COMPARISON OF THE ABILITIES OF IL-10- AND RETINOIC ACID-DIFFERENTIATED DENDRITIC CELLS TO INDUCE ALLERGEN TOLERANCE IN A MOUSE MODEL OF 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 Department of Veterinary Microbiology

University of Saskatchewan

Saskatoon

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ABSTRACT

Dendritic cells (DCs) in different compartments can affect tolerance via distinct mechanisms. Thus, retinoid acid (RA) and integrins expressed by $CD103^+$ dendritic cells in the gut play important roles in regional regulatory T cell induction and trafficking, while IL-10 expression by lung-associated tolerogenic dendritic cells is integral to tolerance in that compartment. Whether RA- and IL-10-differentiated DC (DCRA and DC10, respectively) can reciprocally induce tolerance in either compartment remains largely unexplored. We have shown that DC10 induce asthma tolerance in part by activating $CD25^+Foxp3^+$ Treg (Huang, Dawicki, Zhang, Town, & Gordon, 2010; Lu et al., 2011), but also by recruiting other cells (e.g., endogenous pulmonary DC) into an infectious tolerance pathway. Herein we began to assess whether DCRA can be equally tolerogenic, and whether they employ similar mechanisms, in an OVA/alum mouse model of asthma. On FACS analysis, we found that DCRA expressed significantly higher levels of CD40, CD86 and MHC II than DC10 (i.e., at levels equivalent to fully mature DC). DCRA secreted higher levels of TGF-β1 and IL-27 than DC10, but equivalent levels of IL-10. DCRA and DC10 suppressed *in vitro* Th2 response, but DCRA were more effective than DC10 at suppressing proliferation. Both DCRA and DC10 increased expression of $Foxp3^+$ on effector T cells. DCRA promoted little expansion of $Foxp3^+$ T cells. In contrast, DC10 promoted expansion of $Foxp3^+$ T cells. Treatment of asthmatic mice with DC10 and DCRA reduced airway hyperresponsiveness and serum allergen-specific IgE and IgG1 levels. We previously showed that DC10-induced tolerance is critically dependent on their expression of IL-10. The results of this study showed that both DCRA and DC10 induce tolerance in asthmatic mice through different mechanisms.

ACKNOWLEDGEMENTS

I am very grateful to my supervisor, Dr. John Gordon, who has led me into the amazing field of immunology. Because of his insightful guidance and tremendous support during my journey towards this degree, I have learned essential research skills and have developed an improved understanding of immunology. I also would like to express my gratitude to my external examiner, Dr. Baljit Singh, and my committee members, Dr. Volker Gerdts, and Dr. Philip Griebel, Dr. Janet Hill, and Dr. Jim Xiang. They have given me great advice throughout different stages of my graduate study.

I would like to thank Dr. Wojciech Dawicki and Xiaobei Zhang for their tremendous help and technical support during my study. My appreciation also extends to other fellow lab mates including Dr. Hui Huang, Brittany Klischuk, Nicole Paur, Nathan Wright, Yanna Ma, and Shui Jiang. I would like to thank Mark Boyd for his assistance during my FACS analysis and the staff of the animal care unit for taking a good care of my experimental mice.

I am indebted to my husband who has given me huge support and filled me with the courage to face difficulties. With his unconditional love, I have completed my project. I would like to give special thanks to my parents and parents-in-law for their tremendous support.

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LIST OF ABBREVIATIONS

Ab	antibody
Ag	antigen
AHR	airway hyperresponsiveness
ALDH	(retin) aldehyde dehydrogenase
Alum	aluminum hydroxide
AMs	alveolar macrophages
APC	antigen-presenting cell
ATRA	all-trans retinoic acid
BAL	bronchoalveolar lavage
BALT	bronchus-associated lymphoid tissue
β2 –ARs	Beta-2 adrenergic receptors
BDCA	blood-dendritic cell antigen
BM-DC	bone marrow-derived dendritic cell
CCL	CC chemokine ligand
CCR	CC chemokine receptor
cDCs	conventional DCs
CRTH2	chemoattractant receptor homologous Th2 cells
CTLA-4	cytotoxic T lymphocyte antigen-4
DC	dendritic cell
DC10	IL-10-differentiated tolerogenic DCs
DClps	lipolysaccharide-stimulated DCs

DCRA	retinoic acid-differentiated tolerogenic DCs
DNA	deoxyribonucleic
dsRNA	double-stranded RNA
DTH	delayed-type hypersensitivity
EAR	early asthmatic reaction
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
ELISA	enzyme-linked immunosorbent assay
EPO	eosinophil peroxidase
FACS	fluorescence-activated cell sorter
Fc	crytallizable fragment of Ab (receptor-binding portion of Ig molecule)
FEV_1	forced expiratory volume in 1 second
FITC	fluorescein isothiocynate
FLT3L	FMS-related tyrosine kinase 3 ligand
Foxp3	forkhead box protein 3
GATA-1	GATA-binding protein 1
GATA-3	GATA-binding protein 3
GITR	glucocorticoid-induced TNF-receptor-related protein
GM-CSF	granulocyte/monocyte colony-stimulating factor
HLA-DR	human leukocyte antigen-DR
HRF	histamine-releasing factor
i.p.	intraperitoneal
ICAM-1	intracellular adhesion molecule-1

ICOS	inducible T-cell costimulator
ICOSL	inducible T-cell costimulator ligand
IDO	indoleamine-2, 3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.t.	intratracheal
iTreg	induced Treg
LAG-3	lymphocyte activation gene-3
LAR	late asthmatic reaction
LIR	leukocyte inhibitory receptor
LPS	lipopolysaccharide
LSM	lymphocyte separation medium
LT	leukotriene
LTC4	leukotriene C4
Mab	monoclonal antibodies
MBP	major basic protein (from eosinophils)
Mch	methacholine
MCP	monocyte chemotactic protein
mDC	myeloid dendritic cell
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase

MPO	myeloperoxidase
mRNA	messenger RNA
NO	nitric oxide
OVA	ovalbumin
PAF	platelet-activating factor
PAR	proteinase-activated receptor
PBMC	peripheral blood mononuclear cell
PD-1	programed death-1
PD-L1	programed death-ligand 1
PD-L2	programed death-ligand 2
pDC	plasmacytoid dendritic cell
Pg	picogram
PG	prostaglandin
PSGL	P-selectin glycoprotein ligand
qRT-PCR	quantitative real-time polymerase chain reaction
RA	retinoic acid
RANTES	regulated on activation normal T cell-expressed and secreted
RAR	retinoic acid receptor
RNA	ribonucleic acid
RORα	RAR related orphan receptor α
RORγt	retinoic-acid-related orphan receptor
RSV	respiratory syncytial virus
RXR	retinoid X receptors

RUNX1	Runt-related transcription factor 1
s.c.	subcutaneous
SCF	stem cell factor
SEM	standard error of the mean
Siglec-H	sialic acid-binding immunoglobulin-like lectin H
SIT	specific immunotherapy
SLIT	Sublingual immunotherapy
ILC-2	group 2 innate lymphoid cells
TARC	thymus and activation regulated chemokine
T-bet	T cell-specific T-box transcription factor 21
Tconv	conventional T cells
TcR	T cell receptor
Teff	effector T cell
T_{FH}	follicular helper T
TGF-β	transforming growth factor-beta
Th	T-helper cell
Th2	Type 2 T-helper cell
Th3	TGF-β-producing T-helper cell
TLR	toll-like receptor
TNF	tumor necrosis factor
Tr1	type 1 regulatory T cells
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin

SLIT	sublingual immunotherapy
VCAM-1	vascular cell adhesion molecule-1
VIP	vasoactive intestinal peptide
VLA-1	very late activation-1
μg	microgram
μl	microliter

CHAPTER 1: INTRODUCTION

Dendritic cells (DCs) are professional antigen-presenting cells that can prime T cell responses in peripheral tissues. DCs pick up antigens, digest them to their constituent peptides which are loaded onto cell surface major histocompatibility complex II (MHCII) proteins, transport these to T cell-enriched areas of draining lymph nodes and present the antigen peptides to naïve T cells (Satthaporn & Eremin, 2001). Asthma is attributable to Th2-driven responses that reinforce the disease through their induction of IgE antibody responses, airway hyperresponsiveness (AHR), and airway inflammation (Murdoch & Lloyd, 2010). DCs play an important role in directing Th2 cell differentiation in asthma. They not only induce immune responses but also immune tolerance.

Prior studies have shown that interleukin (IL)-10-differentiated dendritic cells (DC10) are able to induce asthma tolerance. Within 3 weeks of delivery into "asthmatic" mice, tolerogenic IL-10-differentiated DCs abrogate AHR and begin to reduce OVA-specific IgE and IgG1 levels and ameliorate allergen challenge-induced airway eosinophilia, and BAL and lung parenchymal Th2 cytokine secretion (Nayyar et al., 2012). In particular, DC10 are able to induce tolerance by activating $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells (Treg) (Huang et al., 2010). These Tregs subsequently suppress Teff responses in a contact-dependent manner (Li et al., 2010). Together they dampen the asthma phenotype in the treated animals. DC10-induced tolerance, as assessed to date in our lab, can be broken by repeated high-level allergen challenge but not by physiologic levels of allergen (Nayyar et al., 2012). We also hypothesize that there are alternate tolerogenic dendritic cell strategies that are as effective as DC10 therapy.

Retinoic acid (RA) is a metabolite of vitamin A (retinol) that can regulate the functions of

vitamin A, which is required for cell growth and development. $CD103^+$ DCs in the gut have been reported to be naturally occurring tolerogenic DCs (Coombes et al., 2007). RA is synthesized by $CD103^+$ DCs in the gut-associated lymphoid tissues and plays an important role in controlling T cell trafficking (Iwata, 2009). Its functions include increasing TGF-β-induced expression of Foxp3, impressing B and T cells with gut-homing specificity and enhancing the IgA production of B cells (Coombes et al., 2007). The expression of (retin) aldehyde dehydrogenase (ALDH) 1a is critical to RA production (Yokota et al., 2009). CD103 (alpha (E)) integrin expression can be used to distinguish this gut population of DC (Laffont, Siddiqui, & Powrie, 2010). $CD103^+$ DCs can be found in many lymphoid or non-lymphoid organs of normal mice, including the lungs, skin and intestine (Guilliams et al., 2010). $CD103^+$ DCs promote the conversion of naïve T cells into $Foxp3^+$ Treg cells in a TGF-β- and RA-dependent fashion. Intestinal $CD103^+$ DCs can drive induction of α4β7 and CCR9 expression, which are gut-homing receptors on effector T cells. In the steady state, 60%−70% of DCs can express CD103 (Laffont et al., 2010). Retinoic acid expressing DCs in the gut have reported to have regulatory functions (Coombes et al., 2007). However, the function of LPS matured retinoic acid-differentiated DCs (DCRA) has remained largely unexplored.

DCs in different compartments can affect tolerance via distinct mechanisms. IL-10 expression by lung-associated tolerogenic DCs is integral to tolerance in that compartment. Recent studies (Huang et al., 2010; Lu et al., 2011) have shown that DC10 induce asthma tolerance in part not only by activating $CD25^+Foxp3^+$ Treg but also by recruiting other cells (e.g., endogenous pulmonary DC; C. Li and J.R. Gordon, unpublished observation) into an infectious tolerance pathway. A recent study also demonstrated that retinoic acid-producing dendritic cells are present in lung upon exposure to antigen - these pulmonary $CD103^+$ DCs

upregulated retinoic acid expression after exposure to inhaled Ag. $CD103^+$ DCs from tolerized mice were found to express higher levels of MHC II, indicating they were competent for Ag presentation, but low levels of CD80 and CD86, indicating that they had a reduced capacity to activate naïve T cells (Broeren, Gray, Carreno, & June, 2000). They also observed that the absence of TGF-β signaling in $CD4^+$ T cells significantly reduced the induction of Foxp3 expression by the DCs. It was reported that lung $CD103^+$ DCs from OVA-tolerized mice acquired the ability to induce Foxp3 in $CD4^+$ T cells and that under the tolerized condition TGF-β and RA together promoted $Foxp3^+$ induction in $CD4^+$ T cells (Khare et al., 2013). LPS-exposed DCRA express ALDH1a, as well as Treg growth factors such as IL-27 and TGF-β (W. Dawicki, unpublished observation). However, it remains uncertain whether LPS-treated DCRA display suppressive abilities for effector/memory T cells from asthmatic mice and how they compare to other populations of tolerogenic DCs (e.g., DC10). I hypothesize that DCs that have been exposed to retinoic acid and LPS can be tolerogenic and just as effective therapeutically as DC10 in a mouse model of asthma. Therefore, there is a need to understand (i) how those cells work; (ii) how they induce T regulatory cells in asthma; and (iii) how they compare to DC10.

To our knowledge, this is the first study that shows that LPS-matured retinoic acid-differentiated DCs could reduce AHR, and circulating IgG1 and IgE levels in a mouse model of asthma. The results of this study will help to understand the mechanisms by which tolerogenic DCs are effective in asthma and provide an improved theoretical foundation for the development of clinical therapies.

CHAPTER 2: LITERATURE REVIEW

2.1 Asthma

2.1.1 Prevalence of Asthma

Asthma is a chronic allergic disorder of the lower airways characterized by episodic exacerbations of partially reversible airflow limitation. The clinical manifestations of asthma are believed to be caused by the combined effects of bronchial constriction, airway inflammation and airway hyperresponsiveness in response to various antigens (Bloemen et al., 2007) . The spectrum of symptoms includes coughing, wheezing, chest tightness, and dyspnea. The prevalence of asthma is increasing, particularly among children and young adults in western countries (Saleh, 2008). For example, asthma prevalence in the United States increased from 7.3% to 8.4% over the period of 2001-2010 (Akinbami et al., 2012). During 2006-2008, the prevalence of asthma was higher among children (9.3%) compared with adults (7.3%) and among females (8.6%) compared with males (6.9%) (Moorman et al., 2011). The total incremental cost of asthma in the United States was $56 billion, according to a 2007 investigation (Barnett & Nurmagambetov, 2011). This stresses the necessity of more research and work to reduce the economic burden to health care system.

2.1.2 Mouse Model of Asthma

Asthma is a complex disease, the mechanisms of which are not well understood. Studies on human subjects with asthma help us gain a better understanding on the disease mechanism, but there are ethical and practical considerations for researchers to perform detailed studies on the mechanisms of the disease in humans. The use of animal models can help to gain a better

understanding of effects of specific molecules, proteins or cells in asthma. Furthermore, basic mechanisms can be explored using *in vitro* approaches, but the use of animal models allows studies to be carried out in an intact immune system (Zosky & Sly, 2007).

Very few species naturally develop asthma-like symptoms, so responses that replicate asthma-like features must be induced in animals (Shapiro, 2006). Mice are the most commonly used animal model for asthma due to the easy availability of genetically-modified animals and experimental reagents (Nials & Uddin, 2008). Among the strains, BALB/c and C57BL/6 mice are widely used strains to establish asthma-like responses due to their well-characterized immunological responses (Shin, Takeda, & Gelfand, 2009). Previous studies have focused on the methods to induce allergic inflammation via sensitizing and challenging animals with a variety of antigens (S. J. Kim et al., 2009; Zosky et al., 2008). A number of allergens are able to induce allergic responses in animal models of asthma, including ovalbumin (OVA). The benefits of using OVA include that fact that it is inexpensive and that its immunodominant epitopes are well characterized (Shin et al., 2009). In a popular mouse model of asthma mice are sensitized with 2 intraperitoneal (i.p.) injections of ovalbumin (often conjugated to alum) two weeks apart and the mice are subsequently challenged three times via the airway with 1% (w/v) OVA in saline (Wagers, Lundblad, Ekman, Irvin, & Bates, 2004). Other allergens such as house dust mite, cockroach, and ragweed or fungi have also proven to induce asthma-like responses in animal models (Shin et al., 2009). House dust mite has also been chosen as an allergen to induce allergic response in animal models that resemble human asthma (Lu et al., 2011). Assessment of lung function can be accomplished by whole body plethysmography, which is the unrestrained body measurement of lung volume by measuring respiratory or airflow rates (DeLorme & Moss, 2002). The pathogenesis of asthma of the mouse model includes antibody production and airway

eosinophilia, as well as airway Th2 cytokine production in response to allergen challenge (Trivedi & Lloyd, 2007). The development of mouse model of asthma helps us to understand more detailed mechanisms in asthma.

2.1.3 Immunobiology of Asthma

Asthma is considered to be attributable to Th2-driven responses. Th2 cytokines have been reported to promote IgE production (IL-4 and IL-13), improve growth of mast cells (IL-4 and IL-9), increase eosinophil accumulation (IL-5), and enhance mucus hypersecretion and AHR (IL-9 and IL-13) (Umetsu & DeKruyff, 2006). Structural change, termed airway remodeling, is another feature of asthma; many cytokines such as TGF-β, IL-5, -9, -13 and chemokines such as eotaxin-1 lead to airway remodeling (T. Doherty & Broide, 2007). Mast cells are important for airway remodeling because their mediators (such as tryptase and cytokines) can modulate airway smooth muscle cell function and induce goblet cell hyperplasia (Saito, 2007).

Allergen cross-linking of mast cell surface FcεRI-bound IgE molecules triggers responses including early phase asthmatic reactions (EAR) and late phase asthmatic reactions (LAR). The early response peaks within 30 minutes after allergen challenge and, when it occurs, the late phase follows 4 to 6 hours later (Baraniuk, 1997). In the early asthmatic reaction, on allergen re-exposure and cross-linking of mast cell FcεRI-bound IgE molecules (Turner & Kinet, 1999), mast cells release mediators such as histamine, prostaglandins, leukotrienes, which drive the early asthmatic response (Janeway CA Jr, 2001) and cytokines, which recruit inflammatory cells (e.g., eosinophils, lymphocytes and neutrophils) into the late phase asthmatic reaction. These recruited cells release their own mediators, which together mediate the late phase responses (W. W. Busse & Lemanske, 2001; Janeway CA Jr, 2001).

2.1.3.1 Eosinophils

Eosinophils are produced from hematopoietic stem cells in the bone marrow. Transcription factors such as GATA-1 are particularly important in the differentiation of hematopoietic stem cells into eosinophils (Yamaguchi et al., 1998). Cytokines such as IL-5, IL-3 and GM-CSF are also crucial to the development of eosinophils (Sur et al., 1998). Eosinophils secrete proinflammatory cytokines, chemokines, and lipid mediators following triggering via their immunoglobulin, cytokine, and complement receptors, (Kita, 1996).

Eosinophils are tissue-dwelling cells and most of them reside in the bone marrow, mammary glands, adipose tissues, thymus, gut, and uterus (Mishra, Hogan, Lee, Foster, & Rothenberg, 1999). Under steady state, the ratio of human tissue to blood eosinophils is 100:1 or more (Y. M. Park & Bochner, 2010). In healthy human, eosinophils primarily migrate to the gastrointestinal tract after spending a very brief period in the circulation (Rosenberg, Dyer, & Foster, 2013; Weller, 1991). In response to local tissue inflammatory stimuli, eosinophils accumulate in peripheral tissues (Hogan et al., 2008). There are many factors contributing to eosinophilia; for example, IL-5 plays a pivotal role in eosinophil development, activation and survival (Collins, Marleau, Griffiths-Johnson, Jose, & Williams, 1995).

