTES), and chemokine receptors (e.g., CCR2, CCR5), etc. (Chen, Gordon et al. 2002). We now know that a variety of mediators can induce a tolerogenic DC phenotype, including IL-10, dexamethasone, vitamin D3, neuropeptides, rapamycin, corticosteroids, and some cytokines, many of which have been particularly well-studied in human or mouse models (Gordon, Ma et al. 2014).

1.3.1.2 IL-10-induced tolerogenic DCs

IL-10 can induce a tolerogenic phenotype in both human monocyte- and murine bone marrow-derived DCs. As noted, IL-10 differentiated monocyte-derived DCs (DC10) display decreased levels of MHC II and costimulatory markers, but they strongly express CCR7, ILT2 (an inhibitory HLA-G receptor), PD-L1 and PD-L2 , and IL-10. Semi-mature $CD14^+$ monocyte-derived DC10 from atopic asthmatic patients suppress Th2 cell proliferation and cytokine production *ex vivo*, and induce Teff cells to differentiate into $CD25^+Foxp3^+LAG3^+CTLA4^+$ Tregs that, in turn, can suppress allergen-derived Teff cells responses in a contact-dependent fashion. These cells also express high levels of ILT3, ILT4, PD-L1 and PD-L2, respond strongly to the lymph node-homing chemokine CCL19 and induce Tregs to suppress autologous T cell responses in a contact-, but not IL-10 or TGF-β-dependant fashion. Immature human DC10 induce of the development of alloantigen-specific regulatory T cells through IL-10 and TGF-β-dependent mechanism. On the contrary, murine bone marrow-derived DCs that have been virally-transfected to secrete very high levels of IL-10, reverse the asthmatic phenotype in mouse models. IL-10-differentiated mouse bone marrow-derived DC (DC10) express high levels of IL-10, TGF-β, low levels of MHC II,

costimulatory signals, and provide an effective therapy in ovalbumin (OVA) and HDM mouse models of asthma. IL-10 expression, although not sufficient, is critical in tolerance induction by such DC10. It has been shown that DC10 treatments induce $CD4^{+}CD44^{hi}CD69^{hi}CD62L^{lo}CD25^{lo}Foxp3^{-}$ T effector cells to convert into $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells, which in turn suppress Teff cell response, with maximum Treg activation being observed in the asthmatic lung at 3 weeks after intraperitoneal DC10 delivery. In addition, DC10-induced Tregs (iTreg, $CD4^{+}CD25^{+}Foxp3^{+}$) are more effective than naturally-occurring Tregs (nTreg, $CD4^{+}CD25^{+}Foxp3^{+}$) in suppressing the asthma phenotype. DC10-induced Tregs in asthmatic mice express LAG3, ICOS, PD-1, GITR (Huang, Dawicki et al. 2010), CTLA-4 (cytotoxic T lymphocyte antigen-4) and neuropilin-1, with lower levels of Helios. While, DC10-induced Treg in humans express LAG3 and CTLA3 (Gordon, Ma et al. 2014).

1.3.1.3 Vitamin D-induced tolerogenic DCs

There is substantial evidence showing the influence of vitamin D3 (VitD3) and its metabolites on immunologic disorders. Tolerogenic DCs can be induced by VitD3 treatment of mouse bone marrow- or human monocyte-derived DCs (Piemonti, Monti et al. 2000; Gordon, Ma et al. 2014). These cells express low levels of MHC II and costimulatory markers, but higher levels of IL-10 production. Others have reported increased expression of TNF and PD-L1 by semi-mature monocyte-derived DC-VitD3, and that PD-L1 is important in the induction of IL-10-expressing Tregs (Unger, Laban et al. 2009). By adding vitamin D3 to human skin Langerhans cells, TGF-β was induced and thereby lead to $CD25^{+}CD127^{lo}Foxp3^{+}$ iTreg production. However, vitamin D3 treatment of human dermal DCs leads to IL-10-expressing $Foxp3^{-}$ Tr1 cells that secrete high levels of IL-10, indicating that exposure of different cell populations to the same mediator may lead to different Treg outcomes (Gordon, Ma et al. 2014). *In vivo*, treatment of diabetic mice with pancreatic islet antigen-pulsed DC-VitD3 significantly decreases islet rejection following pancreatic islet transplantation (Ferreira, van Etten et al. 2011).

1.3.1.4 Dexamethasone-induced tolerogenic DCs

Corticosteroids have been employed clinically for their anti-inflammatory and immunosuppressive properties (Coutinho and Chapman 2011), but they also induce immature

DCs to adopt a tolerogenic phenotype. Some studies have reported that dexamethasone treatment induces mature DCs to undergo apoptosis *in vitro* and *in vivo*. Dexamethasone-induced DCs (DC-Dex) express low levels of MHC II and costimulatory signals, elevated levels of IL-10 and GILZ, and modestly increased levels of ILT2, ILT3 (Chamorro, García-Vallejo et al. 2009); GILZ is critical to IL-10, ILT3, and PD-L1 expression by these cells. Similar to DC10, dexamethasone-exposed DC2.4 cells (a DC cell line) promote $Foxp3^+$ Treg differentiation *in vitro*, with elevated expression of CD80, CD86, and PD-L1. DC-Dex immunotherapy leads to increased levels of intragraft $Foxp3^+$ T cells, decreased inflammatory cell infiltrates and prolonged graft survival in corneal allografts. Tolerogenic DCs induced by co-treatment with vitamin D3 and dexamethasone (DC-Dex/VitD3) produce much higher levels of IL-10 than either vitamin D3- or dexamethasone-induced tolerogenic DCs (DC-VitD3 or DC-Dex), display higher IL-10/IL-12 ratios and poorly stimulate T cell proliferation responses. DC-Dex/VitD3 are also effective in suppressing colitis pathology in an antigen-dependent fashion in a mouse model (Gordon, Ma et al. 2014).

1.3.1.5 Vasoactive intestinal peptide-induced tolerogenic DCs

Vasoactive intestinal peptide (VIP) is a 28-amino acid immunomodulatory neuropeptide that can induce regulatory T cell responses in both humans and animal models. It also can induce DCs to adopt a regulatory phenotype. For example, consistent with DC-VitD3, DC-Dex and DC10, VIP-differentiated human DCs (DC-VIP) produce IL-10 and induce Treg responses. It has been shown that DC-VIP treatments dampen pathology in mouse models of experimental allergic encephalomyelitis (EAE), colitis, bone marrow transplant-induced GVHD, and rheumatoid arthritis (Gordon, Ma et al. 2014). Interestingly, in some of these models DC-VIP induced $CD4^+CD25^+Foxp3^+$ Tregs (Gonzalez‐Rey, Fernandez‐Martin et al. 2006), while in others Tr1 phenotype regulatory cells were generated (Delgado 2009).

1.3.1.6 Rapamycin-induced tolerogenic DCs

Rapamycin affects both T cells and DCs by binding to the serine/theonine protein kinase mammalian target of rapamycin (mTOR), whereby it dampens DC maturation and regulates cell

growth, proliferation and survival in many systems. Suppression of mTOR leads to induction of $CD25^+Foxp3^+$ Tregs. Clinically, rapamycin has been broadly used to prevent allograft rejection, especially in renal transplant individuals. Rapamycin-differentiated mouse DCs (DC-Rap) induce naive T cells to convert to $CD25^+Foxp3^+$ Tregs. Rapamycin treatment of animals effectively impaired DC activation, and markedly reduced proinflammatory cytokine-TNF-α production. In murine heart transplant models, DC-Rap treatment induces $Foxp3^+$ Treg out-growth and long-term transplant survival (Gordon, Ma et al. 2014).

1.3.2 Regulatory T cells

Tolerance can be categorized as occurring either centrally or in the periphery. Central tolerance relates to the establishment of the thymic T cell repertoire with immature lymphocytes through positive and negative selection. Because thymic depletion is incomplete, the immune system has developed a secondary, peripheral, mechanism of tolerance by which mature lymphocytes found in the primary lymphoid organs and circulation provide necessary immunosuppressive responses (Li and Boussiotis 2008). Regulatory T cells are defined as cells that suppress immune responses by a variety of mechanisms, including suppressive cytokines such as IL-10, IL-35 and/or TGF-β. Granzyme B, but not perforin, is involved in contact-dependent $CD4^+CD25^+$ regulatory T cell suppression, while, in contrast, granzyme A and perforin mediate regulatory T cell-dependent killing (Ozdemir, Akdis et al. 2009). Elevated expression levels of CD25 empowers regulatory T cells to bind or sequester IL-2, resulting in lower levels of IL-2 available for effector T cell survival (de la Rosa, Rutz et al. 2004). Cytotoxic T-lymphocyte antigen-4 (CTLA-4) and lymphocyte activation gene-3 (LAG-3, which is also expressed on $CD4^+$, $CD8^+$ T lymphocytes and a subset of NK cells, are cell surface molecules expressed by regulatory T cells (Vignali, Collison et al. 2008), which are the responsible for maintaining peripheral tolerance (Shevach 2004; Zou 2006).

1.3.2.1 Treg cell markers

CD25, the IL-2 receptor α-chain (IL-2Rα), has been recognized as one phenotypic marker of thymic and some populations of induced $CD4^+$ regulatory T cells (Zou 2006). In the past decade,

Foxp3 has been defined as a key intracellular marker that is also crucial to the functioning of some $CD4^+CD25^+$ Tregs. The discovery of the immune dysfunction-associated Immune dysregulation, polyendocrinopathy, and enteropathy, X-linked (IPEX) gene, attributable to a faulty Foxp3 mutation, confirmed the role of Foxp3 in human tolerance. Foxp3 mutation in mice leads to hyper-IgE syndrome, eosinophil accumulation and allergic airway inflammation (Chatila 2005). Several phenotypically distinct regulatory T cell populations have been identified, including $CD4^+CD25^+Foxp3^+$ (Tregs) cells, $CD4^+IL\text{-}10^+Foxp3^-$ (Tr1) cells, $CD4^+IL\text{-}10^-Foxp3^-$ (Dawicki et al, unpublished observations), $CD4^+$TGF-β (Th3) cells, $CD8^+CD25^+$T cells, $CD8^+CD28^-$ T cells, $CD8^+IL\text{-}10^+$ T cells, and $CD8^+CD62^+CD122^+$ T cells (Zou 2006). The classic, or natural, regulatory T cell is the thymus-derived $CD4^+CD25^+Foxp3^+$ Treg. A growing numbers of candidate Treg surface markers have been proposed, particularly in humans. For instance, because the majority of regulatory T cells from human peripheral blood cells express Foxp3 and CD25, but low or no levels of CD127 (also known as IL-7 receptor). Thus, a combination of CD4, CD25 and CD127 could highly increase the purity of Treg isolation (Liu, Putnam et al. 2006). In addition, a high level of folate receptor 4 (FR4) is expressed on Tregs compared to Teffs (Yamaguchi, Hirota et al. 2007). More markers are expected to be identified in the future.

1.3.2.2 Naturally-occurring and induced Tregs: development and function

Naturally-occurring $CD4^+CD25^+Foxp3^+$ Tregs display diverse TCRs, which are specific for self-antigens expressed in the thymus (Fontenot, Dooley et al. 2005). The inhibitory cytokines IL-10 and TGF-β are important in Treg-mediated suppression. Tregs can be induced from naïve T cells or converted from effector T cells by such inhibitory cytokines *in vivo* or experimentally generated as a possible therapeutic (Grazia Roncarolo, Gregori et al. 2006). For example, Tr1 and Th3 can be induced experimentally through IL-10 and TGF-β, respectively (Chen, Jin et al. 2003). In the presence of TGF-β and retinoic acid, $CD103^+$ DCs stimulate Teff cells to convert to $Foxp3^+$ Treg in gut-associated lymphoid tissue (GALT) *in vivo* (Coombes, Siddiqui et al. 2007). It was shown that both natural and antigen-specific Tregs control allergy and asthma disease in an IL-10-, or, IL-10 and TGF-β-dependent manner (Joetham, Takada et al. 2007). Specific ablation of conditional IL-10 allele expression in Tregs, resulted in increased lung inflammation

and hyperreactivity, indicating that IL-10 was essential for Treg cell immunosuppressive functions in the colon, lung and skin (Rubtsov, Rasmussen et al. 2008). On the contrary, adoptive transfer of allergen-specific Tregs results in significant IL-10 secretion by endogenous host $CD4^+$ T cells in the lungs after allergen sensitization, even if the Treg were from IL-10-knock-out donors, Thus, transfer of IL-10-deficient Tregs suppresses inflammation and airway hyperreactivity, but still upregulates IL-10 production, confirming that IL-10 production by the inducing Tregs is not critical (Kearley, Barker et al. 2005). Thus, the role of IL-10 in regulatory T cells is still controversial.

Similarly, the role of TGF-β in nTreg function has been controversial. For instance, studies in which TGF-β was neutralized with antibodies or in which TGF-β-deficient Tregs were used suggest that TGF-β may not be necessary for nTreg function (Piccirillo, Letterio et al. 2002). While other *in vitro* and *in vivo* studies have indicated that membrane-bound TGF-β of Tregs is required for these cells to realize their maximal regulatory activity. Indeed, TGF-β produced by Tregs is important in suppression of allergic immune diseases, host immune responses to *M. tuberculosis*, and colitis in an inflammatory bowel disease (IBD) model. IL-35, a newly recognized inhibitory cytokine expressed by Tregs, is important for the suppressive activity of Treg (Ozdemir, Akdis et al. 2009). Granzyme B is also a mediator of suppression through its cytolytic activities. Granzyme B-deficient Tregs in murine models display decreased suppression activity *in vitro* (Gondek, Lu et al. 2005). Moreover, others have reported that Tregs suppress B cell functions in a granzyme B-, and partially perforin-dependent fashion (Zhao, Thornton et al. 2006).

In addition to directly affecting Teff function, Treg modulate the function or maturation of DCs. Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is expressed by Tregs , and it is known that Treg interact with DCs via CTLA-4 and CD80 and CD86, which fosters indoleamine 2, 3-dioxygenase (IDO) expression by DC (IDO catabolizes tryptophan as a mechanism to induce suppression of Teffs). Tregs can also downregulate the expression of CD80 and CD86 on DCs *in vitro* (Ozdemir, Akdis et al. 2009). Studies on human Tregs indicated that these cells can also modulate the functions of macrophages, monocytes, or other cells types (e.g., mast cells). For example, Treg-derived IL-9 is essential in the activation and recruitment of mast cells in peripheral allograph tolerance (Lu, Lind et al. 2006).

1.3.3 Regulatory B cells

1.3.3.1 Subsets and distribution of regulatory B cells

In the 1970s, splenic B cells were first reported to suppress immune responses, specifically to impair delayed type hypersensitivity (DTH) responses in guinea pigs (Neta and Salvin 1974). B cells with negative regulatory capabilities were proposed by Mizoguchi and colleagues (Mizoguchi and Bhan 2006). Over the past decade, phenotypically-diverse regulatory B cell subsets have been identified in the context of multiple autoimmune diseases, including $CD5^+$ B1-a cells, IL-10-producing B cells, transitional-2-marginal zone precursor B cells, and $CD1d^+$ marginal zone B cells. Most recently, a numerically rare Breg subset, $CD1d^{hi}CD5^+CD19^{hi}$, with IL-10-producing capacity, was identified in the spleen of both normal and autoimmune mice. These so-called B10 cells are antigen-specific Bregs that affect T cell immune responses through IL-10 production. B10 are potent negative regulators of inflammation and autoimmunity in mouse models (DiLillo, Matsushita et al. 2010).

In mice, B10 cells are predominantly located in the neonatal spleen (14-15%) or the adult peritoneal cavity (8-10%), with a small proportion (2-3%) present in adult mouse bone marrow. $CD1d^{hi}CD5^+$ B cells are very rare in blood and lymph nodes of adults (DiLillo, Matsushita et al. 2010), but about 9-15% of these cells are IL-10-competent B10 cells. Even though phenotypically distinct in wild-type mice, splenic B10 cells share some overlapping features with $CD5^+$ B1-a, $CD21^{hi}CD23^-IgM^{hi}CD1d^{hi}$ marginal zone (MZ), and $CD21^{hi}CD23^+IgM^{hi}CD1d^{hi}$ transitional-2 (T2)-MZ precursor B cell subsets (DiLillo, Matsushita et al. 2010). Most IL-10-competent B cells do not express CD23, and only half of the cells are $CD21^{hi}$, and they do not belong to the T2-MZ precursor B cells subset (DiLillo, Matsushita et al. 2010). Furthermore, $CD1d^{hi}CD5^+$ B10 cells do not secrete other cytokines, except IL-10 (DiLillo, Matsushita et al. 2010). Thus, B10 cells represent a real B cell subset with unique phenotype markers and cytokine production. There is no identified phenotypic marker for human Bregs *in vivo*. A transcription factor specific for Breg differentiation and development has not been identified yet (i.e. one analogous to Foxp3 in nTregs) (Mauri and Ehrenstein 2008).

1.3.3.2 Mechanisms of regulatory B cells

Regulatory B cells were first recognized when μMT mice were backcrossed with TCRα$^{-/-}$ mice, resulting in offspring that developed colitis and exacerbated intestinal inflammation compared to single knockout TCRα mice. Similarly, in a non-obese diabetic (NOD) mouse model of diabetes, LPS-stimulated B cells express Fas ligand and TGF-β in comparison to control B cells, and transfer of these LPS-activated B cells prevents diabetes development in these mice. Later, it was reported that Breg express higher levels of CD1d (relative non-B10 B cells) and secrete IL-10 in chronic inflammatory settings. Breg reduce intestinal inflammation by directly down-regulating inflammation-associated IL-1β and STAT3 activation (Mizoguchi, Mizoguchi et al. 2002). The number of spleen B10 cells in 6 month-old mice is two-fold higher than in 2 month-old mice, indicating that B10 cells may accumulate with age, or memory B cells may exist for continued antigen challenge (DiLillo, Matsushita et al. 2010). In fixed BCR transgenic mice, B10 cells numbers are reduced by approximately 90%, suggesting that the BCR influences B10 cell development (Yanaba, Bouaziz et al. 2009). B10 cell numbers are reduced by 70-80% in mice lacking CD19 (Yanaba, Bouaziz et al. 2008), suggesting that BCR diversity and CD19-generated signals contribute importantly to B10 cell development *in vivo*. Regulatory B cells were identified in the mesenteric lymph nodes of Gαi2-protein-deficient (Gαi2$^{-/-}$) mice, which spontaneously develop Th1-mediated inflammatory colitis, indicating that Bregs may have no effect in a Th1 microenvironment (Mauri and Ehrenstein 2008) or that expression of Gαi2 is important to Breg effector functions. TLR signals and CD40 stimulation induces IL-10 production by polyclonal B cells (DiLillo, Matsushita et al. 2010), while co-stimulation via BCR and CD40 leads to production of the pro-inflammatory cytokines lymphotoxin, IL-6 and TNF-α (Duddy, Alter et al. 2004). BCR ligation and TLR signals induce IL10-competent B10 cells which negatively affect T cell activation, dendritic cells and macrophages and thereby influences both cellular and humoral immunity (DiLillo, Matsushita et al. 2010). However, others reported that the impact of CD40 engagement in B cells depends on the stage of B cell maturation, and the strength and duration of signals between T and B cells, which in turn guide the differentiation of plasma cells, regulatory or memory B cells, and cytokine production (Miyashita, McIlraith et al. 1997; Lee, Haynes et al. 2002). B cell depletion has been considered as a potential long-term

therapy for autoimmune disorders, although it is important to recognize that such treatment might deplete both pathogenic and regulatory B cells (Duddy, Niino et al. 2007).

CHAPTER 2: Hypothesis and objectives

2.1 Hypothesis:

Tolerogenic DC10 can down-regulate allergen-specific IgG1-secreting plasma cell responses by multiple mechanisms.

2.2 Objectives:

i. To characterize the cell surface phenotype and *in vitro* regulatory activities of tolerogenic DC10.

ii. To assess the abilities of DC10 to regulate OVA-specific IgG1 secreting B cell responses of OVA-asthmatic mice.

iii. To establish an OVA-specific IgG1 ELISPOT assay.

iv. To map the lung, bone marrow and global (plasma) kinetics of OVA-specific IgG1-secreting plasma cells responses in untreated and DC10-treated OVA-asthmatic mice.

v. To determine whether DC10 affect IgG1 plasma cell responses directly or indirectly via induction of Treg.

CHAPTER 3: Methodology and Materials

3.1 Animals, reagents and materials

Female BALB/c mice (6~8 week old) were purchased from Charles River Laboratories (Sherbrooke, PQ). Congenic CD45.1$^+$ female C57BL/6 mice and OVA TCR-specific transgenic OT II mice were purchased from Jackson Laboratory (Bar Harbor, ME), and bred in the animal care unit of our institution to obtain CD45.1$^+$ OTII mice. All mice were maintained in the Animal Care Unit located in the Health Sciences Building at the University of Saskatchewan and treated according to the Animal Care Committee guidelines from the University of Saskatchewan.

Recombinant mouse (rm) GM-CSF (Cat. # 34-8331-85) and IL-10 (Cat. # 34-8101-82) were obtained from eBioscience, Inc. (San Diego, CA). OVA was biotinylated as noted previously (Campbell, Canono et al. 1994). Ovalbumin from chicken egg-white (OVA, Cat. # A5503) was purchased from Sigma-Aldrich (St. Louis, MO). MHC II-restricted OVA peptide 323-339 (Cat. # vac-isq) was obtained from InvivoGen (San Diego, CA). Thymidine [methyl-3H] was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO).

FITC-conjugated anti-mouse CD40, CD80, MHC class II (Ia^d), CD69 and CD16/CD32 antibodies (Abs) were purchased from BD Pharmingen Inc. (Mississauga, ON). PE-conjugated anti-mouse programmed death ligand (PD-L) 1 (also known as CD197), PD-L2 (CD273), PE-conjugated anti-mouse CD25, PE cy5-conjugated anti-mouse CD4, CD19, and Foxp3, along with related isotype controls were purchased from eBioscience, Inc. (San Diego, CA). Mouse CD4$^+$ T cell and CD4$^+$CD25$^+$ regulatory T cell magnetic sorting kits were obtained from Miltenyi Biotec (Auburn, CA). An intracellular Foxp3 staining kit was purchased from eBioscience, Inc. (Cat. # 00-5523-00, San Diego, CA) and included fixation/permeabilization diluent, fixation and permeabilization concentrate, and permeabilization buffer (10×).

Capture rat anti-mouse IgE Ab (Clone R35-72; Cat. # 553413) and biotinylated rat anti-mouse-IgG1 were obtained from BD Biosciences (Clone A85-1; Cat. # 553441, Mississauga, ON). Horseradish peroxidase-conjugated streptavidin (SA-5004) was purchased from Vector

Laboratories, Inc. (Burlingame, CA); ABTS peroxidase substrate (Cat. # 50-66-01) was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

Elispot MultiScreen$_{HTS}$ IP 96-well filter plates (0.45 µm) were obtained from Millipore Ltd (Etobicoke, ON). Digestion solution for enzymatic dispersal of lung cells contained 0.1% collagenase (1 mg/ml [w/v]), and 0.05% hyaluronidase (0.5 mg/ml [w/v]) purchased from Worthington Biochemical Corp (Lakewood, NJ) and were diluted in RPMI-1640 medium obtained from Hyclone Laboratories, Inc. (Logan, Utah). Fetal bovine serum was purchased from Mediatech, Inc. (Cat. # 35-010-CV, Manassas, VA). Streptavidin-alkaline phosphatase conjugate (Cat. # SA1008) was obtained from Invitrogen Life Technologies Inc. (Burlington, ON). Nitro-blue Tetrazolium [NBT]/5-bromo-4-chloro-3-indoylphosphate [BCIP] stock solution was purchased from Roche Diagnostics (Cat. # 11681451001; Mannheim, Germany).

