MECHANISMS MEDIATING ASTHMA TOLERANCE INDUCED BY REGULATORY DENDRITIC CELLS THROUGH $CD4^+CD25^+FOXP3^+$ T REGULATORY CELLS

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ABSTRACT

IL-10-treated tolerogenic dendritic cells (DC10) have been used as a powerful tool for the treatment of experimental asthmatic responses. We explored the important role of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) in the induction of tolerance by DC10 in three levels. We first used the siRNA approach to silence IL-10 gene expression in murine DC10 and we generated DC10 from CD80/86 or $H\text{-}2Ia^b$ gene-KO mice. When used to treat asthmatic mice, the IL-10-silenced and MHCII-deficient DC10 had lost their abilities to induce tolerance, as determined by airway hyperresponsiveness (AHR), eosinophilia and Th2 responses, while the $CD80^{-/-}/86^{-/-}$ DC10 had reduced tolerogenic activities. Further studies showed that the OVA-presenting DC10 up-regulated expression of the Treg-associated markers ICOS, PD-1, GITR, LAG-3 and CTLA-4 on pulmonary Treg cells in treated asthmatic mice while DC10 ($CD80^{-/-}CD86^{-/-}$), DC10 ($Ia^{b-/-}$) and DC10 ($IL\text{-}10^{-/-}$) treatments lead to reduced Treg activation. This demonstrated that the activities of tolerogenic DCs are differentially affected by their expression of IL-10, MHC II and costimulatory molecules.

DC10 induce allergen tolerance in asthmatic mice, during which the animals-lung Th2 T effector cells (Teff) are displaced by activated Treg cells. Intestinal DCs have been shown to promote oral tolerance by inducing *antigen-naïve* T cells to differentiate into Treg, but whether DCs can induce Teff to differentiate into Treg remains uncertain. We addressed this question in OVA-asthmatic mice that were treated with DC10. DC10 delivery maximally activated lung Treg in these animals at 3 wk post-treatment, as determined by *in vitro* and *in vivo* Treg-inhibitory assays. In parallel cultures OVA-, but not house dust mite (HDM)-, presenting DC10 induced ≈43% of CFSE-labeled $CD25^{-/lo}Foxp3^-$ Teff cells from asthmatic OVA-TCR transgenic mice to redifferentiate into

Treg. We recapitulated this *in vivo* using OVA-asthmatic mice that were co-injected with OVA- or HDM-presenting DC10 (*i.p.*) and CFSE-labeled $CD4^{+}CD25^{-/lo}Foxp3^{-}$ Teff cells (*i.v.*) from the lungs of asthmatic DO11.10 mice. From ≈7 to 21% of the activated (i.e., dividing) DO11.10 Teff that were recovered from the lungs, lung-draining lymph nodes or spleens of the OVA-DC10 recipients had redifferentiated into Treg. These data indicate that DC10 treatments induce tolerance at least in part by inducing Teff to redifferentiate into Treg.

Finally, we assessed the relative efficiency with which natural Treg cells (nTreg) and induced Treg cells (iTreg) tolerize Teff cells from asthmatic mice. The iTreg were induced either by culture of Teff cells with OVA-presenting DC10 or by injecting these Teff cells into DC10-treated asthmatic mice. We purified nTreg from allergen-naïve mice and also cultured them with DC10 before use, or co-injected them into DC10-treated recipients. The iTreg were 26-41% more effective than analogous nTreg in reducing Teff cell proliferation and IL-4/IL-5 secretion *in vitro*. Neutralization of IL-10, but not TGFβ, eliminated the suppressive activities of iTreg, which also expressed higher levels of PD-1, LAG3 & CTLA-4. Transfer of 5×10^{5} nTreg had no impact on AHR or IgE levels in asthmatic recipients, but reduced the airway eosinophil and IL-4/IL-5 responses by 5-19%, while the iTreg normalized AHR and reduced all airway responses to allergen challenge by 82-96%. Taken together, our data show that DC10 provide three signals to induce Treg differentiation directly from allergen-experienced Teff, and that iTreg and nTreg of the same antigen specificity employ distinct mechanisms to effect tolerance. They also indicate that iTreg are distinctly superior to nTreg in induction of tolerance.