Eosinophils are innate immune leukocytes involved in initiating and maintaining type 2 immune responses (e.g., asthma and allergy) (Spencer et al., 2009). Under inflammatory conditions, a variety of cytokines (e.g., IL-4, IL-5, and IL-13) are involved in the trafficking of eosinophils into inflammatory sites (Horie et al., 1997; Moser, Fehr, & Bruijnzeel, 1992). The interaction between with IL-5 and eotaxin plays an important role in the regulation of eosinophil homing and migration into tissues (Mould, Matthaei, Young, & Foster, 1997). The epithelial

cell-derived cytokines (i.e., TSLP, IL-25 and IL-33) promote eosinophilia by inducing IL-5 production (Rosenberg et al., 2013).

2.1.3.2 Basophils

Basophils constitute a type of leukocyte involved in inflammation and allergic disease in response to a variety of stimuli. Basophils also develop under the influence of IL-3 from myeloid lineage precursors in the bone marrow, but survive for approximately 5 days after entering the circulation (Spencer et al., 2009). They strongly express the FcεRI on their cell surface; the IgE-dependent stimulation induces release of a variety of pro-allergic inflammatory mediators (i.e., histamine, leukotriene C4 and Th2 cytokines) (Patil & Shreffler, 2012), and expression of a number of primary activation markers (e.g, CD63, CD69, and CD203c)(Gober et al., 2007; Siracusa, Kim, Spergel, & Artis, 2013). The basophil activation test is a relatively common method for predicting food allergy in susceptible patients (Kumar, Verma, Das, & Dwivedi, 2012).

Many studies have indicated that basophils are antigen-presenting cells and interact with $CD4^+$ T cells to promote Th2 immunity; basophils express MHC II, CD40, CD80, and CD86, and secret IL-4, IL-13 and TSLP (Sokol et al., 2009). After activation by protease antigens and cross-linking of cell surface IgE, basophils upregulate their expression of MHC II, CD40, CD80 and CD86 (Yoshimoto et al., 2009). Using a co-culture system consisting of basophils, DCs and T cells, the direct interaction between basophils and T cells has been found to be an crucial factor in Th2 skewing *in vitro* (Sokol & Medzhitov, 2010). TSLP promotes the differentiation of basophils containing few granules, but they also expresses high levels of IL-18Rα, IL33R, IL4, IL6, CCL3, CCL4, CCL12, and CXC-chemokine ligand 2 (CXCL2), compared with IL-3-

elicited basophils (Voehringer, 2013). A protein called histamine-releasing factors (HRF) can stimulate the release of histamine and the production of IL-4 and IL-13 from IgE-sensitized basophils (Kawakami, Kashiwakura, & Kawakami, 2014).

2.1.3.3 Epithelial Cells

When foreign antigens are inhaled into the airway, epithelial cells are the first line of defense, acting not only function as a physical barrier, but also modulating immune responses (Eastburn & Mostov, 2010). Activated in response to exposure to stimuli, airway epithelial cells synthesize a variety of mediators (e.g., cytokines, chemokines, lipid mediators, and peptide mediators) (Proud & Leigh, 2011), which contribute to the activation and the recruitment of immune cells to inflammatory sites and further airway inflammatory responses (Lambrecht & Hammad, 2012). Upon exposure to different stimuli, epithelial cells produce cytokines such as TSLP, GM-CSF IL-8, eotaxin, Regulated on Activation Normal T cell Expressed and Secreted (RANTES) and Thymus and Activation Regulated Chemokine (TARC) (Proud & Leigh, 2011). TSLP is largely produced by epithelial cells and it affects DCs to promote induction of Th2 cell responses (Huston & Liu, 2006). Eotaxin is an agonist for the CCR3 receptor expressed on eosinophils, so is integrally involved in the recruitment of eosinophils to airway inflammatory sites (Takizawa, 2005). TARC is the ligand for the CCR4 receptors expressed on the majority of Th2 lymphocytes (Imai et al., 1999), such that epithelial expression of TARC fosters $CCR4^+$ T cells recruitment to the airway (Andrew et al., 2001).

2.1.3.4 Dendritic Cells

DCs are professional antigen-presenting cells. They capture and process antigens, migrate

to tissue-draining lymphoid organs and there they present their processed antigens to T cells. DCs are able to activate $CD4^+$ T cells, and do so using three classical APC signals: antigen peptide/MHCII complex molecule signals (signal 1), costimulatory molecule signals (e.g., CD40, CD80/CD86, and CD54; signal 2), and cytokine signals (signal 3; Figure 2.1) (Gutcher & Becher, 2007). In addition to activation of T cells, DCs can also contribute to tolerance through expression of inhibitory signal 2-like molecules (e.g., PD-L1, ICOS-L) (C. Y. Chung, Ysebaert, Berneman, & Cools, 2013). Costimulatory molecule and cytokine signals provided by DCs direct the differentiation of $CD4^+$ T cells into different subtypes of T helper cells (i.e., Th1, Th2, Th9, Th17, or Tregs) (Gutcher & Becher, 2007).

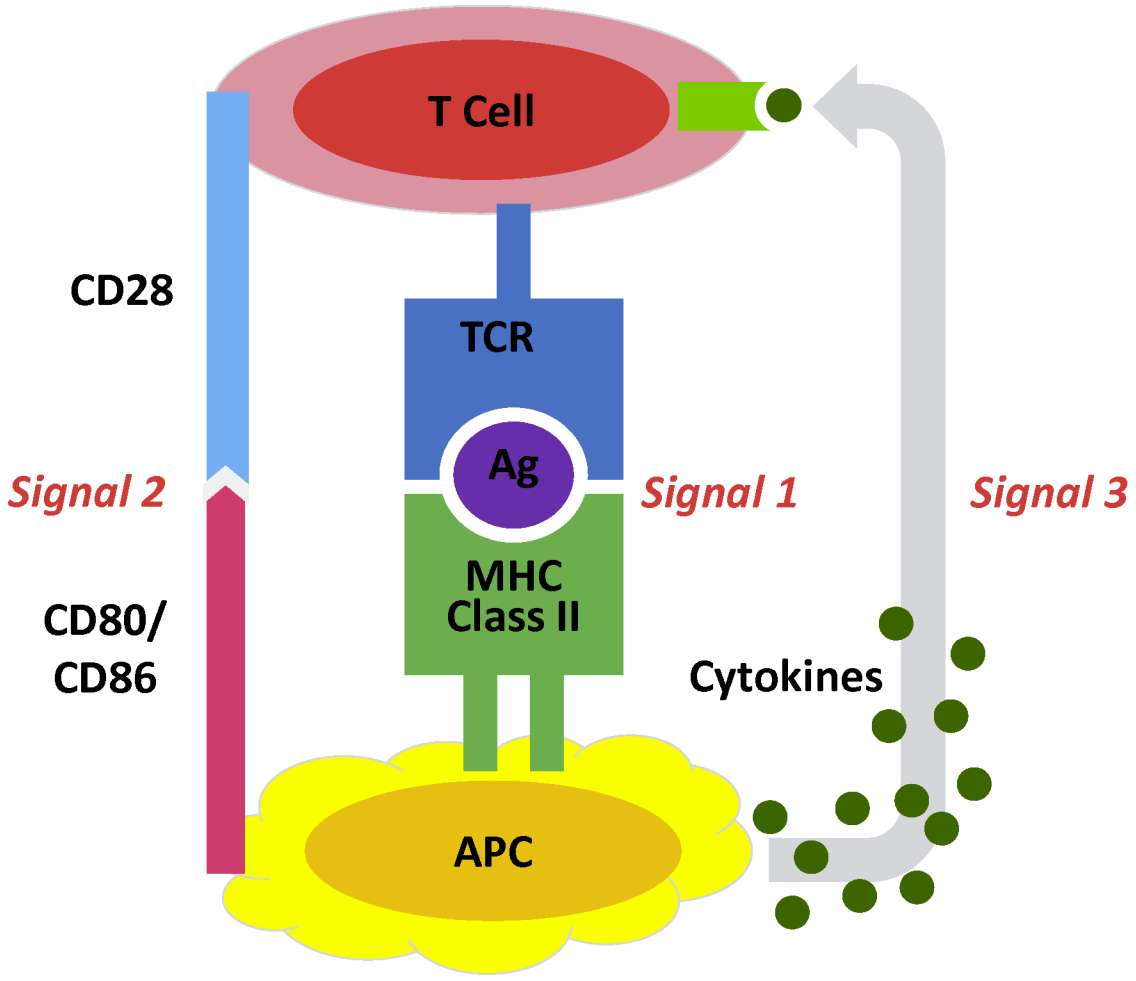


Figure 2.1 Three signals are required for activation and differentiation of naïve T cells. Signal 1 is an antigen peptide/MHCII complex molecule signal. Signal 2 is a cumulative signal related to the various costimulatory molecules and counter-receptors expressed by DC and T cells. Signal 3 is a cytokine signal that induces differential T cell polarization (e.g., Th1 versus Th2). The figure is adapted from (Gutcher & Becher, 2007).

Both DC and macrophages are integral to normal and pathologic lung biology, although there are many differences between these cells in maintaining lung homeostasis and inflammatory responses. Alveolar macrophages (AMs) are found in the alveolar lumen and represent more than 90% of the alveolar cells (Holt, Strickland, Wikstrom, & Jahnsen, 2008; Tournier & Mohamadzadeh, 2008). Dendritic cells also can be found but in smaller populations in alveolar space (Holt et al., 1993). A number of studies demonstrate that the depletion of AMs enhances the antigen presentation activities of lung DCs, suggesting that one role for AMs could be an ongoing suppression of antigen presentation by lung DCs, perhaps as a means to maintain tolerance (Holt et al., 1993; Holt et al., 2008; Thepen, Van Rooijen, & Kraal, 1989). For example, DCs can maintain an immature phenotype when acquiring harmless antigens (Novak & Bieber, 2008). On the other hand, AMs can increase the production of pro-inflammatory cytokines in asthmatic subjects (Gosset et al., 1999). Resting AMs express low levels of the low-affinity FcεR II (IgE receptor), but express them at increased levels in asthma subjects (Williams et al., 1992). Such FcεR II-activated AMs release pro-inflammatory cytokines such as IL-1, TNF-β, and leukotriene (Lane, Sousa, & Lee, 1994) and thereby contribute to innate immune responses. Compared with AMs, DCs can also modulate immune responses through more direct interactions with the adaptive immune systems (Ferenbach & Hughes, 2008).

Many studies have demonstrated the importance of DC to induction of Th2 responses to inhaled antigen and in the pathogenesis of asthma – they play an important role in sensitization to allergens. When CD11c-positive DCs have been deleted from mice, OVA aerosol exposure does not lead to the production of interleukin IL-4, IL-5, and IL-13 by endogenous or adoptively transferred $CD4^+$ Th2 cells (van Rijt et al., 2005), while adoptive transfer of $CD11c^+$ DCs into CD11c-depleted mice restores eosinophilic inflammation and Th2 cytokine secretion (van Rijt et

al., 2005). Moreover, recent studies have found that after taking up various allergens, including ovalbumin, house dust mite or cockroach *in vivo*, $CD103^+$ DCs isolated from the lungs or lung-draining lymph nodes induce Th2 differentiation *in vitro* (Nakano et al., 2012). It was also found that in the absence of $CD103^+$ DCs, mice show reduced Th2 priming to a variety of inhaled allergens and do not develop asthmatic responses after further allergen challenge (Nakano et al., 2012; van Rijt et al., 2005).

2.1.3.5 Th2 Cells

Asthma pathogenesis involves distinct functions of $CD4^+$ T cell subsets, but it is traditionally considered to be a 'Th2 immune' disease. In early studies, $CD4^+$ Th2 cells found in the lung of asthmatic patients expressed more Th2 cytokines than T cells from non-asthmatic patients (Xiao, Yu, & Zheng, 2000). In experimental mouse models of asthma, mice received OVA-specific DO11.10 TCR-transgenic Th2 cells and developed hallmark traits of asthma (e.g., eosinophilic airway inflammation and AHR after OVA challenge) (Maezawa et al., 2004). Moreover, the development of allergic lung inflammation could be prevented in OVA-sensitized wild-type mice by the depletion of $CD4^+$ T cells prior to allergen challenge (T. A. Doherty, Soroosh, Broide, & Croft, 2009).

Th2 cells secrete the IL-4, IL-5 and IL-13 that drive the key pathological features of this disease. IL-4 is essential for allergic sensitization and IgE production (Steinke & Borish, 2001). IL-5 is crucial for development, differentiation, and trafficking of eosinophils (Hamelmann & Gelfand, 2001). IL-13 is another important effector cytokine in asthma, which not only causes airway fibrosis, but also promotes epithelial damage, mucus production, and eosinophilia (Cohn, Elias, & Chupp, 2004). Airway remodeling is another feature of asthma; many cytokines (e.g.,

IL-5, -9, & -13) and chemokines (e.g., eotaxin-1) can lead to airway remodeling (T. Doherty & Broide, 2007). Transgenic mice that over-express IL-13, IL-9 and IL-5 have shown AHR and collagen deposition, suggesting that Th2 cytokines promote airway remodeling (Cohn et al., 2004).

Recent studies have also indicated that that thymic stromal lymphopoietin (TSLP), IL-33, and IL-25, produced by activated epithelial cells, can promote Th2 immune responses, including the production of eosinophilia, IL-4, IL-5, IL-13, and IgE antibody (Ohno, Morita, Arae, Matsumoto, & Nakae, 2012; Tamachi et al., 2006; Ziegler, 2012). Through the effective activation of myeloid DCs, TSLP can promote the induction of inflammatory responses associated with little IL-10 and high TNF-α production; in the absence of IL-12, TSLP can induce OX40 ligand that can trigger inflammatory Th2 differentiation (Liu et al., 2007). IL-33 is a protein that binds to cellular surface receptor ST2 and thus activates intracellular signaling pathways (Ciccia et al., 2013; Palmer & Gabay, 2011). It is released upon cell injury as an alarmin, the production of which is upregulated in inflamed tissue (G. Chen et al., 2010; Ciccia et al., 2013). IL-33 can induce allergic airway inflammation with or without inputs from the adaptive immune system (Kaiko & Foster, 2011). IL-33 has shown the ability to increase release of IL-13, IL-4 and histamine in response to IgE cross-linking (Smith, 2010). As a new member of the IL-17 cytokine family, IL-25 can promote $CD4^+$ Th2 inflammatory responses in the lung, potentially sustaining AHR and acute pulmonary inflammation with eosinophilia (Sharkhuu et al., 2006). Neutralization of IL-25 is associated with decreased IL-13 levels, increased IL-17A production, and a reduction in AHR (Barlow, Flynn, Ballantyne, & McKenzie, 2011).

The development of group 2 innate lymphoid cells (ILC-2, also known as natural type-2 helper cells) requires many transcription factors (e.g., RAR related orphan receptor α (RORα),

GATA-3, T cell factor 1) (Halim et al., 2012; Hoyler et al., 2012; Yang et al., 2013). These cells reside in the mucosa and release large amounts of IL-13 and IL-5 in response to distress signals sent by epithelial cells (Martinez & Vercelli, 2013). ILC-2 are critical to the mounting of robust Th2-mediated allergic lung inflammation in response to the protease-allergen papain (Halim et al., 2014). The transcription factor GATA-3 is also important for differentiation of Th2 cells and regulation of Th2 cytokine expression (Zhou & Ouyang, 2003), such that its expression is increased in the airways in asthma patients compared with normal controls (Nakamura et al., 1999). GATA-3 promotes Th2 type immune responses through its direct binding to inhibitory sites within the Foxp3 promoter, which suppresses Foxp3 expression (Mantel et al., 2007) and thereby regulatory T cell responses.

It has been proposed that reduced exposure to microbial stimuli may facilitate a shift towards Th2 immune response in allergic disease. The *hygiene hypothesis* suggests that microbe-induced Th1 responses may dampen Th2 responses in allergic disease, with the Th1 cytokine IFN-γ inhibiting IgE production and eosinophilia (Bruselle et al., 1997). Deletion of the T-bet transcription factor in mouse models automatically leads to the development of Th2 responses (Finotto et al., 2002; Tang, Desierto, Chen, & Young, 2010). While IFN- γ may counterbalance the Th2 response in allergic disease, administration of recombinant human IFN-γ does not improve the condition of asthma patients compared with controls (Boguniewicz et al., 1995). Indeed, IFN-γ has been shown to work simultaneously with Th2 cytokines in sustaining chronic inflammatory responses in allergic disease (Ngoc, Gold, Tzianabos, Weiss, & Celedon, 2005).

2.1.3.6 Th17 Cells

Recent studies have found Th17 cells, as newly identified T helper cells, are involved in

the pathogenesis of asthma. Th17 cells secrete various types of cytokines, with IL-17 being the most important one among them. IL-17 can induce other cells to secrete cytokines such as IL-8, IL-6, and GM-CSF, as well as chemokines such as CCL2, CCL7, CXCL1, and CCL20 (S. H. Chang & Dong, 2007). There are numerous IL-17 family members, including IL-17A through IL-17F (Wright et al., 2008), with IL-17A being 10-fold more potent than IL-17F (Dubin & Kolls, 2009). Human Th17 cells express the transcription factor ROR-γt and the IL-23 receptor and promote the recruitment of neutrophils (Cosmi, Liotta, Maggi, Romagnani, & Annunziato, 2011). IL-23 promotes IL-17 production by Th17 cells, but IL-6 together with TGF-β, rather than IL-23, have been identified as an important combination for differentiation of Th17 cells *in vitro* and *in vivo* in mice (Croxford, Mair, & Becher, 2012; Oboki, Ohno, Saito, & Nakae, 2008; Zuniga, Jain, Haines, & Cua, 2013). Moreover, orphan nuclear receptor ROR-γt has been identified as a key transcription factor for Th17 differentiation (Ivanov et al., 2006) inasmuch as it plays an essential role in the differentiation of Th17 cells (Ivanov et al., 2006). The family of IL-17 receptors is made of IL17RA-IL17RE (Haines & Cua, 2011).