3.2 Generation of tolerogenic (IL-10 differentiated, DC10) and immunostimulatory (DCLPS) dendritic cells and induced regulatory T (iTreg) cells *in vitro*

3.2.1 DC10 differentiation

Bone marrow cells were differentiated into dendritic cells by culturing for 10 days in complete medium (RPMI1640, 1% antibiotics/antimycotics, 50 µM 2-mercaptoethanol, and 10% heat-inactivated FBS (according to Appendices-1 and -2)) containing 20 ng/ml rmGM-CSF. On day 10, the non-adherent DCs were resuspended in complete medium with 50 ng/ml rm IL-10 and 7.5 ng/ml rm GM-CSF, as a means of increasing the purity of the DC population and decreasing GM-CSF-dependent outgrowth of granulocytes (Lutz, Kukutsch et al. 1999). A phenotypic characterization of the cells from such DC10 cultures has been reported on previously (Nayyar 2009). On day 12, the DC were pulsed with allergen (50 µg/ml OVA) for 24 h at 37℃and washed prior to use (Lutz, Kukutsch et al. 2000).

3.2.2 DCLPS differentiation

Bone marrow cells were grown for 7 days in 20 ng/ml rm GM-CSF in complete medium. On

day 7, DCs were pulsed with 1 μg/ml LPS (*Escherichia coli* serotype 0127: B8) and 50 μg/ml OVA for 24 h at 37℃, and washed extensively before use. These LPS-pulsed DC were used as immunostimulatory cells for activation of effector T cells (Huang, Dawicki et al. 2010).

3.2.3 Generation of iTreg cells *in vitro*

$CD4^+CD25^-Foxp3^-$ T effector cells were magnetically sorted from the spleens and lymph nodes of otherwise untreated asthmatic OT II donor mice (asthma was induced as in §3.6, below) using CD4-specific paramagnetic beads. We co-cultured these Teff cells at 10^5/well with 3×10^4 OVA-presenting DC10/well and 0.2 ng/ml recombinant human (rh) IL-2 in complete medium in 96-well round bottom plates for 5 days at 37℃(Huang, Ma et al. 2013). The magnetically-sorted CD4 cells from these cultures comprised iTreg, as reported previously (Huang, Dawicki et al. 2010).

3.3 Flow cytometry for DCs and T cell characterization

Cells were assessed by FACS using a FACScan (Becton Dickinson, Mountain View, CA) for the expression of the noted markers. DC10 and DCLPS were stained with FITC-conjugated anti-mouse CD40, CD80, or MHC II, PE-conjugated anti-mouse PD-L1 and PD-L2 antibodies or isotype control antibodies. $CD4^+$ T cells, total lung cells, or $CD4^-$ T cells from saline or DC10-treated asthmatic mice were stained with FITC-conjugated anti-mouse CD4, CD69, and PE-conjugated anti-mouse CD25, PE-cy5-conjugated anti-mouse CD4, CD19, Foxp3, or isotype control antibodies. After washing with FACS buffer (PBS, 0.5% BSA and 2mM EDTA, pH7.2) the cells were incubated with Fc block (CD16/CD32, to block non-specific binding of our fluorophores to Fc receptors) for 10 min, and stained with the marker-specific or isotype control antibodies for another 20 min on ice. Some cells were fixed in 1% paraformaldehyde and analyzed by FACS for surface markers, while other cell markers, such as Foxp3, were analyzed following intracellular staining. To do this, cells were permeabilized overnight at 4℃with fixation and permeabilization buffer, washed thoroughly and treated with Fc block for 10 min, after which they were stained with antibodies for 20 min on ice, fixed in 1% paraformaldehyde and analyzed by FACS.

3.4 Magnetic sorting of $CD4^+$ T cell and iTregs

$CD4^+$ T cell isolation. $CD4^+$ T cells were isolated from spleens and lungs of saline- or DC10-treated asthmatic mice using CD4 positive-selection magnetic sorting, according to the manufactures' instructions (Miltenyi Biotec, Auburn, CA). Briefly, organ single cell suspensions were suspended in 90 µl of MACS buffer (0.5% BSA, 2 mM EDTA in PBS, pH 7.2), and incubated with 10 µl of microbeads (CD4 [L3T4]) per 10^7 total cells for 15 min on ice. Cells were washed with 2ml of MACS buffer per 10^7 total cells and sedimented at 250×g for 5 min. The cells were resuspended at $\leq 10^8$ cells in 500 µl buffer, applied to magnetic sorting LS columns (Miltenyi Biotec, Auburn, CA) held in the magnetic field of a MACS Separator, and rinsed with three times with 3 ml of buffer. The columns were then removed from the magnetic field and flushed with 5 ml buffer to elute the labeled $CD4^+$ T cells.

iTreg cell isolation DC10-exposed $CD4^+CD25^+$ iTreg are positively selected by magnetic sorting using the mouse $CD4^+CD25^+$ regulatory T cell isolation kits, according to the supplier's instructions (Miltenyi Biotec, Auburn, CA). Briefly, cells were suspended in MACS buffer and incubated with 10 µl Biotin-Antibody Cocktail per 10^7 total cells for 10 min at 4℃, after which 20 µl of anti-biotin microbeads and 10 µl of CD25-PE antibody were added per 10^7 total cells. The cells were added to LD sorting columns (Miltenyi Biotec, Auburn, CA), used as above, with the total effluent comprising the unlabeled $CD4^+$ T cell fraction. The eluted $CD4^+$ T cells were washed and resuspended in 90 µl of buffer and 10 µl of anti-PE microbeads for magnetic sorting of the $CD25^{hi}$ Treg using MS columns (Miltenyi Biotec, Auburn, CA), using the procedure noted above.

3.5 Proliferation and suppression assay

$CD4^+$ Th2 Teff cells were magnetically sorted from splenocytes of asthmatic OT II mice. We had previously optimized our DC10 and Treg suppression assays (Huang, Dawicki et al. 2010). Thus, we co-cultured OVA-presenting immunostimulatory DCLPS (3.7×10^3) with $CD4^+$ T (10^5) cells for three days to obtain the baseline outcomes of Teff proliferation. To assess the impact of DC10 on $CD4^+$ T cell responses, we co-cultured OVA-presenting DC10 and DCLPS with $CD4^+$

T cells for three days. In order to get high-level proliferation in this *in vitro* study, we also added 3 nM $OVA_{323-339}$ peptide into the co-culture system for presentation by both DC10 and DCLPS. In the iTreg suppression assay, we co-cultured varying numbers of iTregs (10^5, 5×10^4, 2.5×10^4, or 10^3) with DCLPS (3.7×10^3) and $CD4^+$ T (10^5) cells for 2 days. In the proliferation assay, ^3H thymidine was added for the final 24 h of a 3-day culture, after which we harvested the cellular DNA onto glass-fibre filters using routine methods. The proliferation responses were determined by measuring the levels of ^3H thymidine incorporation into cellular DNA by liquid scintillation counting (Huang, Dawicki et al. 2010).

3.6 Establishment of asthma mouse model and DC10 treatments

The establishment of the asthma phenotype in mice has been described previously (Gordon, Li et al. 2005). BALB/c mice were injected i.p. with 2 μg OVA conjugated onto 1 mg of alum on days 0 and 14, and then on days 28, 30, and 32 they were challenged by airway exposure to nebulized aerosols of 1% OVA for 20 min/day, as described previously (Schneider, Li et al. 2001; Gordon, Li et al. 2005). This model induces an asthma-like phenotype, characterized by strong OVA-specific IgG1 and IgE responses, high level pulmonary Th2 cytokine production (IL-4, -5, -9, -13), and airway eosinophil responses to recall allergen challenge, and airway hyperresponsiveness (AHR). Two weeks after the last airway challenge, when the airway inflammation has subsided somewhat, the asthmatic mice were treated (i.p.) with 1×10^6/mouse OVA-presenting DC10 (Nayyar, Dawicki et al. 2012) and sacrificed for assessment of their asthma phenotype at varying times thereafter (Fig. 3.1); all mice were given a recall allergen challenge with nebulized 1% OVA (20 min) 2 days before sacrifice.

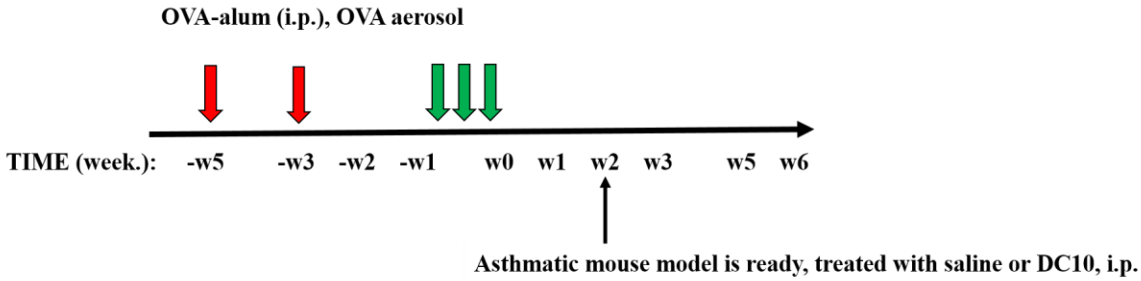


Figure 3.1 Establishment of asthma mouse model and DC10 treatment. BALB/c mice were injected i.p. with 2 µg OVA conjugated onto 1 mg of alum on days 0 and 14, and then on days 28, 30, and 32 they were challenged by airway exposure to nebulized aerosols of 1% OVA for 20 min/day, as described previously (Schneider, Li et al. 2001; Gordon, Li et al. 2005). Two weeks after the last airway challenge, the asthmatic mice were treated (i.p.) with 1×10^6 OVA-presenting DC10 (Nayyar, Dawicki et al. 2012).

3.7 ELISA to detect OVA specific IgG1 and IgE antibody

Heparin-anti-coagulated plasma was obtained by cardiac puncture. A reference standard of pooled plasma from OVA-sensitized asthmatic mice, with an arbitrarily-assigned value of 10,000 experimental units/ml, was used; 1:5 and 1:10 dilutions in PBST of the standard plasma was used for evaluation of IgE and IgG1, respectively, while experimental plasma samples were diluted 1:100 and 1:10 in PBST for IgG1 and IgE detection, respectively.

For the OVA-specific IgG1 ELISA, the immunolon-4 96-well flat-bottom plates were coated with OVA (10 µg/ml) in coating buffer (1M $NaHCO_3$, 1M Na_2CO_3; pH 9.6) overnight at 4℃. For the OVA-specific IgE, the plate was coated with anti-IgE (1 µg/ml) capture reagent. The plates were washed twice with PBST and blocked with 200 µl/well of PBST supplemented with 10% heat-inactivated FBS (56℃for 0.5 h) for 2 h at room temperature. Following another 4 washes, 100 µl/well of standards and samples were added to the wells; blank wells were filled with 100 µl/well of PBST. The plates were covered and incubated overnight at 4℃, washed with PBST, and then 100 µl of biotinylated anti-mouse IgG1 (1 µg/ml, for OVA-specific IgG1) or biotinylated OVA (10 µg/ml, for OVA-specific IgE) were added to the plates, which were incubated at room temperature for 1 h. The wells were washed for 6 times with PBST, and then 100 µl/well of streptavidin-conjugated horseradish peroxidase, diluted 1:1000 in PBS/10% FBS, were added and incubated at room temperature for another 2 h. The plates were washed 8 times with PBST, and then 100 µl/well of 2, 2'-azinobis (3-ethylbenthiazoline-sulfonic acid) (ABTS) peroxidase substrate was added to each well and allowed to develop for 3-15 min. The plates were read at an wavelength of 405 nm by a computer-assisted ELISA plate reader (Bio-Rad Model 3550 Microplate reader, Bio-Rad Laboratories, Hercules, CA) utilizing the Microplate Manager program for the Macintosh (Campbell, Canono et al. 1994; Nayyar 2009). These data were expressed as relative levels compared to serum from asthmatic mice.

3.8 Bronchoalveolar lavage (BAL) and assessment of BAL cells

To collect BAL fluids, the mice were first sacrificed with CO_2 and their tracheae were cannulated using small bore Tygon tubing ligated into the trachea. The lungs were washed with 1.5 ml of PBS (flushed into and out of the lungs 5 times); usually, we recovered 1.5 ml BAL

fluid from each mouse. The BAL cells were re-suspended at a concentration of 5×10^5 cells/ml in PBS, and deposited onto centrifuge slides (5×10^4 cells/slide) using a cytocentrifuge (Shandon Elliott). The slides were allowed to air-dry before staining with Wright solution for 5 min and buffer for another 5 min (Wright solution: Wright stain 1.049 g, methanol 360 ml, Hyceral 10.4 ml; Wright stain buffer: NaH_2PO_4 3.32 g, Na_2HPO_4 1.28 g in 500 ml dH_2O). Then slides were rinsed in cold tap water, dried overnight and cover-slipped before differential cell counts were performed by direct examination of 100 cells per sample.

3.9 Generation of lung single cell suspensions

The lungs were removed from each CO_2-euthanized mouse, minced mechanically into $\leq$1 mm^3 pieces, and digested in 1 mg/ml collagenase and 0.5 mg/ml hyaluronidase in RPMI 1640 medium at 37℃for three 15 min periods. For each lung were incubated in 4 ml digest solution, after which the tissue suspension was aspirated 10 times through a 10 ml syringe equipped with an 18 ga needle attached. For the third incubation we added an additional 2 ml of digest solution, and the digested tissues were then sieved through a 70 μm cell strainer (Cat. # 352350, Corning Incorporated, Coning, NY). Red blood cells in the lung, spleen, or bone marrow single cell suspensions (the latter two generated by simple mechanical dispersal) were depleted using hypotonic lysis, wherein cell pellets were resuspended in 9 vol of dH_2O for 17 s, followed by addition of 1 vol of 10×PBS to normalize osmolarity; cell viabilities were invariably >95%.

3.10 IgG1 ELISPOT

Polyvinylpyrrolidone-free (PVDF) membranes on ELISPOT plates were pre-washed using 15 μl/well of 35% ethanol and washed with twice with 150 μl/well PBST, then the wells were coated overnight with 0.5% OVA at 4℃. The wells were washed thrice with 150 μl/well PBST to remove residual OVA and the wells blocked with 200 μl/well complete medium (RPMI1640, 1% antibiotics/antimycotics, 50 μM 2-mercaptoethanol, and 10% heat-inactivated FBS) for 2 h at 37℃.

In general, in our assay, either the cells were simultaneously added to the ELISPOT wells and incubated for 5 hr before developing the plates, or the cells were co-cultured for 24 h in 96-well U-bottom plates to allow the populations to interact prior to being placed on the ELISPOT plates, as above. In each case, total single cell suspensions from lung, spleen or bone marrow, containing 10^6, 5×10^5, or 2×10^5 cells/well in 200 µl volumes of complete medium were added to the ELISPOT plate wells (4 wells/sample). In cell co-cultures, negative control splenocytes from normal mice were added when appropriate to make the total number of cells/well equivalent in all wells; when cultured alone, these splenocyte assays yielded 0 or 1 nonspecific spot/well in our assays. After the 5 h culture period, the plates were washed twice with 150 µl/well PBST, after which the cells were lysed by addition of 100 µl/well of dH_2O (1 min), and the plates washed again four times with 150 µl/well PBST to remove cell debris. Biotinylated anti-mouse IgG1 (1 ng/ml in PBS), used to detect IgG1 antibody-secreting plasma cells, was added to the plates overnight at 4℃, then the plates were washed eight times with 150 µl/well PBST. Streptavidin-alkaline phosphatase enzyme conjugate (1:5000) was added to the wells for 1.5 h at room temperature, then the wells were washed eight times with 150 µl/well PBST. Finally, BCIP/NBT substrate (1:50 diluted in 0.1M Tris-Hcl pH 9.5, 0.1M NaCl, 0.05M $MgCl_2$) was used to develop the spots, which were counted directly using an Olympus binocular microscope (SZX9, Tokyo, Japan).

3.10.1 Impact of DC10 on IgG1 production by OVA-specific B cells from the lungs of asthmatic mice

Total lung single cell suspensions from asthmatic mice (10^6 or 2×10^5 cells /well) were either not pre-cultured with varying numbers of DC10 (lung cell: DC10 ratios of 1:1, 3:1, 10:1, or no DC10) prior to the ELISPOT assay, or the cells were co-cultured for 24 h prior to the assay, as noted in §3.10. Then OVA-specific IgG1-secreting plasma cell numbers were assessed by ELISPOT assay.

3.10.2 Assessing whether asthmatic Th2 T helper cells play a role in DC10-dependent suppression of OVA-specific IgG1 secretion by asthmatic lung plasma cells

To determine whether pulmonary $CD4^+$ T cells are required for DC10 to alter B cell responses, we depleted $CD4^+$ T cells from single cell suspensions of enzymatically-dispersed lung cells from asthmatic mice by negative selection magnetic sorting and then co-cultured the CD4 T cell-depleted lung cells (as a source of allergen-specific B cells) with varying numbers of DC10 (i.e., ratios of 1:1, 3:1, 10:1, or no DC10) for 24 h, and then assessed OVA-specific IgG1-secreting plasma cell numbers using ELISPOT assays. We also assessed the impact of adding the magnetically sorted $CD4^+$ T cells back into the co-cultures to determine whether their 'helper' activity had any impact on B cell responses, using numbers of $CD4^+$ T cells equivalent to their original representation in the asthmatic lung cells (19%, as determined in three experiments).

3.10.3 Assessment of the impact of DC10-induced Treg on OVA-specific IgG1 secretion by plasma cells

To determine whether DC10-induced pulmonary Tregs (which our lab previously documented to be maximally induced at 3 week after DC10 treatment (Huang, Dawicki et al. 2010)) can directly suppress antibody-producing plasma cells, we purified $CD4^+$ T cells from the lungs of DC10-treated asthmatic mice at 3 week after DC10 treatment, and co-cultured these with total lung cells from asthmatic mice as a source of plasma cells, using 5×10^5 or 2×10^5 total lung cells and 1:1, 1:3 or 1:10 ratios of lung cells and Treg-enriched $CD4^+$ cells, respectively. We assessed the numbers of OVA-specific IgG1-secreting plasma cells using ELISPOT assays. We had previously documented that the lungs of untreated asthmatic mice contain minimal Treg activity (Huang, Dawicki et al. 2010).

3.11 Statistical analysis

All data were analyzed using the software program GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). Statistical significance was assessed using t tests or One-way ANOVA assays, with Tukey's post-hoc testing. *, **, and *** signify $P<0.05$, <0.01, and <0.005, respectively, versus the indicated controls.

CHAPTER 4: Results

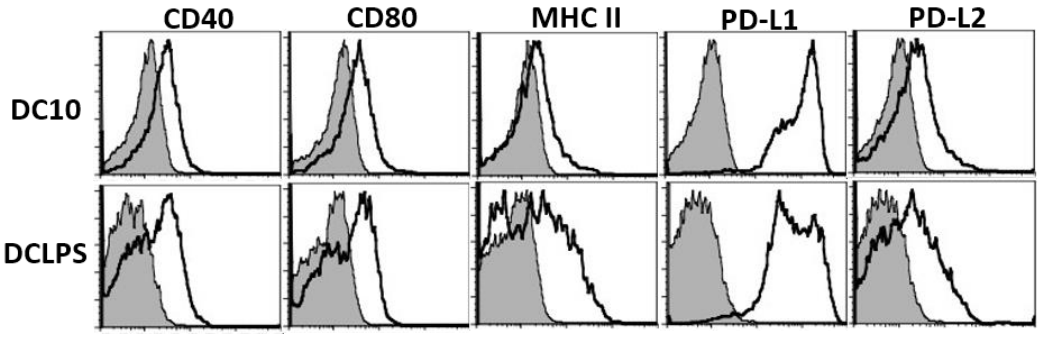
4.1 DC10 express a tolerogenic phenotype and suppress Th2 responses

Phenotypically-immature and -mature bone marrow-derived DCs were differentiated in the presence of IL-10 or LPS, respectively. DCLPS have been reported previously to be highly immunostimulatory, whereas DC10 express a regulatory phenotype (Li, Yang et al. 2010; Lu, Dawicki et al. 2011). In this study, we characterized the antigen presentation-associated surface markers of DC10 and DCLPS *(*Fig. 4.1). We observed that DC10 expressed reduced levels of costimulatory surface molecules, such as CD40, MHC II and PD-L1, with modest reductions of PD-L2 relative to the DC-LPS, suggesting that the DC10 were less mature immunologically than the DCLPS and therefore potentially tolerogenic.

To assess the tolerogenic activities of DC10, they were co-cultured with $CD4^+$ T cells that were being activated by allergen-presenting DC-LPS. $CD4^+$ T cells were obtained from the spleens and lymph nodes of OVA-asthmatic mice by positive-selection magnetic sorting; the purified cells were $\geqslant$90% $CD4^+$, as determined by FACS (Fig. 4.2A). In a preliminary experiment, we titrated the numbers of DCLPS required to induce half-maximal $CD4^+$ T cell proliferation (data not shown). For the experiments reported herein, half-maximal numbers of DCLPS (3.7×10^3 cells/well) were used as a control against the possibility that addition of equal numbers of DC10 could suppress the Th2 response simply by virtue of overcrowding (Li, Yang et al. 2010). We found that DC10 strongly suppressed DCLPS-induced T effector (Teff) cell proliferation (Fig. 4.2B).

We know that the lung cells from DC10-treated asthmatic mice contain large numbers of activated $CD25^+Foxp3^+$ Treg at week 4 after treatment (Lu, Dawicki et al. 2011) - such 4-week $CD4^+CD25^{hi}$ cells reduce DCLPS-driven Teff cell proliferation and Th2 cytokine production by 54 and 35-40%, respectively (Huang, Dawicki et al. 2010). To assess the functions of putative Tregs generated *in vitro*, we co-cultured DC10 with $CD4^+$ Teff cells in the presence of IL-2 for 5 days, and then MACS-purified $CD4^+CD25^+$ T cells from these cocultures using a Treg-sorting kit. Subsequently, we co-cultured the indicated numbers of the induced Tregs (iTreg) with Teff cells to assess the influence of the iTreg on proliferation (Fig. 4.3). Our data indicated that

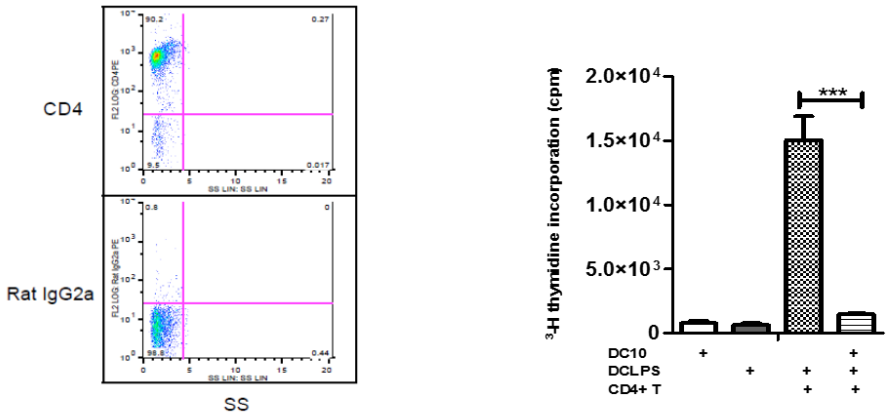
DC10-induced Treg generated *in vitro* could significantly suppress Teff proliferation in a dose-dependent manner.