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Dedicated to

Baba and Mama for their love and for supporting my education

My wife Hong Yu and my son Peidong Huang for their love and support

My sister and brother for their love and care

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LIST OF ABBREVIATIONS

Ab	antibody
AHR	airway hyperresponsiveness
APC	antigen-presenting cell
ATRA	all-trans retinoic acid
BAL	bronchoalveolar lavage
BCR	B cell receptor
BMDCs	bone marrow-derived DCs
Breg	regulatory B cell
CBP	cAMP-responsive element-bing protein-binding protein
CCL	CC chemokine Ligand
CCR	CC chemokine receptor
cDCs	conventional DCs
COX	cyclooxygenase
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte antigen-4
DC	dendritic cell
DC10	IL-10-treated tolerogenic DCs
DCIR-2	dendritic cell inhibitory receptor-2
dsRNA	double-stranded RNA
DTH	delayed-type hypersensitivity
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
ELISA	enzyme-linked immunosorbent assay
EPO	eosinophil peroxidase
FACS	fluorescence-activated cell sorter
FasL	Fas ligand
Fc	crytallizable fragment of Ab
FITC	fluorescein isothiocynate

Foxp3	forkhead box protein 3
FRET	fluorescence (Förster) resonance energy transfer
GCs	glucocorticoids
GFP	green fluorescent protein
GILZ	glucocorticoid-induced leucine zipper
GITR	glucocorticoid-induced TNF-receptor-related protein
GM-CSF	granulocyte/monocyte colony-stimulating factor
GRs	glucocorticoid receptors
Gs protein	stimulatory G protein
GVHD	graft-versus-host disease
HDM	house dust mite
HEVs	high endothelial venules
IBD	inflammatory bowel disease
i.p.	intraperitoneal
ICAM-1	intracellular adhesion molecule-1
ICOS	inducible costimulatory molecule
ICS	inhaled corticosteroids
IDO	indoleamine-2, 3-dioxygenase
IFN	interferon
Ig	immunoglobin
IL	interleukin
iTreg	induced Treg
KO	knockout
LAG3	lymphocyte activation gene 3
LCs	Langerhans cells
LNs	lymph nodes
LPS	lipopolysaccharide
LSM	lymphocyte separation medium
LT	leukotriene
MAdCAM	mucosal addressin cell adhesion molecule
MBP	major basic protein

MAb	monoclonal antibodies
Mch	methacholine
MHC	major histocompatability complex
MIP	macrophage inflammatory protein
MKP-1	MAPK phosphatase-1
mRNA	messenger RNA
MZ	marginal zone
NSAID	non-steroidal anti-inflammatory drugs
nt	nucleotide
nTreg	natural Treg or naturally occurring Treg
OVA	ovalbumin
PCR	polymerase chain reaction
PD-1	programmed death-1
PDL-1	programmed death ligand 1
pDCs	plasmacytoid DCs
pg	picogram
PGs	prostaglandins
PI	propidium iodine
PPD	purified protein derivative
PRR	pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1
RA	retinoic acid
RANTES	regulated on activation normal T cell expressed and secreted (chemokine)
RNA	ribonucleic acid
RORγt	retinoic-acid-related orphan receptor
RSV	respiratory syncytial virus
SCF	stem cell factor
SEM	standard error of the mean
siRNA	small interfering RNA
SLPI	secretory leukoprotease inhibitor
T2-MZP	transitional 2-marginal zone precursor

T-bet	T cell-specific T-box transcription factor 21
Tconv	conventional T cells
TCR	T cell receptor
tDCs	tolerogenic DCs
Teff	effector T cell
T_{FH}	follicular helper T
TGFβ	transforming growth factor-beta
Th	T-helper cell
Th2	Type 2 T-helper cell
Th3	TGFβ-producing T helper cell
TLR	toll-like receptor
TNF	tumor necrosis factor
Tr1	regulatory type 1 T cells
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin
VCAM-1	vascular cell adhesion molecule-1
VLA	very late antigen
µg	microgram
µl	microlitre