Th17 cells can induce neutrophil-mediated inflammation in the airway. Prior studies have found that the numbers of Th17 lymphocytes and the level of IL-17 protein expression in sputum and bronchoalveolar lavage (BAL) fluids increased in patients with asthma (Molet et al., 2001; Morishima et al., 2013). In addition, compared with healthy age-matched controls, the plasma concentrations of IL-17 and IL-17 mRNA expression in sputum are increased in asthmatic patients (Bullens et al., 2006; Zhao, Yang, Gao, & Guo, 2010). Evidence has shown that the numbers of neutrophils tend to increase in severe asthma (Jatakanon et al., 1999), such that anti-IL-17 antibody remarkably reduces the numbers of neutrophils in BAL fluids in a murine model of asthma (S. J. Park et al., 2010). IL-17 increases the release of IL-8 by bronchial epithelial cells

and venous endothelial cells and thus recruits neutrophils into the airways in humans (Laan et al., 1999), where increased production of IL-17 is correlated with increases in levels of IL-8 mRNA, neutrophilic inflammation and asthma severity (Bullens et al., 2006). Both Th2 and Th17 cells are able to induce AHR (McKinley et al., 2008), but airway inflammation and AHR mediated by Th17 cells are steroid-resistant, suggesting that Th17 cells may play a potential role in steroid-resistant asthma (McKinley et al., 2008). The evidence described above suggests that Th17 lymphocytes are involved in the pathogenesis of neutrophil-mediated asthma and thus serve as a potential target for asthma therapeutics.

In addition to Th2 cells and Th17 cells, other $CD4^+$ Th cell subsets, such as Th9 cells, also participate in the pathogenesis of asthma. Expression of transcription factor PU.1 is important for differentiation of Th9 cells, which secrete a large amount of IL-9 (H. C. Chang et al., 2010). IL-9 is detected in airway and promotes airway inflammation in asthma (Shimbara et al., 2000), although the pathway by which Th9 cells affect pathological changes in asthma remains unclear.

2.1.3.7 Treg Cells

IL-2 receptor α chain (CD25) was the first surface marker discovered as a Treg marker on $CD4^+$ T cells, such that $CD4^+CD25^+$ T cells are thought to maintain immunologic self-tolerance (Sakaguchi, Sakaguchi, Asano, Itoh, & Toda, 1995). Upon activation, $CD4^+$ T cells (i.e., Th1, Th2 and Th17) express moderate levels of CD25, while $Foxp3^+$ Treg express high levels of CD25 (Atfy et al., 2009). Recently, forkhead box protein 3 (Foxp3) has been described as the most specific marker for Tregs and is considered a master transcription factor for the development and function of $CD4^+CD25^+Foxp3^+$ Tregs (Randolph & Fathman, 2006).

Foxp3 is crucial for the function of some, but not all, regulatory T cells and is regulated by many molecules. IL-2 is an important activator of $CD4^+CD25^+Foxp3^+$ Treg cells (Brandenburg et al., 2008). In the absence of IL-2, TGF-β is not able to induce conventional $CD4^+$ T cells to convert into Tregs (Adalid-Peralta, Fragoso, Fleury, & Sciutto, 2011). In addition, TCR signal promotes microRNA family member miR-126 molecule expression (C. Z. Chen, Schaffert, Fragoso, & Loh, 2013), high expression of which acts through its target gene p85 to downregulate p23k/Akt signalling, and promote Foxp3 expression and induction of iTregs (Qin et al., 2013). This suggests an essential control for at least some miRNAs in the induction of iTregs in the periphery (C. Z. Chen et al., 2013).

Natural regulatory Treg cells (nTregs) are the thymus-derived T cells and important for tolerance and immune homeostasis (Bilate & Lafaille, 2012). nTregs express Foxp3, which is a master regulator for development and function of nTreg (Workman, Szymczak-Workman, Collison, Pillai, & Vignali, 2009). Recent studies have shown that nTreg Foxp3 interacts with Runt-related transcription factor 1 (RUNX1) which inhibits production of IL-2 and IFN-γ (Palomares et al., 2010). nTregs represent 5-10% of $CD4^+$ T cells in the peripheral blood of human (Baraut et al., 2014). Prior studies have shown that nTregs are generated in the thymus through high-avidity interactions between MHC class II molecules and T cell receptors (Curotto de Lafaille & Lafaille, 2009). Foxp3 is important for the function of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Fontenot, Gavin, & Rudensky, 2003). There are no specific markers to distinguish nTregs from iTregs; common markers on both nTregs and $CD25^+Foxp3^+$ iTregs express include Foxp3, CD25, glucocorticoid-induced TNF-receptor-related protein (GITR), cytotoxic T lymphocyte antigen-4 (CTLA-4), programed death-1 (PD-1), Helios, and neuropilin-1 (Nrp1) (Lin et al., 2013). In DC10-induced tolerance in a mouse model of asthma, DC10-

induced Foxp3^{+} Tregs exhibit higher expression of GITR, CTLA-4, and PD-1 in comparison with nTregs (Huang, Dawicki, et al., 2013). "Induced" Tregs can include Foxp3^{+} (iTregs) and Foxp3^{-} populations, such as Th3, Tr1, iTr35, and CD8^{+}CD28^{-} (Povoleri et al., 2013), the primary function of which is to regulate immune responses. Th3 cells secrete a large amount of TGF-β (Carrier, Yuan, Kuchroo, & Weiner, 2007), while Tr1 cells secrete a large amount of IL-10, such that immune regulation by these cells is not dependent on direct cell-cell interactions (Andolfi et al., 2012). IL-27, a potent inducer of Tr1 cells (Pot et al., 2009), is composed of two subunits: EBI3 and p28 (Kastelein, Hunter, & Cua, 2007). Three elements regulate IL-27 production, including the transcription factor c-Maf, IL-21 and the costimulatory receptor ICOS, which collectively promote Tr1 cell differentiation (Pot et al., 2009). IL-27 was initially reported to be pro-inflammatory, but it was subsequently found to have anti-inflammatory functions (Carl & Bai, 2008). Pretreatment of DC with IL-27 diminishes their abilities to induce the differentiation of Th1 and Th17 cells and increases their ability to generate Tregs (Mascanfroni et al., 2013).

Many studies have found changes in the numbers of Tregs and/or the function of Tregs in asthma. Decreased numbers of Tregs in the peripheral blood were found in asthmatic subjects compared with healthy subjects (Abdulamir et al., 2009). The transfer of antigen-specific Tregs from transgenic mice that express ovalbumin- (OVA) specific T cell receptors suppresses AHR and airway inflammation in an OVA-induced mouse model of asthma (Huang et al., 2010; Kearley, Barker, Robinson, & Lloyd, 2005). Evidence also shows that a large number of Foxp3^{+} Tregs accumulate in local lymph nodes, and that is associated with spontaneous resolution of chronic asthma in a mouse model (Carson et al., 2008). Several studies have reported that CD4^{+}CD25^{+} Tregs and IL-10-producing Tregs demonstrate suppressive function to Th2

responses in asthma. Human peripheral blood $CD4^+CD25^+$ T cells from non-atopic subjects suppress autologous allergen-stimulated $CD4^+CD25^-$ T cells proliferation (Ling et al., 2004). In addition, the numbers of Th2 cells in peripheral blood from atopic individuals is increased when the numbers of IL-10-producing Tregs decreases (Robinson, 2009), suggesting that IL-10-producing Tregs were suppressive to allergen-stimulated Th2 cytokine production by Th2 Teff cells. It has been reported that effector $CD4^+$ T cells in the periphery could be converted to $Foxp3^+$ iTregs by DC10 in both human and mouse models of asthma (Huang et al., 2010; Li et al., 2010), while others had reported that naïve $CD4^+$ T cells can be converted into $Foxp3^+$ Treg (Bilate & Lafaille, 2012; Q. Chen, Kim, Laurence, Punkosdy, & Shevach, 2011; Curotto de Lafaille et al., 2008; Finney, Taylor, Wilson, & Maizels, 2007; Kretschmer et al., 2005). Together with this, Tregs are essential to control the immune responses to allergen in asthma. One of the goals for immunological therapy in asthma is to increase the number and/or upregulate the function of Tregs.

2.1.4 Current Therapy of Asthma

The current treatments for asthma include primarily steroids and bronchial muscle relaxants (Gupta & Agrawal, 2010). Inhaled glucocorticoids are commonly used to reduce inflammation, swelling and mucus production in asthmatic patients (Holgate & Polosa, 2008). There are several mechanisms involved in the anti-inflammatory activities of glucocorticoids, including inhibition of cytokine and inflammatory mediator production, down-modulation of adhesion molecule and interference with cellular signaling events (Coutinho & Chapman, 2011). Beta-2 adrenergic receptor (β_2 –ARs) agonists are widely used as bronchodilators, or rescue medications, in individuals suffering from acute asthmatic attacks. Their receptors, which belong

to the G-protein-coupled family of receptors, transfer signals from outside of cells to the inside (McGraw & Liggett, 2005), activating adenylate cyclase and thus increasing cyclic adenosine monophosphate (cAMP) levels (McGraw & Liggett, 2005). These drugs are used to manage asthma, but they do not prevent disease progression or exacerbations (Holgate & Davies, 2009).

IgE antibody is produced by B cells and binds to high-affinity IgE receptors expressed on mast cells and basophils in allergic diseases, where it plays a predominant role in triggering activation of these cells, as noted above (Stone, Prussin, & Metcalfe, 2010). There is a close association between serum levels of IgE and the expression of the IgE receptors so that reductions in circulating IgE can result in significant decreases in receptor expression (MacGlashan et al., 1998). Omalizumab is a recombinant humanized anti-IgE monoclonal antibody that prevents free or serum IgE from binding to the receptors expressed on mast cells and basophils. Among patients diagnosed with moderate-severe allergic asthma, omalizumab treatment reduces asthma exacerbations and corticosteroid requirements. In these studies, the patients were given subcutaneous injections of omalizumab in combination with inhaled corticosteroid, and it was seen that the omalizumab co-treatment helped to reduce the doses of inhaled corticosteroids needed to control the subject's asthma (W. Busse et al., 2001; Hochhaus et al., 2003; Milgrom et al., 2001; Soler et al., 2001). Recent studies have focused on anti-cytokine therapies, such as anti-IL-4Rα, anti-IL-5 and anti-TNF-α (Berry et al., 2006; Corren et al., 2010; Flood-Page et al., 2007). Anti-IL-4Rα antibodies can be used to effectively block both the IL-4 and IL-13 pathway's contributions to pathogenesis (Corren et al., 2010). Anti-IL-5 can effectively suppress airway eosinophilia (Flood-Page et al., 2007), while anti-TNF-α has effects on suppression of AHR and reduction of airway histamine levels (Berry et al., 2006). Combinations of anti-cytokine therapies might lead to improved performance of these therapies

on asthma.

Many therapeutic approaches have been developed to treat asthma. Allergen-specific immunotherapy (SIT) is the treatments that aim to restore the loss of tolerance in allergic patients (Durham et al., 1999; Gabrielsson et al., 2001), but its effectiveness has been largely confined to treatment of venom hypersensitivities and allergic rhinitis or conjunctivitis. SIT is performed by repeated (eg, daily or weekly) subcutaneous or sublingual administration of gradually increasing doses of specific allergens over months to years (Grundmann, Mosters, & Brehler, 2014). The mechanisms of SIT may include the induction of IgG4 antibody to compete with IgE for allergens, which prevents IgE crosslinking and the degranulation of mast cells (Flicker et al., 2008; Niederberger et al., 2004; van Neerven et al., 1999). In addition, the mechanisms of SIT may involve the modulation of DCs to be tolerogenic DCs and thus the induction of Tregs (Larche, Akdis, & Valenta, 2006). SIT has proved effective in many cases, but it has a long duration and its potential for induction of anaphylaxis brings high health risks (Akdis, 2006; Bousquet, Demoly, & Michel, 2001). There is an increasing need to develop more effective and reliable therapies for asthma.

2.2 Dendritic Cell in Asthma

2.2.1 Dendritic Cell Biology

DCs are professional antigen-presenting cells that provide a link between innate and adaptive immunity (Guermonprez, Valladeau, Zitvogel, Thery, & Amigorena, 2002; Trombetta & Mellman, 2005). Due to their specialized abilities of antigen uptake and presentation, DCs induce not only immunity but also induce immune tolerance. DCs sample all forms of antigen in the airways and process them to peptides, which are presented by DCs on their cell surface,

complexed to MHC-II molecules. DCs migrate into lymphoid organs and interact with T cells, with their MHC-II allergen peptide complexes interacting with the T cell receptors (TCR) on cognate T cells to form pMHC-TCR complexes. In particular, DCs require three signals to activate naïve or circulating T cells: antigen peptide/MHCII complex molecule signals, costimulatory molecule signals, and cytokine signals. As noted above, DC costimulatory molecules are needed in this process to fully stimulate the T cells, as are the DCs secreted cytokines, some of which can favor Th2 differentiation of naïve T cells, a prevalent process in the development of asthma (Banchereau et al., 2000).

DCs can be divided into two types, myeloid DC (mDC) and plasmacytoid DC (pDC). mDCs are the major antigen-presenting cells in the body, whereas pDCs play an important role in modulating immune responses (Grayson, 2006). In different tissues, mDCs can be further subdivided based on their expression of specific markers and functions (Steinman & Banchereau, 2007); all mDCs express the integrin CD11c in mice, but their subsets can be characterized according to their additional expression of the myeloid marker CD11b and their anatomical location in the lung (del Rio, Rodriguez-Barbosa, Kremmer, & Forster, 2007; van Rijt et al., 2005). $CD14^+$ monocytes are a popular source of experimental human DC precursors and can differentiate into mDC under the influence of GM-CSF and IL-4 (Kuwana, 2002), while culture of mouse bone marrow in GM-CSF expands an immature mDC population (Lutz et al., 1999).

2.2.2 Lung Dendritic Cell Subsets

Pulmonary DCs are found within and below the airway epithelium, lung parenchyma, visceral pleura and in the alveolar air spaces (Lambrecht & Hammad, 2009; Vermaelen & Pauwels, 2005). They are responsible for immune surveillance under steady condition and can

rapidly respond to environmental and microbial antigens (Ruckwardt, Malloy, Morabito, & Graham, 2014). There are many subsets of pulmonary DCs. Conventional $CD11c^+$ DCs are found in the deeper interstitial compartments and lining the conducting airways (Lambrecht & Hammad, 2009). Based on expression of integrin CD11b, cDCs comprise two subsets. $CD11b^-$ cDCs identify viral particles in the airway lumen, while $CD11b^+$ cDCs pick up antigens passing through the basal membrane (GeurtsvanKessel & Lambrecht, 2008). CD11b is expressed on the surface of many leukocytes, including myeloid DCs, macrophages, natural killer cells, and neutrophils (Solovjov, Pluskota, & Plow, 2005). It is a member of the integrin family and associates with CD18 to form Mac-1 (Berton & Lowell, 1999). As such it is involved the adhesion and migration of leukocytes to inflammatory sites and thus the mediation of inflammatory responses (Streuli, 2009). Markers for pDCs in humans include blood dendritic cell antigen (BDCA)-2, BDCA-4, and the IL-3 receptor (Sapoznikov et al., 2007; Shortman & Liu, 2002). In mice, pDCs are identified as CD11clow cells that co-express B220, Gr-1, mPDCA-1 and 120G8 (Asselin-Paturel, Brizard, Pin, Briere, & Trinchieri, 2003; Sapoznikov et al., 2007; Shortman & Liu, 2002) and selectively express sialic acid-binding immunoglobulin-like lectin H (Siglec-H) (Blasius et al., 2004; Zhang et al., 2006). pDCs have been reported to secrete a large amount of IFN-α in response to viral infection (Siegal et al., 1999); on the other hand, pDCs induce airway tolerance in asthma (de Heer et al., 2004). $CD103^+$ DCs migrate from the airway mucosa to the lung draining lymph nodes under the influence of CCR7 (a lymph node-homing chemokine) (Gordon, Ma, Churchman, Gordon, & Dawicki, 2014). In lymphoid tissue, there is one subset of DCs that uniquely expresses CD8α; in the steady state, $CD8\alpha^+$ DCs display immune regulatory properties and facilitate the maintenance of tolerance to self-antigens (Shortman & Heath, 2010). It has been reported that immunotherapy with quiescent allergen-

presenting $CD8a^+$ DC can partially reverse the asthma phenotype in a mouse model of asthma (Gordon, Li, Nayyar, Xiang, & Zhang, 2005).

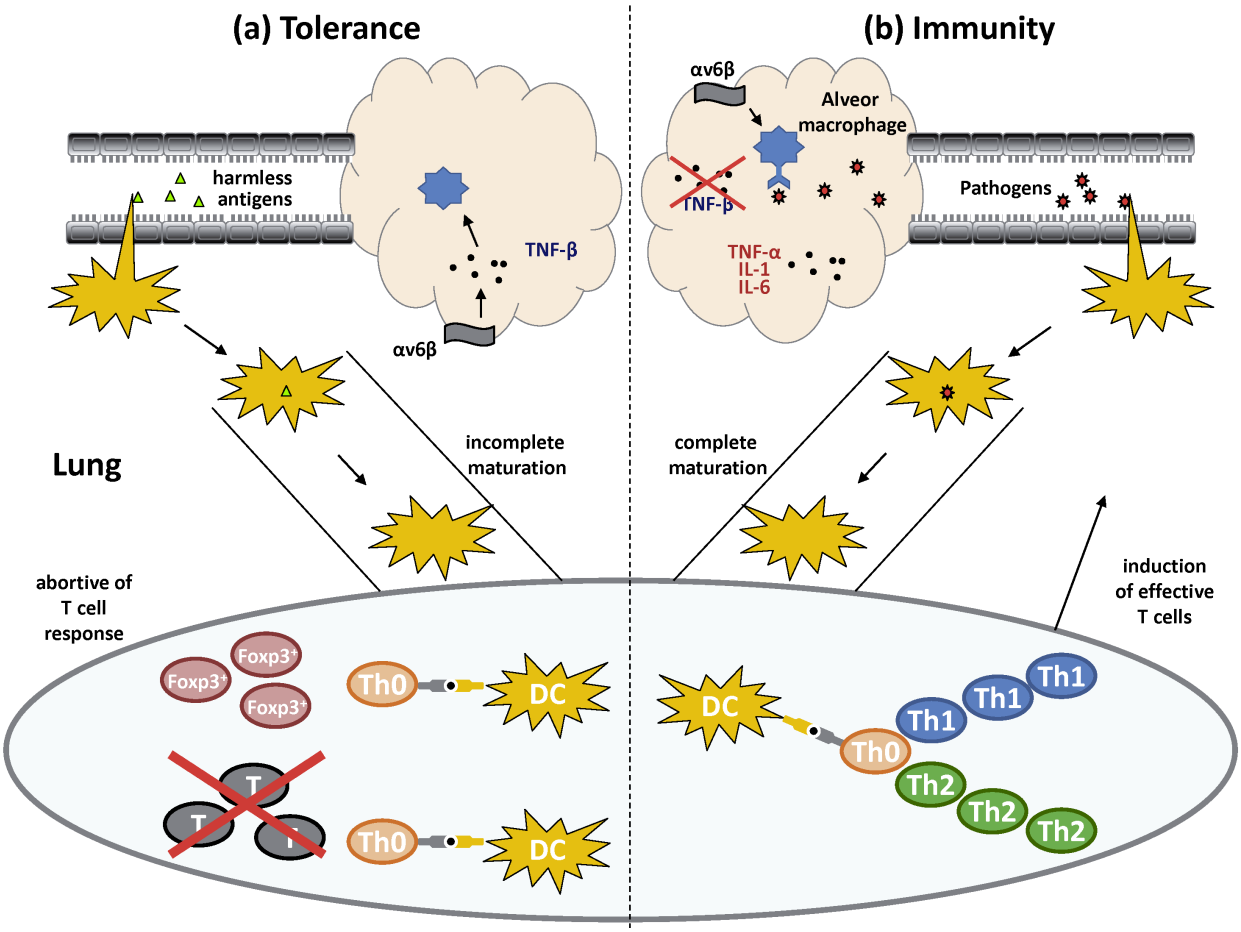


Figure 2.2 Immunity and tolerance induction by dendritic cells in the lung. (A) Under steady state conditions, DCs remain immature. When harmless antigens are inhaled into the airway, these lung DCs acquire these antigens under non-inflammatory conditions, such that they remain immature and fail to activate Teff in the lymph nodes. Furthermore, IL-10-producing DCs in the lung contribute to generation of $Foxp3^+$ Treg cells, leading to immune tolerance in asthma. (B) Under inflammatory condition, lung DCs undergo maturation and direct T cells into Th2 cell responses. This figure is adapted from (Novak & Bieber, 2008).