MFI fold change

	DC10	DCLPS
CD40	1.6	5
CD80	2.2	3.2
MHC II	1	4.1
PD-L1	119.9	134.7
PD-L2	5.1	6.5

Figure 4.1 FACS analysis of selected cell surface markers on putatively tolerogenic IL-10-differentiated dendritic cells. IL-10-differentiated dendritic cells (DC10) were generated from bone marrow cells in vitro by culture for 10 days in complete medium containing 20 ng/ml rm GM-CSF. On day 10, the non-adherent DCs were resuspended in complete medium with 50 ng/ml rm IL-10 and 7.5 ng/ml rm GM-CSF. On day 12, the DC were pulsed with allergen (50 µg/ml OVA) for 24 h at 37℃.
LPS-differentiated dendritic cells (DCLPS) were generated from bone marrow in vitro by culture for 7 days in 20 ng/ml rm GM-CSF in complete medium. On day 7, DCs were pulsed with 1 µg/ml *Escherichia coli* (serotype 0127: B8) LPS and 50 µg/ml OVA for 24 h at 37℃. DC10 and DCLPS were analyzed by flow cytometry for the indicated surface markers, using antibodies specific for MHC II, costimulatory molecules CD40 or CD80, as well as the inhibitory markers PD-L1 and PD-L2, or isotype-matched antibodies. Shaded and solid line histograms represent isotype control and positive staining, respectively. This data is representative of the outcomes of three repeat experiments.



A: FACS analysis of sorted CD4⁺ T cells **B: Suppressive assay**

Figure 4.2 OVA-presenting DC10 are able to suppress immunostimulatory DC-induced cognate CD4⁺ T effector cell proliferative responses in vitro. (A). $CD4^+$ effector T cells were magnetically sorted from the spleens of OVA-TCR transgenic OTII mice and assessed for purity by FACS, using marker-specific or isotype control antibodies. The selected population of cells was 90% $CD4^+$ cells. **(B).** For our assay of the suppressive activities of DC10, DC10 and DCLPS were generated as noted in Fig. 4.1, while we used the magnetically sorted $CD4^+$ Th2 Teff cells from panel A as the indicator cells. We co-cultured immunostimulatory DCLPS (3.7×10^3 cells/well) with $CD4^+$ T (10^5 cells/well) cells for 2 days to obtain the baseline outcomes, and then assessed the impact of adding DC10 (10^5) into these cultures. To get high level proliferation in this *in vitro* study, we put $OVA_{323-339}$ peptide into the co-culture system (3 nM). On day 3, ^3H thymidine was added for the final 24 h. Cells were harvested and ^3H thymidine incorporation was determined by liquid scintillation counting (Huang, Dawicki et al. 2010). This data is representative of three identical experiments performed, with four replicate wells in each group; each bar represents the mean (±SEM) of the 4 wells. *** Signifies $P<0.005$ versus DCLPS-stimulated $CD4^+$ T cell proliferation, as determined by one-way ANOVA, with Tukey's post-hoc testing.

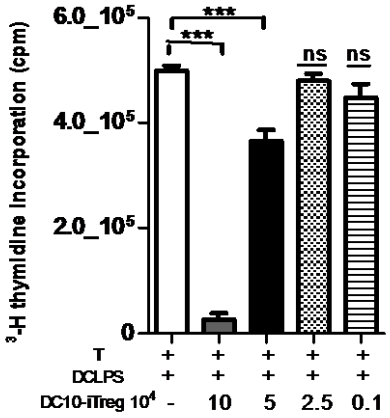


Figure 4.3 Impact of DC10-induced regulatory T cells (iTreg) on proliferative responses of Th2 cells from asthmatic mouse donors. DC10 iTreg were magnetically sorted as $CD4^+$ T cells from 5-dy rh IL-2-supplemented (0.2 ng/ml) co-cultures of DC10 (3×10^4 cells/well) and Teff cells purified as in Fig. 4.2 (10^5 cells/well), as noted in the Materials and Methods section (§3.2.3). The indicated numbers of DC10-iTreg (i.e., 0, 10^5, 5×10^4, 2.5×10^4, or 10^3 cells) were added to co-cultures of DCLPS (3.7×10^3 cells/well) and $CD4^+$ T (10^5 cells/well) cells for 3 days, with ^{3}H thymidine being added for the last 24 h of culture. ^{3}H thymidine incorporation was determined by liquid scintillation, as noted in Fig. 4.2. This data is representative of three identical experiments, with four wells in each treatment; each bar represents the mean (±SEM) of four wells. One-way ANOVA, Tukey's post-hoc testing, *** signifies $P<0.005$.

4.2 Impact of DC10 treatment on the disease phenotype in asthmatic mice

We previously reported that OVA-DC10 treatment reduced recall allergen-induced pulmonary eosinophilia and BAL fluid levels of Th2 cytokines, circulating allergen-specific IgE and IgG1 levels, and AHR of asthmatic mice, and that this effect was progressive and long lasting. For example, OVA-DC10 treatment reduces eosinophil infiltrates by ≈60% compared to saline-treated asthmatic mice, and circulating of IgE and IgG1 antibody levels were also dramatically reduced (Nayyar, Dawicki et al. 2012). Our observation that DC10 treatment reduced serum antigen-specific antibody levels, albeit more slowly than it dampens Th2 responses (Nayyar, Dawicki et al. 2012) led us to question how it accomplishes this. For example, is this through a direct effect of DC10 or DC10-induced Treg on antigen-specific antibody-producing plasma cells, or simply through a loss of T cell help for the plasma cell response? First, however, we wanted to reconfirm that our DC10 could indeed effect Th2 tolerance in asthmatic mice, as reported previously, simply by looking at airway eosinophil responses to recall allergen challenge as a prototypical asthmatic response and at serum OVA-specific IgE and IgG1 levels in DC10- versus saline-treated asthmatic mice. The DC10-treatment down-regulated eosinophil accumulation by about 55% (Fig. 4.4A), and reduced the mean values for serum IgG1 and IgE levels (Fig. 4.4B). To address the impact of the DC10 treatment on pulmonary plasma cell antibody production, we examined the abilities of plasma cells present in single cell suspensions prepared by enzymatically-digesting lung tissues from asthmatic mice to secrete OVA-specific IgG1, as determined by ELISPOT assay. We found that the plasma cells from the DC10-treated animals displayed strongly downregulated OVA-specific IgG1 release compared to analogous cells from saline-treated asthmatic mice (Fig. 4.5A). Although IgE is a key mediator in the maintenance of allergen-responsiveness in allergic individuals (Dullaers, De Bruyne et al. 2012), it is expressed at very low levels relative to IgG isotype antibodies, such that we were unable to detect IgE-secreting plasma cells by ELISPOT. Indeed, serum allergen-specific IgE cannot be detected in the sera of asthmatic mice using a standard ELISA. In seeking to optimize our assay, we queried whether antibody production by plasma cells within a freshly-isolated total lung cell population would increase if the lung cells, including APC and T helper cells, were exposed to allergen *ex vivo*. Thus, we again generated lung single cell suspensions from asthmatic mice and incubated them with or without allergen,

either adding OVA directly to the cells or placing the cells in wells that had been pre-coated with OVA (0.5%) for 4.5 h, after which we analyzed the culture supernatants for OVA-specific IgG1 by ELISA (Fig. 4.5B). While we were readily able to detect IgG1 in supernatants of cells that were not exposed *ex vivo* to allergen, we were unable to detect any IgG1 production by cells that had been exposed to allergen *ex vivo*.

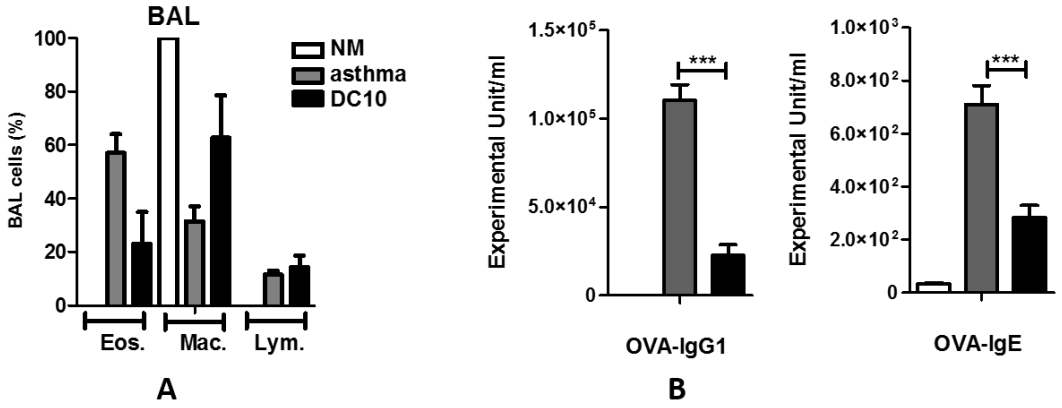


Figure 4.4 Impact of DC10 treatment on the airway inflammatory cell response to recall allergen challenge and on circulating allergen-specific IgE and IgG1 levels in asthmatic mice. Asthma was induced in BALB/c mice by i.p. administration of OVA-alum and repeated exposure to OVA aerosols, as noted in the Materials and Methods section. The asthmatic mice were treated with 1×10^{6} DC10 or saline, and six weeks later re-challenged for 20 min with nebulized OVA aerosols, and then sacrificed 48 h later to assess their responses to the DC10 treatment. **(A).** Bronchoalveolar lavage (BAL) fluids were collected from normal mice (NM), asthmatic mice and DC10-treated asthmatic mice (DC10-asthma) on wk 8 and differential cell counts done using Wright's solution-stained BAL cell cytocentrifuge slides. Differential counting of eosinophils (Eos.), monocytes/macrophages (Mac.) and lymphocytes (Lym.) were determined by counting 100 cells/sample, with three mice per group. **(B).** Impact of DC10 immunotherapy on circulating OVA-specific IgE and IgG1 levels in asthmatic mice. Asthma was induced in BALB/c mice, which were treated with DC10 as in Fig. 3.1. At 3 weeks after DC10 treatment, sera were obtained from each mouse and analysed for OVA-specific IgE and IgG1. The data depict the relative level of antibody compared to serum standard, with each bar representing the mean (±SEM) of either two mice (normal controls) or three (asthmatic treatment recipient) mice. This data is representative of three experiments. *** signifies $P<0.005$.

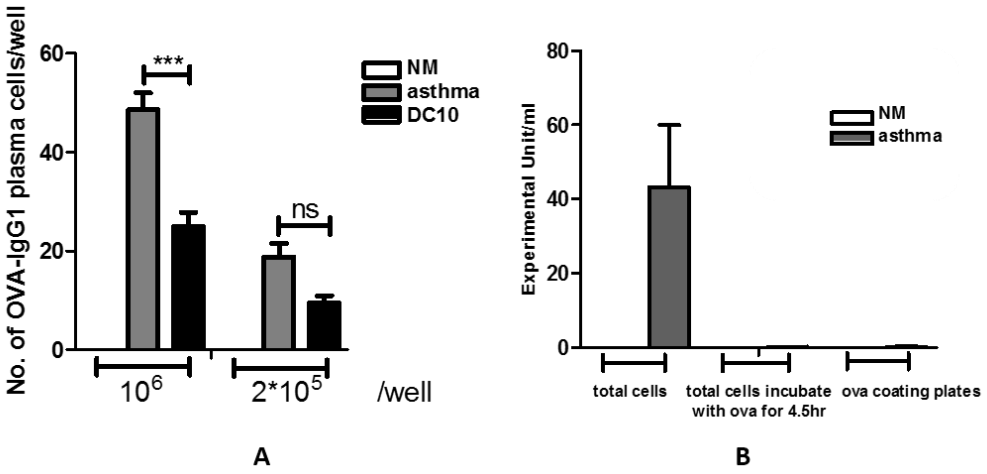


Figure 4.5 Impact of DC10 treatment on frequency of OVA-specific IgG1 secreting cells in the lungs of asthmatic mice. Asthma was induced in BALB/c mice, which were treated with DC10, both as in Fig. 4.4, and then the lungs of the mice were enzymatically dispersed as noted in the Materials and Methods. **(A)** Cells in single cell suspensions from lungs of normal mice and saline- or DC10-treated asthmatic mice 6 weeks after DC treatment were cultured for 5 h at either 10^6 or 2×10^5 cells /well in ELISPOT plates and the number of OVA-specific IgG1-secreting plasma cells was determined by ELISPOT. Each bar represents the mean (±SEM) of 4 wells, with three mice in each group. One-way ANOVA, with Tukey's posthoc testing, *, ** signifies $P<0.05$ and 0.01, respectively. **(B)** The impact of adding exogenous OVA to the lung cells prior to assessment of OVA-specific antibody analysis was also assessed. Lung cells (10^6 cells/well) were cultured in 24-well plates either without exogenous allergen, or exposed to OVA either by culture with exogenous soluble OVA (0.5%) for 4.5 h at 37℃, or by coating the plates with 0.5% OVA prior to adding the cells to the wells. Supernatants were taken after 3 days and assessed for OVA-specific IgG1 by ELISA. The data depicts the relative level of antibody compared to an asthmatic serum standard, as noted. Each bar represents the mean (±SEM) of two wells. This experiment was repeated two times.

4.3 Assessing lung and bone marrow of asthmatic mice as reservoirs for OVA-specific IgG1-secreting plasma cells

We wished to know the natural history of allergen-specific IgG1-secreting plasma cells in asthmatic mice. As such, we assessed the numbers of OVA-specific IgG1-secreting plasma cells in the lungs versus bone marrow of asthmatic mice at varying times after their last airway exposure to allergen during the asthma induction phase of the experiment (Fig. 4.6). We found that the numbers of OVA-specific IgG1-secreting plasma cells in the lungs of asthmatic mice that were not again exposed to aerosolized allergen fell by ≈85% from week 1 to 3, after which they remained at very low levels. In contrast, the numbers of OVA-specific IgG1-secreting plasma cells in bone marrow increased by ≈47% between week 1-2, and then fell very slowly thereafter (e.g., by ≈15% at week 5) (Fig. 4.7), suggesting that the bone marrow was a major reservoir for IgG1-secreting plasma cells in asthmatic mice. The OVA-specific IgG1 levels in the animal's plasma continued to increase by ≈80% between weeks 1-3 after cessation of allergen exposure, but then decreased rapidly by week 6. Given the kinetics of plasma cell accumulation in the lungs versus bone marrow, this data would suggest that the bone marrow, or some other, non-lung, compartment, became the major source of plasma IgG1 in these mice after week 2 (Fig. 4.8, left panel).

IgE is a key mediator in the allergen-responsiveness of asthmatic humans and mice, with the majority of IgE secreting-B cells reportedly residing in the bone marrow after antigen challenge (Luger, Fokuhl et al. 2009). While we were not able to detect any IgE-secreting plasma cells by ELISPOT in either the lungs or bone marrow (data not shown), we did, however, evaluate the kinetics of the serum IgE responses by ELISA and found OVA-specific IgE levels continually decreased from week 1-3 after the last sensitizing dose of allergen exposure, and then maintained a steady low level thereafter to week 6 (Fig. 4.8, right panel).

While these data suggested that allergen avoidance leads to significant reductions in serum IgE and IgG1, we have shown that asthmatic animals such as these retain full allergen-responsiveness for at least 8 months after sensitization (Nayyar, Dawicki et al. 2012). We know from our previous reports that DC10 treatment has a significant impact on the serum antibody levels in asthmatic mice, and we confirmed this herein. As mentioned, we measured the OVA-specific serum IgE and IgG1 levels in normal mice, as well as in saline- and DC10-treated

asthmatic mice at 3 wk after DC10 treatment, and found that DC10 significantly down-regulated serum levels of OVA-specific IgG1 and IgE antibodies compared to those seen in saline-treated asthmatic animals (Fig. 4.4B; $p \leqslant 0.005$).

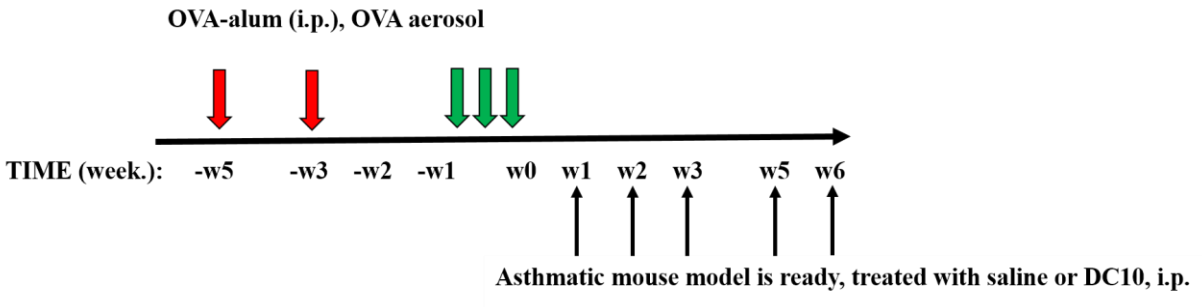


Figure 4.6 Flow chart for the allergen sensitization and experimental phases of the plasma cell study. To establish asthma, BALB/c mice were given 2 µg OVA-alum i.p. on day 0 and 14, and then on days 28, 30, and 32 they were challenged by airway exposure to nebulized aerosols of 1% OVA for 20 min/day. All mice were exposed to a recall allergen challenge with aerosolized 1% OVA on wk. 1, 2, 3, 5 or 6, and 48 h later (at day 9, 16, 23, 37 or 44) the mice were harvested. Each group has three or five mice.

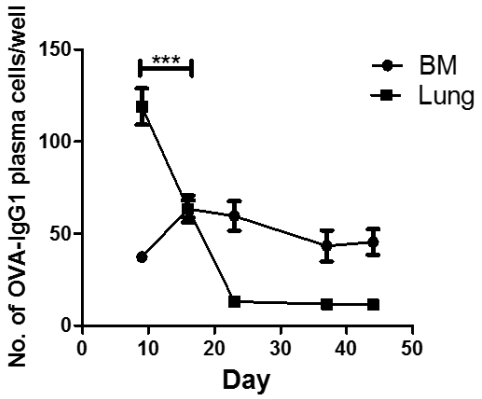


Figure 4.7 Enumeration of lung and bone marrow allergen-specific plasma cells in asthmatic mice across time after asthma induction. BALB/c mice were rendered asthmatic as in Fig 4.6 At the indicated times after disease induction, we challenged the mice with an allergen aerosol 48 h before sacrifice, then generated single cell suspensions of their lungs and bone marrow (BM), and assayed the numbers of OVA-specific IgG1-secreting plasma cells by ELISPOT assay, using 5×10^5 or 2×10^5/well. The data depicts the numbers of OVA-specific IgG1-secreting plasma cells in the 2×10^5 cells/well format (the wells containing 5×10^5 cells had too many spots to count), and are presented as the mean (±SEM) of 4 wells/sample (n=3 or 5 mice/group). *** signifies $P<0.005$. This data was representative of two replicate experiments.

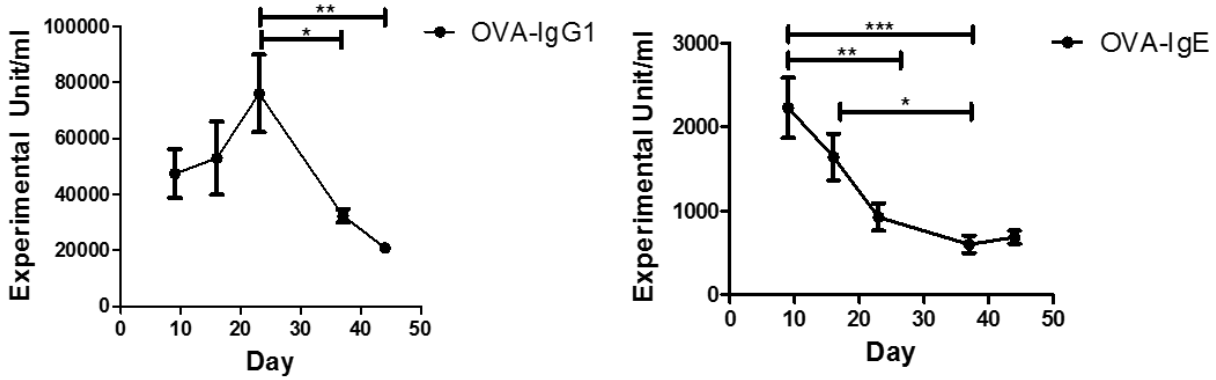


Figure 4.8 OVA-specific IgE and IgG1 levels in the plasma of OVA-asthmatic mice as a function of time after asthma induction. Plasma was collected from mice immunized as shown in Fig 4.6 and assayed for OVA-specific IgG1 and IgE antibody by ELISA with duplicate wells per sample (n= 3 or 5 mice/group). The data are presented as relative levels of antibody compared to a standard of pooled plasma from asthmatic mice that was arbitrarily assigned a value of 10000 experimental units/ml. Each point represents the mean (±SEM). This data was representative of two experiments. *, **, and *** signify P<0.05, <0.01, and <0.005, respectively.

4.4 The interactions of DC10, DC10-induced Treg and IgG1-secreting plasma cells

Having observed that DC10 treatment suppressed allergen-specific IgG1 and IgE antibody production in asthmatic mice, we investigated next which cell population(s) were regulating this process. In principle, the three populations most likely to play a direct role could be the Th2 cells that provide help for the plasma cell response (i.e., removal of 'help' could attenuate antibody production), the DC10 themselves, and/or DC10-induced Treg. Our lab has tracked treatment DC10 in asthmatic mice using DC10 from CD45.1$^+$ congenic B6 mice to treat asthmatic CD45.2$^+$ B6 mice, and found that DC10 appear to sequentially and increasingly accumulate in the airways, lung tissues and lung-draining lymph nodes of the recipient mice (Huang 2012), giving these cells an opportunity to impact lung-resident B cells. We have also reported previously that maximal induction of iTreg occurs in the lungs of DC10-treated mice at 3 wk post-treatment (Huang, Dawicki et al. 2010). Before beginning this study we assessed the CD4 (T cells) and CD19 (B cells) profiles of our pulmonary single cell suspensions using FACS. As one of our first questions, we generated lung cell populations that were depleted of potential Th2 helper cells using negative selection magnetic sorting to test whether Th2 T cell help or attrition contributes to the suppression effects.

4.4.1 DC10 can suppress antibody production by OVA-specific IgG1-secreting plasma cells from asthmatic mice

Since OVA-DC10 treatments had a significant impact on OVA-specific IgE and IgG1 responses in asthmatic mice, we next asked whether DC10 have direct and/or indirect effects on antibody-producing B cells. We used an *in vitro* approach, co-culturing varying numbers of DC10 with our single cell suspensions of lung cells from asthmatic mice and using ELISPOT assays to assess the numbers of OVA-specific IgG1-secreting plasma cells. We found that DC10 significantly decreased the number of OVA-IgG1 plasma cells we could detect in our assay when co-cultured at a 1:1 ratio with lung cells from asthmatic mice. DC10 did not discernibly affect the plasma cell response when we used fewer DC10 in this assay (Fig. 4.9A). We also assessed whether allowing the DC10 to interact with the lung cells for a longer period would increase

their impact on the plasma cell response. As such, we co-cultured the DC10 and lung cells together for 24 h before transferring the cells into the ELISPOT plates. We found that pre-culture of the DC10 with the lung cells for one day substantially increased the sensitivity of our assay, such that the DC10 decreased the OVA-IgG1 plasma cell responses in a dose-dependent manner, whether we used high (10^6 cells/well) or low (2×10^5 cells/well) density cultures (Fig. 4.9B). Based on this outcome, for all subsequent experiments we retained this 24 h pre-culture period approach.