CHAPTER 1: LITERATURE REVIEW

1.1. IMMUNOPATHOLOGY OF ASTHMA

1.1.1. Definition and overall asthmatic response

Asthma is a chronic inflammatory disorder of the airways that causes episodic wheezing and difficulty breathing. Despite significant pharmacological advances in asthma therapy, asthma rates for Canadian children have jumped four-fold over the past 20 years to a level where more than one in ten people have been diagnosed with this respiratory ailment. The estimated prevalence of asthma is 300 million persons across the world. It affects 16.2 million adults and 6.7 million children in the United States according to 2007 investigation (Locksley 2010). In North America asthma has become the most common chronic childhood disease; it now costs Canadians and Americans-Health Care Systems around $600 million and $20 billion per year respectively (Busse and Lemanske 2001; Cohn, Elias et al. 2004 Locksley 2010).

Type 2 T-helper cell (Th2) immune responses play a key role in the initiation, progression and persistence of asthma. Allergens (e.g., animal dander, cockroach particles, house dust mites, grass, tree pollen, cat dander) enter the airways and cross the epithelial surface of the endobronchial tree via uptake by dendritic cells (DCs), which are antigen presenting cells (APCs). These APCs process their ingested allergens and present them to naïve Th cells in the lung-draining lymph nodes (LNs). Allergen-specific activation of naïve Th cells induces their differentiation into Th2 cells. Finally, the Th2 responses culminate in the tightened airways and airway hyperresponsiveness (AHR) that typify asthma, the symptoms of which include shortness of breath, chest tightness,

wheezing and coughing (Cohn and Ray 2000; Busse and Lemanske 2001; Lambrecht and Hammad 2003; Hawrylowicz and O'Garra 2005).

1.1.2. Immunobiology of asthma

The immunology of asthma is complex and involves various immune and non-immune cells and molecular components. Inhaled allergens recognize the surface-bound immunoglobulin-E (IgE) to activate sensitized mast cells, resulting in the release of several bronchoconstrictor mediators, including leukotrienes and prostaglandin. Allergens are taken up and processed by DCs, while the epithelium secrete the pro-Th2 cytokines interleukin (IL)-25, IL-33 and thymic stromal lymphopoietin (TLSP), which act on both T cells and non-T cells to promote Th2 responses. Th2 cells play a dominant role in inflammatory responses in asthma via secretion of cytokines IL-4, IL-5, IL-6, IL-9 and IL-13. IL-4 and IL-13 stimulate B cells to produce IgE, IL-5 is required for eosinophilic inflammation and IL-9 induces mast-cell proliferation. The deficiency of regulatory T cell (Treg) may favour further Th2-cell proliferation (Barnes 2008).

1.1.2.1. Mast cells

Mast cells are widely present in many tissues, including the skin, mucosa of the respiratory, genitourinary and gastrointestinal tracts, and the lymphoid organs. Fibroblast-derived stem cell factor (SCF), which engage its c-kit receptor expressed on Mast cells, promotes the development of mast cells, while IL-3 secreted by T cells increases the development of mucosal mast cells but not connective tissue mast cells. Mast cells

express an array mediators and co stimulatory molecules. They are "tuneable", and will respond variably based on their physiologic or pathologic environment. Mast cells are best known as effector cells in IgE-mediated allergic responses, although the multifunction ability of mast cells in innate and adaptive immunity have been extensively reported recently. They can function as effector cells that elicit inflammation or as regulatory cells that suppress responses (Sayed, Christy et al. 2008).