2.2.3 Function of Lung Dendritic Cells in Allergic Airway Disease

DCs not only induce immune stimulation, but they can also contribute to immune tolerance (Fig 2.2). The immature DCs with low expression of costimulatory molecules (e.g., CD40, CD80 and CD86) and MHC II have a reduced ability to activate naïve T cells (Lambrecht, 2005; Steinman, Hawiger, & Nussenzweig, 2003). In contrast, activated or mature DCs have less ability to endocytose Ags, but they can efficiently activate T cells (Asselin-Paturel et al., 2003). In addition to the tolerance induced by immature mDCs, even relatively mature pDCs can induce tolerance. Murine DCs transiently produce IL-10 and can induce regulatory T cells production through activation of the ICOS-ICOSL pathway (Akbari & Umetsu, 2005). As such, depletion of pDCs prior to inhalation of the normally tolerogenic allergen OVA leads instead to development of all the cardinal features of asthma (i.e., IgE production and Th2 cytokine secretion) (de Heer et al., 2004). It has been reported that FLT3-L treatment of asthmatic mice, which effectively induces differentiation of pDCs, inhibits asthmatic symptoms (Edwan, Perry, Talmadge, & Agrawal, 2004). On the other hand, the asthma phenotype is aggravated in asthmatic mice following treatment with GM-CSF, which enhances mDC differentiation (Stampfli et al., 1998); mDC numbers are seen to increase in acute asthma, whereas mDC deletion abrogates the features of asthma (Vermaelen & Pauwels, 2003). Therefore, the balance between pDCs and mDCs is important in the development of asthma disease.

It has also been reported that tolerization of the asthma phenotype in affected mice by treatment with DC10 is associated with increases in the numbers and activities of regulatory T cells (Huang et al., 2010; Huang, Ma, Dawicki, Zhang, & Gordon, 2013; Lu et al., 2011) and regulatory DC populations in the lungs (C. Li, unpublished observation). However, it was not

clear whether these latter, regulatory DC were the treatment DC10 that we know take up residence in the lung (Huang, Dawicki, et al., 2013) or they were endogenous DC that had been rendered regulatory by the treatments.

2.3 Tolerogenic Dendritic Cell and Asthma

2.3.1 Tolerogenic Dendritic Cell

Like all cells, DCs receive signals via inputs through their myriad receptors. Signaling by many molecules (e.g., CD40 and TNF-α) can lead to activation of DCs, leading to a transition from immature DCs that capture antigens to mature DCs that present antigens (Banchereau et al., 2000; Brunner et al., 2000; Caux et al., 1994). This process could be induced and/or regulated by many factors (e.g., pathogen-associated molecular patterns [PAMPs], the local balance between proinflammatory and antiinflammatory signals, and T cell–derived signals), as well as several coordinated events (i.e., upregulation of costimulatory molecules, change in morphology and change in class II MHC compartments (Banchereau et al., 2000; Li et al., 2012; Wallet, Sen, & Tisch, 2005). However, the maturation of DCs can also occur under steady state conditions initiated by changes in E-cadherin-mediated DC-DC adhesion, and this maturation process is characterized by increased expression of MHC class II, costimulatory molecules, and chemokine receptors as well as the failure to release immunostimulatory cytokines (Jiang et al., 2007). Incompletely matured DCs express lower levels of MHC, costimulatory molecules and IL-12, resulting in abortive T cell responses (Banchereau & Steinman, 1998). Similarly, these immature DCs also produce anti-inflammatory cytokine IL-10 and thus induce regulatory T cell differentiation, leading to tolerance in a cytokine-dependent manner (Gordon et al., 2014). On the other hand, as shown in recent studies using DC10 treatment of asthmatic mice, the treated

mice lose most signs of asthma within three to four weeks of DC10 treatment (Nayyar et al., 2012) These DCs-induced Tregs express Foxp3 and reportedly suppress Teff responses in a contact-dependent manner (Li et al., 2010). In addition, pDCs in the lungs can induce respiratory tolerance (de Heer et al., 2004). Many studies have shown that DCs induce different regulatory T cell populations (Ray, Khare, Krishnamoorthy, Qi, & Ray, 2010) and that Treg cells can also influence DCs and re-program them into tolerogenic cells (Misra, Bayry, Lacroix-Desmazes, Kazatchkine, & Kaveri, 2004). The generation of DC with a tolerogenic phenotype could be realized through the incubation of Treg purified from tolerant recipients with DC progenitors (Min et al., 2003). DCs grown with $CD4^+$T cells from tolerant mice showed lower levels of CD40, CD86 and MHC II and weak allostimulatory ability in mixed lymphocyte responses (MLR), while DCs co-cultured with $CD4^+$ T cells from immune mice showed higher levels of CD40, CD86 and MHC II and strong allostimulatory ability (Min et al., 2003). Furthermore, it has been reported that Treg induce DC indoleamine-2, 3-dioxygenase (IDO) expression through CTLA-4 and B7 interactions (Fallarino et al., 2003), although CCL22 and CCR4 interactions may also play a role in this IDO response (Onodera et al., 2009).

DCs, including both naturally-occurring and induced populations, can induce tolerance under different conditions. In the intestine, promotion of regulatory T cells is dependent on TGF-β and retinoic acid (Coombes et al., 2007). After antigen activation in the intestine, $CD103^+$ DC can promote the conversion of naïve mesenteric lymph node (MLN) $CD4^+$ T cells into $Foxp3^+$ T cells (Coombes et al., 2007), although this induction of tolerance by $CD103^+$ DCs is plastic. Thus, inflammation dampens the tolerogenic properties of MLN $CD103^+$ DC, including their expression of TGF-β2 and ALDH1a2 (Laffont et al., 2010). Accordingly, $CD103^+$ DCs taken from mice with colitis are impaired in their abilities to induce $Foxp3^+$ Treg and instead favor the

emergence of IFN-γ-producing $CD4^+$ T cells (Laffont et al., 2010). This dichotomy of function makes sense, inasmuch as the intestinal immune system must initiate strong immunity against harmful pathogens while at the same time restraining immune responses against harmless commensal microbes and dietary antigens (Denning, Wang, Patel, Williams, & Pulendran, 2007). Recent studies have also shown that Tr1 production is dependent on IL-27 (Kushwah & Hu, 2011). IL-27 can limit ongoing immune responses under different conditions (A. O. Hall, Silver, & Hunter, 2012). IL-27 promotes IL-10 transcription in T cells through the activation of STAT1 and STAT3, which facilitates differentiation of Tr1 cells (Iyer, Ghaffari, & Cheng, 2010; C. Wu et al., 2013).

2.3.2 Dendritic Cell Exposure to Anti-Inflammatory Mediators

A variety of mediators can endow DCs with regulatory functions, including IL-10, TGF-β, PGE2, RA, Vitamin D3, rapamycin, and neuropeptides (Gordon et al., 2014; Mascanfroni et al., 2013; Popov & Schultze, 2008). This study focuses on the roles of TGF-β, IL-10 and RA to induce regulatory DCs. Epithelial cell production of TGF- β facilitates the induction of regulatory DCs and thus, indirectly, the induction of Tregs. *In vitro* data and transgenic mouse models have shown that TGF-β signaling in DCs can prevent autoimmunity at least in part by maintaining DCs in an immature and tolerogenic state that is characterized by low expression of MHC II and costimulatory molecules and increased IDO expression (Geissmann et al., 1999; Ramalingam et al., 2012). In addition, TGF-β suppresses E-cadherin expression on mucosal inflammatory DCs (Siddiqui, Laffont, & Powrie, 2010). The roles of IL-10 and RA in the induction of regulatory DC will be discussed in the following sections (see 2.3.3 and 2.3.4).

2.3.3 IL-10, Dendritic Cells and Tolerance

IL-10 is an important anti-inflammatory cytokine in terms of reducing antigen presentation and inhibiting T cell activation (Grutz, 2005). IL-10 can be expressed by many cells of adaptive and innate immune systems (e.g., T helper cell subsets, $CD8^+$ T lymphocytes, Tregs, B cells, DCs, macrophages, eosinophils, natural killer cells, and neutrophils) (Saraiva & O'Garra, 2010). IL-10 functions as an immunosuppressive cytokine that is involved in the downregulation of CD80/CD86-mediated costimulation and reduction of MHC class II expression (Asadullah, Sterry, & Trefzer, 2002). For example, human or mouse DCs that have been exposed to IL-10 express lower levels of co-stimulatory molecules and MHC class II (Li et al., 2010; Nayyar et al., 2012). In addition, IL-10 reduces the production of pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, IL-12, and TNF-α (Lyke et al., 2004). IL-10 is also critical to the induction of IDO in DCs. For example, the presence of IL-10 or TGF–β inhibits IFN-γ–induced downregulation of IDO in DCs, causing sustained expression of functional IDO even in mature, IFN-γ–activated DCs (Munn et al., 2002). Furthermore, IL-10 plays an important role in the inhibition of airway inflammation. In allergic individuals, antigen-specific Treg cells that produce IL-10 are present in much lower frequencies than they are in non-allergic individuals (Akbari & Umetsu, 2005). For example, IL-10 could inhibit the function of macrophages and thus suppress the production of inflammatory cytokines (F. Chung, 2001). Pulmonary IL-10-producing DCs play an important role in induction of tolerance in asthma (Akbari, DeKruyff, & Umetsu, 2001). Production of IL-10 by some populations of DCs in human and mouse have been found to promote generation of Tr1 cells (Akbari et al., 2001; Fu, Chuang, Huang, & Chiang, 2008; Gregori et al., 2010; Levings et al., 2005), although otherwise quiescent murine lung DC that present innocuous antigens to naïve T cells secrete IL-10 while doing so and instead induce $Foxp3^+$ Treg (Dumitriu, Dunbar,

Howie, Sethi, & Gregory, 2009; Yamazaki et al., 2007). The ability of this type of DCs to induce Tr1 cells could be inhibited by using antibody to block IL-10 signaling (Gravano & Vignali, 2012), indicating that IL-10 is critical in induction of Tr1. These DCs are immature DCs that require IL-10 to generate Tr1 cells. This type of Tr1 cells suppress immune responses in an IL-10-dependent manner (Levings et al., 2005). Recent study in our lab found that semi-mature IL-10-differentiated DCs induced $CD4^+CD25^+Foxp3^+$ Tregs to suppress effector responses in a cell-contact dependent manner (Li et al., 2010). IL-10-producing DCs purified from bronchial lymph nodes can induce Treg cells through ICOS-ICOS-L mediated signal (Akbari et al., 2002). In addition, IL-10-differentiated DCs have been reported to induce and maintain airway tolerance in asthma. Human DC10 derived from human blood $CD14^+$ monocytes in asthmatic subjects lead to the development of $CD4^+$ $CD25^+Foxp3^+$ Tregs *in vitro* (Li et al., 2010). Murine DC10 also convert Teff cells to $CD4^+CD25^+Foxp3^+$ T cells (Huang et al., 2010).

2.3.4 Retinoic Acid, Dendritic Cells and Tolerance

Retinoic acid (RA) is a metabolite of vitamin A (Fig 2.3). Retinoic acid can be produced in the body by conversion of retinol to retinaldehyde, which is then converted to RA (Molotkov et al., 2002). The enzymes responsible for generation of RA include retinol dehydrogenases and retinaldehyde dehydrogenases (RALDH1, RALDH2 and RALDH3) that metabolize retinol to retinaldehyde and retinaldehyde to RA, respectively (Pino-Lagos, Benson, & Noelle, 2008).

Retinoic acid, derived from vitamin A (retinol), mediates the growth and development functions of cells (Kam, Deng, Chen, & Zhao, 2012). Retinoic acid, produced by $CD103^+$ DCs in the gut-associated lymphoid organs, is important in controlling T cell trafficking (Iwata, 2009). It can increase TGF-β-induced expression of Foxp3, impress B cells with gut-homing specificity,

and facilitate their IgA production (Coombes et al., 2007). The expression of RALDH is critical to RA production (Yokota et al., 2009).

All trans-RA exclusively binds retinoid X receptors (RXR) via heterodimers they form with members of the RA receptor (RAR) family, including RAR-alpha, -beta or -gamma (Matt, Ghyselinck, Wendling, Chambon, & Mark, 2003). It has been found that RA induces expression of the $\alpha 4$ subunit of $\alpha 4\beta 7$ by binding together with the RARα to responding elements within the regulatory region of the $\alpha 4$ gene (J. A. Hall, Grainger, Spencer, & Belkaid, 2011). Mucosal DC can trigger $\alpha 4\beta 7$ and CCR9 expression through their capacity to synthesize RA (Evans & Reeves, 2013; Ruane et al., 2013). Recent study also demonstrated that RA-producing DCs are present in the lung upon exposure to antigen in an OVA-induced tolerance mouse model. Specifically, pulmonary $CD103^+$ DCs upregulate RA expression after exposure to inhaled Ag. It has been observed that these DCs locally promote induction of $Foxp3^+$ Tregs through upregulated expression of RA together with TGF-β signaling (Khare et al., 2013).

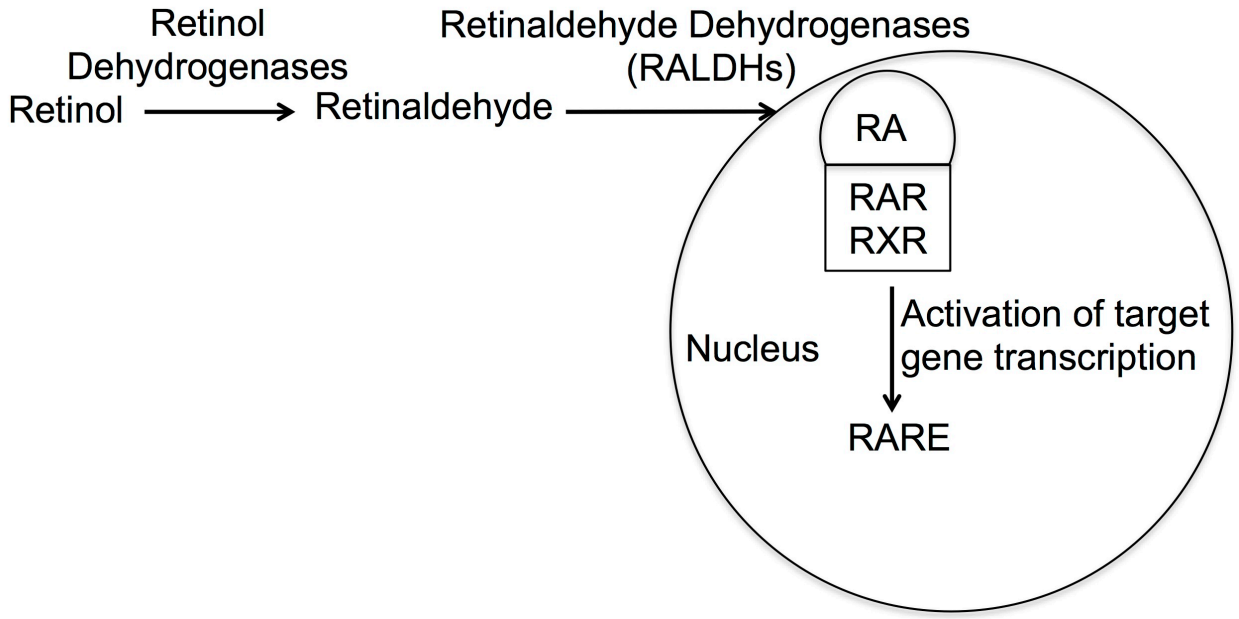


Figure 2.3 The retinoic acid metabolic pathway and cellular retinoic acid signalling. Retinol is converted to retinaldehyde through the actions of retinol dehydrogenases, and then to retinoic acid (RA) through retinaldehyde dehydrogenases. RA binds to nuclear receptors such as RAR and RXR, which in turn bind to RA-response elements (RARE) within the promotors of target genes, leading to transcriptional activation of these targets.

Gut $CD103^+$ DCs are capable of synthesizing RA (Coombes et al., 2007). GM-CSF and vitamin A play important roles in inducing and maintaining of RALDH2 expression in gut $CD103^+$ DCs (Yokota et al., 2009), with vitamin A being essential for the production of RALDH in DCs (Jaensson-Gyllenback et al., 2011; Molenaar et al., 2011). Prior studies have shown that blocking Wnt-β-catenin-mediated signalling attenuates RALDH expression in DCs (Manicassamy et al., 2010), while LPS exposure enhances RALDH expression in DCs (J. Chang et al., 2013).

Several studies have explored the role of LPS in DCs maturation. A wide variety of stimuli contribute to DCs maturation; they include proinflammatory cytokines (e.g., TNF, IFN, IL-1, CD40L, and LPS) (Brossart et al., 1998; Sallusto & Lanzavecchia, 1994). LPS is a major component of gram-negative bacterial wall and contains pathogen-associated molecule patterns (PAMP). PAMP can be identified by pathogen-recognized receptors (PRR), e.g., toll-like receptor (TLR)-4 (Akira, Takeda, & Kaisho, 2001). IL-4- and GM-CSF-differentiated human DCs undergo maturation in response to LPS stimulation (Brosbol-Ravnborg, Bundgaard, & Hollsberg, 2013). LPS not only induces Th1 and Th17 responses, but also initiates Th2 responses and drives Th2 cytokine production (Y. Kim et al., 2013). Low doses of LPS induce Th2 responses through TLR4 and MyD-88-dependent mechanisms (Piggott et al., 2005). LPS-containing HDM allergen activates airway structural cells to produce Th2 cytokines (e.g., TSLP, IL-33 and IL-25) through TLR4 signaling and these cytokines influence DCs to induce Th2 responses (Hammad et al., 2009).