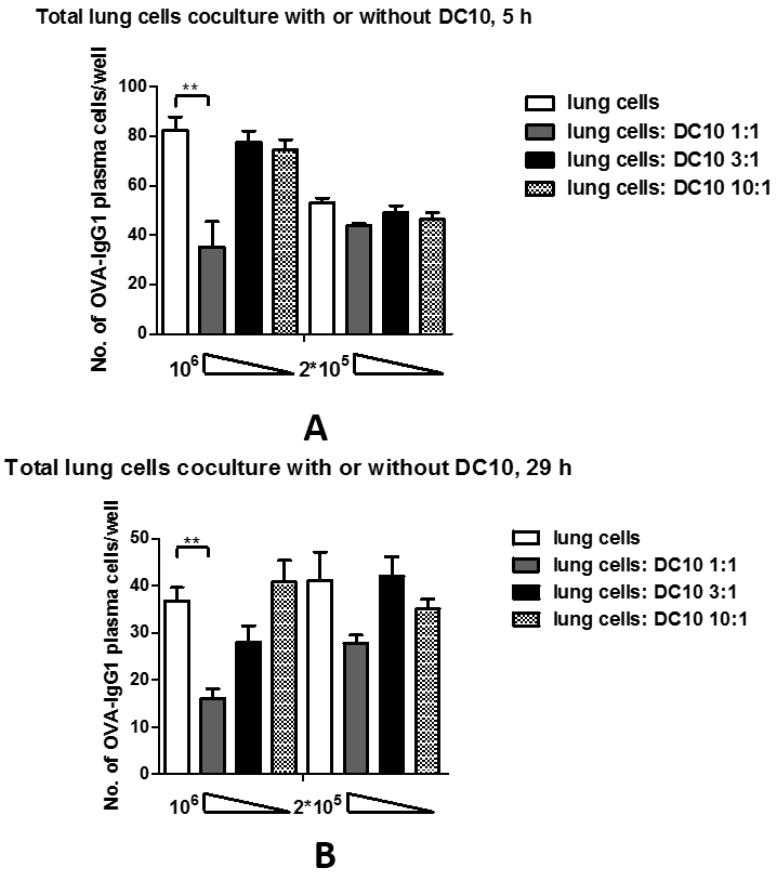


Figure 4.9 Impact of DC10 on in vitro OVA-specific IgG1 secreting plasma cells from the lungs of asthmatic mice. Single cell suspensions were generated from the lungs of asthmatic mice (n=4) at 2 weeks after asthma induction, as in Fig. 3.1; all mice were given an OVA recall allergen challenge 2 days before sacrifice. **(A)** The single cell suspensions were aliquoted into ELISPOT plates at 10^6 or 2×10^5 cells/well, and co-cultured with varying numbers of DC10 (none, or at a lung cell: DC10 ratio of 1:1, 3:1, or 10:1) in a standard 5 h in ELISPOT assay. Subsequently, the plates were processed to detect IgG1-secreting plasma cells. **(B)** Alternately, the lung cells and DC10 were cultured together in 96-well u-bottom plates for 24 h, and another 5 h in ELISPOT plates, as in panel A. Each bar represents the mean (±SEM) of 4 wells. ** signifies $p<0.01$. This data was representative of three identical experiments.

4.4.2 Absence of $CD4^+$ Th2 cells does not diminish the impact of DC10 on the in vitro OVA-specific IgG1-secreting plasma cells response

As noted above, we have reported previously that DC10 treatments induce $CD4^+CD25^-Foxp3^-CD69^+$ T cells from asthmatic mice to transdifferentiate into $CD4^+CD25^+Foxp3^+$ regulatory T cells, thereby both reducing the pool of T helper cells available to support B cell responses but also increasing the pool of Treg (Huang, Dawicki et al. 2010). Thus, we next assessed whether depletion of putative T helper cells from the lungs of asthmatic mice would impact the abilities of DC10 to suppress B cell responses. The CD4-depleted population was 22% $CD19^+$ (i.e., B) cells, with 3% contaminating $CD4^+$ cells (Fig. 4.10). The CD4-enriched fraction was 84% $CD4^+$ cells, 31% of which were activated as determined by CD69 expression (Fig. 4.10). We cocultured CD4-depleted lung cells with varying numbers of DC10 (i.e., ratios of 1:1, 3:1, 10:1) for 24 h before measuring OVA-specific IgG1-secreting plasma cell numbers by ELISPOT, running these experiments at both 2×10^5 B cells/ml and at 5×10^5 plasma cells/ml. We added splenocytes from naïve BALB/c mice into the assay to ensure that all wells contained the same total numbers of cells. We found that at both plasma cell concentrations, the DC10 down-regulated the OVA-specific IgG1-secreting plasma cell frequency in a dose-dependent fashion, with $\approx$65% (5×10^5) and 57% (2×10^5) suppression being observed when the two populations were cultured together at a 1:1 ratio. Moreover, adding $CD4^+$ Teff cells (i.e., from the CD4-enrichment sort) back into these DC10-plasma cell co-cultures in their original numbers did not have any discernible impact on the numbers of OVA-specific IgG1-secreting plasma cells detected under these conditions (Fig. 4.11). These data suggested that Th2 T helper activity, or lack thereof, did not significantly contribute to the ability of DC10 to diminish allergen-specific plasma cell responses.

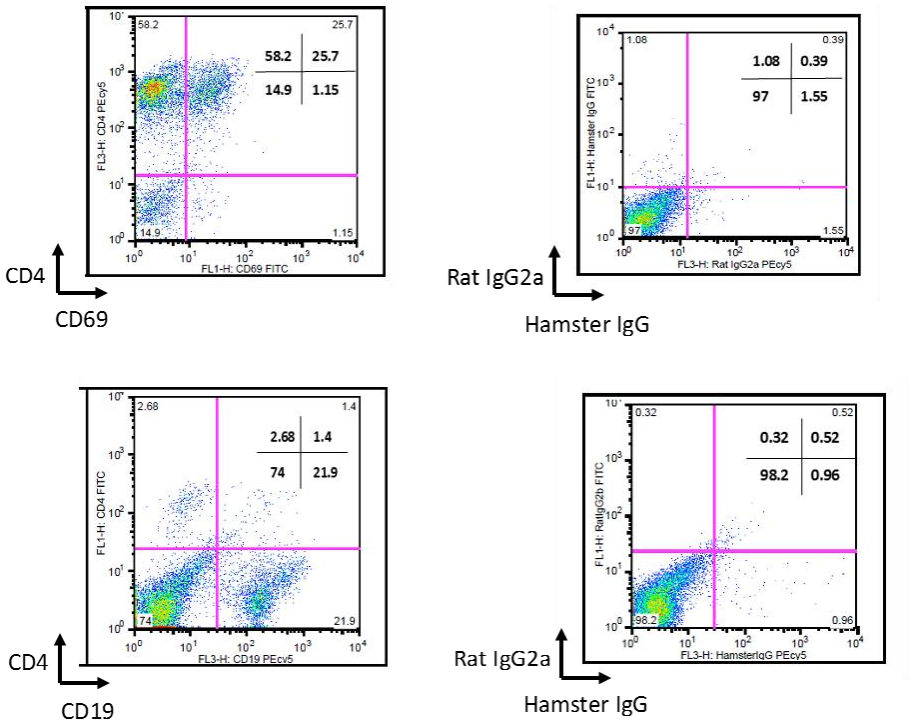


Figure 4.10 FACS analysis of CD4-enriched and CD4-depleted single cell suspensions from the lung of asthmatic mice. Magnetic sorting was used to fractionate single cell suspensions from the lungs of asthmatic mouse into CD4-enriched (top panels) and CD4-depleted (bottom panels) fractions. After purification, $CD4^+$ and $CD4^-$ lung cells were stained with FITC or PEcy5-conjugated anti-mouse CD4, CD69, CD19, or their relevant (rat or hamster) isotype control antibodies (right two panels). This data was representative of three experiments (n=4 mice/experiment).

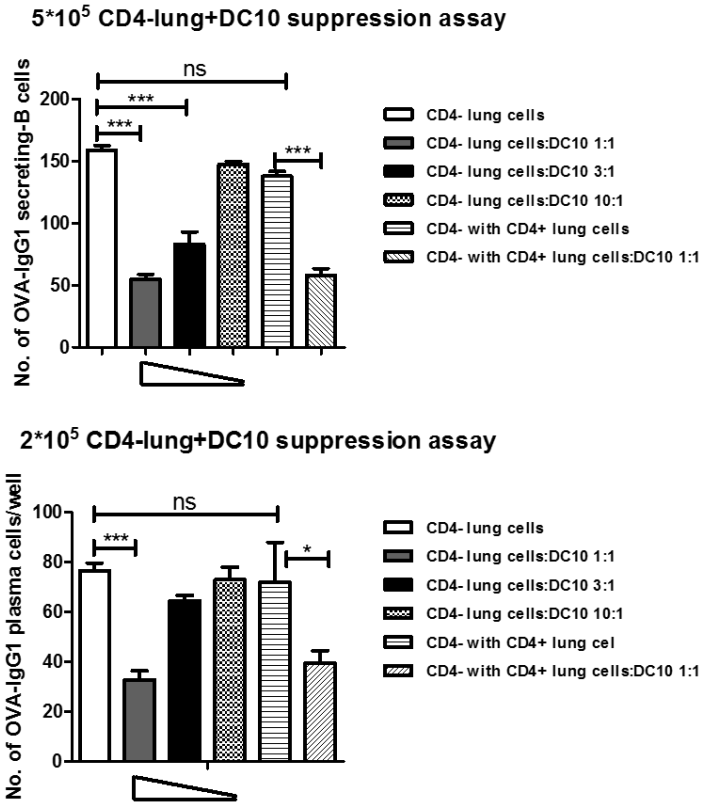


Figure 4.11 CD4$^+$ T helper cells do not contribute significantly to DC10-dependent inhibition of OVA-specific IgG1-secreting plasma cell responses. We depleted CD4$^+$ cells (putative Th2 T helper cells) from the lung single cell suspensions of asthmatic mice and used these as a source of B cells, co-culturing these (at either 5×10^5 or 2×10^5 cells/ml) for 24 h with varying numbers of OVA-presenting DC10 (CD4$^-$ lung cells: DC10, 1:1, 3:1, 10:1) before enumerating IgG1-secreting plasma cells by ELISPOT. We also assessed whether adding the either 5×10^5 or 2×10^5 purified CD4$^+$ Th2 helper cells back into the DC10/B cell cultures had any effect on the B cell response. Each bar represents the mean (±SEM) of 4 wells. Splenocytes from normal mice were used to normalize cell numbers across all treatment groups. Each experiment was repeated 3 times, with n=4 mice/experiment.

4.4.3 Impact of DC10-induced Treg on OVA-specific IgG1-secreting plasma cell responses.

Not only could the DC10 that migrate through the lungs of DC10-treated asthmatic mice in principle impact lung B cell responses, but so too could the DC10-induced Treg that reside in that compartment (Huang, Dawicki et al. 2010). To determine whether iTregs can directly suppress antibody-producing plasma cells, we sorted $CD4^+$ T cells from the lungs of asthmatic mice at week 3 after DC10 treatment (when maximal numbers of iTreg are present [347]) and co-cultured these $CD4^+$ 'Treg' - enriched T cells with B cells. The $CD4^+$ "iTreg" population from the lungs of saline- or DC10-treated asthmatic mice contained 9% and 6% $CD25^+Foxp3^+$ Treg, respectively (Fig. 4.12.3, Fig. 4.12.4). This is consistent with our previous observations that the proportions of $CD4^+CD25^+Foxp3^+$ cells in the lungs of normal, asthmatic and DC10-treated asthmatic mice are approximately equivalent, although the regulatory activities of the cells from the DC10-treated mice are significantly increased relative to $CD25^+Foxp3^+$ from the lungs of normal or asthmatic mice (Huang 2012). The CD4 T cell-depleted cells (i.e., B cell-enriched populations) from the lungs of the saline- or DC10-treated asthmatic mice contained 43% and 46% $CD19^+$ B cells, respectively (Fig. 4.12.1, Fig. 4.12.2). We found that the DC10-induced Tregs also were effective in suppressing OVA-specific IgG1-secreting plasma cells, and they did so in a dose-dependent manner. The $CD4^+$ lung cells from saline-treated asthmatic mice did not have any influence on antibody secretion. The positive control wells that included total lung cells from DC10-treated asthmatic mice also displayed fewer OVA-specific IgG1-secreting plasma cells compared to asthmatic mice (Fig. 4.12.1). Taken together, this data indicated that both DC10 and DC10-induced Treg have significant, and approximately equivalent, regulatory effects on allergen-specific IgG1 antibody secretion by lung cells of asthmatic mice.

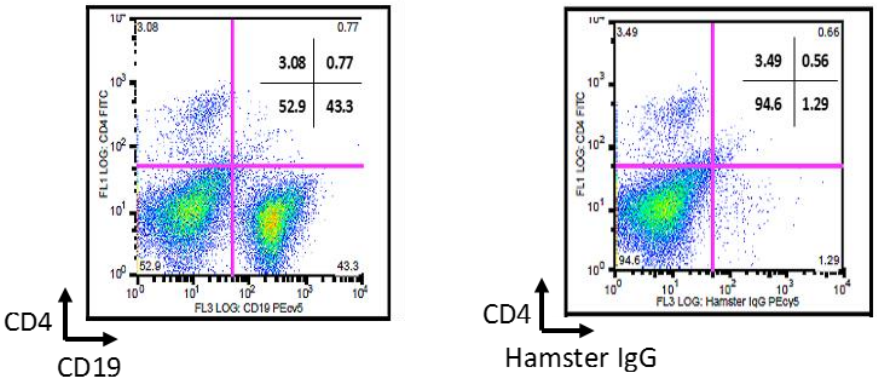


Figure 4.12.1 CD4- lung cells from asthmatic mouse

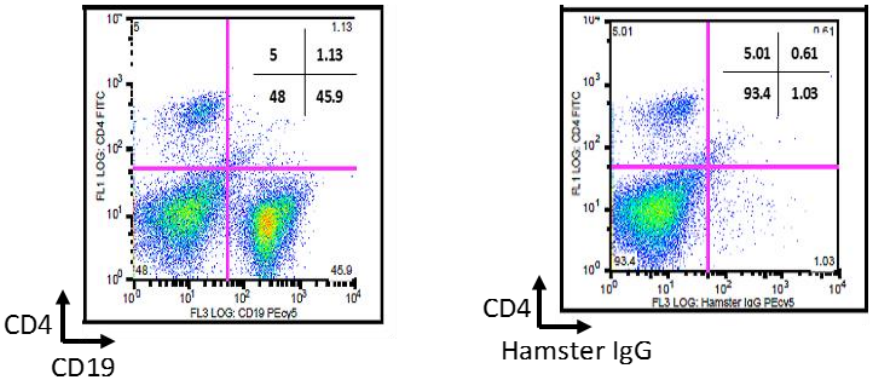


Figure 4.12.2 CD4- lung cells from DC10-treated asthmatic mouse on week 5

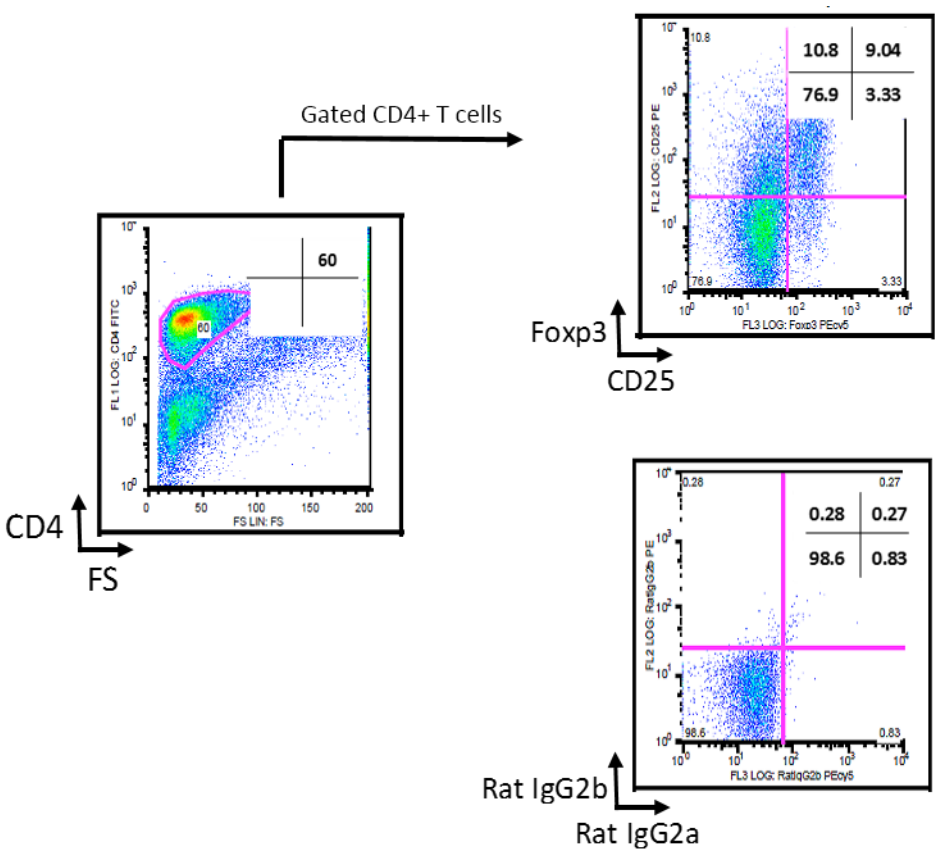


Figure 4.12.3 CD4⁺ lung cells from asthmatic mouse

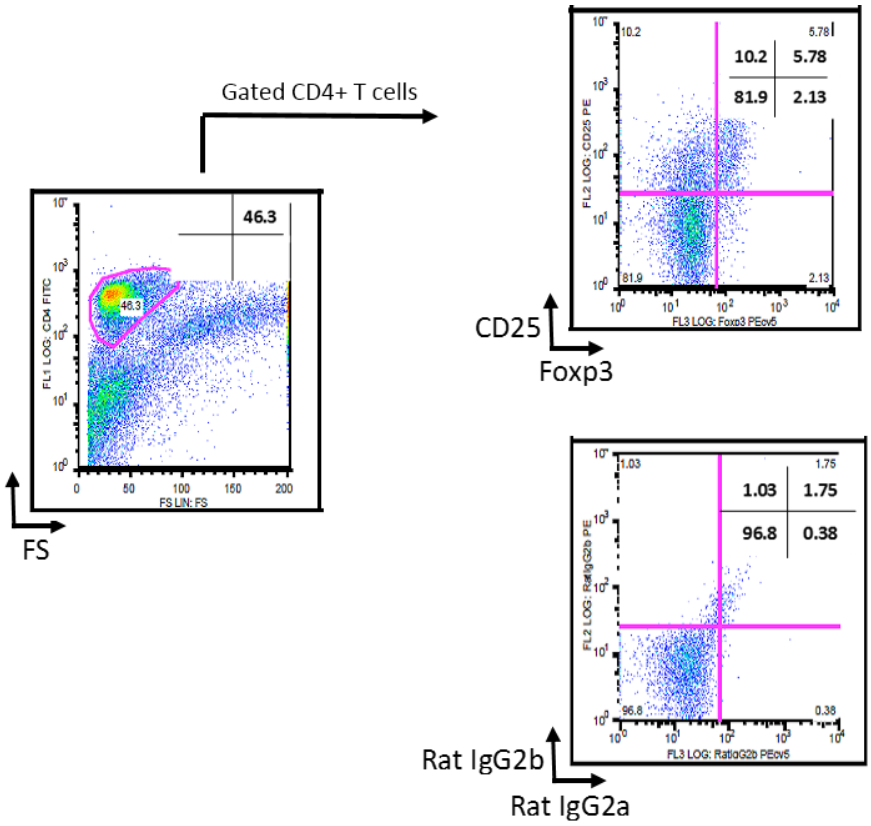


Figure 4.12.4 CD4⁺ lung cells from DC10-treated asthmatic mouse

Figure 4.12 Flow cytometric assessment of the purity of CD4⁺ cells from the lungs of saline- or DC10-treated asthmatic mice. $CD4^+$ lung cells were magnetically sorted from lung single cell suspensions of saline-or DC10-treated asthmatic mice (5 wk after DC10 treatment). The CD4-depleted lung cells were stained with FITC or PEcy5-conjugated anti-CD4 and CD19, or isotype control antibodies (Figure 4.12.1, 4.12.2), while the $CD4^+$ lung cells were stained with FITC, PE or PEcy5-conjugated anti-CD4, -CD25 and -Foxp3, or isotype control antibodies (Figure 4.12.3, 4.12.4). This data was representative of three experiments.

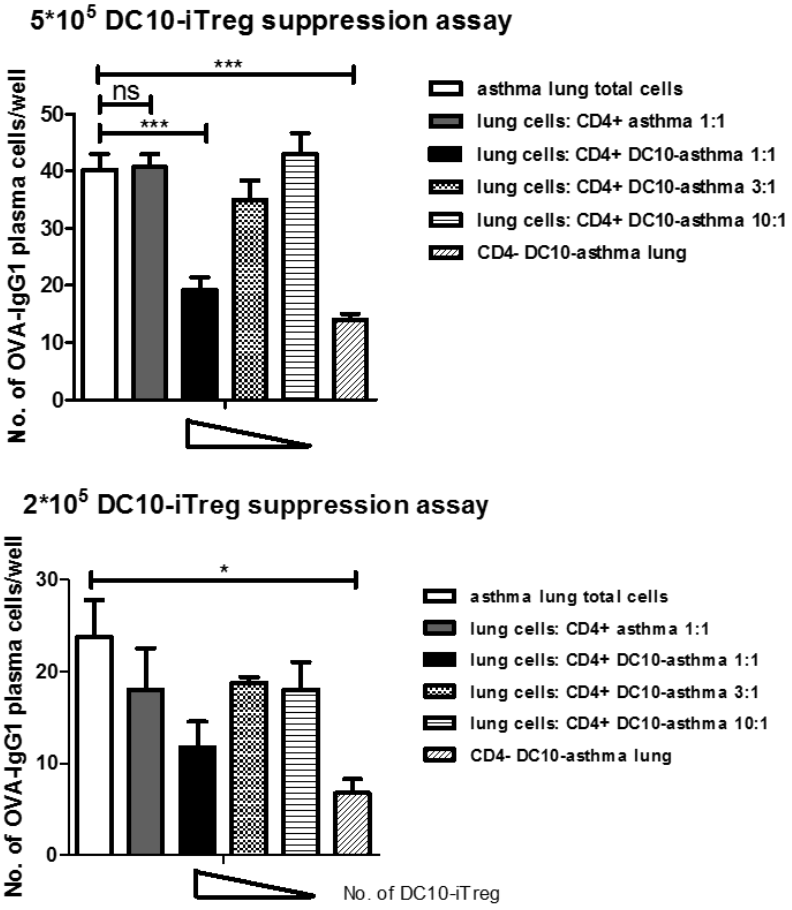


Figure 4.13 Effect of DC10-induced regulatory T cells on OVA-specific IgG1-secreting plasma cells. To assess whether DC10-induced Treg suppress IgG1-secretion by pulmonary plasma cells, we added $CD4^+$ T cells from saline- or DC10-treated asthmatic mice (i.e., iTreg-enriched cells) to cultures of asthmatic total lung cells for 24 h, and then assessed IgG1 secreting plasma cells as above. Each bar represents the mean (±SEM) of 4 wells. Splenocytes from normal mice were used to bring the total cell numbers in each well to equivalent values. Each experiment was repeated three times, with n=4 mice/group in each experiment.