1.1.2.1.1. Mast cell activation

Mast cells highly express FcεRI, the high-affinity receptor for IgE. Antigen-specific IgE produced by B cells binds with FcεRI on mast cells to prime them. When primed mast cells encountered their cognate antigen, the antigen-specific IgE on the mast cells interacts with the antigen which results in the activation of mast cells. The activated mast cells secrete three classes of mediators: (1) preformed mediators that are stored in the cells and immediately released through degranulation; (2) newly-synthesized proinflammatory lipid mediators such as prostaglandins and leukotrienes; (3) and cytokines and chemokines such as IL-1, -2, -3, -4, -5, -6, -7, -8, -10, -12, -13, -15, -18, -21, -23, IFN-α,-β,-γ, and TSLP and TNF (Kalesnikoff and Galli 2008). Activated mast cells also increase expression of costimulatory molecules. Upon secretion by mast cells these mediators subsequently activate others cells, such as T cells, B cells, eosinophils, and smooth muscle cells, which induce continuation of the cycle and further release of the mediators. Therefore, IgE plays a key role in the acute reaction to antigens and in the induction of airway inflammation. Allergic asthma patients have high level of FcεRI-bound IgE on mast cells (Andersson, Tufvesson et al. 2011).

Mast cells can also be activated to perform important functions by IgE-independent pathways (Sayed, Christy et al. 2008). Human mast cells express high-affinity IgG receptors FcγRI and low-affinity IgG receptors FcγRII and FcγRIII. Cross-linking of IgG receptors on mast cells through antigen-IgG complexes results in degranulation, which is associated with Type II and III hypersensitivity reactions. Mast cells also can be activated by a variety of stimuli through receptor-mediated pathways such as TLRs or complement receptors and thereby exert extremely versatile functions.

1.1.2.1.2. Mast cell influence on immune responses

Mast cells and DCs are closely co-located in all tissues. Both DCs and mast cells can be activated by the same microbial signals through (e.g.TLRs). Activated mast cells promote DC maturation or suppression and control T cell differentiation pathways through cytokine release and costimulatory molecules expression. For example, histamine, the best-known mast cell mediator, has potent influences on DCs and Th cells, both of which express histamine receptors. Histamine suppresses the production of IL-12 and increases the secretion of IL-10 from DCs, thereby inhibiting production of Th1 (e.g. IFN-γ) and promoting the production of Th2 (e.g. IL-4) cytokine, by T cells (Mazzoni, Siraganian et al. 2006). It was reported that the co-culture of human DCs and activated mast cells in vitro lead to significantly increases of MHC I and II, CD80, CD86, CCR7 expression on DCs, which promote Th2 responses in allergy (Kitawaki, Kadowaki et al. 2006).

1.1.2.1.3. Mast cell and immunosuppression

In addition to being promoters of inflammation, mast cells can have immunosuppressive effects on immune cells. An early study demonstrated that the systemic immunosuppression of contact hypersensitivity responses induced by ultraviolet B irradiation of skin was mast cell-dependent (Hart, Grimbaldeston et al. 1998). The chemokine receptor CXCR4 on mast cells mediated ultraviolet irradiation-induced migration of mast cells from the skin to the draining LNs, which is a crucial step for the immunosuppressive induction (Byrne, Limon-Flores et al. 2008). A study conducted by Lu et al showed that the maintenance of the immunosuppression in a murine skin transplant model was mast cell-dependent and Treg-mediated. Mast cell-deficient mice were unable to maintain skin allografts. Graft tolerance relied on interaction between mast cells and T cells. On the one hand, Treg secrete IL-9, a mast cell growth factor that enhances mast cell recruitment and activation in the allograft but, on the other hand, mast cells produce IL-10 to mediate negative immunomodulatory suppression (Lu, Lind et al. 2006). In addition, proteases (e.g. β-tryptase) released by mast cells can cleave IgE (Rauter, Krauth et al. 2008), which may limit IgE-dependant allergic hypersensitivity reactions such as allergic asthma and food allergies.

1.1.2.2. Basophils

Basophils represent less than 0.5% of blood cells in the blood circulation in the steady state. Being the least common leukocyte, it has received the little recognition other than as the source of intracellular mediators such as histamine and leukotrienes released

in IgE-mediated allergic reaction. Recently it has been reported that basophils are the primary source of IL-4 for initiation of Th2 responses and professional APC to prime naive T cell in allergic inflammation (Min, Brown et al. 2012). Although DCs are sufficient and necessary to mediate asthmatic response to many allergens, in some situations basophils present antigen for induction of Th2 immune response to some allergens (Karasuyama, Mukai et al. 2011).