TLR2 or TLR4 on DCs may induce anti-inflammatory responses and even further induce Treg cells and peripheral tolerance. TLR have been found to preferentially induce Th1 and Th2 responses, although the impact of LPS on tolerogenic DCs is not well understood. For example,

tolerogenic DCs stimulated by TLR stimuli upregulated TLR2 expression (Chamorro et al., 2009). Also, TLR signaling stimulated splenic DCs to induce Tregs due to high-level IL-10 production (Manicassamy et al., 2009). In another study, when DCs were induced by vitamin D3, LPS-induced maturation was prevented by Vit-D3 (Brosbol-Ravnborg et al., 2013). On the other hand, it has been shown that LPS-enhanced RALDH2 expression on tolerogenic RA-differentiated DCs (J. Chang et al., 2013). TLR2 stimulation (Pam-3-cys) activates DCs to produce less IL-12 but more IL-10 (Dillon et al., 2004).

TLR2- or TLR4-induced tolerance features on DCs promote Treg growth cytokines such as IL-10 and IL-27 (Molle et al., 2007; Yanagawa & Onoe, 2007). LPS-stimulated hepatic DCs preferentially produced IL-10 and IL-27 and promote T cell hyporesponsiveness, which is associated with Tregs (Y. Chen et al., 2009). Vit-D3 inhibited DCs-maturation stimulated by LPS (Brosbol-Ravnborg et al., 2013). LPS-induced RA-differentiated DCs do not promote $Foxp3^+$ Tregs (J. Chang et al., 2013).

Features of RA-differentiated DCs have also been studied. It was found that RA-differentiated DCs derived from peripheral blood of pigs acquired characteristics of mucosal-type DCs: IgA secretion in B cells, CCR9 expression and $\alpha 4\beta 7$ integrin expression on lymphocytes (Saurer, McCullough, & Summerfield, 2007). Few studies have analyzed the features of LPS-induced RA differentiated DCs. The impact of LPS on RA-differentiated DCs may not lead to immune responses. RA-differentiated DCs expressed RALDH2; however, LPS-induced RA differentiated DCs enhanced RALDH2 expression (J. Chang et al., 2013; Yokota et al., 2009).

Overall, there is a lack of understanding on the impact of LPS-treated DCRA on peripheral tolerance. Our lab has found that DCRA secrete high levels of IL-27 and that DCRA

without IL-27 expression cannot induce tolerance in a mouse model of food allergy (Dr. Wojciech, unpublished observation). Built on these findings, the present study is the first attemp to explore the impact of LPS-treated DCRA on airway tolerance.

2.3.5 Dendritic Cell Modulation in Asthma Therapy

Although a variety of therapies have been developed to reduce allergic responses in asthma patients, asthma continues to represent a substantial burden to the health care system. This indicates an urgent need to improve therapeutic strategies through innovative research, and the utilization of DCs could provide a new solution. DCs can play a crucial role in directing Th2 responses, but also in initiating immune tolerance. HDM allegens activate airway epithelial cells through TLR4-mediated signals to produce Th2 cytokines that further activate DCs to induce Th2 responses in HDM-sensitized asthmatic mice; TLR4 antagonism reduces airway eosinophilia and AHR in this mouse model (Hammad et al., 2009). On the other hand, the modulation of DC function through TLR can induce tolerance. A TLR2 ligand (e.g., zymosan) has been shown to induce tolerogenic DCs which secrete abundant IL-10, and thereby suppress Th2 responses (Dillon et al., 2006). Similarly, another TLR2 ligand (e.g., Pam3CSK4) endowed DCs with tolerogenic properties and decreased AHR in OVA-sensitized mouse model (Lombardi et al., 2008).

Vitamin D may improve asthma control through enhancing production of IL-10 by Tregs and DCs (Luong & Nguyen, 2012). It has also been shown that vitamin D3 can induce differentiation of tolerogenic DCs and Tregs (Gordon et al., 2014). Semi-mature Vitamin D3-induced tolerogenic DCs express lower level of costimulatory molecules and MHC II levels, but produce high levels of IL-10 (Griffin et al., 2001). Concomitant administration of vitamin D3

with OVA decreased AHR and lung eosinophilia in a murine model of allergic asthma (Lombardi & Akbari, 2009).

CHAPTER 3: RESEARCH HYPOTHESIS AND OBJECTIVES

3.1 Hypothesis

Specific allergen-presenting LPS-matured, retinoic acid-differentiated dendritic cells (DCRA) are tolerogenic and as effective therapeutically as allergen-presenting IL-10-differentiated dendritic cells (DC10) in a mouse model of asthma.

3.2 Objectives

(1) To characterize the phenotype of LPS-matured DCRA and immature DC10 compared with LPS-matured immunostimulatory DC;

(2) To examine the impact of these DCRA versus DC10 on *in vitro* Th2 responses and their mechanisms of action; and

(3) To assess the impact of these DCRA versus DC10 immunotherapy on the asthma phenotype in a mouse model of asthma.

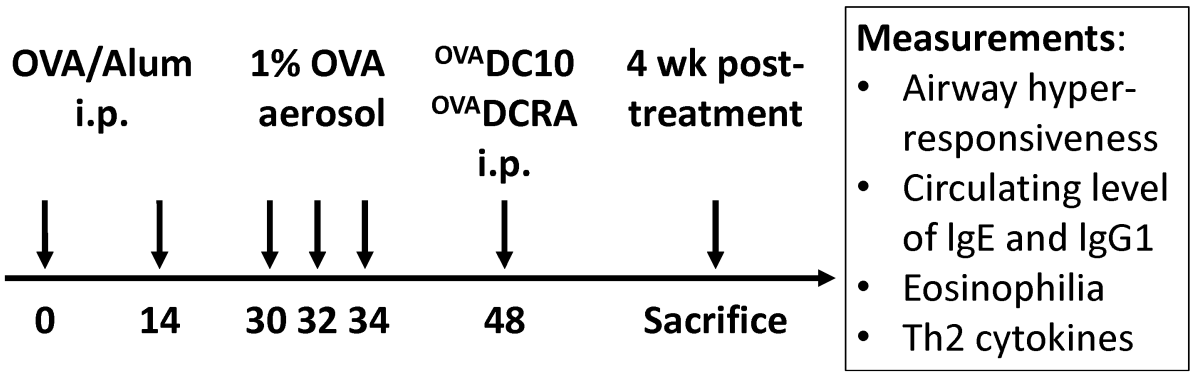


Figure 3.1 Experimental Design. In order to induce an asthma phenotype, female BALB/c mice were given two i.p. injections of 2 μg OVA/alum on dy 0 and 14, and then exposed to 1% (w/v) OVA in saline aerosols on day 30, 32, and 34 (20 min/day) (Schneider, Li, Zhang, & Gordon, 2001). The mice were treated with 1×10^6 OVA-presenting DCRA or DC10 two weeks later (Day 48), and four weeks after that the asthma phenotype was assessed, including airway hyperresponsiveness (AHR), circulating levels of OVA-specific IgE and IgG1, and airway eosinophilia and Th2 cytokine recall responses 48 h after a final allergen challenge.

CHAPTER 4: MATERIALS AND METHODS

4.1 Mouse Model of Asthma

BALB/c mice were chosen because of their development of vigorous Th2 responses and high levels of serum IgE following sensitization and challenge with OVA (Jiang et al., 2007). Mice were sensitized for asthma-like disease using a standard protocol reported in prior studies (Gordon et al., 2005; Schneider et al., 2001). Mice were given two i.p. injections of OVA-alum (2 μg of OVA/mg alum, in 200 μl of saline) on days 0 and 14, and 1% (w/v) OVA in saline aerosols on days 30, 32, and 34 (20 min/day). By day 35, such mice routinely display 40–70% airway eosinophilia 48 h after airway OVA rechallenge, strong airway hyperresponsiveness (AHR) to methacholine, high levels of circulating OVA-specific IgE and IgG1 antibodies, and airway Th2 cytokine (IL-4, -5, -9, and -13) responses (Gordon et al., 2005; Schneider et al., 2001). Although BALB/c asthma would be the preferred model for this study, OT-II OVA-specific TCR- transgenic mice were on a C57BL/6 (B6) genetic background, such that a B6 asthma model was also used in this study.

Six to eight week-old female BALB/c mice were purchased from the Animal Resources Center of the University of Saskatchewan. OT-II OVA-specific TCR- transgenic mice were from the Jackson Laboratory (Bar Harbor, ME). The mice were housed in groups of 5-10 animals in the animal facility at the Western College of Veterinary Medicine (University of Saskatchewan). The asthma phenotype was induced in the mice as noted above, and then they were treated with 1×10^6 OVA-DCRA or OVA-DC10 two weeks later (Day 48). All experiments were performed according to the guidelines of the Canadian Council on Animal Care.

4.2 Generation of DCs from Bone Marrow Progenitors

Mice were sacrificed and their femurs and tibiae were removed and disinfected by immersion in 70% ethanol for 1 to 2 minutes. The marrow channel was exposed by cutting both ends of each bone and then the bone marrow was flushed from the bone shaft with PBS. The cell culture medium was complete RPMI, consisting of RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with penicillin (100 U/ml), streptomycin (100 go/ml), L-glutamine (2mM), 2-mercaptoethanol (50 μM; each from Sigma Chemical Co, Mississauga, ON), and 10% heat-inactivated fetal bovine serum (FBS) (Gibco). The cells were resuspended in 24 ml of complete RPMI supplemented with recombinant mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF [20 ng/ml]; R&D Systems, Minneapolis, MN), and seeded in 6-well plates (VWR, Mississauga, ON); every 48 hours half of the medium in each well was exchanged. At day 10 the levels of rmGM-CSF were reduced to 7.5 ng/ml to reduce granulocyte growth and the cells were cultured for an additional 3 days in this reduced GM-CSF medium, which was also supplemented with IL-10 (50 ng/ml; R&D Systems) to generate “tolerogenic dendritic cells” (DC10). For treatment of asthmatic mice, the DC10 were pulsed with 50 μg/ml OVA (Sigma Chemical Co) overnight, and then cells were harvested and washed 2 times before use.

To generate immunostimulatory DCs, 1 μg/ml *E.coli* serotype 0127:B8 lipopolysaccharide (LPS, MD Biosciences, St Paul, MN) was added to the cells (DClps), which were then pulsed overnight with 50 μg/ml OVA (Sigma Chemical Co). The cells were subsequently harvested and washed 2 times before use.

4.3 Generation of DCRA

To generate DCRA, the bone marrow progenitors were cultured in presence of rmGM-

CSF (20ng/ml), rmIL-4 (10ng/ml; eBioscience, San Diego, CA) and retinoic acid (1μM; Sigma Chemical Co) for 8 days (Feng, Cong, Qin, Benveniste, & Elson, 2010; Yokota et al., 2009). During the final day of culture the cells were pulsed with 1μg/ml LPS (Huang et al., 2010), then they were pulsed overnight with OVA (50 μg/ml; Sigma Chemical Co) and washed 2 times before use, as noted above for our other DC populations.

4.4 Characterization of DCs

DCs were collected and analyzed for the expression of co-stimulatory molecules and MHC II by FACS (Counter EPICS XL Flow cytometer, Beckman coulter, Mississauga, ON). The cells were stained with FITC-labeled anti-mouse CD40 (clone: HM 40-3; eBioscience), CD86 (clone: GL1; BD, Mississauga, ON) or MHC II (clone: M5/114.15.2; eBioscience), or with appropriate isotype control antibodies (CD40, hamster IgM; CD86, rat IgG2a; and MHC II, rat IgG2b) for 20 minutes on ice. After being washed twice, the cells were fixed with 1% paraformaldehyde for flow cytometry analysis within 48 h of fixation.

4.5 DC-T Cell Co-Culture

4.5.1 Lung Single Cell Suspensions

Asthmatic or healthy control mice were sacrificed and their ventral skin reflected cranially and caudally. The ribcage was removed and the entire cardio-respiratory tree was removed aseptically and rinsed to remove free superficial blood. The lung tissues were diced finely in RPMI-1640-10% FBS medium in a petri dish using two scalpel blades. The tissues were then transferred into digestion solution (RPMI-1640/10% FBS, containing 0.75 mg/ml Type 4 collagenase and 2 mg/ml hyaluronidase; Worthington Biochemical Corporation, Freehold, NJ)

and were incubated at 37 °C for 30 minutes. Any small, undigested fragments of tissue were transferred to and ground through a cell strainer using a syringe plunger to further disrupt the tissues. The dispersed tissues were filtered through four layers of sterile gauze into 15 ml centrifuge tubes and the cells were washed with RPMI-1640-10% FBS medium (Gordon et al., 2005) and counted in the presence of 1% trypan blue (to assess viability) using a hemocytometer.

4.5.2 Splenocyte Isolation

The mice were sacrificed and their spleens were collected asceptically into petri dishes containing RPMI-1640-10% FBS medium on ice. The spleens were trimmed of any superficial fatty tissues and cut into small pieces, which were transferred into cell strainers; the cells were passed through the strainer using plungers, then the cells were washed and resuspended in fresh medium. The red blood cells were lysed using 10 ml lysis solution (ammonia chloride: to 900 ml of H2O, add 2.42 g Tris and 7.56 g NH_4Cl, pH to 7.2 with HCl; add H_2O to 1 litre & filter sterilize) for 5 minutes. The cells were washed again and the cell numbers were determined as above.

4.5.3 $CD4^+$ T cell Isolation

Lung cells suspensions generated as above were layered onto 10 ml cushions of lymphocyte separation medium (LSM; Valeant Pharmaceuticals, CA) and centrifuged at 400 xg for 15 min. After centrifugation, the mononuclear cells were harvested from the LSM/medium interface and the numbers of cells determined as above. The cells were washed and then resuspended in 90 μl of PBS/EDTA MACS buffer (PBS [pH=7.2], 5 % FBS and 2 mM EDTA) per 10^7 total cells, then 10 μl of CD4-specific microbeads (Miltenyi Biotec, Auburn, CA) per 10^7

total cells were added to the suspension, which was then mixed and incubated for 15 minutes at 4-8°C. The cells were then washed with 1-2 ml of PBS/EDTA buffer per 10^7 cells and centrifuged at 300 x g for 10 minutes and the cells resuspended to 10^8 cells in 500 µl buffer. Type 'LS' MACS columns were prepared by rinsing with 3 ml buffer and placed onto the magnet, the cell suspension was applied onto the column and the columns were washed 3 times with 3 ml volumes of buffer. The flow-through cells were collected as marker-negative cells. The columns were then removed from the magnet, transferred to suitable test tubes and the bound cell fractions were eluted immediately using 5 ml volumes of buffer and a plunger.

4.5.4 DC-T Cell Co-culture

To test the abilities of the DCRA and DC10 to tolerize Th2 responses, we used pulmonary $CD4^+CD25^-$ Th2 Teff/Tm cells from asthmatic mice (taken one month after their last allergen exposure) and OVA-presenting DClps. The irradiated (3000 rads) putative tolerogenic DCs (5×10^4/well), $CD4^+$ T cells (1×10^5/well) and irradiated (3000 rads) OVA-DClps (4×10^3/well) were seeded together into round bottom 96-well plates and cultured for 72 h at 37°C in a 5% CO_2 atmosphere. To assess cell proliferation, we used a standard ^{3}H-thymidine uptake assay, wherein 1 µCi/well of ^{3}H-thymidine was added for the last 17 hours of culture and the cells harvested onto a glass fiber filters for analysis of ^{3}H incorporation into the cellular DNA by liquid scintillation counting.

4.5.5 *In vitro* Induction of $Foxp3^+$ Treg

$CD4^+$ T cells were isolated from the lungs of OTII OVA-asthmatic mice and cultured with tolerogenic DCs generated from bone burrow progenitors from normal mice. The $CD4^+$ T

effector cells were labeled with CFSE (2 μM; Invitrogen) for 20 minutes at 37°C, washed, and then co-cultured (10^5 T cells/well) with OVA-pulsed DCRA or DC10, in the presence of IL-2 (10 U/ml; Peprotech, NJ) as a growth factor. After 5 days, cells were fixed, permeabilized and stained with PE-Cy5-labeled anti-mouse Foxp3 antibody (eBioscience) and PE anti-mouse CD4 antibody (eBioscience). This test was used to assess the expression of Foxp3 and the proliferation of $CFSE^+$ cells. Control cultures included $CD4^+$ T cells stimulated with immunostimulatory OVA-pulsed DClps.

4.6 Assessment of Airway Hyperresponsiveness and Differential Airway Cell Counts

AHR was assessed in asthmatic animals by head-out, whole-body plethysmography. Briefly, air supply was provided to the body compartment of a plethysmograph using a small animal ventilator, and we monitored the changes in the airflow through the body compartment. Doubling doses of nebulized methacholine (0.75-25 mg/ml) were sequentially delivered to the head compartment. The data, accurately reflecting bronchiolar constriction, was collected as running 1s means of the airflow at the 50% point in the expiratory cycle (Flow@50%TVe1). Bronchoalveolar lavages (BAL) were performed to obtain the airway cells from mice challenged 48 h earlier with 1% OVA in saline aerosols (20 min challenges). The BAL fluid cells were deposited on slides using a cytocentrifuge, the slides stained with Giemsa-Wright solution and differential cell counts were performed for each mouse.

4.7 OVA-Specific Antibody by Enzyme-linked Immunosorbant Assay (ELISA)

For the OVA-specific IgG1 antibody assays, the wells were coated with 50 μl OVA (20 μg/ml) in coating buffer as the capture reagent. The serum samples were diluted in 1:10 in PBST

and 100 μl sample were added to the plate. Biotinylated anti-mouse IgG1 was diluted in PBST (1 μg/ml) as the detection antibody and 100 μl/well was added to the plate. The levels of OVA-specific IgG1 were presented as OD values. IgE was detected using anti-IgE (2 μg/ml) as the capture reagent. Biotinylated OVA (2 μg/ml) as the detection reagent was added to the coated plates. The levels of OVA specific IgE were presented as OD values. The biotinylated detection antibodies for IgG1 and IgE assays were obtained from BD (Franklin Lakes, NJ).

4.8 Cytokine ELISA

Our cytokine ELISA protocol has been reported previously (Gordon et al., 2005). The concentrations of capture and biotinylated antibodies were pre-optimized. The purified capture antibody in 50 μl/well of coating buffer (1M NaHCO3, 1M Na2CO3; pH 9.6) was added to the wells of Immunolon-4 96-well flat-bottom plates (Dynatech laboratories Inc., Chantilly, VA), and the plates coated overnight at 4 °C. The plates were then washed three times with PBS/Tween (PBS-0.5% Tween 20; Polyoxyethylene-sorbitan monolaurate, Sigma Chemical Co.) (PBST) and blocked at room temperature for 2 hours with 200 μl/well of blocking buffer (PBS-10% FBS), then again washed four times with PBST. Various concentrations of recombinant cytokine protein standards (100 μl/well) or samples (100μl/well) were then added to the plates and incubated overnight at 4 °C, before the plate were again washed four times. The biotinylated detection antibodies (100 μl/well) were added to the wells, incubated for 1 hour at room temperature and then the plats washed 6 times with PBST before 100 μl of a 1:1000 dilution of streptavidin-conjugated horseradish peroxidase (Vector Laboratories Inc., Burlingame, CA) in PBST was added to each well. The plates were incubated for 1 hour, washed eight times with PBST and finally 100 μl of l2, 2′-azinobis (3-ethylbenthiazoline-sulfonic acid) (ABTS)

peroxidase substrate solution (Gibco, Gaithersburg, MD) was added to each well. The reaction products were visualized by incubating the plates for 5-15 min at room temperature for color development and the colorimetric products detected using a NOVOSTAR plate reader (BMG LABTECH, ON).