CHAPTER 5: Discussion

Our laboratory has reported results from multiple models indicating that DC10 treatments reverse the asthma phenotype, and that induction of $CD4^+CD25^+Foxp3^+$ cells is an important part of this. Tolerance is associated with reductions in airway hyperresponsivenss (AHR) to methacholine, airway Th2 and eosinophilic inflammatory responses to recall allergen challenge, and circulating levels of antigen-specific IgG1 and IgE (Ansotegui, Arruda et al. 2011; Huang 2012; Nayyar, Dawicki et al. 2012). Moreover, we documented that DC10 induce $CD4^+CD25^{-/lo}Foxp3^-$ Th2 effector cells to transdifferentiate into $CD4^+CD25^{hi}Foxp3^+$ Tregs which themselves can reverse asthma phenotype on passive transfer (Huang 2012). While the peak of Treg activation occurs at $\approx$3 week after DC10 treatment, the tolerance induced by a single DC10 treatment is progressive over $\approx$8 months (Nayyar, Dawicki et al. 2012). The impact of the DC10 treatment on the allergen-specific IgE/IgG1 responses lags somewhat behind their impact on airway recall responses or AHR, but nevertheless the levels of IgE and IgG1 in DC10-treated mice approaches background at $\approx$4 months (Nayyar 2009). Nevertheless, the mechanisms by which this effect occurs have not been defined.

The maturation status of DC, as judged by the levels at which they express MHC II, costimulatory markers, and cytokines, can regulate APC and naive T cells interactions. In this study, we generated IL-10-differentiated bone marrow-derived tolerogenic DC, DC10, pulsed these cells with OVA, and confirmed that they, as well as DC10-induced Tregs, can significantly suppress OVA-pulsed immunostimulatory (DCLPS) DC-stimulated OVA-specific T effector cell proliferation. In addition, we characterized the expression of antigen presentation markers by both DC10 and control DCLPS, and found that DC10 expressed moderately to markedly lower levels of CD40, MHC II and the inhibitory receptor programmed death ligand-1 (PD-L1), than mature DCLPS, although there were no significant differences in expression of CD80 or PD-L2. This reduced expression of MHC II and costimulatory signals in itself could contribute to the cells' tolerogenic capabilities (Huang 2012), perhaps by inducing effector T cell anergy. This result was in accord with previous observations in our lab relating to DC10 therapy in mouse models of OVA or house dust mite-asthma (Lu, Dawicki et al. 2011) and human monocyte-derived DC10 (Li, Yang et al. 2010). PD-L1 (B7-H1, CD274) (Freeman, Long et al.

2000) and PD-L2 (B7-DC; CD273) (Latchman, Wood et al. 2001) are two ligands of PD-1 and inhibitors of T cell activation. The roles played by PD-L1 or PD-L2 in T cell activation are controversial, with some reporting that both stimulate T cell proliferation and cytokine secretion (Dong, Strome et al. 2002), while others have reported that B7-1/PD-L1 interactions can inhibit T cell proliferation and cytokine production (Butte, Keir et al. 2007).

In confirming the biological activity of the DC10, we treated asthmatic recipients with 1×10^6 OVA-pulsed DC10 and found 6 week later that the DC10-treated mice displayed significantly reduced numbers of pulmonary OVA-specific IgG1 secreting plasma cells, and modestly decreased OVA-IgG1/IgE responses and airway eosinophilia, as noted previously (Li, Yang et al. 2010; Nayyar, Dawicki et al. 2012). In optimizing these assays we found that providing additional, exogenous, allergen to the cells negated our abilities to detect IgG1-secreting plasma cells in the ELISPOT assay, likely because the free allergen adsorbed to the walls of the plastic wells or free in the culture medium bound the OVA-specific antibodies that had been secreted by the plasma cells before they could bind to the ELISPOT well nitrocellulose filters, as required for plasma cell detection.

In the model system analyzed here, systemic sensitization was followed by a series of airway challenges with antigen aerosol, thereby inducing the allergen-driven lung inflammation that defines allergic asthma. It has been reported that plasmablasts (i.e., antibody-secreting B cells) are able to migrate from secondary lymph organs to the sites of inflammation, where they subsequently reside (Radbruch, Muehlinghaus et al. 2006). We previously reported that substantial numbers of allergen-specific IgA-secreting B cells reside in asthmatic lungs (Schneider, Li et al. 2001), but we did not assess whether allergen-specific B cells take up residence in that compartment for extended periods of time, or whether they eventually involute *in situ* or emigrate after cessation of allergen exposure to the bone marrow, which is reported to be a major depot for antibody-secreting B (plasma) cells (Cyster 2003). To determine the natural history of the plasma cell response in our model we examined lung and bone marrow OVA-specific IgG1 secretion from 1-6 week after challenge with the last sensitizing dose of allergen. Our data demonstrated that the numbers of OVA-specific IgG1-secreting plasma cells in the lungs of asthmatic mice diminished rapidly within 3 weeks, and then remained at a stable level for at least another 3 week. That suggests that antigen-specific IgG1-secreting plasma cells

are either short-lived in the lung or that they emigrate elsewhere. This is consistent with other reports that the persistence of plasma cells in the lung depends on continued antigen stimulation, although plasma cells can still be found in other organs, such as the spleen and bone marrow (Luger, Fokuhl et al. 2009). It has also been reported that when mice are treated with cyclophosphamide to eliminate proliferating, short-lived plasma cells, long-lived IgG1, IgA, and IgE plasma cells remain in the bone marrow and to a lesser extent in spleen, but essentially none are found in the lung (Luger, Fokuhl et al. 2009), suggesting that long-lived plasma cells survive preferentially in the bone marrow. This is consistent with our data, wherein we detected increased bone marrow antibody-secreting plasma cells for a couple of weeks after allergen challenge, after which the population remained relatively stable for at least one more month.

It has been reported that both $CD44^+$ and $CD138^+$ B cells have the ability to differentiate into long-lived plasma cells, while $CD44^+CD138^+$ double-positive and $CD44^-CD138^-$ double-negative cells generate short-lived bone marrow-resident plasma cells that immigrate into the bone marrow in the absence of antigen stimulation (O'Connor, Cascalho et al. 2002). In our study, we detected about 13% $CD19^+CD138^+$ cells in the lungs shortly after allergen challenge (Y. Ma, W. Dawicki, J. R. Gordon, unpublished observations). We did a recall allergen challenge to all of our mice 2 days before sacrifice, so we cannot tell whether the small numbers of plasma cells detected in the lungs arose as a result of that allergen recall challenge, or whether they were indeed long-lived plasma cells. To address this question, we could take the established asthmatic mouse, rest them for a few weeks to generate long-lived plasma cells, effectively deleting short-lived plasma cells, and then compare groups of mice that had or had not been allergen-challenged.

In our model, serum levels of OVA-specific IgG1 peaked at week 3 after the last sensitizing dose of allergen and decreased rapidly thereafter. It has been reported that the long-lived plasma cells that reside in bone marrow are the source of 80% of the antibody found in the serum (O'Connor, Cascalho et al. 2002). In our system, we did not observe a direct correlation between bone marrow plasma cells numbers and the serum titer of OVA-specific IgG1, although the general trends for these two parameters were not dissimilar (i.e., an early peak with a decline thereafter). Nevertheless, the question remains as to whether our tolerogenic DC10 could have suppressed antibody secretion by regulating plasma cells in lung - it has been reported that

long-lived plasma cells are resistant to immunosuppression, at least in terms of susceptibility to cyclophosphamide regulation, anti-CD20 antibodies (Edwards, Szczepański et al. 2004), or X-irradiation (Holt, Sedgwick et al. 1984). We know that DC10 delivered i.p. do not migrate in substantial numbers to the bone marrow (H. Huang, W. Dawicki, and J. R. Gordon, unpublished observations), so our working hypothesis was that the DC10 interacted directly, or indirectly via Treg, for example, with antibody-secreting B cells in the inflamed lung tissue, the target organ of asthma.

As noted, IgE has a key role in asthmatic reactivity, despite the fact that we were unable to detect OVA-specific IgE secreting B cells by ELISPOT. We found that to detect allergen-specific IgE in mice we needed to use a modified ELSIA protocol (i.e., employing anti-IgE as the capture reagent and biotinylated allergen as the detection agent) (Schneider, Li et al. 2001; Nayyar 2009). In this way we found that serum OVA-specific IgE antibody levels continually dropped from week 1 to week 6 after cessation of allergen exposure, suggesting that allergen avoidance is sufficient to discernibly down-regulate the IgE response, although allergen-responsiveness remains in DC10-treated asthmatic mice in the absence of allergen challenge for ≥8 mo (Nayyar, Dawicki et al. 2012). It is well recognized that low levels of IgE are sufficient to fully sensitize mast cells, as discussed above (Section 1.1.2.3). To confirm the efficacy of our DC10 treatment, we assessed its impact on OVA-specific IgG1 and IgE responses. DC10 significantly decreased OVA-specific IgG1 and IgE antibody levels in asthmatic mice, and this agrees with data we reported previously (Lu, Dawicki et al. 2011; Nayyar, Dawicki et al. 2012). One research question we want to ask going forward relates to precisely what happens to the lung antibody-secreting plasma cells we detected early on? Do they die by apoptosis or do they immigrate to niches such as the bone marrow and spleen? This has ramifications for the efficacy of immunotherapy protocols if the treatment cells may not have direct access to such alternate compartments.

We next assessed whether DC10 have a direct effect on lung IgG1-secreting plasma cells. We used total lung cells collected from asthmatic mice at 2 weeks after their last sensitizing dose of OVA (but also 2 day after an OVA recall challenge) as a source of B cells, and co-cultured these cells with DC10 prior to assessing OVA-specific IgG1 secretion by ELISPOT. DC10 decreased the number of OVA-IgG1 plasma cells in both short-term cultures and, better yet, after a 24 h

pre-incubation period for the DC10 and plasma cells. We believe that this pre-incubation period allowed the DC10 to have more time to interact with the plasma cells, although we do not know whether this would have been a direct interactions (e.g., via IL-10 secretion by the DC10) or an indirect one (via an effect of DC10 on T helper cells). We next examined the interactions between DC10, DC10-induced Treg and OVA-specific IgG1-secreting plasma cells. We asked whether pulmonary $CD4^+$ T cells (a source of putative T helper cells) were required for DC10-mediated suppression of plasma cell responses, incubating CD4-depleted pulmonary cells (as our source of B cells) with varying numbers of DC10. As noted, DC10 did dampen the IgG1 plasma cell response but, interestingly, adding $CD4^+$ T helper cells back to the DC10-B cell co-cultures did not affect the frequency of OVA-IgG1 plasma cells. This observation suggested $CD4^+$ T cells did not provide input to the antibody secretion responses at this stage, although it is possible that the small numbers of residual $CD4^+$ T cells in our CD4-depleted lung cell populations (i.e., $\approx$3%) were sufficient to provide help for the antibody-secreting B cells. To figure address this question, we could add 3% CD4+ T cells into purified CD19 B cells of asthmatic mouse to assess the influence of low numbers of T cells on antibody secreting B cells.

We previously reported that DC10-induced pulmonary Treg achieve maximal activity in that compartment at 3 weeks after treatment of asthmatic mice (Huang, Dawicki et al. 2010). So in assessing whether DC10-induced Tregs can suppress plasma cells, we used Treg purified from the lung of DC10-treated asthmatic mice at week 3, coculturing these with B cells from asthmatic mice. Our FACS results indicated that $CD4^+CD25^+Foxp3^+$ cells comprised 9% and 6% of $CD4^+$ T cells in the lungs of asthmatic and DC10-treated asthmatic mice, respectively, which in consistent with our published results (Li, Yang et al. 2010; Lu, Dawicki et al. 2011; Huang 2012). While the CD4-depleted lung cells from asthmatic donors contained $\approx$45% $CD19^+$ B cells. We found that DC10-induced Treg decreased OVA-specific IgG1 secretion by plasma cells, but that adding $CD4^+$ putative T helper cells did not have further affect the plasma cell response. We know from previous work that injected DC10 traffic through the lungs of asthmatic mice across the time-frames when, in the present study, we saw plasma cell numbers decline in DC10-treated asthmatic mice. Taken together, our data suggests that there are at least two mechanisms for DC10-induced suppression of plasma cell responses following DC10 immunotherapy. These mechanisms include an iTreg-dependent mechanism and a

DC10-dependent one. This raises an intriguing question, and that is whether DC10 directly interact with plasma cells (i.e., in a manner requiring cell-cell contact) and, if so, precisely what molecular interactions may regulate this process.

IL-10 is a cytokine with its own suppressive activity. We know that murine DC10, human $CD14^+$ monocyte-derived DC10, and DC10-induced Treg secrete elevated levels of IL-10 compared to control DCs (Huang, Dawicki et al. 2010; Li, Yang et al. 2010; Lu, Dawicki et al. 2011), and that DC10 delivered i.p. quickly accumulate in the lungs of treated mice (Huang 2012). We have also shown that IL-10-silenced DC10 do not retain their therapeutic activities (e.g., relating to AHR, eosinophilia, or Th2 cytokines) in a mouse model of asthma (Huang 2012), while neutralization of IL-10 prevents suppression of Th2 effector cell activity by DC10-induced Treg (Huang, Dawicki et al. 2010). IL-10 is reported to maintain APCs in an immature state, even in the face of exogenous maturation signals (Lang, Patel et al. 2002), and it has been reported that IL-10 can completely inhibit human B cell IgE synthesis (de Waal Malefyt, Hans et al. 1992), although contrary results have also been reported (Rousset, Garcia et al. 1992). Nevertheless, we assume that IL-10 locally released by DC10 or their induced Tregs in the lung could, at least in part, ameliorate antibody-secreting B cell responses. Recently, IL-35-producing $CD138^+$ B cells were identified to be a key player in negative immune regulation. Mice lacking B cell IL-35 display high-level macrophage activation, with increased B cell APC activity and augmented inflammatory T cell responses (Shen, Roch et al. 2014). This raises questions regarding IL-35 in the functions of $CD138^+$ plasma cells in our model, and whether DC10 treatments impact this putative B cell IL-35 response. To address this question, we could purify $CD138^+$ cells from asthmatic mice, and rest them for a few days to detect IL-35 production *in vitro*. In addition, to evaluate the role of IL-35 expression by B cells *in vivo,* we also could use DC10 to treat asthmatic mice with a B cell-restricted deficiency in p35, or EBI3 (Shen, Roch et al. 2014), or control wild-type asthmatic mice, and then to see the effect of DC10 on antibody secreting B cells.

There is a constant cross talk between DCs and B cells under physiological conditions. Maddur reported that BCR-activated and TLR9 (CpG)-stimulated B cells significantly reduce the expression of multiple immunoregulatory surface markers on DCs, including dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), CD83, CD40,

CD80, CD86, HLA-DR and CD58. Such human B cell-DC interactions were reported to be cell contact-dependant (Maddur, Kaveri et al. 2012). Morva also reported that DC maturation and function can be regulated by human B cells, and that inefficient B cell regulation might lead to improper balance between the inflammatory and tolerance responses (Morva, Lemoine et al. 2012). The DC of B cell-deficient μMT mice secrete high levels of IL-12p70 compared to wild-type mice, with no IL-4 production, fostering DC-mediated inflammatory response (Moulin, Andris et al. 2000). But, in addition, in the absence of IL-10^{high} secreting B cells (i.e., putative Breg), DC similarly augment inflammatory responses (Moulin, Andris et al. 2000) -we know that co-activation of CD40 and TLR9 on B cell induces IL-10 secretion by these cells (Morva, Lemoine et al. 2012).

We know from studies in our DC10 immunotherapy models that DC10-induced Tregs subsequently and induce endogenous lung dendritic cells to take on a regulatory phenotype, such that full asthma tolerance can be passively transferred with these induced DCreg (C. Li, W. Dawicki, H. Huang and J. R. Gordon, unpublished observations). It was shown that T cells are not required for B10 (IL-10 producing regulatory B cells) cell development (DiLillo, Matsushita et al. 2010), nor is MHC class I or II molecule signalling required (Yanaba, Bouaziz et al. 2009). While we have shown that DC10 can directly and indirectly impact the allergen-specific B cell response, we don't know whether these tolerogenic DC10 convert B cells to a regulatory phenotype or not, but this will be an important point for future investigation in asthma immunotherapy. An alternate approach in asthma immunotherapy is the elimination of IgE-producing plasma cells, but we really don't have a full appreciation of the long-term consequences of ablating IgE-producing plasma cells. IgE secretion is tightly regulated and associated with the cytokine network, which might indicate that the IgE responses form part of a wider, beneficial immune response. Studies in past decades have shown a critical role for IgE in mast cells survival, growth and maturation (Kashiwakura, Kawakami et al. 2009), such that while the total elimination of IgE-producing plasma cells may relief symptoms, its long-term impact remains unknown (Firer 2014). Tolerogenic DC10 immunotherapy could offer advantages in that they may down-regulate allergen-specific IgE responses without unduly influencing other cell types.

The respiratory tract is routinely in contact with microbial and other environmental antigens. Bronchus-associated lymphoid tissue (BALT), found between the bronchi and arteries and under the muscularis mucosa, is not constitutively present in all mammals. In adult mice, the frequency of BALT formation is about 43% (Pabst and Gehrke 1990), although pulmonary exposure of neonatal mice to inflammatory stimuli, such as LPS, induces the development of a more organized inducible BALT (iBALT) (Rangel-Moreno, Carragher et al. 2011) than is found in adults. BALT expands significantly in response to immunostimulation and infection, resulting in the recruitment of antigen-primed "mucosal-homing" T and B lymphocytes into the inflamed bronchial mucosa (Holt 1993). Once formed, BALT can also contain follicular dendritic cells (FDC), resident dendritic cells, high endothelial venules (the main entry sites for lymphocytes into BALT (Otsuki, Ito et al. 1989)), and formed lymphatics (Rangel-Moreno, Carragher et al. 2011). We knew that after i.p. delivery tolerogenic DC10 increasingly accumulate in the lung and lung-draining lymph nodes of asthmatic animals (Huang 2012), and we suspect DC10 might migrate to iBALT of asthmatic mice where they could regulate effector B and T cells, as well as Treg and plasma cells. It is known that memory B and T lymphocytes and plasma cells are maintained in iBALT and respond locally to secondary challenge (Moyron-Quiroz, Rangel-Moreno et al. 2006), and certainly allergen-presenting DC10 would provide such a secondary challenge, albeit a tolerogenic one. It would be interesting to determine by imaging analysis precisely where in the lungs of DC10-treated asthmatic animals these plasma cell-T cell-DC10 interactions occur.

In conclusion, our observations confirmed that IL-10-differentiated DCs and DC10-induced regulatory T cells both impact the frequency of antigen-specific IgG1 secreting cells in the lung, suggesting that therapeutic application of tolerogenic DCs in the clinic could possibly bring disease relief through multiple mechanisms.

References

Adema, G. J., F. Hartgers, et al. (1997). "A dendritic-cell-derived CC chemokine that preferentially attracts naive T cells." Nature **387**(6634): 713-716.

Afkarian, M., J. R. Sedy, et al. (2002). "T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells." Nature Immunology **3**(6): 549-557.

Ahrens, R., A. Waddell, et al. (2008). "Intestinal macrophage/epithelial cell-derived CCL11/eotaxin-1 mediates eosinophil recruitment and function in pediatric ulcerative colitis." The Journal of Immunology **181**(10): 7390-7399.

Akkoc, T., M. Akdis, et al. (2011). "Update in the mechanisms of allergen-specific immunotheraphy." Allergy, Asthma & Immunology Research **3**(1): 11-20.

Allam, J.-P., N. Novak, et al. (2003). "Characterization of dendritic cells from human oral mucosa: A new Langerhans' cell type with high constitutive FcεRI expression." Journal of Allergy and Clinical Immunology **112**(1): 141-148.

Allam, J.-P., W.-M. Peng, et al. (2008). "Toll-like receptor 4 ligation enforces tolerogenic properties of oral mucosal Langerhans cells." Journal of Allergy and Clinical Immunology **121**(2): 368-374. e361.

Allman, D. and S. Pillai (2008). "Peripheral B cell subsets." Current Opinion in Immunology **20**(2): 149-157.

Alt, F. W., A. L. Bothwell, et al. (1980). "Synthesis of secreted and membrane-bound immunoglobulin mu heavy chains is directed by mRNAs that differ at their 3' ends." Cell **20**(2): 293-301.

Amadi-Obi, A., C.-R. Yu, et al. (2007). "TH17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STAT1." Nature Medicine **13**(6): 711-718.

Anderson, C. L. and H. L. Spiegelberg (1981). "Macrophage receptors for IgE: binding of IgE to specific IgE Fc receptors on a human macrophage cell line, U937." The Journal of Immunology **126**(6): 2470-2473.

Ansotegui, I., L. K. P. Arruda, et al. (2011). "WAO white book author affiliations." WAO White Book on Allergy: 209.

Aujla, S. J. and J. F. Alcorn (2011). "TH 17 cells in asthma and inflammation." Biochimica et Biophysica Acta (BBA)-General Subjects **1810**(11): 1066-1079.

Balhara, J. and A. Gounni (2012). "The alveolar macrophages in asthma: a double-edged sword." Mucosal Immunology **5**(6): 605-609.

Bamboat, Z. M., L. M. Ocuin, et al. (2010). "Conventional DCs reduce liver ischemia/reperfusion injury in mice via IL-10 secretion." The Journal of Clinical Investigation **120**(2): 559-569.

Bamboat, Z. M., J. A. Stableford, et al. (2009). "Human liver dendritic cells promote T cell hyporesponsiveness." The Journal of Immunology **182**(4): 1901-1911.

Banchereau, J., F. Briere, et al. (2000). "Immunobiology of dendritic cells." Annual Review of Immunology **18**(1): 767-811.

Banchereau, J. and R. M. Steinman (1998). "Dendritic cells and the control of immunity." Nature **392**(6673): 245-252.

Batista, F. D. and N. E. Harwood (2009). "The who, how and where of antigen presentation to B cells." Nature Reviews Immunology **9**(1): 15-27.

Baumjohann, D. and K. M. Ansel (2013). "MicroRNA-mediated regulation of T helper cell differentiation and plasticity." Nature Reviews Immunology **13**(9): 666-678.

Berry, M. A., B. Hargadon, et al. (2006). "Evidence of a role of tumor necrosis factor α in refractory asthma." New England Journal of Medicine **354**(7): 697-708.
Besnard, A. G., D. Togbe, et al. (2011). "IL-33-activated dendritic cells are critical for allergic airway inflammation." European Journal of Immunology **41**(6): 1675-1686.
Bettelli, E., Y. Carrier, et al. (2006). "Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells." Nature **441**(7090): 235-238.
Bieneman, A. P., K. L. Chichester, et al. (2005). "Toll-like receptor 2 ligands activate human basophils for both IgE-dependent and IgE-independent secretion." Journal of Allergy and Clinical Immunology **115**(2): 295-301.
Black, J. L. and P. R. Johnson (2000). "What determines asthma phenotype? Is it the interaction between allergy and the smooth muscle?" American Journal of Respiratory and Critical Care Medicine **161**: S207-S210.
Blanchard, C. and M. E. Rothenberg (2009). "Biology of the eosinophil." Advances in Immunology **101**: 81-121.
Bochenek, G., K. Nagraba, et al. (2001). "Prostaglandin D2: biosynthesis, biological properties and participation in bronchial asthma." Polskie Archiwum Medycyny Wewnetrznej 106(1): 601-609.
Bochner, B. S. (2009). "Siglec-8 on human eosinophils and mast cells, and Siglec-F on murine eosinophils, are functionally related inhibitory receptors." Clinical & Experimental Allergy **39**(3): 317-324.
Bousquet, J., P. Scheinmann, et al. (1999). "Sublingual-swallow immunotherapy (SLIT) in patients with asthma due to house-dust mites: a double-blind, placebo-controlled study." Allergy **54**(3): 249-260.
Braunstahl, G.-J., C.-W. Chen, et al. (2013). "The eXpeRience registry: the 'real-world'effectiveness of omalizumab in allergic asthma." Respiratory Medicine **107**(8): 1141-1151.
Breitfeld, D., L. Ohl, et al. (2000). "Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production." The Journal of Experimental Medicine **192**(11): 1545-1552.
Brightbill, H. D., S. Jeet, et al. (2010). "Antibodies specific for a segment of human membrane IgE deplete IgE-producing B cells in humanized mice." The Journal of Clinical Investigation **120**(120 (6)): 2218-2229.
Bruce, C. and P. S. Thomas (2005). "The effect of marimastat, a metalloprotease inhibitor, on allergen-induced asthmatic hyper-reactivity." Toxicology and Applied Pharmacology **205**(2): 126-132.
Bryant, V. L., C. S. Ma, et al. (2007). "Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells." The Journal of Immunology **179**(12): 8180-8190.
Burchill, M. A., J. Yang, et al. (2007). "IL-2 receptor β-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells." The Journal of Immunology **178**(1): 280-290.
Busse, W. W., W. J. Morgan, et al. (2011). "Randomized trial of omalizumab (anti-IgE) for asthma in inner-city children." New England Journal of Medicine **364**(11): 1005-1015.
Butte, M. J., M. E. Keir, et al. (2007). "Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses." Immunity **27**(1): 111-122.