BAL fluids or DC culture supernatants for detection of IL-4, IL-5, IL-9, IL-13, IL-27 or TGF-β levels were not diluted for the ELISAs, and the data are presented as picograms per milliliter (pg/ml) according to the recombinant protein standard curves. All assays were sensitive to 5-10 pg/ml. All capture and biotinylated detection antibody pairs and recombinant proteins for ELISA were obtained from R&D Systems, Inc.

4.9 RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

Total RNA from DCRA or DC10 was isolated using RNeasy Mini Kits (Qiagen, Mississauga, ON) according to the manufacturer's instructions. Briefly, 350 µl RLT lysis buffer and 2-mercaptoethanol were added to disrupt the cells, then 350 µl of 70% ethanol was added and mixed well by pipetting before the samples were transferred to RNeasy spin columns (placed in 2 ml collection tubes) and centrifuged for 0.5 minutes at 8000 x g. The flow-through was discarded and 700 µl of buffer RW1 was added to the RNeasy spin column, which was again spun as described above. The flow-through was again discarded and 500 µl Buffer RPE was added to the spin column that was further centrifuged for 2 minutes at 8000 x g to wash the column membrane. The column was placed in a new 1.5 ml collection tube and 30-50 µl RNase-free water was added directly to the spin column membrane. Lastly, the RNeasy spin column was spun for 1 min at 8000 x g to elute the RNA, which was reverse-transcribed to DNA using quantitative real time (qRT)-PCR. qRT-PCR analysis was carried out using Brilliant II SYBR

Green qRT-PCR Master Mix Kits (Stratagene, La Jolla, CA) according to the manufacturer's instructions. qRT-PCR were set up using a Mx3005P Instrument (Stratagene). The primers for mouse IL-10 were as follows: the forward primer AAGCCTTATCGGAAATGATCCA; the reverse primer GCTCCACTGCCTTGCTCTTATT. The reactions were carried out according to the following parameters: 50˚C for 30 min and 95˚C for 10 min (1 cycle); 95˚C for 30 sec (denaturation); 63˚C for 30 sec (annealing); and 72˚C for 30 sec (extension) (40 cycles); the reading was taken at 72˚C during the 30-sec plateau. IL-10 mRNA levels were normalized to mouse β-actin levels.

4.10 Statistics

All data were expressed as the mean ± SEM. Group differences were analyzed by ANOVA with Bonferroni *post-hoc* testing. Anti-inflammatory cytokine production was assessed by unpaired t test. Variances of groups in AHR analyses were assessed by two-way ANOVA. Differences were considered significant when P-values were less than 0.05.

CHAPTER 5: RESULTS

5.1 *In Vitro* Characterization of Tolerogenic Dendritic Cells

5.1.1 Characterization of IL-10- and Retinoic Acid/LPS-differentiated DC

IL-10- (DC10) and retinoic acid/LPS- (DCRA) differentiated DC were generated from mouse bone marrow progenitors using standard protocols, but also in the presence of IL-10 and retinoic acid/LPS, respectively. In order to examine the maturation status of these DC, we investigated the expression levels of co-stimulatory molecules and MHC II on DCRA and DC10 and compared each of them with mature DC, i.e., lipopolysaccharide-stimulated DC (DClps).

The expression of CD40, CD86 and MHC II were assessed by flow cytometry. DC10 expressed lower levels of CD40, CD86 and MHC II compared with DClps (Figure 5.1.1). In contrast to DC10, DCRA showed a different phenotype and expressed higher levels of CD40, CD86 and MHC II. Compared with DClps, DCRA expressed similar levels of CD40 and MHC II, and modestly lower levels of CD86. This suggested that DCRA exhibited a more mature phenotype than DC10, not unlike that of the DClps, potentially related to their exposure to LPS during differentiation.

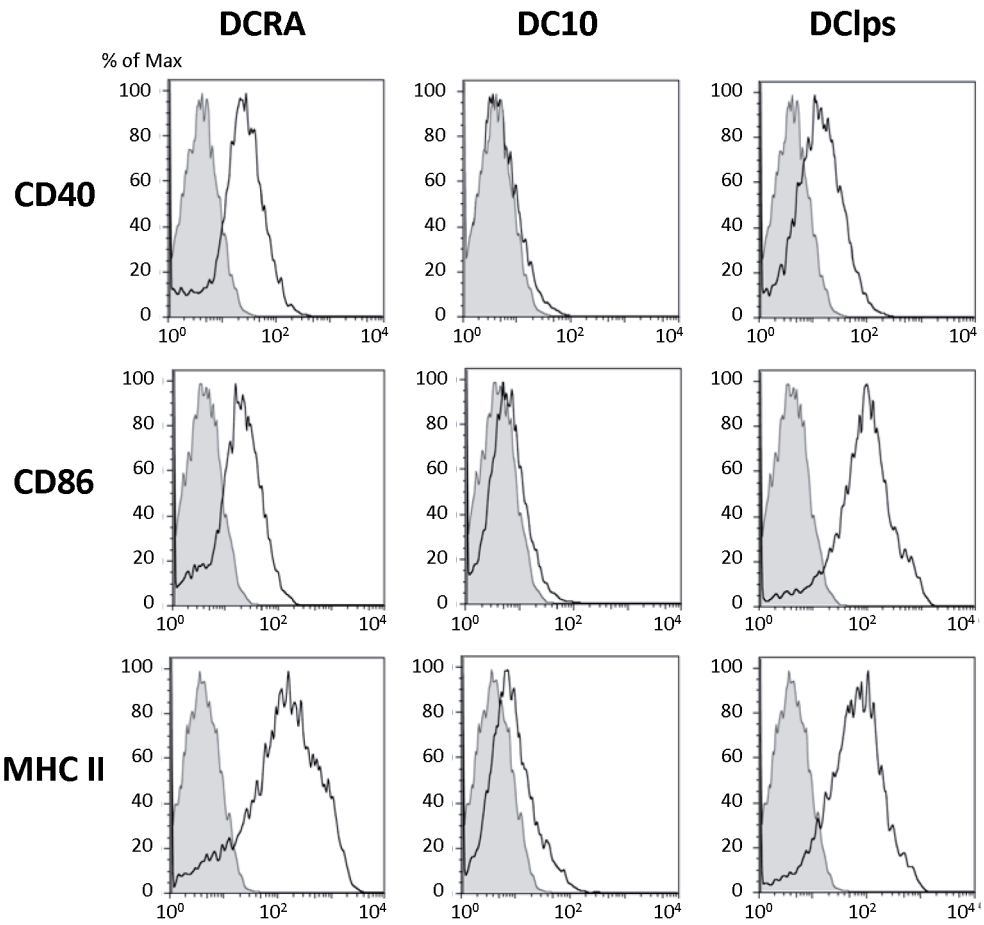


Figure 5.1.1 Comparative study of cell surface CD40, CD86 and MHCII expression by retinoic acid/LPS-, IL-10- and bacterial endotoxin (LPS)-differentiated DCs. Retinoic acid/LPS- (DCRA), IL-10- (DC10) and bacterial endotoxin (DClps)-differentiated DC were generated from bone marrow progenitors as noted in the Materials and Methods. Cell surface expression of CD40, CD86 and MHCII by these cells was analyzed by flow cytometry using FITC-labeled marker-specific and isotype control antibodies. The shaded histograms represent staining with the appropriate isotype control antibody. The depicted data are representative of two repeated experiments.

5.1.2 Cytokine Production by DCRA and DC10

The release of cytokines by DCs might determine the outcome of tolerance induction or immunogenic response (Rutella, Danese, & Leone, 2006). This study compared the abilities of DCRA and DC10 to produce anti-inflammatory cytokines as determined by ELISA and qRT-PCR. As described before, DCRA were cultured according to our standard protocol. In order to avoid RA, LPS and/or IL-10 in the culture medium, DCRA and DC10 were washed twice with PBS at the end of the DC differentiation cultures and further cultured for 48 hours. The supernatants from these cultures were used to assess IL-27 and TGF-β levels, while the cells were used to assess IL-10 mRNA expression. I did not observe a significant difference in IL-27, TGF-β, or IL-10 production (for each, $p \geq 0.05$) between DCRA and DC10.

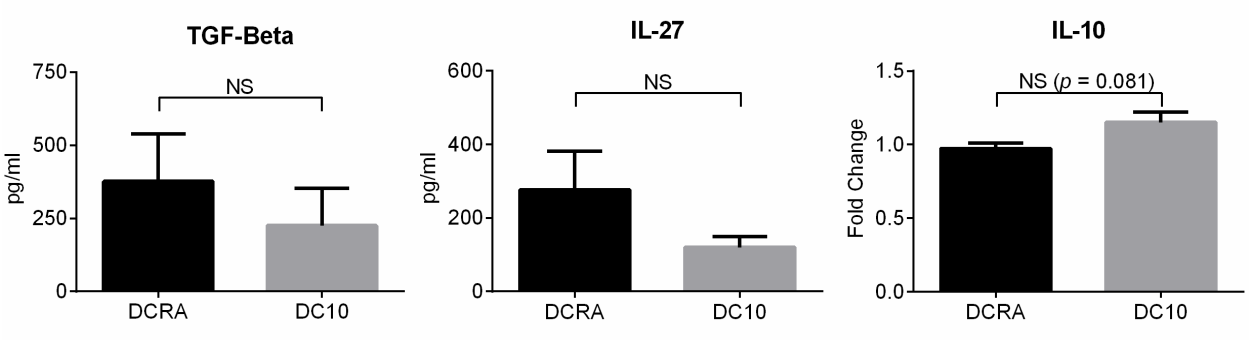


Figure 5.1.2 Expression of TGF-β, IL-27 and IL-10 by DCRA and DC10. DCRA and DC10 were generated from bone marrow progenitors from BALB/c mice as in Fig. 5.1.1, and then they were cultured for a further 48 hours (in the absence of differentiating factors) with no additional manipulations. Expression of these anti-inflammatory cytokines was assessed by ELISA (TGF-β, IL-27) or qRT-PCR (IL-10). The depicted data represents mean with SEM of two repeated experiments. NS, not significant.

5.2 Comparisons of the Tolerance-associated Phenotypes of DCRA and DC10

5.2.1 Assessment of the Abilities of DCRA and DC10 to Suppress Th2 Teff Cell Responses *In Vitro*

As DCRA and DC10 express different tolerance-associated markers, they could also have distinct abilities to suppress Th2 Teff responses. As such, we adopted an *in vitro* system to assess this. Irradiated DCRA or DC10 were co-cultured with $CD4^+$ T cell isolated from asthmatic mice and irradiated immunostimulatory OVA-presenting DClps as noted in the Materials and Methods. Our results showed that DCRA and DC10 both suppressed the ability of Th2 Teff cells to proliferate in response to DClps activation *in vitro* (Figure 5.2.1), and that the DCRA were better than DC10 at suppressing this Th2 Teff cell response ($p<0.05$). With both DCRA and DC10, we observed a cell number-dependent suppression of the Th2 cell proliferation response. In conclusion, DCRA and DC10 both suppressed *in vitro* Th2 Teff cell responses, although DCRA were more effective in this regard. This data indicated that both DCRA and DC10 were tolerogenic DCs.

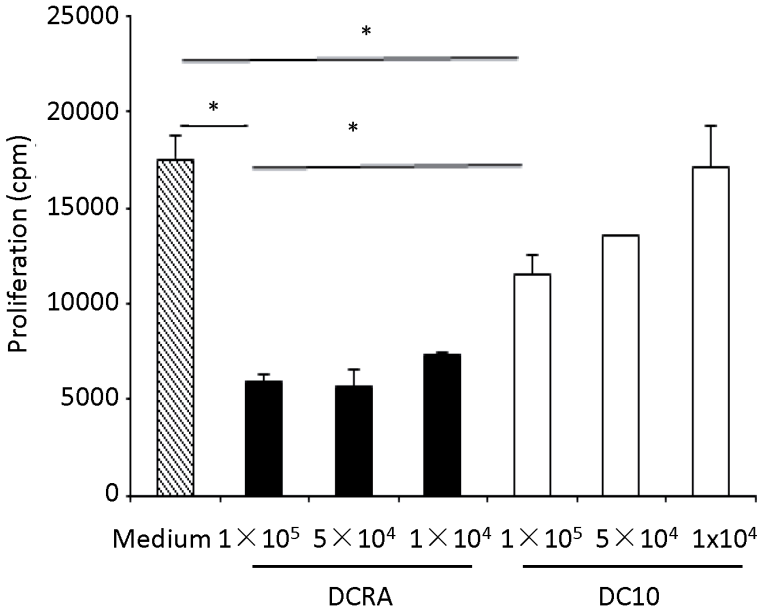


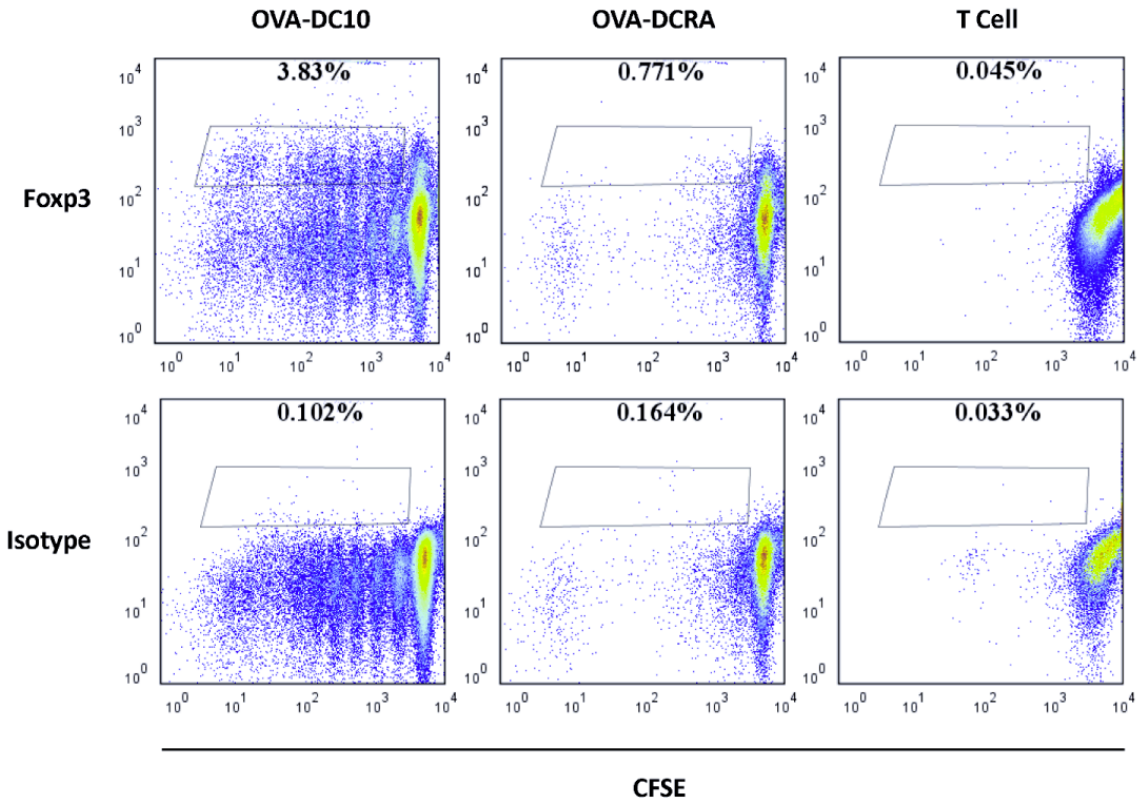
Figure 5.2.1 Comparison of the suppressive abilities of DCRA and DC10 to Th2 responses *in vitro*. OVA-presenting DClps, DCRA and DC10 were generated as in Fig 5.1.1 and then irradiated (3000 rad) before use. DClps were used as stimulator cells for magnetically-sorted pulmonary $CD4^+$ T cells from asthmatic mice, while DCRA and DC10 were titrated into this system to assess their abilities to suppress activated Th2 cell responses. The cells were seeded in triplicate in 96-well plates. Both DCRA and DC10 suppressed $CD4^+$ T cell proliferation in this assay, although the DCRA were more effective in this regard than the DC10 on a cell-per-cell basis. *$p < 0.05$. The data presented are representative of two experiments.

5.2.2 Assessment and Characterization of Regulatory T Cell Induction by DCRA and DC10

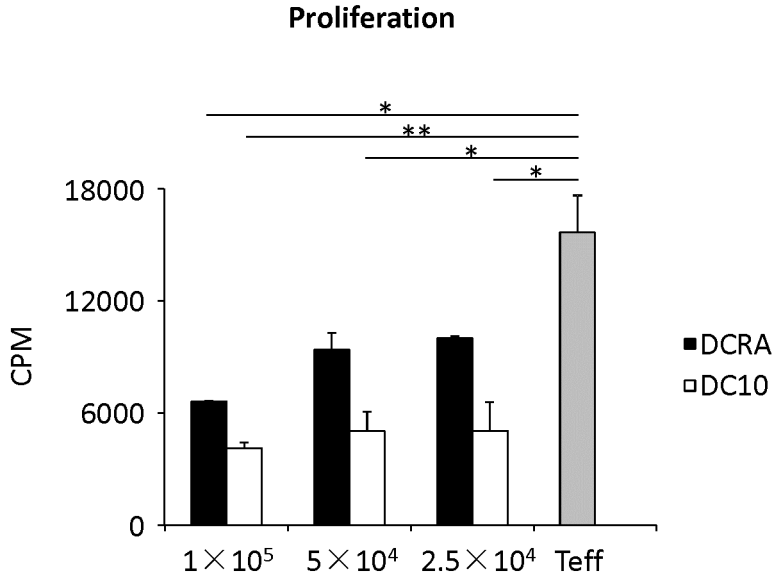
Previous works showed that DC10 play an important role in converting Th2 cells into $Foxp3^+$ Treg cells (Huang et al., 2010; Li et al., 2010; Lu et al., 2011) and thereby induce peripheral tolerance (Huang et al., 2010). Therefore we questioned whether DCRA could similarly induce the differentiation of effector T cells into $CD4^+Foxp3^+$ Treg cells. We set up an *in vitro* culture system to examine this question using $CD4^+$ T cells isolated from spleens of OVA-sensitized transgenic OT II mice. The cells were stained with CFSE and co-cultured with OVA-DCRA or OVA-DC10 for 5 days, after which we assessed the Teff cell proliferative responses and the T cell's expression of Foxp3. Our results showed that OVA-DC10 strongly promoted the proliferation of $CD4^+$ T cells and induced effector T cells to differentiate into $CD4^+Foxp3^+$ cells, while they also induced the proliferation of $Foxp3^-$ T cells. In contrast, OVA-DCRA poorly induced $CD4^+Foxp3^+$ T cells and similarly induced little Teff cell proliferation. This indicated that DC10 might induce tolerance by the induction of $Foxp3^+$ T regs (and potentially some Tr1 cells), while DCRA employed a different mechanism to induce tolerance.

We further assessed the abilities of the putative Treg induced by DCRA or DC10 to suppress the proliferation of effector Th2 phenotype $CD4^+$ T cells in response to DClps stimulation. The putative regulatory $CD4^+$ T cells were isolated from DC10- or DCRA-Teff cell co-cultures by magnetic sorting, then the indicated numbers of these cells were titrated onto splenic $CD4^+$ T cells from OVA-sensitized transgenic OT II mice that were in co-culture with OVA-presenting immunostimulatory DClps, after which we assessed the Th2 Teff proliferation responses. Our results indicated that the induced putative $CD4^+$ Treg from both the DCRA- and DC10-Teff cell co-cultures were effective in suppressing Th2 Teff cell response, and did so in a cell number-dependent fasion ($p<0.05$). Taken together, our data indicates that both DCRA and

DC10 were able to induce tolerance and to suppress *in vitro* Th2 responses by induction of Tregs, but that these two populations of DC employ different mechanisms in induce tolerance *in vitro*.


OVA-DC10
OVA-DCRA
T Cell
Foxp3
Isotype
3.83%
0.771%
0.045%
0.102%
0.164%
0.033%
CFSE

(a)


Proliferation
CPM
18000
12000
6000
0
1×10^5
5×10^4
2.5×10^4
Teff
DCRA
DC10
*
**
*
*

(b)

Figure 5.2.2 Assessment of Treg induction by DCRA and DC10. $CD4^+$ Teff cells were magnetically sorted from the spleens and an array of lymph nodes of OT II mice. These donor mice had been sensitized by i.p. injection of OVA/alum on days 0 and 12, and then they were sacrificed as T cell donors on day 14. **(A)** The Teffs were stained with CFSE and co-cultured with OVA-presenting DC10 or DCRA (10^5 Teffs $^+$ 3×10^4 DC/well) for 5 days; 10 μg/ml IL-2 was added to the culture system. The cells were analyzed by FACS for assessments of cell proliferation (CFSE dilution) and Foxp3 expression. The data shown is representative of the outcomes from three experiments. **(B)** In order to confirm the T cells from these cultures had regulatory roles, the T cells were irradiated, then added into cultures of $CD4^+$ Teff cells and irradiated OVA-presenting DClps for 72 hours. Proliferation was assessed as in Fig 5.2.1. The data shown are representative of two experiments. * or **: $p<0.05$ or $p<0.01$

5.3 Assessing the Efficacy of DCRA and DC10 as a Therapeutic Approach in a Mouse Model of Asthma

Our studies showed that DCRA and DC10 are functionally suppressive to Th2 Teff cell responses *in vitro*, and that they induce the differentation of Treg cells, but it remained unclear whether DCRA could tolerize Th2 responses *in vivo*. We established a BALB/c mouse model of asthma to answer this question. BALB/c mice were chosen because they are reported to develop vigorous Th2 responses and high levels of serum IgE following sensitization and challenge with OVA (Sugita et al., 2003). Mice were sensitized for asthma-like disease using a standard protocol (Nayyar et al., 2012). Briefly, mice were given two i.p. injections of OVA-alum (days 0 and 14), followed by exposure to 1% OVA in saline aerosols on days 30, 32, and 34 (20 min/day). 1×10^6 OVA-DC10 or OVA-DCRA, or an equal volume of saline, were delivered i.p. to asthmatic mice 2 weeks after the establishment of asthma. The mice were assessed for airway hyperresponsiveness (AHR) to methachoine 4 weeks later, then exposed the next day to an aerosolized OVA challenge and sacrificed 48 h later, when we assessed airway inflammation (eosinophils, Th2 cytokines) and serum OVA-specific IgE and IgG1 antibody levels.

5.3.1 Airway Hyperresponsiveness

Airway hyperresponsiveness, a characteristic of asthma, is attributable to reversible smooth muscle constriction and attendant airway narrowing. Head-out, whole body plethysmography was chosen to assess AHR to methacholine in conscious animals at weekly intervals after the DC treatments. Our results indicated that both DCRA and DC10 inhibited AHR in our asthmatic mice ($p < 0.001$), but there were no differences observed between the DCRA- and DC10-treated asthmatic mice ($p > 0.05$).

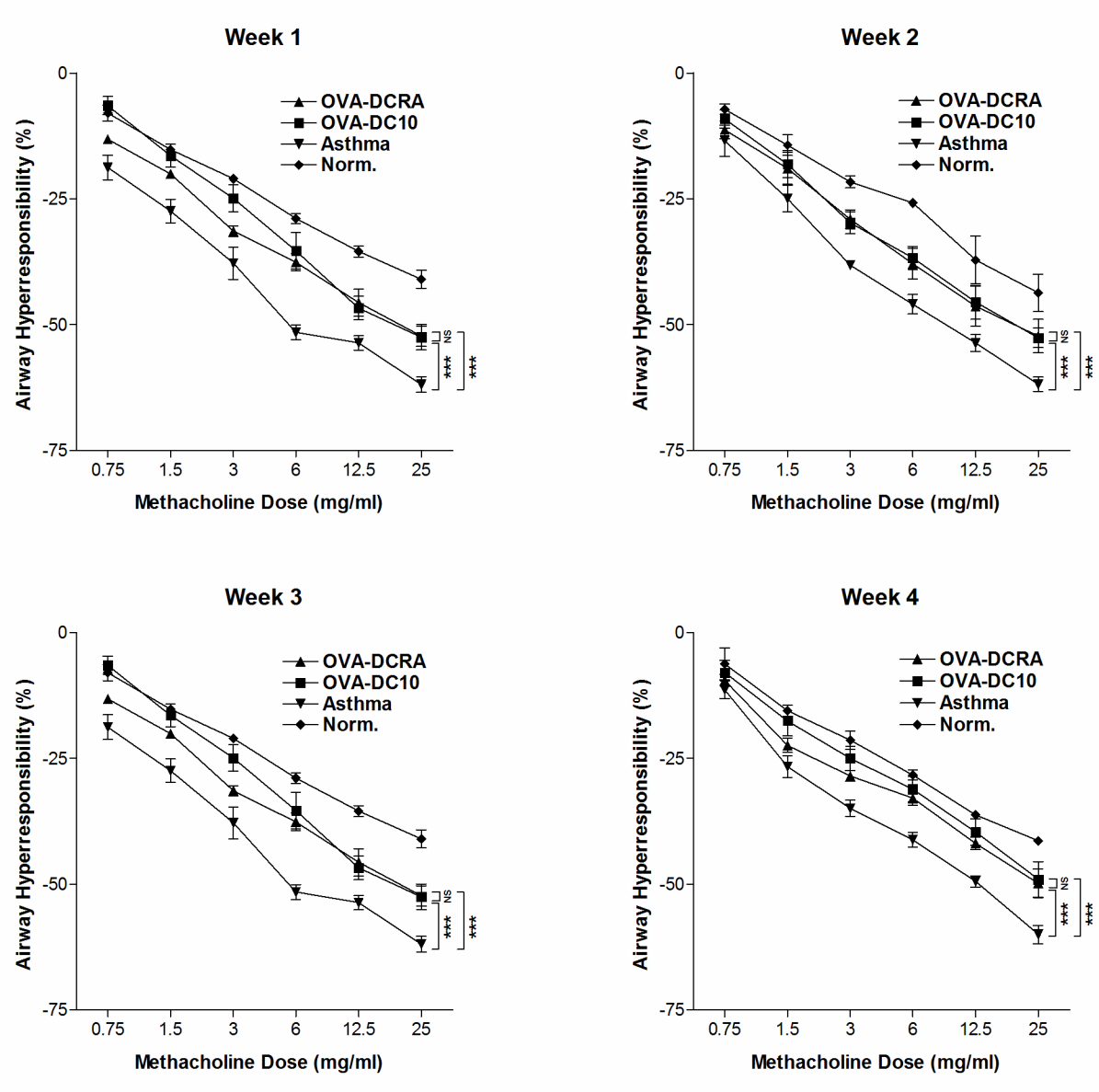
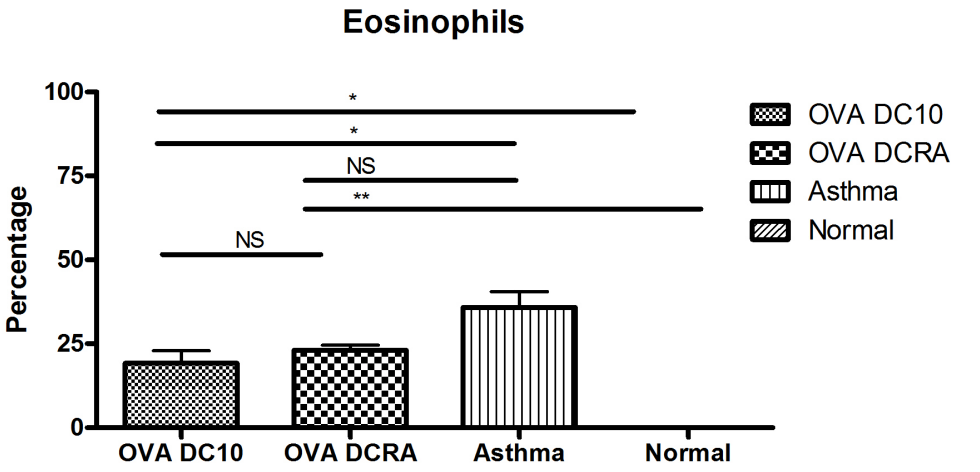
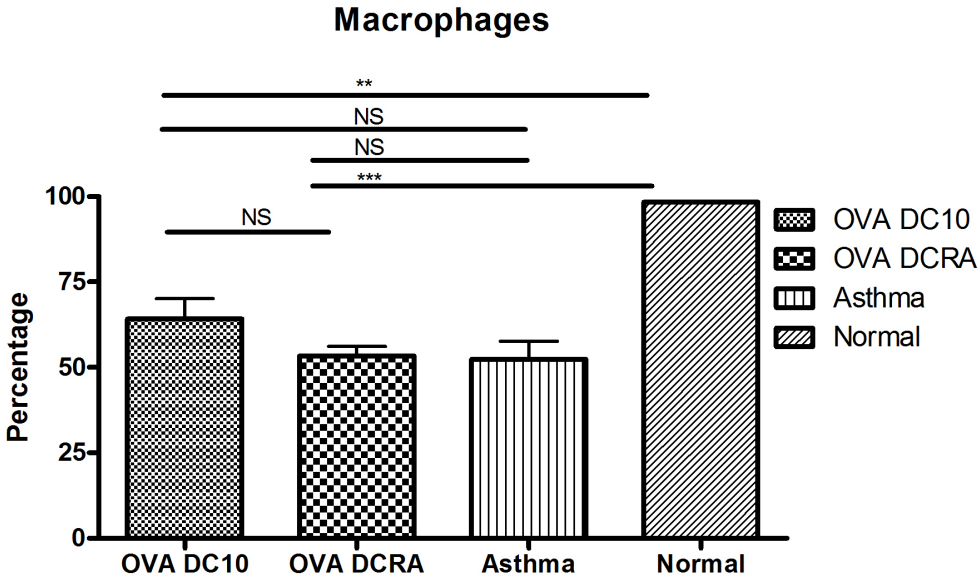

Week 1
Week 2
Week 3
Week 4
OVA-DCRA
OVA-DC10
Asthma
Norm.
Airway Hyperresponsibility (%)
0
-25
-50
-75
0.75
1.5
3
6
12.5
25
Methacholine Dose (mg/ml)
NS
***
***

Figure 5.3.1. Effect of DCRA and DC10 on airway hyperresponsiveness in asthmatic mice. The asthma phenotpe were established using OVA/alum and OVA aerosol exposures, as noted in the Materials and Methods. OVA-presenting DC10 or DCRA (1×10^6 cells/mouse; n=5) were given to the asthmatic mice on day 48, after which airway hyperresponsiveness to methacholine was assessed weekly by head out whole body plethysmography. The data were collected as running 1-sec means of the airflow rate at 50% point in the expiratory cycle. Normal mice were included as a negative control group (n=3). The data shown are representative of three experiments. ***, $p\leq0.001$.

5.3.2 Airway Eosinophilia

We further assessed the effects of these DCRA and DC10 on eosinophilic inflammatory responses to aerosolized recall allergen challenge, as determined at 4 wk after DC treatment. The airways of the mice given OVA-presenting DC10 contained fewer eosinophils than those of the asthma phenotype mice ($p < 0.05$, Figure 5.3.2). The eosinophilia of the DCRA-treated asthmatic mice was 36% lower than that of asthmatic mice that did not receive DC ($p \leq 0.05$), but there were no differences in the eosinophil responses of the DCRA and DC10 treatment groups ($p > 0.05$).


Eosinophils
Percentage
100
75
50
25
0
*
*
NS
**
NS
OVA DC10
OVA DCRA
Asthma
Normal
OVA DC10
OVA DCRA
Asthma
Normal


Macrophages
Percentage
100
75
50
25
0
**
NS
NS
***
NS
OVA DC10
OVA DCRA
Asthma
Normal
OVA DC10
OVA DCRA
Asthma
Normal

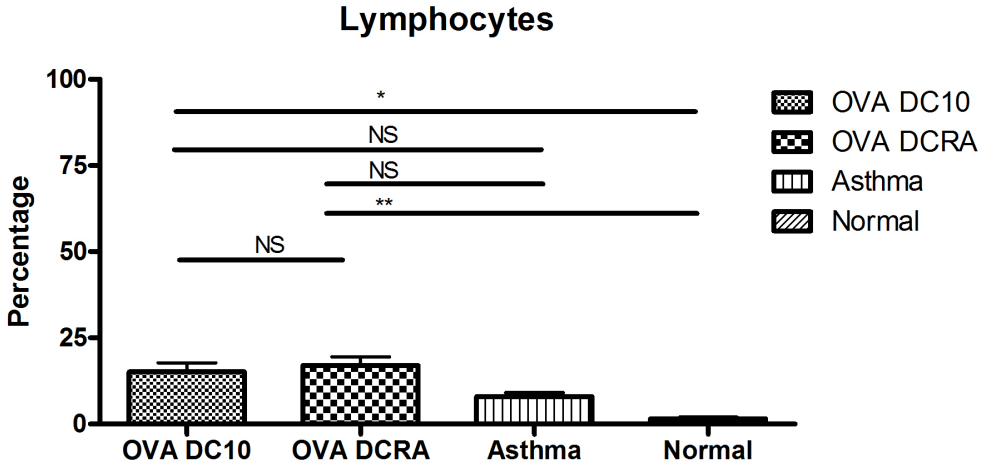

Lymphocytes
Percentage
100
75
50
25
0
*
NS
NS
**
NS
OVA DC10
OVA DCRA
Asthma
Normal
OVA DC10
OVA DCRA
Asthma
Normal

Figure 5.3.2 Effects of DC10 and DCRA treatment on BAL differential cell counts in responses to recall allergen challenge in asthmatic mice. Asthmatic mice were treated with OVA-DC10 (n=5), OVA-DCRA (n=5), or saline (n=5), while healthy control mice remained untreated (n=3); all mice were were exposed to OVA aerosols 4 wk later, then sacrificed after a further 48 hours. Airway cells were recovered by bronchoalveolar lavage (BAL) and differential counts done using Giemsa solution-stained cytocentrifuge preparations of the BAL cells. The data depicts the proportions of eosinophils, macrophages, and lymphocytes among the BAL cells and is representative of three repeat experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

5.3.3 Pulmonary Th2 Cytokine Response

We further assessed the effects of the DC10 and DCRA on Th2 recall responses in the airways of asthmatic mice. Bronchoalveolar lavage (BAL) was done on each mouse and the BAL fluid was analyzed for IL-4, -5, -9 and -13 by ELISA. There were no statistically significant reductions IL-4 and IL-5 in DCRA-treated mice versus the saline-treated mice. Airway IL-9 ($P=0.019$) and IL-13 ($P=0.051$) levels were lower in the DCRA-treated asthmatic mice than in the saline-treated mice. Asthmatic mice with treatment of DC10 did not show statistically significant reduction of Th2 cytokines. But DC10 and DCRA have found to relieve AHR and to reduce circular IgG1 and IgE levels and eosinophils migration into airway in the asthmatic mice with treatment in my experiments. My experiments also showed a trend of reduced levels of Th2 cytokines in BAL in asthmatic mice with treatment of DC10.

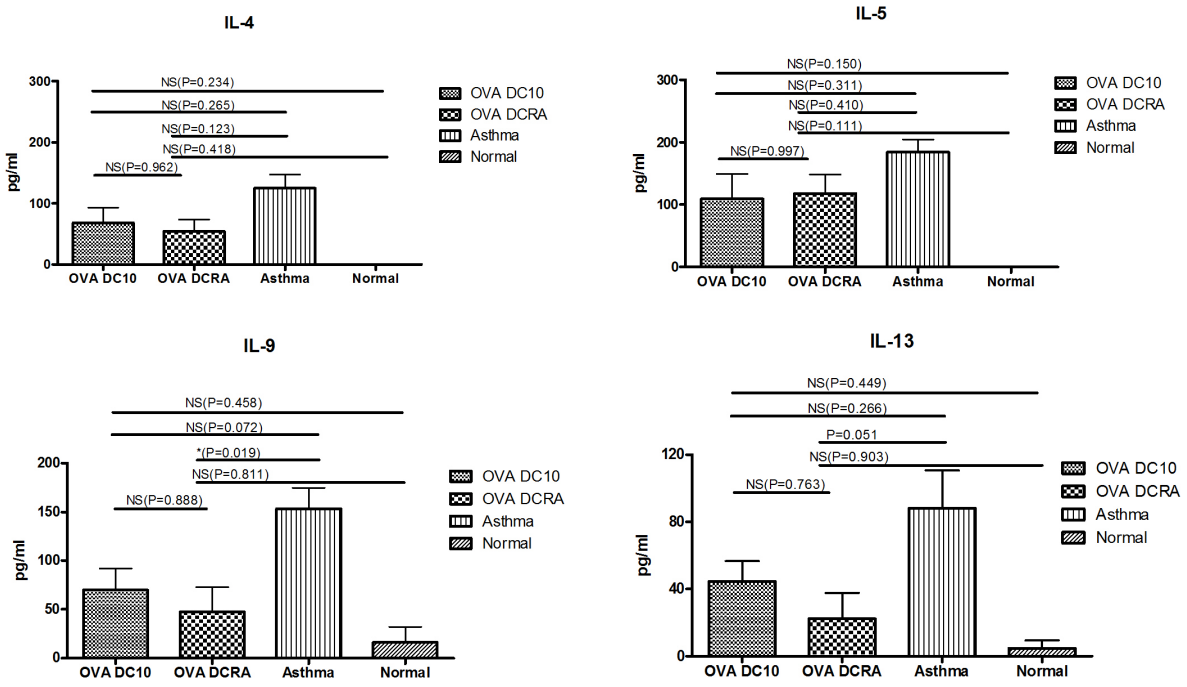


Figure 5.3.3 Effects of OVA-presenting DCRA and DC10 on airway Th2 recall responses in OVA-sensitized asthmatic mice. Asthmatic mice were treated with OVA-DC10 (n=5), OVA-DCRA (n=5), or saline (n=5), while healthy control mice remained untreated (n=3); BAL fluids were analyzed for the indicated cytokines by ELISA. The data shown is representative of three experiments. *, $p<0.05$.