Camateros, P., M. Tamaoka, et al. (2007). "Chronic asthma–induced airway remodeling is prevented by toll-like receptor-7/8 ligand S28463." American Journal of Respiratory and Critical Care Medicine **175**(12): 1241-1249.
Campbell, P., B. Canono, et al. (1994). "Measuements of bacterial ingestion and killing by macorphages." In Current Protocols in Immunology Eds. Coligan, JE, Kruisbeek, AM, Margulies, DH, Shevach, EM, Strober, W., 1994. 3:14.6.1-14.6.13.
Carrasco, Y. R. and F. D. Batista (2006). "B cell recognition of membrane-bound antigen: an exquisite way of sensing ligands." Current Opinion in Immunology **18**(3): 286-291.
Castro, M., S. Mathur, et al. (2011). "Reslizumab for poorly controlled, eosinophilic asthma: a randomized, placebo-controlled study." American Journal of Respiratory and Critical Care Medicine **184**(10): 1125-1132.
Cella, M., A. Engering, et al. (1997). "Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells." Nature **388**(6644): 782-787.
Chamorro, S., J. J. García-Vallejo, et al. (2009). "TLR triggering on tolerogenic dendritic cells results in TLR2 up-regulation and a reduced proinflammatory immune program." The Journal of Immunology **183**(5): 2984-2994.
Chatila, T. A. (2005). "Role of regulatory T cells in human diseases." Journal of Allergy and Clinical Immunology **116**(5): 949-959.
Chen, W., W. Jin, et al. (2003). "Conversion of peripheral CD4+ CD25− naive T cells to CD4+ CD25+ regulatory T cells by TGF-β induction of transcription factor Foxp3." The Journal of Experimental Medicine **198**(12): 1875-1886.
Chen, Y., G. Jiang, et al. (2009). "Distinct response of liver myeloid dendritic cells to endotoxin is mediated by IL-27." Journal of Hepatology **51**(3): 510-519.
Chen, Z., J. R. Gordon, et al. (2002). "Analysis of the gene expression profiles of immature versus mature bone marrow-derived dendritic cells using DNA arrays." Biochemical and Biophysical Research Communications **290**(1): 66-72.
Chorro, L. and F. Geissmann (2010). "Development and homeostasis of 'resident'myeloid cells: the case of the Langerhans cell." Trends in Immunology **31**(12): 438-445.
Chowdhury, P. S., Y. Chen, et al. (2012). "Targeting the junction of CεmX and ε-migis for the specific depletion of mIgE-expressing B cells." Molecular Immunology **52**(3): 279-288.
Chustz, R. T., D. R. Nagarkar, et al. (2011). "Regulation and function of the IL-1 family cytokine IL-1F9 in human bronchial epithelial cells." American Journal of Respiratory Cell and Molecular Biology **45**(1): 145-153.
Coeffier, M., A. Lorentz, et al. (2005). "Epsilon germ-line and IL-4 transcripts are expressed in human intestinal mucosa and enhanced in patients with food allergy." Allergy **60**(6): 822-827.
Coffman, R. L., D. A. Lebman, et al. (1989). "Transforming growth factor beta specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes." The Journal of Experimental Medicine **170**(3): 1039-1044.
CONLEY, M. E. and D. L. Delacroix (1987). "Intravascular and mucosal immunoglobulin A: two separate but related systems of immune defense?" Annals of Internal Medicine **106**(6): 892-899.
Coombes, J. L., K. R. Siddiqui, et al. (2007). "A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-β–and retinoic acid–dependent mechanism." The Journal of Experimental Medicine **204**(8): 1757-1764.

Corren, J., T. Casale, et al. (2009). "Safety and tolerability of omalizumab." Clinical & Experimental Allergy **39**(6): 788-797.
Corren, J., R. F. Lemanske Jr, et al. (2011). "Lebrikizumab treatment in adults with asthma." New England Journal of Medicine **365**(12): 1088-1098.
Coutinho, A. E. and K. E. Chapman (2011). "The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights." Molecular and Cellular Endocrinology **335**(1): 2-13.
Crabbe, P., A. Carbonara, et al. (1965). "The normal human intestinal mucosa as a major source of plasma cells containing gamma-A-immunoglobulin. " Laboratory Investigation **14**: 235-248.
Crawford, A., M. MacLeod, et al. (2006). "Primary T cell expansion and differentiation in vivo requires antigen presentation by B cells." The Journal of Immunology **176**(6): 3498-3506.
Crimi, E., S. Voltolini, et al. (1991). "Local immunotherapy with dermatophagoides extract in asthma." Journal of Allergy and Clinical Immunology **87**(3): 721-728.
Cumberbatch, M., R. Dearman, et al. (2000). "Langerhans cell migration." Clinical and Experimental Dermatology **25**(5): 413-418.
Cyster, J. G. (2003). "Homing of antibody secreting cells." Immunological Reviews **194**(1): 48-60.
D'Amato, G., A. Stanziola, et al. (2014). "Treating severe allergic asthma with anti-IgE monoclonal antibody (omalizumab): a review." Multidisciplinary Respiratory Medicine **34**: 44.
Davies, D. R. and S. Chacko (1993). "Antibody structure." Accounts of Chemical Research **26**(8): 421-427.
de Heer, H. J., H. Hammad, et al. (2005). Dendritic cell subsets and immune regulation in the lung. Seminars in Immunology **17**(4): 295-303.
de la Rosa, M., S. Rutz, et al. (2004). "Interleukin-2 is essential for CD4+ CD25+ regulatory T cell function." European Journal of Immunology **34**(9): 2480-2488.
de Waal Malefyt, R., Y. Hans, et al. (1992). "Interleukin-10." Current Opinion in Immunology **4**(3): 314-320.
Del Rio, M. L., G. Bernhardt, et al. (2010). "Development and functional specialization of CD103+ dendritic cells." Immunological Reviews **234**(1): 268-281.
Delgado, M. (2009). "Generating tolerogenic dendritic cells with neuropeptides." Human Immunology **70**(5): 300-307.
Demedts, I. K., G. G. Brusselle, et al. (2005). "Matrix metalloproteinases in asthma and COPD." Current Opinion in Pharmacology **5**(3): 257-263.
Demedts, I. K., G. G. Brusselle, et al. (2005). "Identification and characterization of human pulmonary dendritic cells." American Journal of Respiratory Cell and Molecular Biology **32**(3): 177-184.
Denzel, A., U. A. Maus, et al. (2008). "Basophils enhance immunological memory responses." Nature Immunology **9**(7): 733-742.
Depoil, D., S. Fleire, et al. (2007). "CD19 is essential for B cell activation by promoting B cell receptor–antigen microcluster formation in response to membrane-bound ligand." Nature Immunology **9**(1): 63-72.
DiLillo, D. J., T. Matsushita, et al. (2010). "B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer." Annals of the New York Academy of Sciences **1183**(1): 38-57.

Domachowske, J. B., K. D. Dyer, et al. (1998). "Recombinant human eosinophil-derived neurotoxin/RNase 2 functions as an effective antiviral agent against respiratory syncytial virus." Journal of Infectious Diseases **177**(6): 1458-1464.
Dong, H., S. E. Strome, et al. (2002). "Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion." Nature Medicine **8**(8): 793-800.
Dubois, B., G. Joubert, et al. (2009). "Sequential role of plasmacytoid dendritic cells and regulatory T cells in oral tolerance." Gastroenterology **137**(3): 1019-1028.
Duddy, M., M. Niino, et al. (2007). "Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis." The Journal of Immunology **178**(10): 6092-6099.
Duddy, M. E., A. Alter, et al. (2004). "Distinct profiles of human B cell effector cytokines: a role in immune regulation?" The Journal of Immunology **172**(6): 3422-3427.
Dullaers, M., R. De Bruyne, et al. (2012). "The who, where, and when of IgE in allergic airway disease." Journal of Allergy and Clinical Immunology **129**(3): 635-645.
Durham, S. R., S. M. Walker, et al. (1999). "Long-term clinical efficacy of grass-pollen immunotherapy." New England Journal of Medicine **341**(7): 468-475.
Edwards, J. C., L. Szczepański, et al. (2004). "Efficacy of B-cell–targeted therapy with rituximab in patients with rheumatoid arthritis." New England Journal of Medicine **350**(25): 2572-2581.
Erin, E. M., B. R. Leaker, et al. (2006). "The effects of a monoclonal antibody directed against tumor necrosis factor-α in asthma." American Journal of Respiratory and Critical Care Medicine **174**(7): 753-762.
Eyerich, S., K. Eyerich, et al. (2009). "Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling." The Journal of Clinical Investigation **119**(12): 3573-3585.
Fagarasan, S. and T. Honjo (2003). "Intestinal IgA synthesis: regulation of front-line body defences." Nature Reviews Immunology **3**(1): 63-72.
Fairfax, K. A., A. Kallies, et al. (2008). "Plasma cell development: from B-cell subsets to long-term survival niches." Seminars in Immunology **20**(1): 49-58.
Falcone, F. H., H. Haas, et al. (2000). "The human basophil: a new appreciation of its role in immune responses." Blood **96**(13): 4028-4038.
Fanucchi, M. V., E. S. Schelegle, et al. (2004). "Immunostimulatory oligonucleotides attenuate airways remodeling in allergic monkeys." American Journal of Respiratory and Critical Care Medicine **170**(11): 1153-1157.
Farache, J., I. Koren, et al. (2013). "Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation." Immunity **38**(3): 581-595.
Fayette, J., B. Dubois, et al. (1997). "Human dendritic cells skew isotype switching of CD40-activated naive B cells towards IgA1 and IgA2." The Journal of Experimental Medicine **185**(11): 1909-1918.
Fernandes, D. J., R. W. Mitchell, et al. (2003). "Invited review: do inflammatory mediators influence the contribution of airway smooth muscle contraction to airway hyperresponsiveness in asthma?" Journal of Applied Physiology **95**(2): 844-853.
Ferreira, G. B., E. van Etten, et al. (2011). "1, 25-Dihydroxyvitamin D3 alters murine dendritic cell behaviour in vitro and in vivo." Diabetes/Metabolism Research and Reviews **27**(8): 933-941.

Finkelman, M. A., S. J. Lempitski, et al. (2006). "β-Glucans in standardized allergen extracts." Journal of Endotoxin Research **12**(4): 241-245.
Finotto, S., M. F. Neurath, et al. (2002). "Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet." Science **295**(5553): 336-338.
Firer, M. (2014). "Might selective B-cell depletion have a place in targeted allergy therapy?". Journal of Hematology Research 1: 11-15.
Flaishon, L., S. Becker-Herman, et al. (2004). "Expression of the chemokine receptor CCR2 on immature B cells negatively regulates their cytoskeletal rearrangement and migration." Blood **104**(4): 933-941.
Fong, E. W. and R. H. Levin (2007). "Inhaled corticosteroids for asthma." Pediatrics in Review **28**(6): e30-e35.
Fontenot, J. D., J. L. Dooley, et al. (2005). "Developmental regulation of Foxp3 expression during ontogeny." The Journal of Experimental Medicine **202**(7): 901-906.
Fontenot, J. D., M. A. Gavin, et al. (2003). "Foxp3 programs the development and function of CD4+ CD25+ regulatory T cells." Nature Immunology **4**(4): 330-336.
Foroughi, S., B. Foster, et al. (2007). "Anti-IgE treatment of eosinophil-associated gastrointestinal disorders." Journal of Allergy and Clinical Immunology **120**(3): 594-601.
Förster, R., A. Schubel, et al. (1999). "CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs." Cell **99**(1): 23-33.
Fort, M. M., J. Cheung, et al. (2001). "IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo." Immunity **15**(6): 985-995.
Freeman, G. J., A. J. Long, et al. (2000). "Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation." The Journal of Experimental Medicine **192**(7): 1027-1034.
Fulkerson, P. C. and M. E. Rothenberg (2013). "Targeting eosinophils in allergy, inflammation and beyond." Nature Reviews Drug Discovery **12**(2): 117-129.
Galli, S. J., J. Kalesnikoff, et al. (2005). "Mast cells as "tunable" effector and immunoregulatory cells: recent advances." Annual Review of Immunology **23**: 749-786.
Galli, S. J., S. Nakae, et al. (2005). "Mast cells in the development of adaptive immune responses." Nature Immunology **6**(2): 135-142.
Galli, S. J. and M. Tsai (2012). "IgE and mast cells in allergic disease." Nature Medicine **18**(5): 693-704.
GAUVREAU, G. M., J. M. LEE, et al. (2000). "Increased numbers of both airway basophils and mast cells in sputum after allergen inhalation challenge of atopic asthmatics." American Journal of Respiratory and Critical Care Medicine **161**(5): 1473-1478.
Gauvreau, G. M., P. M. O'Byrne, et al. (2014). "Effects of an anti-TSLP antibody on allergen-induced asthmatic responses." The New England Journal of Medicine **2014**(370): 2102-2110.
Genestier, L., M. Taillardet, et al. (2007). "TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses." The Journal of Immunology **178**(12): 7779-7786.
Gessner, A., K. Mohrs, et al. (2005). "Mast cells, basophils, and eosinophils acquire constitutive IL-4 and IL-13 transcripts during lineage differentiation that are sufficient for rapid cytokine production." The Journal of Immunology **174**(2): 1063-1072.

Gidaro, G., F. Marcucci, et al. (2005). "The safety of sublingual-swallow immunotherapy: an analysis of published studies." Clinical & Experimental Allergy **35**(5): 565-571.
Girardi, M. (2006). "Immunosurveillance and immunoregulation by γδ T cells." Journal of Investigative Dermatology **126**(1): 25-31.
Gondek, D. C., L.-F. Lu, et al. (2005). "Cutting edge: contact-mediated suppression by CD4+ CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism." The Journal of Immunology **174**(4): 1783-1786.
Gonzalez-Espinosa, C., S. Odom, et al. (2003). "Preferential signaling and induction of allergy-promoting lymphokines upon weak stimulation of the high affinity IgE receptor on mast cells." The Journal of Experimental Medicine **197**(11): 1453-1465.
Gonzalez-Rey, E., A. Fernandez-Martin, et al. (2006). "Vasoactive intestinal peptide induces CD4+, CD25+ T regulatory cells with therapeutic effect in collagen-induced arthritis." Arthritis & Rheumatism **54**(3): 864-876.
Goodnow, C. C., C. G. Vinuesa, et al. (2010). "Control systems and decision making for antibody production." Nature Immunology **11**(8): 681-688.
Gordon, J. R., F. Li, et al. (2005). "CD8α+, but not CD8α−, dendritic cells tolerize Th2 responses via contact-dependent and-independent mechanisms, and reverse airway hyperresponsiveness, Th2, and eosinophil responses in a mouse model of asthma." The Journal of Immunology **175**(3): 1516-1522.
Gordon, J. R., Y. Ma, et al. (2014). "Regulatory dendritic cells for immunotherapy in immunologic diseases." Frontiers in Immunology **5:7**.
Gould, H. J. and B. J. Sutton (2008). "IgE in allergy and asthma today." Nature Reviews Immunology **8**(3): 205-217.
Gould, H. J., B. J. Sutton, et al. (2003). "The biology of IgE and the basis of allergic disease." Annual Review of Immunology **21**(1): 579-628.
Gowans, J. (1959). "The recirculation of lymphocytes from blood to lymph in the rat." The Journal of Physiology **146**(1): 54-69.
Grazia Roncarolo, M., S. Gregori, et al. (2006). "Interleukin-10-secreting type 1 regulatory T cells in rodents and humans." Immunological Reviews **212**(1): 28-50.
Greenlee, K. J., Z. Werb, et al. (2007). "Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted." Physiological Reviews **87**(1): 69-98.
Guermonprez, P., J. Valladeau, et al. (2002). "Antigen presentation and T cell stimulation by dendritic cells." Annual Review of Immunology **20**(1): 621-667.
Haldar, P., C. E. Brightling, et al. (2009). "Mepolizumab and exacerbations of refractory eosinophilic asthma." New England Journal of Medicine **360**(10): 973-984.
Hammad, H., M. Chieppa, et al. (2009). "House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells." Nature Medicine **15**(4): 410-416.
Hammad, H., M. Plantinga, et al. (2010). "Inflammatory dendritic cells—not basophils—are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen." The Journal of Experimental Medicine **207**(10): 2097-2111.
Harris, L. J., S. B. Larson, et al. (1992). "The three-dimensional structure of an intact monoclonal antibody for canine lymphoma." Nature **360**, 369-372.
Hart, D. N. (1997). "Dendritic cells: unique leukocyte populations which control the primary immune response." Blood **90**(9): 3245-3287.

Hauser, A. E., G. F. Debes, et al. (2002). "Chemotactic responsiveness toward ligands for CXCR3 and CXCR4 is regulated on plasma blasts during the time course of a memory immune response." The Journal of Immunology **169**(3): 1277-1282.
Hedlin, G. and M. van Hage (2012). "Severe asthma and allergy: mechanisms, diagnostics and treatment." Journal of Internal Medicine **272**(2): 104-107.
Heinemann, A., R. Schuligoi, et al. (2003). "Δ12-Prostaglandin J2, a plasma metabolite of prostaglandin D2, causes eosinophil mobilization from the bone marrow and primes eosinophils for chemotaxis." The Journal of Immunology **170**(9): 4752-4758.
Henri, S., D. Vremec, et al. (2001). "The dendritic cell populations of mouse lymph nodes." The Journal of Immunology **167**(2): 741-748.
Hertz, C. J., Q. Wu, et al. (2003). "Activation of Toll-like receptor 2 on human tracheobronchial epithelial cells induces the antimicrobial peptide human β defensin-2." The Journal of Immunology **171**(12): 6820-6826.
Hesslein, D. G. and D. G. Schatz (2001). "Factors and forces controlling V (D) J recombination." Advances in Immunology **78**: 169-232.
Heufler, C., F. Koch, et al. (1996). "Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon- γ production by T helper 1 cells." European Journal of Immunology **26**(3): 659-668.
Hirai, H., K. Tanaka, et al. (2001). "Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2." The Journal of Experimental Medicine **193**(2): 255-262.
Ho, I.-C., T.-S. Tai, et al. (2009). "GATA3 and the T-cell lineage: essential functions before and after T-helper-2-cell differentiation." Nature Reviews Immunology **9**(2): 125-135.
Hogan, S. P., H. F. Rosenberg, et al. (2008). "Eosinophils: biological properties and role in health and disease." Clinical & Experimental Allergy **38**(5): 709-750.
Holgate, S. T. (2012). "Innate and adaptive immune responses in asthma." Nature Medicine **18**(5): 673-683.
Holt, P. (1993). "Development of bronchus associated lymphoid tissue (BALT) in human lung disease: a normal host defence mechanism awaiting therapeutic exploitation?" Thorax **48**(11): 1097.
Holt, P., J. Sedgwick, et al. (1984). "Long-lived IgE-and IgG-secreting cells in rodents manifesting persistent antibody responses." Cellular Immunology **89**(2): 281-289.
Holt, P. and W. Thomas (2005). "Sensitization to airborne environmental allergens: unresolved issues." Nature Immunology **6**(10): 957-960.
Holt, P. G., S. Haining, et al. (1994). "Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways." The Journal of Immunology **153**(1): 256-261.
Holt, P. G. and J. W. Upham (2004). "The role of dendritic cells in asthma." Current Opinion in Allergy and Clinical Immunology **4**(1): 39-44.
Hoshino, M. and J. Ohtawa (2012). "Effects of adding omalizumab, an anti-immunoglobulin E antibody, on airway wall thickening in asthma." Respiration **83**(6): 520-528.
Howarth, P. H., K. S. Babu, et al. (2005). "Tumour necrosis factor (TNFα) as a novel therapeutic target in symptomatic corticosteroid dependent asthma." Thorax **60**(12): 1012-1018.
Hu-Li, J., C. Pannetier, et al. (2001). "Regulation of expression of IL-4 alleles: analysis using a chimeric GFP/IL-4 gene." Immunity **14**(1): 1-11.

Huang, H. (2012). "Mechanisms mediating asthma tolerance induced by regulatory dendritic cells through CD4+CD25+Foxp3+ T regulatory cells." Doctoral dissertation, University of Saskatchewan.
Huang, H., W. Dawicki, et al. (2010). "Tolerogenic dendritic cells induce CD4+ CD25hiFoxp3+ regulatory T cell differentiation from CD4+ CD25−/loFoxp3− effector T cells." The Journal of Immunology **185**(9): 5003-5010.
Huang, H., Y. Ma, et al. (2013). "Comparison of induced versus natural regulatory T cells of the same TCR specificity for induction of tolerance to an environmental antigen." The Journal of Immunology **191**(3): 1136-1143.
Hubo, M., B. Trinschek, et al. (2013). "Costimulatory molecules on immunogenic versus tolerogenic human dendritic cells." Frontiers in Immunology **4: 82**.
Igyártó, B. Z., K. Haley, et al. (2011). "Skin-resident murine dendritic cell subsets promote distinct and opposing antigen-specific T helper cell responses." Immunity **35**(2): 260-272.
Imaoka, H., H. Campbell, et al. (2011). "TPI ASM8 reduces eosinophil progenitors in sputum after allergen challenge." Clinical & Experimental Allergy **41**(12): 1740-1746.
Ivanov, I. I., B. S. McKenzie, et al. (2006). "The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17+ T helper cells." Cell **126**(6): 1121-1133.
Jacobs, H. and L. Bross (2001). "Towards an understanding of somatic hypermutation." Current Opinion in Immunology **13**(2): 208-218.
Jawdat, D. M., E. J. Albert, et al. (2004). "IgE-mediated mast cell activation induces Langerhans cell migration in vivo." The Journal of Immunology **173**(8): 5275-5282.
Joetham, A., K. Takada, et al. (2007). "Naturally occurring lung CD4+ CD25+ T cell regulation of airway allergic responses depends on IL-10 induction of TGF-β." The Journal of Immunology **178**(3): 1433-1442.
Johansson-Lindbom, B., M. Svensson, et al. (2005). "Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing." The Journal of Experimental Medicine **202**(8): 1063-1073.
Johnson, P. R., M. Roth, et al. (2001). "Airway smooth muscle cell proliferation is increased in asthma." American Journal of Respiratory Critical Care Medicine **164**(3): 474-477.
Jonuleit, H., E. Schmitt, et al. (2000). "Induction of interleukin 10–producing, nonproliferating CD4+ T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells." The Journal of Experimental Medicine **192**(9): 1213-1222.
Jutel, M., M. Akdis, et al. (2003). "IL-10 and TGF- β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy." European Journal of Immunology **33**(5): 1205-1214.
Jutel, M., L. Jaeger, et al. (2005). "Allergen-specific immunotherapy with recombinant grass pollen allergens." Journal of Allergy and Clinical Immunology **116**(3): 608-613.
Jutel, M., T. Watanabe, et al. (2001). "Histamine regulates T-cell and antibody responses by differential expression of H1 and H2 receptors." Nature **413**(6854): 420-425.
Kabashima, K., D. Sakata, et al. (2003). "Prostaglandin E2–EP4 signaling initiates skin immune responses by promoting migration and maturation of Langerhans cells." Nature Medicine **9**(6): 744-749.
Kallies, A. (2008). "Distinct regulation of effector and memory T-cell differentiation." Immunology and Cell Biology **86**(4): 325-332.

Kallies, A., E. D. Hawkins, et al. (2006). "Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance." Nature Immunology **7**(5): 466-474.
Kampen, G. T., S. Stafford, et al. (2000). "Eotaxin induces degranulation and chemotaxis of eosinophils through the activation of ERK2 and p38 mitogen-activated protein kinases" Blood **95**(6): 1911-1917.
Kaplan, M. H., U. Schindler, et al. (1996). "Stat6 is required for mediating responses to IL-4 and for the development of Th2 cells." Immunity **4**(3): 313-319.
Kaser, A., S. Dunzendorfer, et al. (1999). "A role for IL-16 in the cross-talk between dendritic cells and T cells." The Journal of Immunology **163**(6): 3232-3238.
Kashiwakura, J.-i., Y. Kawakami, et al. (2009). "Polyclonal IgE induces mast cell survival and cytokine production." Allergology International **58**(3): 411-419.
Kawabe, T., T. Naka, et al. (1994). "The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation." Immunity **1**(3): 167-178.
Kawakami, T. and S. J. Galli (2002). "Regulation of mast-cell and basophil function and survival by IgE." Nature Reviews Immunology **2**(10): 773-786.
Kay, A. (2001). "Allergy and allergic diseases. Second of two parts." The New England Journal of Medicine **344**(2): 109.
Kearley, J., J. E. Barker, et al. (2005). "Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+ CD25+ regulatory T cells is interleukin 10 dependent." The Journal of Experimental Medicine **202**(11): 1539-1547.
King, C., S. G. Tangye, et al. (2008). "T follicular helper (TFH) cells in normal and dysregulated immune responses." Annual Reviews of Immunology **26**: 741-766.
Klaus, G., J. Humphrey, et al. (1980). "The follicular dendritic cell: its role in antigen presentation in the generation of immunological memory." Immunological Reviews **53**(1): 3-28.
KleinJan, A., J. Vinke, et al. (2000). "Local production and detection of (specific) IgE in nasal B-cells and plasma cells of allergic rhinitis patients." European Respiratory Journal **15**(3): 491-497.
Kolbeck, R., A. Kozhich, et al. (2010). "MEDI-563, a humanized anti–IL-5 receptor α mAb with enhanced antibody-dependent cell-mediated cytotoxicity function." Journal of Allergy and Clinical Immunology **125**(6): 1344-1353. e1342.
Koopman, G., H. K. Parmentier, et al. (1991). "Adhesion of human B cells to follicular dendritic cells involves both the lymphocyte function-associated antigen 1/intercellular adhesion molecule 1 and very late antigen 4/vascular cell adhesion molecule 1 pathways." The Journal of Experimental Medicine **173**(6): 1297-1304.
Koppel, E. A., C. W. Wieland, et al. (2005). "Specific ICAM-3 grabbing nonintegrin-related 1 (SIGNR1) expressed by marginal zone macrophages is essential for defense against pulmonary Streptococcuspneumoniae infection." European Journal of Immunology **35**(10): 2962-2969.
Korn, T., E. Bettelli, et al. (2007). "IL-21 initiates an alternative pathway to induce proinflammatory TH17 cells." Nature **448**(7152): 484-487.
Kumar, R. K., C. Herbert, et al. (2003). "Inhibition of inflammation and remodeling by roflumilast and dexamethasone in murine chronic asthma." Journal of Pharmacology and Experimental Therapeutics **307**(1): 349-355.

Kurata, H., H. J. Lee, et al. (1999). "Ectopic expression of activated Stat6 induces the expression of Th2-specific cytokines and transcription factors in developing Th1 cells." Immunity **11**(6): 677-688.
Kushnir, N., L. Liu, et al. (1998). "Dendritic cells and resting B cells form clusters in vitro and in vivo: T cell independence, partial LFA-1 dependence, and regulation by cross-linking surface molecules." The Journal of Immunology **160**(4): 1774-1781.
Lambrecht, B. N. and H. Hammad (2009). "Biology of lung dendritic cells at the origin of asthma." Immunity **31**(3): 412-424.
Lambrecht, B. N. and H. Hammad (2012). "The airway epithelium in asthma." Nature Medicine **18**(5): 684-692.
Lambrecht, B. N. and H. Hammad (2012). "Lung dendritic cells in respiratory viral infection and asthma: from protection to immunopathology." Annual Review of Immunology **30**: 243-270.
Lang, R., D. Patel, et al. (2002). "Shaping gene expression in activated and resting primary macrophages by IL-10." The Journal of Immunology **169**(5): 2253-2263.
Langenkamp, A., M. Messi, et al. (2000). "Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells." Nature Immunology **1**(4): 311-316.
Larché, M. and D. C. Wraith (2005). "Peptide-based therapeutic vaccines for allergic and autoimmune diseases." Nature Medicine **11**: S69-S76.
Latchman, Y., C. R. Wood, et al. (2001). "PD-L2 is a second ligand for PD-1 and inhibits T cell activation." Nature Immunology **2**(3): 261-268.
Lechler, R. and J. R. Batchelor (1982). "Restoration of immunogenicity to passenger cell-depleted kidney allografts by the addition of donor strain dendritic cells." The Journal of Experimental Medicine **155**(1): 31-41.
Lee, B. O., L. Haynes, et al. (2002). "The biological outcome of CD40 signaling is dependent on the duration of CD40 ligand expression reciprocal regulation by interleukin (IL)-4 and IL-12." The Journal of Experimental Medicine **196**(5): 693-704.
Lee, J. J., D. Dimina, et al. (2004). "Defining a link with asthma in mice congenitally deficient in eosinophils." Science **305**(5691): 1773-1776.
Lee, S. Y., J. Y. Cho, et al. (2006). "Immunostimulatory DNA inhibits allergen-induced peribronchial angiogenesis in mice." Journal of Allergy and Clinical Immunology **117**(3): 597-603.
Leung, S. Y., A. Niimi, et al. (2006). "Effect of transforming growth factor-β receptor I kinase inhibitor 2, 4-disubstituted pteridine (SD-208) in chronic allergic airway inflammation and remodeling." Journal of Pharmacology and Experimental Therapeutics **319**(2): 586-594.
Li, L. and V. Boussiotis (2008). "Control and regulation of peripheral tolerance in allergic inflammatory disease: therapeutic consequences." Chemical Immunology and Allergy **94**: 178-188.
Li, X., A. Yang, et al. (2010). "Induction of type 2 T helper cell allergen tolerance by IL-10–differentiated regulatory dendritic cells." American Journal of Respiratory Cell and Molecular Biology **42**(2): 190-199.
Lighvani, A. A., D. M. Frucht, et al. (2001). "T-bet is rapidly induced by interferon-γ in lymphoid and myeloid cells." Proceedings of the National Academy of Sciences **98**(26): 15137-15142.

Lin, T.-J., L. H. Maher, et al. (2003). "Selective early production of CCL20, or macrophage inflammatory protein 3α, by human mast cells in response to Pseudomonas aeruginosa." Infection and Immunity **71**(1): 365-373.
Linterman, M. A., L. Beaton, et al. (2010). "IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses." The Journal of Experimental Medicine **207**(2): 353-363.
Lipworth, B. J. (1999). "Systemic adverse effects of inhaled corticosteroid therapy: a systematic review and meta-analysis." Archives and Internal Medicine **159**(9): 941-955.
Liu, W., A. L. Putnam, et al. (2006). "CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells." The Journal of Experimental Medicine **203**(7): 1701-1711.
Liu, Y., D. Joshua, et al. (1989). "Mechanism of antigen-driven selection in germinal centres."
Liu, Y. J., S. Oldfield, et al. (1988). "Memory B cells in T cell-dependent antibody responses colonize the splenic marginal zones." European Journal of Immunology **18**(3): 355-362.
Lordan, J. L., F. Bucchieri, et al. (2002). "Cooperative effects of Th2 cytokines and allergen on normal and asthmatic bronchial epithelial cells." The Journal of Immunology **169**(1): 407-414.
Lu, L.-F., E. F. Lind, et al. (2006). "Mast cells are essential intermediaries in regulatory T-cell tolerance." Nature **442**(7106): 997-1002.
Lu, M., W. Dawicki, et al. (2011). "Therapeutic induction of tolerance by IL-10-differentiated dendritic cells in a mouse model of house dust mite-asthma." Allergy **66**(5): 612-620.
Luger, E. O., V. Fokuhl, et al. (2009). "Induction of long-lived allergen-specific plasma cells by mucosal allergen challenge." Journal of Allergy and Clinical Immunology **124**(4): 819-826. e814.
Lutz, M. B., N. Kukutsch, et al. (1999). "An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow." Journal of Immunological Methods **223**(1): 77-92.
Lutz, M. B., N. A. Kukutsch, et al. (2000). "Culture of bone marrow cells in GM-CSF plus high doses of lipopolysaccharide generates exclusively immature dendritic cells which induce alloantigen-specific CD4 T cell anergy in vitro." European Journal of Immunology **30**(4): 1048-1052.
Macfarlane, A. J., O. M. Kon, et al. (2000). "Basophils, eosinophils, and mast cells in atopic and nonatopic asthma and in late-phase allergic reactions in the lung and skin." Journal of Allergy and Clinical Immunology **105**(1): 99-107.
MacGlashan, D., B. S. Bochner, et al. (1997). "Down-regulation of Fc (epsilon) RI expression on human basophils during in vivo treatment of atopic patients with anti-IgE antibody." The Journal of Immunology **158**(3): 1438-1445.
Maddur, M. S., S. V. Kaveri, et al. (2012). "Regulation of human dendritic cells by B cells depends on the signals they receive." Blood **119**(16): 3863-3864.
Malaviya, R., N. J. Twesten, et al. (1996). "Mast cells process bacterial Ags through a phagocytic route for class I MHC presentation to T cells." The Journal of Immunology **156**(4): 1490-1496.
Maldonado-López, R., T. De Smedt, et al. (1999). "CD8α+ and CD8α− subclasses of dendritic cells direct the development of distinct T helper cells in vivo." The Journal of Experimental Medicine **189**(3): 587-592.

Mandels, T., R. Phippsi, et al. (1980). "The follicular dendritic cell: long term antigen retention during immunity." Immunological Reviews **53**(1): 29-59.
Manis, J. P., M. Tian, et al. (2002). "Mechanism and control of class-switch recombination." Trends in Immunology **23**(1): 31-39.
Maraskovsky, E., K. Brasel, et al. (1996). "Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified." The Journal of Experimental Medicine **184**(5): 1953-1962.
Marone, G., M. Triggiani, et al. (2005). "Mast cells and basophils: friends as well as foes in bronchial asthma?" Trends in Immunology **26**(1): 25-31.
Marshall, J. S. (2004). "Mast-cell responses to pathogens." Nature Reviews Immunology **4**(10): 787-799.
Martin, F. and J. F. Kearney (2002). "Marginal-zone B cells." Nature Reviews Immunology **2**(5): 323-335.
Masopust, D. and J. M. Schenkel (2013). "The integration of T cell migration, differentiation and function." Nature Reviews Immunology **13**(5): 309-320.
Matsukura, S., C. Stellato, et al. (2001). "Interleukin-13 upregulates eotaxin expression in airway epithelial cells by a STAT6-dependent mechanism." American Journal of Respiratory Cell and Molecular Biology **24**(6): 755-761.
Mauad, T., E. H. Bel, et al. (2007). "Asthma therapy and airway remodeling." Journal of Allergy and Clinical Immunology **120**(5): 997-1009.
Mauri, C. and M. R. Ehrenstein (2008). "The 'short'history of regulatory B cells." Trends in Immunology **29**(1): 34-40.
McFadden Jr, E. (1995). "Perspectives in β 2-agonist therapy: Vox clamantis in deserto vel lux in tenebris?" Journal of Allergy and Clinical Immunology **95**(3): 641-651.
McIlroy, D., B. Autran, et al. (1995). "Infection frequency of dendritic cells and CD4+ T lymphocytes in spleens of human immunodeficiency virus-positive patients." Journal of Virology **69**(8): 4737-4745.
McWilliam, A. S., D. Nelson, et al. (1994). "Rapid dendritic cell recruitment is a hallmark of the acute inflammatory response at mucosal surfaces." The Journal of Experimental Medicine **179**(4): 1331-1336.
Melén, E. and G. Pershagen (2012). "Pathophysiology of asthma: lessons from genetic research with particular focus on severe asthma." Journal of Internal Medicine **272**(2): 108-120.
Merad, M., F. Ginhoux, et al. (2008). "Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells." Nature Reviews Immunology **8**(12): 935-947.
Miyashita, T., M. J. McIlraith, et al. (1997). "Bidirectional regulation of human B cell responses by CD40-CD40 ligand interactions." The Journal of Immunology **158**(10): 4620-4633.
Mizoguchi, A. and A. K. Bhan (2006). "A case for regulatory B cells." The Journal of Immunology **176**(2): 705-710.
Mizoguchi, A., E. Mizoguchi, et al. (2002). "Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation." Immunity **16**(2): 219-230.
Molet, S., Q. Hamid, et al. (2001). "IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines." Journal of Allergy and Clinical Immunology **108**(3): 430-438.

Mori, Y., H. Iwasaki, et al. (2009). "Identification of the human eosinophil lineage-committed progenitor: revision of phenotypic definition of the human common myeloid progenitor." The Journal of Experimental Medicine **206**(1): 183-193.
Morva, A., S. Lemoine, et al. (2012). "Maturation and function of human dendritic cells are regulated by B lymphocytes." Blood **119**(1): 106-114.
Moulin, V., F. Andris, et al. (2000). "B lymphocytes regulate dendritic cell (Dc) function in vivo increased interleukin 12 production by DCs from B cell–deficient mice results in T helper cell type 1 deviation." The Journal of Experimental Medicine **192**(4): 475-482.
Moyron-Quiroz, J. E., J. Rangel-Moreno, et al. (2006). "Persistence and responsiveness of immunologic memory in the absence of secondary lymphoid organs." Immunity **25**(4): 643-654.
Mukai, K., K. Matsuoka, et al. (2005). "Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells." Immunity **23**(2): 191-202.
Müller, U., C. A. Akdis, et al. (1998). "Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom." Journal of Allergy and Clinical Immunology **101**(6): 747-754.
Muniz, V. S., P. F. Weller, et al. (2012). "Eosinophil crystalloid granules: structure, function, and beyond." Journal of Leukocyte Biology **92**(2): 281-288.
Nakajima, H., H. Sano, et al. (1994). "Role of vascular cell adhesion molecule 1/very late activation antigen 4 and intercellular adhesion molecule 1/lymphocyte function-associated antigen 1 interactions in antigen-induced eosinophil and T cell recruitment into the tissue." The Journal of Experimental Medicine **179**(4): 1145-1154.
Nakajima, T., N. Inagaki, et al. (2002). "Marked increase in CC chemokine gene expression in both human and mouse mast cell transcriptomes following Fcε receptor I cross-linking: an interspecies comparison." Blood **100**(12): 3861-3868.
Nakano, T., T. Sonoda, et al. (1985). "Fate of bone marrow-derived cultured mast cells after intracutaneous, intraperitoneal, and intravenous transfer into genetically mast cell-deficient W/Wv mice. Evidence that cultured mast cells can give rise to both connective tissue type and mucosal mast cells." The Journal of Experimental Medicine **162**(3): 1025-1043.
Nathan, A. T., E. A. Peterson, et al. (2009). "Innate immune responses of airway epithelium to house dust mite are mediated through β-glucan–dependent pathways." Journal of Allergy and Clinical Immunology **123**(3): 612-618.
Naureckas, E. T., I. Maurice N., et al. (1999). "Bronchoalveolar lavage fluid from asthmatic subjects is mitogenic for human airway smooth muscle." American Journal of Respiratory and Critical Care Medicine **160**(6): 2062-2066.
Nayyar, A. (2009). Therapeutic immunomodulation of allergic lung disease using regulatory dendritic cells in a mouse model of asthma. Doctoral dissertation, University of Saskatchewan.
Nayyar, A., W. Dawicki, et al. (2012). "Induction of prolonged asthma tolerance by IL-10–differentiated dendritic cells: differential impact on airway hyperresponsiveness and the Th2 immunoinflammatory response." The Journal of Immunology **189**(1): 72-79.
Neill, D. R., S. H. Wong, et al. (2010). "Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity." Nature **464**(7293): 1367-1370.

Neta, R. and S. Salvin (1974). "Specific suppression of delayed hypersensitivity: the possible presence of a suppressor B cell in the regulation of delayed hypersensitivity." The Journal of Immunology **113**(6): 1716-1725.
Nurieva, R. I., Y. Chung, et al. (2009). "Bcl6 mediates the development of T follicular helper cells." Science **325**(5943): 1001-1005.
O'Connor, B. P., M. Cascalho, et al. (2002). "Short-lived and long-lived bone marrow plasma cells are derived from a novel precursor population." The Journal of Experimental Medicine **195**(6): 737-745.
Obata, K., K. Mukai, et al. (2007). "Basophils are essential initiators of a novel type of chronic allergic inflammation." Blood **110**(3): 913-920.
Oh, S.-W., C. I. Pae, et al. (2002). "Tryptase inhibition blocks airway inflammation in a mouse asthma model." The Journal of Immunology **168**(4): 1992-2000.
Oldfield, W. L., M. Larche, et al. (2002). "Effect of T-cell peptides derived from Fel d 1 on allergic reactions and cytokine production in patients sensitive to cats: a randomised controlled trial." The Lancet **360**(9326): 47-53.
Otsuki, Y., Y. Ito, et al. (1989). "Lymphocyte subpopulations in high endothelial venules and lymphatic capillaries of bronchus-associated lymphoid tissue (BALT) in the rat." American Journal of Anatomy **184**(2): 139-146.
Oxelius, V., L. Hanson, et al. (1985). "IgG3 deficiency: common in obstructive lung disease. Hereditary in families with immunodeficiency and autoimmune disease." Monographs in Allergy **20**: 106-115.
Ozdemir, C., M. Akdis, et al. (2009). "T regulatory cells and their counterparts: masters of immune regulation." Clinical & Experimental Allergy **39**(5): 626-639.
Pabst, R. and I. Gehrke (1990). "Is the bronchus-associated lymphoid tissue (BALT) an integral structure of the lung in normal mammals, including humans?" American Journal of Respiratory Cell and Molecular Biology **3**(2): 131-135.
Page, K., J. R. Ledford, et al. (2010). "Research Mucosal sensitization to German cockroach involves protease-activated receptor-2." Respiratory Research **11**:62.
Park, C., S. Li, et al. (2000). "Immune response in Stat2 knockout mice." Immunity **13**(6): 795-804.
Pawankar, R., G. Canonica, et al. (2011). "WAO white book on allergy." Milwaukee, WI: World Allergy Organization: 1-216.
Pawankar, R., G. W. Canonica, et al. (2012). "Allergic diseases and asthma: a major global health concern." Current Opinion in Allergy and Clinical Immunology **12**(1): 39-41.
Pecaric-Petkovic, T., S. A. Didichenko, et al. (2009). "Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33." Blood **113**(7): 1526-1534.
Phillips, J., M. Everson, et al. (1990). "Synergistic effect of IL-4 and IFN-gamma on the expression of polymeric Ig receptor (secretory component) and IgA binding by human epithelial cells." The Journal of Immunology **145**(6): 1740-1744.
Piccirillo, C. A., J. J. Letterio, et al. (2002). "CD4+ CD25+ regulatory T cells can mediate suppressor function in the absence of transforming growth factor β1 production and responsiveness." The Journal of Experimental Medicine **196**(2): 237-246.
Piemonti, L., P. Monti, et al. (2000). "Vitamin D3 affects differentiation, maturation, and function of human monocyte-derived dendritic cells." The Journal of Immunology **164**(9): 4443-4451.

Pilette, C., S. R. Durham, et al. (2004). "Mucosal immunity in asthma and chronic obstructive pulmonary disease: a role for immunoglobulin A?" Proceedings of the American Thoracic Society **1**(2): 125-135.
Polat, G., J. Laufer, et al. (1993). "Cross-linking of monocyte plasma membrane Fc alpha, Fc gamma or mannose receptors induces TNF production." Immunology **80**(2): 287.
Pope, S. M., P. C. Fulkerson, et al. (2005). "Identification of a cooperative mechanism involving interleukin-13 and eotaxin-2 in experimental allergic lung inflammation." Journal of Biological Chemistry **280**(14): 13952-13961.
Poynter, M. E., C. G. Irvin, et al. (2002). "Rapid activation of nuclear factor-κB in airway epithelium in a murine model of allergic airway inflammation." The American Journal of Pathology **160**(4): 1325-1334.
Prieto, J., C. Lensmar, et al. (2000). "Increased interleukin-13 mRNA expression in bronchoalveolar lavage cells of atopic patients with mild asthma after repeated low-dose allergen provocations." Respiratory Medicine **94**(8): 806-814.
Puchelle, E., J. Jacqot, et al. (1986). "In vitro restructuring effect of human airway immunoglobulins A and lysozyme on airway secretions." European Journal of Respiratory Diseases. Supplement **153**: 117-122.
Pulendran, B., J. Smith, et al. (1999). "Distinct dendritic cell subsets differentially regulate the class of immune response in vivo." Proceedings of the National Academy of Sciences **96**(3): 1036-1041.
Pulendran, B., H. Tang, et al. (2010). "Programming dendritic cells to induce TH2 and tolerogenic responses." Nature Immunology **11**(8): 647-655.
Radbruch, A., G. Muehlinghaus, et al. (2006). "Competence and competition: the challenge of becoming a long-lived plasma cell." Nature Reviews Immunology **6**(10): 741-750.
Randolph, G. J., J. Ochando, et al. (2008). "Migration of dendritic cell subsets and their precursors." Annual Reviews of Immunology **26**: 293-316.
Rangel-Moreno, J., D. M. Carragher, et al. (2011). "The development of inducible bronchus-associated lymphoid tissue depends on IL-17." Nature Immunology **12**(7): 639-646.
Reinhardt, R. L., H.-E. Liang, et al. (2009). "Cytokine-secreting follicular T cells shape the antibody repertoire." Nature Immunology **10**(4): 385-393.
Riccio, A., R. Dal Negro, et al. (2011). "Omalizumab modulates bronchial reticular basement membrane thickness and eosinophil infiltration in severe persistent allergic asthma patients." International Journal of Immunopathology & Pharmacology **25**(2): 475-484.
Rissoan, M.-C., V. Soumelis, et al. (1999). "Reciprocal control of T helper cell and dendritic cell differentiation." Science **283**(5405): 1183-1186.
Robbiani, D. F., R. A. Finch, et al. (2000). "The leukotriene C 4 transporter MRP1 regulates CCL19 (MIP-3β, ELC)–dependent mobilization of dendritic cells to lymph nodes." Cell **103**(5): 757-768.
Rock, K. L., B. Benacerraf, et al. (1984). "Antigen presentation by hapten-specific B lymphocytes. I. Role of surface immunoglobulin receptors." The Journal of Experimental Medicine **160**(4): 1102-1113.
Romani, N., S. Koide, et al. (1989). "Presentation of exogenous protein antigens by dendritic cells to T cell clones. Intact protein is presented best by immature, epidermal Langerhans cells." The Journal of Experimental Medicine **169**(3): 1169-1178.