5.3.4 Antibody Response

We also examined the effects of OVA-presenting DCRA and DC10 on the circulating levels of OVA-specific IgE and IgG1 in asthmatic mice. Our results showed decreased levels of circulating IgE and IgG1 in both the DC10- and DCRA-treated groups compared with asthmatic mice ($p \leq 0.001$). There were no differences in the levels of either isotype of antibody between the DCRA and DC10 treatment groups. Therefore, compared to asthmatic mice, both DC10 and DCRA reduced the circulating IgE and IgG1 levels.

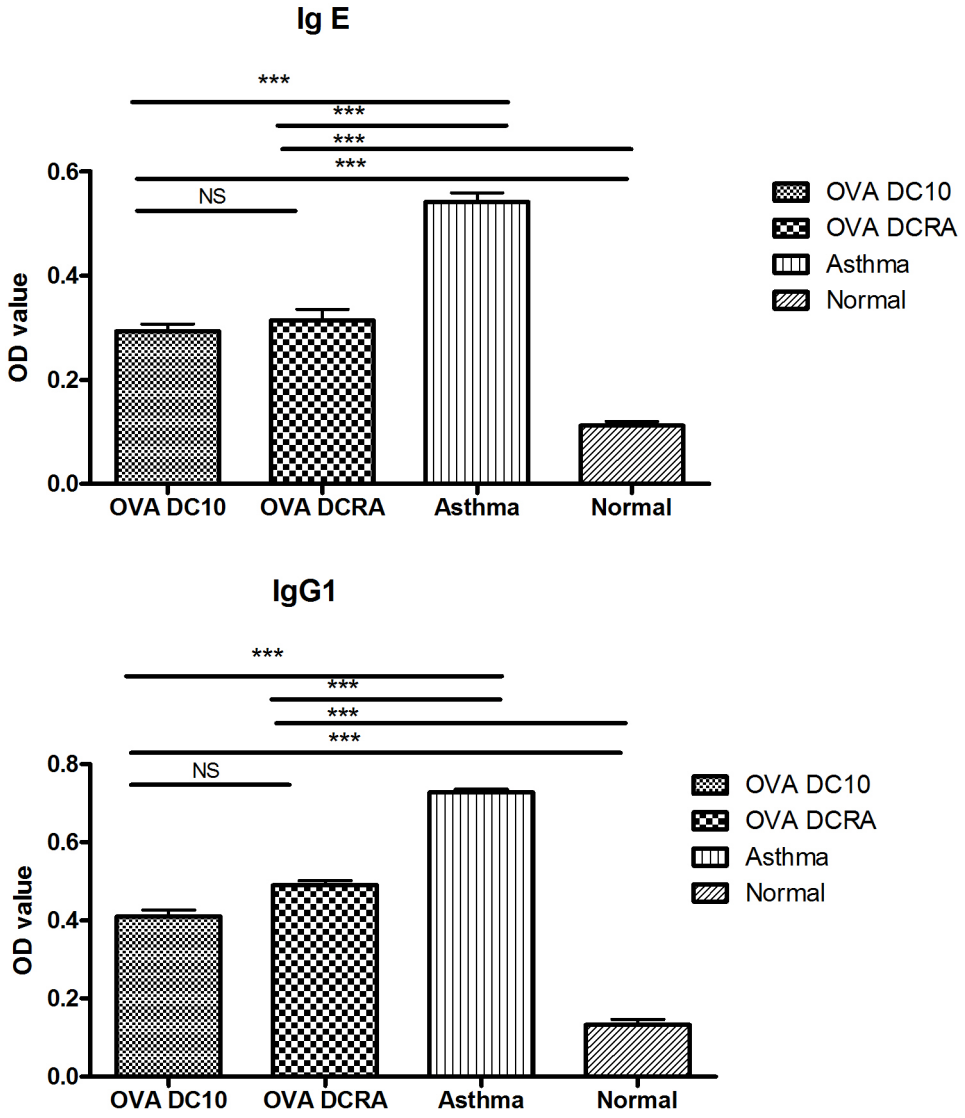


Figure 5.3.4 Effects of OVA-presenting DCRA and DC10 on circulating OVA-specific IgE and IgG1 levels in OVA-sensitized asthmatic mice. Asthmatic mice were treated with OVA-DC10 (n=5), OVA-DCRA (n=5), or saline (n=5), while healthy control mice remained untreated (n=3); Plasma OVA-specific IgE and IgG1 levels were assessed by ELISA as indicated in the Materials and Methods section. The data shown is representative of three experiments. ***, $p<0.001$.

CHAPTER 6: DISCUSSION AND CONCLUSIONS

6.1 Discussion

Prior studies in our lab have indicated that DC10 treatment in a mouse model of asthma reverses airway hyperresponsiveness and reduces airway inflammation (Huang et al., 2010; Lu et al., 2011; Nayyar et al., 2012). Our lab's data clearly indicates that antigen-specific $Foxp3^+$ Tregs are critical to DC10-induced tolerance in this model of asthma (Huang, Dawicki, et al., 2013; Huang et al., 2010; Huang, Ma, et al., 2013; Lu et al., 2011). Retinoic acid (RA) is a metabolite of vitamin A and can be produced by the body. The enzymes that regulate the generation of RA include RALDH1, RALDH2, and RALDH3, which metabolize retinaldehyde to RA (Duester, 2008). RA serves as a cofactor with TGF- β for the generation of induced Treg (iTreg) cells in the gut; *in vitro* findings have confirmed that relative to splenic DCs, mesenteric lymph node (MLN) and gut lamina propria (LP) DCs are strong inducers of iTreg cell differentiation (J. A. Hall et al., 2011). Airway tolerance, a specialized form of immunological surveillance (Andreev, Graser, Maier, Mousset, & Finotto, 2012), is necessary to avoid the development of asthma, which is characterized by airway inflammation, airway hyperresponsiveness, and airway remodeling (Zuyderduyn, Sukkar, Fust, Dhaliwal, & Burgess, 2008). A prior study demonstrated that in antigen-induced tolerized mice, RA-expressing DCs were crucial to induction of airway tolerance locally (Khare et al., 2013), suggesting that RA plays an important role in the tolerogenic activities of pulmonary DCs and thereby the induction of airway tolerance. The generation of iTregs is one of the mechanisms contributing to airway tolerance, and tolerogenic DCs play an important role in generation of these cells. There is an increasing need to gain a better understanding of therapeutic tolerance induced in asthma by

tolerogenic DCs.

Current therapies control asthma symptoms but they do not target its underlying immunologic basis. Tolerogenic DCs could offer a new opportunity for asthma treatment. The induction of peripheral T cell tolerance and promotion of Treg differentiation is an important mechanism in allergen-specific immunotherapy (Akdis, Blaser, & Akdis, 2005). DCs play an important role in directing Th2 cell responses in asthma (Lambrecht et al., 2000). However, DCs can also initiate allergen-specific immune tolerance. Indeed, there are natural populations of tolerogenic DCs that contribute to this tolerance. In the gut, $CD103^+$ dendritic cells were found to induce $Foxp3^+$ Treg cells and this is dependent on retinoic acid and TGF-β1 (Coombes et al., 2007). Plasmacytoid DCs were suggested to be tolerogenic DCs, inasmuch as they possess a regulatory phenotype and prime IL-10-producing Tregs (de Heer et al., 2004; Ito et al., 2007). Aimed at gaining a better understanding of tolerogenic DC therapy, in this study we differentiated DCs in the presence of the tolerogenic agents RA/LPS or IL-10 and explored the abilities of the induced DCs to induce allergen tolerance. We identified some relevant characteristics of these DCs, including their cytokine and cell surface antigen-presenting cell marker expression profiles, suppressive functions related to Th2 responses, and their abilities to promote Treg cell differentiation. We also evaluated the capabilities of DCRA and DC10 to suppress allergic airway inflammation.

DCRA and DC10 exhibit different phenotypes based on their expression of cellular surface markers *in vitro*. Results of this study show that DC10 expressed lower levels of CD40, CD86 and MHC II, and this is consistent with prior studies. For example, DC10 were reported to express lower levels of costimulatory molecules and MHC II than mature DCs (Nayyar et al., 2012). DC10 are similar to immature DCs in this phenotype, which generally are characterized

as expression lower levels of costimulatory and MHC II molecules (Manicassamy & Pulendran, 2011). On the contrary, our study showed that LPS-matured DCRA expressed higher levels of CD40, CD86 and MHC II molecules compared with DC10, suggesting that DCRA are more competent at antigen presentation and activating T cells than immature DCs. Having said that, DCRA expressed similar levels of MHC II but lower levels of CD86 than fully mature DC, suggesting that they have lower stimulatory ability than these latter cells.

LPS is a major component of the gram-negative bacterial cell wall. LPS contains pathogen-associated molecular patterns (PAMP) that are recognized by the TLR4 receptor. LPS not only induces Th1 and Th17 responses, but also under appropriate circumstances Th2 responses (Y. Kim et al., 2013). The impact of LPS on DCs may not always result in immunity. Few studies have investigated the characteristics of LPS-matured DCRA, particularly their tolerogenic properties (e.g., LPS-treated tolerogenic DCs produced more IL-10). LPS-stimulated hepatic DCs reportedly produce Treg cell growth factors such as IL-10 and IL-27 (Y. Chen et al., 2009). In this study, LPS-matured DCRA and DClps expressed similar levels of CD40 and MHCII, but lower levels of CD86, which indicates a lower capacity to induce differentiation of naïve T cells. Unlike DC10 or mature DCs, LPS-matured DCRA strongly expressed the RALDH2 (W. Dawicki, unpublished data; (J. Chang et al., 2013).

It has been reported that IL-27 acts as anti-inflammatory cytokine, possibly by inducing IL-10 expression and thereby limiting inflammatory responses (Bosmann & Ward, 2013). Signaling via the transcription factor c-maf and IL-21 expression are important factors in the generation of IL-27-driven Tr1 (Pot, Apetoh, Awasthi, & Kuchroo, 2010). IL-27, mainly produced by activated monocyte-derived DCs, is upregulated by LPS-activation of DCs (Larousserie et al., 2004). My results showed that DCRA promote little expansion of $Foxp3^+$ T

cells, and this fits with a prior report that when T cells were differentiated in presence of IL-27 there was a significant reduction in $Foxp3^+$ Tregs (Huber et al., 2008). This could be attributed to the IL-27 production by DCRA, which is different from the mechanisms in DC10-induced $Foxp3^+$ Tregs. Although our lab has found that LPS-activated DCRA secrete a large amount of IL-27 (W. Dawicki, unpublished observation), the results of my two experiments showed that DCRA express higher levels of IL-27 than DC10 but this was not a statistically-significant difference. This lack of significance herein could be due to the small number of samples tested.

DCRA and DC10 both suppress the responses of Th2 Teff cells from the lungs of asthmatic mice, although the former cells were modestly more effective in this regard. In the present study, OVA-DC10 inhibited Th2 Teff responses, and this is consistent with previous findings of our lab wherein OVA-DC10 (either mouse or human) induce $CD25^+Foxp3^+LAG3^+$ $CTLA\text{-}4^+$ Treg from autologous Th2 Teff cell populations (Huang et al., 2010; Li et al., 2010). In the human system, these Treg subsequently suppressed autologous Teff responses in a contact-dependent manner (Li et al., 2010). In terms of DCRA, our study showed that OVA-DCRA were also able to suppress Th2 Teff responses. DCRA promoted little if any expansion of $Foxp3^+$ Tregs but potently suppressed Teff Th2 responses. It is well known that IL-27 can promote production of IL-10 from different T cell subsets (e.g., Th1, Th2, Th17, and Tr1) through transcription factors (i.e., STAT-1 and STAT-3) and costimulatory molecules (i.e., ICOS) (Pot et al., 2009). In addition, IL-27 is essential to the generation and expansion of IL-10-producing Tr1 cells (Awasthi et al., 2007). Overall, DCRA were more effective than DC10 in reducing T cell proliferation, but both DCRA and DC10 suppressed the proliferation of Th2 Teff cells, indicating that they function as tolerogenic DCs.

OVA-DCRA and OVA-DC10 are different in their abilities to induce or expand $Foxp3^+$

T cells. Although both OVA-DCRA- and OVA-DC10-induced regulatory T cells that displayed inhibitory abilities to Teff Th2 responses, their related mechanisms for tolerance induction were different. As noted, OVA-DCRA promoted little expansion of $Foxp3^+$ Tregs (0.771%), although the T cells remained viable in culture and acquired a regulatory phenotype, although when cultured for 5 days in the absence of DC, Teff died rapidly in culture. OVA-DC10 more strongly promoted the expansion of $Foxp3^+$ Tregs in our study as well as in analogous studies in our lab. Similarly, human OVA-DC10 caused induction of Foxp3 expression on T cells and suppressed Teff responses dependent in a cell-contact-dependent manner (Boks, Zwaginga, van Ham, & ten Brinke, 2010). A prior study in our lab also showed that human OVA-DC10 led to the development of $CD4^+CD25^+Foxp3^+$Tregs among peripheral blood $CD4^+$ Th2 cells from asthmatic donors (Li et al., 2010). Murine OVA-DC10 also converted Teff cells into $CD4^+CD25^+Foxp3^+$ Tregs, which played an important role in OVA-DC10 induced tolerance.

RA by itself improves the ability of DCs to induce Foxp3 expression from $CD4^+$ T cells in periphery (J. A. Hall et al., 2011). Similarly, the induction of Foxp3 expression by $CD103^+$ DCs is dependent on RA and TGF-β (Khare et al., 2013) but, unlike traditional RA-differentiated DCs, LPS matured-OVA-DCRA, which produce abundant IL-27, induced little T cell Foxp3 expression. Such a difference could be caused by many factors. The frequency of Foxp3-expressing Tregs is known to decreases significantly when T cells were differentiated in presence of IL-27 (Huber et al., 2008). DCRA produced a large amount of IL-27, which is known to play an important role in promoting Tr1 differentiation (Neufert et al., 2007; Pot et al., 2009; Stumhofer et al., 2006). Furthermore, negating IL-27 signaling, by knocking out the IL-27R, resulted in enhanced conversion of T cells into $Foxp3^+$ Tregs in the animal model of colitis (Cox et al., 2011), indicating that IL-27 may act to decrease the expression of Foxp3.

Based on *in vitro* experiments that have identified DCRA and DC10 as tolerogenic DCs, we have tested their regulatory functions in a mouse model of asthma. It has been reported that serum levels of vitamin A, an essential micronutrient, are inversely correlated with the asthma phenotype. Thus, asthmatic children have lower serum vitamin A levels compared with health controls (Arora, Kumar, & Batra, 2002). Administration of all-trans RA in a mouse model of asthma has been reported to reduce airway inflammation (Wu, Zhang, Liu, Zhong, & Xia, 2013). In this study, AHR started to decrease in asthmatic mice with treatment of DCRA or DC10. Both DCRA and DC10 significantly reduced AHR, suggesting that constriction of smooth muscle in asthmatic mice was relieved after treatment of DCRA or DC10. There is no difference between DCRA and DC10 in reducing AHR in a mouse model of asthma. It was reported that AHR was restored after giving ATRA supplement in animal model (McGowan, Holmes, & Smith, 2004). Our lab also found that AHR was normalized in DC10-treated asthmatic mice. To our knowledge, the present study is the first work to identify that DCRA could relieve AHR in asthmatic mice. In addition to the AHR, this study also tests the ability of DCRA or DC10 to reduce IgG1 and IgE levels in the peripheral blood at 4 weeks after treatment. DCRA reduced serum IgE and IgG1 levels in our treated asthmatic mice, possibly through its regulation of B cells. Other studies also found that RA reduced IgE production by CD40-stimulated B cells (Scheffel, Heine, Henz, & Worm, 2005; Worm, Krah, Manz, & Henz, 1998). In terms of DC10, serum level of OVA-specific IgE and IgG1 level are significantly decreased in DC10-treated asthmatic mice, which is similar to previous results in our lab. Results of this study show that DCRA reduces airway levels of IL-9 and IL-13. There was also a trend towards decreased airway Th2 cytokine levels in our DC10-treated asthmatic mice, although this was not statistically significant.

Finally, although both DC10 and DCRA established tolerance in the periphery, it was not clear which one of them was more effective in inducing tolerance *in vivo*. This study revealed for the first time that DCRA were tolerogenic and partially induced peripheral tolerance *in vivo* in a mouse model of asthma. This would provide a solid basis to further explore new asthma therapies, although a number of challenges could affect tolerogenic DCs-based asthma treatments. For example, it could be necessary to assess the combined use of DCRA and DC10 aimed at an enhanced ability of tolerogenic DCs to induce tolerance in asthma.

6.2 Conclusions and Future Directions

This study led to the following conclusions:

(1) DC10 expressed lower levels of costimulatory molecules and MHC II molecules than DCRA.

(2) DCRA and DC10 suppressed *in vitro* Th2 response, but DCRA were more effective than DC10 at suppressing proliferation.

(3) DC10 promoted expansion of $Foxp3^+$ T cells, whereas DCRA promoted little expansion of $Foxp3^+$ T cells.

(4) Treatment of asthmatic mice with DC10 and DCRA reduced AHR, and serum IgE and IgG1 levels.

Together this indicates that, DCRA are tolerogenic and have a distinct mechanism by which they induce tolerance in asthma in comparison with DC10.

The present study could be enhanced by inclusion of control DCs that had not been exposed to RA or to LPS. Such control groups might provide better insights into the activities of LPS-activated DCRA. We found that DCRA induced no discernible Foxp3 expression among Teff cells, indicating that LPS-activated DCRA might induce $Foxp3^-$ Tregs such as Tr1 cells from Teff cells. Future studies would address additional topics including: (1) whether DCRA locally induce tolerance in lung or lung draining lymph node, (2) whether DCRA similarly reduce the asthma phenotype in alternate mouse models (e.g., house dust mite asthma), (3) whether human monocyte-derived DCRA would suppress Teff cell responses of asthmatic patients, as determined in *in vitro* experiments, and (4) whether IL-27 is involved in Treg population induced by LPS-activated DCRA in mouse models of asthma.

CHAPTER 7: BIBLIOGRAPHY

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