Rousset, F., E. Garcia, et al. (1992). "Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes." Proceedings of the National Academy of Sciences **89**(5): 1890-1893.
Rubtsov, Y. P., J. P. Rasmussen, et al. (2008). "Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces." Immunity **28**(4): 546-558.
Rutella, S. and R. M. Lemoli (2004). "Regulatory T cells and tolerogenic dendritic cells: from basic biology to clinical applications." Immunology Letters **94**(1): 11-26.
Saenz, S. A., M. C. Siracusa, et al. (2010). "IL25 elicits a multipotent progenitor cell population that promotes TH2 cytokine responses." Nature **464**(7293): 1362-1366.
Sallusto, F., M. Cella, et al. (1995). "Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products." The Journal of Experimental Medicine **182**(2): 389-400.
Sallusto, F., J. Geginat, et al. (2004). "Central memory and effector memory T cell subsets: function, generation, and maintenance." Annual Reviews of Immunology **22**: 745-763.
Sallusto, F., D. Lenig, et al. (1999). "Two subsets of memory T lymphocytes with distinct homing potentials and effector functions." Nature **401**(6754): 708-712.
Salvi, S. and S. Holgate (1999). "Could the airway epithelium play an important role in mucosal immunoglobulin A production?" Clinical & Experimental Allergy **29**(12): 1597-1605.
Samitas, K., J. Lötvall, et al. (2010). "B cells: from early development to regulating allergic diseases." Archivum Immunologiae et Therapiae Experimentalis **58**(3): 209-225.
Sánchez-Sánchez, N., L. Riol-Blanco, et al. (2006). "The multiple personalities of the chemokine receptor CCR7 in dendritic cells." The Journal of Immunology **176**(9): 5153-5159.
Sayama, K., M. Diehn, et al. (2002). "Transcriptional response of human mast cells stimulated via the FcεRI and identification of mast cells as a source of IL-11." BMC immunology **3**(1): 5.
Scheeren, F. A., M. Naspetti, et al. (2005). "STAT5 regulates the self-renewal capacity and differentiation of human memory B cells and controls Bcl-6 expression." Nature Immunology **6**(3): 303-313.
Schneider, A. M., F. Li, et al. (2001). "Induction of pulmonary allergen-specific IgA responses or airway hyperresponsiveness in the absence of allergic lung disease following sensitization with limiting doses of ovalbumin–alum." Cellular Immunology **212**(2): 101-109.
Schneider, E., A.-F. Petit-Bertron, et al. (2009). "IL-33 activates unprimed murine basophils directly in vitro and induces their in vivo expansion indirectly by promoting hematopoietic growth factor production." The Journal of Immunology **183**(6): 3591-3597.
Sehmi, R., S. Dorman, et al. (2003). "Allergen-induced fluctuation in CC chemokine receptor 3 expression on bone marrow CD34+ cells from asthmatic subjects: significance for mobilization of haemopoietic progenitor cells in allergic inflammation." Immunology **109**(4): 536-546.
Semlali, A., E. Jacques, et al. (2010). "Thymic stromal lymphopoietin–induced human asthmatic airway epithelial cell proliferation through an IL-13–dependent pathway." Journal of Allergy and Clinical Immunology **125**(4): 844-850.
Shen, P., T. Roch, et al. (2014). "IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases." Nature **507**(7492): 366-370.

Shevach, E.M. (2004). "Fatal attraction: tumors beckon regulatory T cells." Nature Medicine **10**(9): 900-901.
Shortman, K. and Y.-J. Liu (2002). "Mouse and human dendritic cell subtypes." Nature Reviews Immunology **2**(3): 151-161.
Sigmundsdottir, H., J. Pan, et al. (2007). "DCs metabolize sunlight-induced vitamin D3 to'program'T cell attraction to the epidermal chemokine CCL27." Nature Immunology **8**(3): 285-293.
Siracusa, M. C., S. A. Saenz, et al. (2011). "TSLP promotes interleukin-3-independent basophil haematopoiesis and type 2 inflammation." Nature **477**(7363): 229-233.
Sornasse, T., V. Flamand, et al. (1992). "Antigen-pulsed dendritic cells can efficiently induce an antibody response in vivo." The Journal of Experimental Medicine **175**(1): 15-21.
Soumelis, V., P. A. Reche, et al. (2002). "Human epithelial cells trigger dendritic cell–mediated allergic inflammation by producing TSLP." Nature Immunology **3**(7): 673-680.
Spiegelberg, H. (1989). "Biological role of different antibody classes." International Archives of Allergy and Immunology **90**(Suppl. 1): 22-27.
Sprent, J., J.-H. Cho, et al. (2008). "T cell homeostasis." Immunology and Cell Biology **86**(4): 312-319.
Stämpfli, M. R., R. E. Wiley, et al. (1998). "GM-CSF transgene expression in the airway allows aerosolized ovalbumin to induce allergic sensitization in mice." Journal of Clinical Investigation **102**(9): 1704.
Steinman, R. M. (2012). "Decisions about dendritic cells: past, present, and future." Annual Review of Immunology **30**: 1-22.
Steinman, R. M. and J. Banchereau (2007). "Taking dendritic cells into medicine." Nature **449**(7161): 419-426.
Steinman, R. M. and Z. A. Cohn (1974). "Identification of a novel cell type in peripheral lymphoid organs of mice II. Functional properties in vitro." The Journal of Experimental medicine **139**(2): 380-397.
Stone, K. D., C. Prussin, et al. (2010). "IgE, mast cells, basophils, and eosinophils." Journal of Allergy and Clinical Immunology **125**(2): S73-S80.
Straumann, A., S. Conus, et al. (2009). "Anti-interleukin-5 antibody treatment (mepolizumab) in active eosinophilic oesophagitis: a randomized, placebo-controlled, double-blind trial." Gut:178558.
Süss, G. and K. Shortman (1996). "A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand-induced apoptosis." The Journal of Experimental Medicine **183**(4): 1789-1796.
Szabo, S. J., S. T. Kim, et al. (2000). "A novel transcription factor, T-bet, directs Th1 lineage commitment." Cell **100**(6): 655-669.
Takatsu, K. (2011). "Interleukin-5 and IL-5 receptor in health and diseases." Proceedings of the Japan Academy. Series B, Physical and Biological Sciences **87**(8): 463.
Takhar, P., C. J. Corrigan, et al. (2007). "Class switch recombination to IgE in the bronchial mucosa of atopic and nonatopic patients with asthma." Journal of Allergy and Clinical Immunology **119**(1): 213-218.
Tangye, S. G., D. T. Avery, et al. (2003). "Intrinsic differences in the proliferation of naive and memory human B cells as a mechanism for enhanced secondary immune responses." The Journal of Immunology **170**(2): 686-694.

Trifilieff, A., C. Walker, et al. (2002). "Pharmacological profile of PKF242 - 484 and PKF241 - 466, novel dual inhibitors of TNF- α converting enzyme and matrix metalloproteinases, in models of airway inflammation." British Journal of Pharmacology **135**(7): 1655-1664.
Tsai, M., S.-Y. Tam, et al. (2002). "Mast cells derived from embryonic stem cells: a model system for studying the effects of genetic manipulations on mast cell development, phenotype, and function in vitro and in vivo." International Journal of Hematology **75**(4): 345-349.
Tsujimura, Y., K. Obata, et al. (2008). "Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis." Immunity **28**(4): 581-589.
Uehara, A., Y. Fujimoto, et al. (2007). "Various human epithelial cells express functional Toll-like receptors, NOD1 and NOD2 to produce anti-microbial peptides, but not proinflammatory cytokines." Molecular Immunology **44**(12): 3100-3111.
Ueno, H., E. Klechevsky, et al. (2011). "Targeting human dendritic cell subsets for improved vaccines." Seminars in Immunology **23**(1): 21-27.
Unger, W. W., S. Laban, et al. (2009). "Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: Differential role for PD-L1." European Journal of Immunology **39**(11): 3147-3159.
Usui, T., J. C. Preiss, et al. (2006). "T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on IFNG gene acetylation and transcription." The Journal of Experimental Medicine **203**(3): 755-766.
Varney, V., J. Edwards, et al. (1997). "Clinical efficacy of specific immunotherapy to cat dander: a double-blind placebo-controlled trial." Clinical & Experimental Allergy **27**(8): 860-867.
Vignali, D. A., L. W. Collison, et al. (2008). "How regulatory T cells work." Nature Reviews Immunology **8**(7): 523-532.
Vinuesa, C. G. and P.-P. Chang (2013). "Innate B cell helpers reveal novel types of antibody responses." Nature Immunology **14**(2): 119-126.
Vliagoftis, H., A. Schwingshackl, et al. (2000). "Proteinase-activated receptor-2–mediated matrix metalloproteinase-9 release from airway epithelial cells." Journal of Allergy and Clinical Immunology **106**(3): 537-545.
von Garnier, C., L. Filgueira, et al. (2005). "Anatomical location determines the distribution and function of dendritic cells and other APCs in the respiratory tract." The Journal of Immunology **175**(3): 1609-1618.
Vremec, D., J. Pooley, et al. (2000). "CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen." The Journal of Immunology **164**(6): 2978-2986.
Vroman, H., B. van den Blink, et al. (2014). "Mode of dendritic cell activation; the decisive hand in Th2/Th17 cell differentiation. Implications in asthma severity?" Immunobiology **220** (2): 254-261.
Walter, M. J., N. Kajiwara, et al. (2001). "Interleukin 12 p40 production by barrier epithelial cells during airway inflammation." The Journal of Experimental Medicine **193**(3): 339-352.
Wang, Y.-H., P. Angkasekwinai, et al. (2007). "IL-25 augments type 2 immune responses by enhancing the expansion and functions of TSLP-DC–activated Th2 memory cells." The Journal of Experimental Medicine **204**(8): 1837-1847.

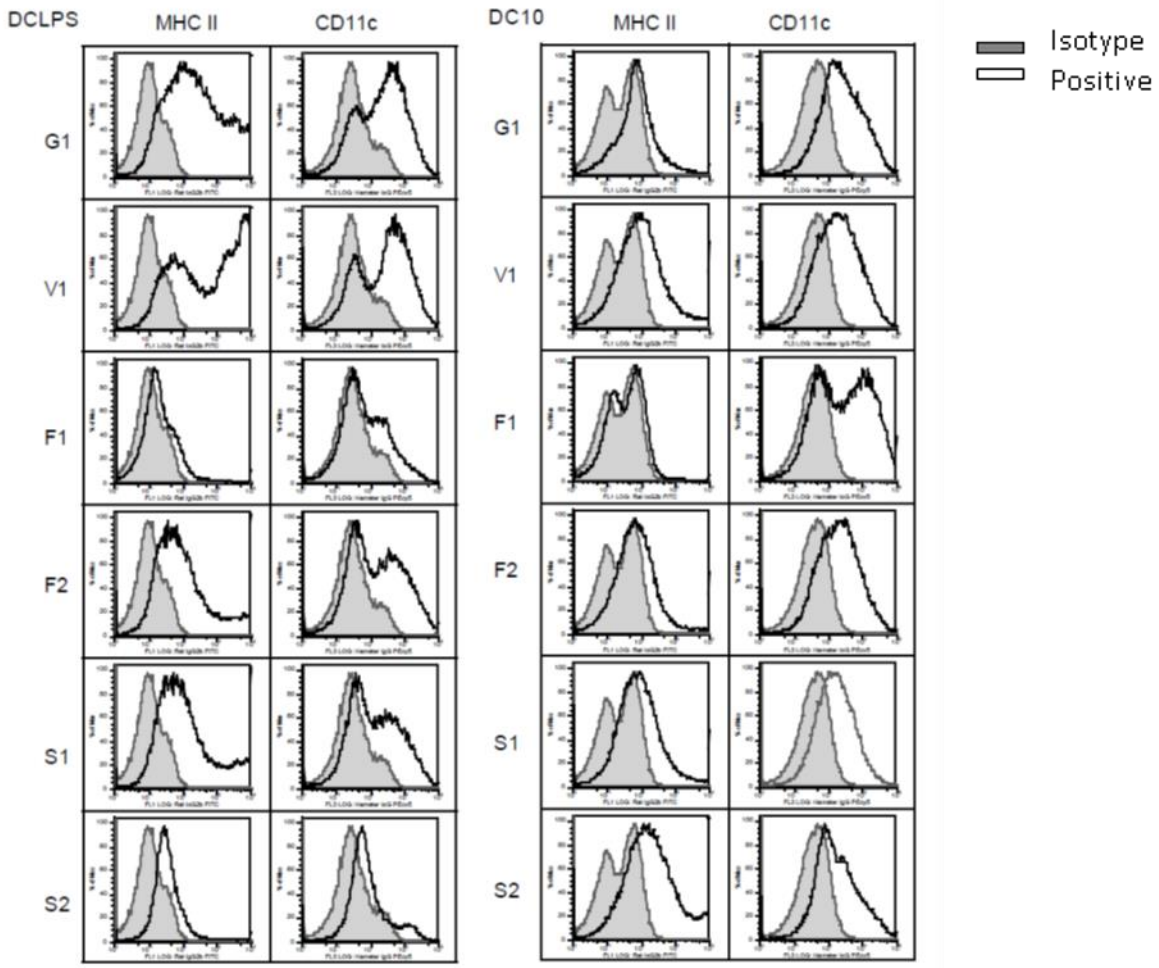
Wegmann, M., R. Goggel, et al. (2007). "Effects of a low-molecular-weight CCR-3 antagonist on chronic experimental asthma." American Journal of Respiratory Cell and Molecular Biology **36**(1): 61-67.
Weiner, H. L., A. P. da Cunha, et al. (2011). "Oral tolerance." Immunological Reviews **241**(1): 241-259.
Wershil, B. K., Z.-S. Wang, et al. (1991). "Recruitment of neutrophils during IgE-dependent cutaneous late phase reactions in the mouse is mast cell-dependent. Partial inhibition of the reaction with antiserum against tumor necrosis factor-alpha." Journal of Clinical Investigation **87**(2): 446.
Williams, C. M. and S. J. Galli (2000). "Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice." The Journal of Experimental Medicine **192**(3): 455-462.
Williams, L. M. and A. Y. Rudensky (2007). "Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3." Nature Immunology **8**(3): 277-284.
Witmer-Pack, M. D., W. Olivier, et al. (1987). "Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells." The Journal of Experimental Medicine **166**(5): 1484-1498.
Woerly, G., N. Roger, et al. (1999). "Expression of Cd28 and Cd86 by human eosinophils and role in the secretion of type 1 cytokines (interleukin 2 and interferon γ) Inhibition by Immunoglobulin a Complexes." The Journal of Experimental Medicine **190**(4): 487-496.
Wong, C. K., S. Hu, et al. (2010). "Thymic stromal lymphopoietin induces chemotactic and prosurvival effects in eosinophils: implications in allergic inflammation." American Journal of Respiratory Cell and Molecular Biology **43**(3): 305-315.
Woolhiser, M. R., K. Brockow, et al. (2004). "Activation of human mast cells by aggregated IgG through FcγRI: additive effects of C3a." Clinical Immunology **110**(2): 172-180.
Wykes, M., A. Pombo, et al. (1998). "Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response." The Journal of Immunology **161**(3): 1313-1319.
Xia, S., Z. Guo, et al. (2008). "Hepatic microenvironment programs hematopoietic progenitor differentiation into regulatory dendritic cells, maintaining liver tolerance." Blood **112**(8): 3175-3185.
Xiao, C., S. M. Puddicombe, et al. (2011). "Defective epithelial barrier function in asthma." Journal of Allergy and Clinical Immunology **128**(3): 549-556. e512.
Yamaguchi, T., K. Hirota, et al. (2007). "Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor." Immunity **27**(1): 145-159.
Yanaba, K., J.-D. Bouaziz, et al. (2008). "A regulatory B cell subset with a unique CD1d hi CD5+ phenotype controls T Cell-dependent inflammatory responses." Immunity **28**(5): 639-650.
Yanaba, K., J.-D. Bouaziz, et al. (2009). "The development and function of regulatory B cells expressing IL-10 (B10 cells) requires antigen receptor diversity and TLR signals." The Journal of Immunology **182**(12): 7459-7472.
Yanagihara, Y., K. Kajiwara, et al. (1998). "Cultured basophils but not cultured mast cells induce human IgE synthesis in B cells after immunologic stimulation." Clinical & Experimental Immunology **111**: 136-143.

Yang, X. O., B. P. Pappu, et al. (2008). "T helper 17 lineage differentiation is programmed by orphan nuclear receptors RORα and RORγ." Immunity **28**(1): 29-39.
Yssel, H., S. Lecart, et al. (2001). "Regulatory T cells and allergic asthma." Microbes and Infection **3**(11): 899-904.
Zhang, D.-H., L. Cohn, et al. (1997). "Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene." Journal of Biological Chemistry **272**(34): 21597-21603.
Zhang, J., F. Alt, et al. (1995). "Regulation of class switch recombination of the immunoglobulin heavy chain genes." Immunoglobulin Genes **235**.
Zhao, D.-M., A. M. Thornton, et al. (2006). "Activated CD4+ CD25+ T cells selectively kill B lymphocytes." Blood **107**(10): 3925-3932.
Zheng, W.-p. and R. A. Flavell (1997). "The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells." Cell **89**(4): 587-596.
Zhou, B., M. R. Comeau, et al. (2005). "Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice." Nature Immunology **6**(10): 1047-1053.
Zhou, L., I. I. Ivanov, et al. (2007). "IL-6 programs TH-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways." Nature Immunology **8**(9): 967-974.
Zhu, J., J. Cote-Sierra, et al. (2003). "Stat5 activation plays a critical role in Th2 differentiation." Immunity **19**(5): 739-748.
Zhu, J., B. Min, et al. (2004). "Conditional deletion of Gata3 shows its essential function in TH1-TH2 responses." Nature Immunology **5**(11): 1157-1165.
Zhu, J., H. Yamane, et al. (2006). "GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors." Cell Research **16**(1): 3-10.
Zhu, J., H. Yamane, et al. (2010). "Differentiation of effector CD4 T cell populations." Annual Review of Immunology **28**: 445.
Zimmermann, N., M. McBride, et al. (2008). "Siglec-F antibody administration to mice selectively reduces blood and tissue eosinophils." Allergy **63**(9): 1156-1163.
Zou, W. (2006). "Regulatory T cells, tumour immunity and immunotherapy." Nature Reviews Immunology **6**(4): 295-307.

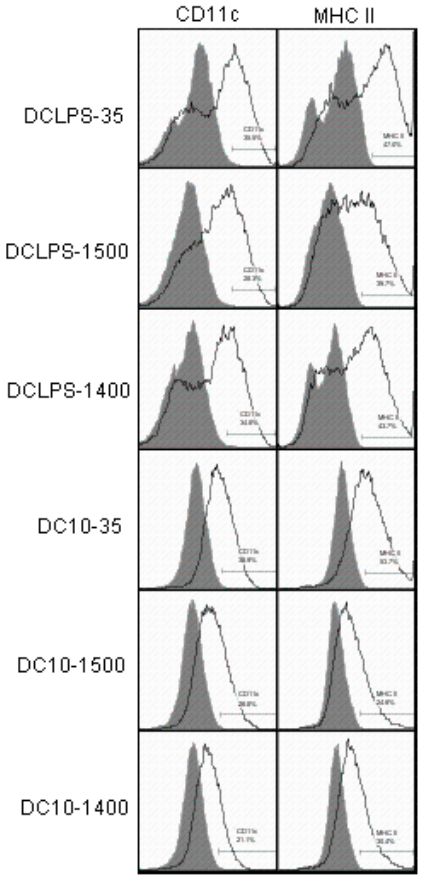
Appendices

Serum test in culturing dendritic cells

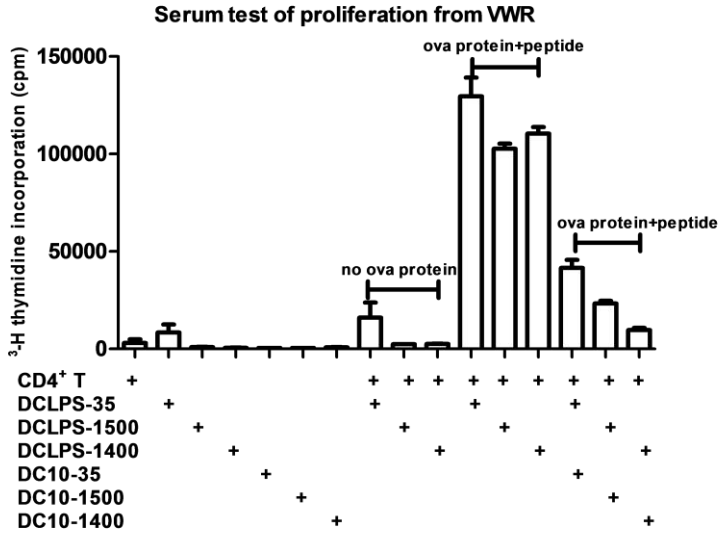
While I have been working on my project for some 3 year now, in reality the data presented in this thesis was realized in the last ≈14 month period. The reason for this delay is that we had issues in the lab that blocked our progression. For example, from June 2012 to August 2013, when we cocultured immunostimulatory OVA-pulsed DCLPS with OVA-specific $CD4^+$T cells, we did not see T cell proliferative responses and we didn't know why - we had reliably seen these responses over several years before that time. Some possibilities were that the GM-CSF we were using to induce dendritic cell differentiation was defective, such that we were not getting differentiation of *bona fide* dendritic cells, so we tested different batches and a range of concentrations of GM-CSF (0, 20, 40, 60, 80 ng/ml; usually we use 7.5 and 20 ng/ml). We found that this didn't make any difference in the T cell proliferative response (data not shown). Another issue was that without informing other lab personnel, a summer student in the lab had used the last of our meticulously optimized sera and ordered a replacement batch of untested serum that went into general lab use. When this was discovered well after the fact, we spent a great deal of time assessing the abilities of several new batches of fetal calf serum to support our *in vitro* DC and T cell responses. This problem was compounded by the company running out of the reserved lot we chose after our first 3-4 month testing phase, so that we had to start the testing process again. We cultured DC10 and DCLPS in complete medium with different serum samples and used expression of CD11c and MHC II as measures of *bone fide* DC induction. As can be seen in Fig. 4.1, the different sera had a major impact on CD11c and MHC II expression. We also assessed the abilities of these cultured DC to support T cell proliferation, and for DC10 to suppress this response (Fig. 4.2). In the end, we identified one serum that worked well in terms of supporting DCLPS-induced Teff cell proliferation, but only if the DC had been pulsed with OVA peptides. It appears that these issues have now been resolved, although we are not certain that we fully identified the problems.



Appendices-1 Surface phenotype of DCLPS and DC10 cultured with different sera.



Appendices-1 Surface phenotype of DCLPS and DC10 cultured with different sera. Identification of commercial sera that would support DC10 and DCLPS differentiation, as determined by expression of CD11c and MHCII. DC10 and DCLPS that were differentiated in cultures containing different serum (i.e., G1, V1, F1, F2, S1, S2 were obtained from different company. Lots 35, 1500 and 1400 were from the same company, but represent different lot numbers) were stained with MHC II and CD11c. Shaded and solid line histograms represent isotype control, and positive staining, respectively.



Appendices-2 Serum test of proliferation and suppression of DC10. Assessment of the abilities of different commercial sera to support DC10-induced suppression of DCLPS-driven $CD4^+$ T cell proliferation. The indicated cells were cocultured for 2 days and ^3H thymidine was added for an additional 24 h. After harvest ^3H thymidine incorporation was determined by liquid scintillation. Each bar represents the mean (±SEM) of 4 wells.