1.1.2.2.1. Basophils and their features

Basophils are derived from bone marrow precursors, but they are released into the peripheral blood as fully mature cells. The cytokines IL-3 is involved in basophil differentiation. The frequency of basophils significantly increases in Th2-related responses, including allergic inflammation and parasitic infection, suggesting that they may contribute to the induction of Th2 type responses (Gauvreau, Lee et al. 2000; Macfarlane, Kon et al. 2000). Three reports have demonstrated that basophils function as potent antigen presenting cells in draining LNs to prime specific Th2 and IgE responses against allergic proteins and protease released from parasitic helminths. Basophils secrete IL-4 upon encounter with allergens thereby contribute to the initiation of Th2 immune responses (Perrigoue, Saenz et al. 2009; Sokol, Chu et al. 2009; Yoshimoto, Yasuda et al. 2009).

There are some evidences that basophils possess classical features of antigen presenting cells for Th2 immunity. (1) express MHC II molecules and costimulatory molecules such as CD80, CD86, CD40 and CD54; (2) express FcεRI to capture IgE-antigen complexes; (3) endocytose antigens and present peptide-MHC II complexes to

CD4 T cells; and (4) express CD62L to migrate to lymphoid tissues. Uniquely and importantly, basophils secrete cytokines IL-4, IL-13 and TSLP to polarize Th2 response. TSLP inhibits IL-12 production by DCs and promote Th2 cell differentiation (Perrigoue, Saenz et al. 2009; Sokol, Chu et al. 2009; Yoshimoto, Yasuda et al. 2009).

1.1.2.2.2. The role of DCs and basophils in airway infection

DCs have a broader, more potent capacity to induce anti-pathogen immune responses in term of quantity and quality when compared with basophils. First at all, DCs can take up particulate antigens while basophils do not (Sokol, Chu et al. 2009). Airway infections with viruses, bacteria or fungi cause full maturation of DCs, at least partially through toll like receptors (TLRs). Mature DCs highly express MHC class II, costimulatory molecules and homing receptors (e.g. CCR7), while even activated basophils have lower level expression of MHC class II and costimulatory molecules than mature DCs. Thus, as professional antigen presenting cells, DCs possess much greater capacity to present antigen to naïve T cells, and they migrate into the lung-draining LN to stimulate naïve T cells. Mature DCs can secrete the type 1 cytokines IL-12 and IFN-γ to stimulate the expression of transcription factor T-cell-specific T-box transcription factor 21 (T-bet) and thereby induce Th1 cells for resistance to viruses and intracellular bacteria (Huang, Hao et al. 2007). DC can also directly induce cytotoxic T lymphocytes (CTLs) to kill cells infected with these pathogens. DCs prompt the expression of retinoic-acid-related orphan receptor (RORγt) to induce Th17 cells with IL-6, IL-23 and TGF-β for resistance to extracellular bacteria. On the other hand, DCs can stimulate the expression of Foxp3 to induce $CD4^+CD25^+Foxp3^+$ Treg with IL-10 and TGF-β to counter-regulate

adaptive immunity (Reiner 2007; Yamazaki, Bonito et al. 2007; Dumitriu, Dunbar et al. 2009; Reiner 2009). The role of basophils as APCs in airway infection has not been clear. It is reported that during respiratory syncytial virus (RSV) infection, basophils are the primary source of IL-4 which contributes to the Th2 immunopathology induced by the infection (Moore, Newcomb et al. 2009).

1.1.2.2.3. Basophils rather than dendritic cells play a dominant role as APCs in the allergen-induced Th2 responses.

Th2 immunoinflammatory responses play a key role in the initiation, progression and persistence of allergic reactions. Allergens (e.g., house dust mites, grass and tree pollens and cat dander) enter the mucosal system and cross the epithelial surface via active APCs uptake. These APCs process their ingested allergens and present allergen related peptides to naïve Th cells. Allergen-specific activation of naïve Th cells induces their differentiation into the Th2, but not Th1 pathway, characterized by T cell proliferation, Th2 cytokine (IL-4, -5, -9, -10, -13) production, inflammation, eosinophilia and IgE production. These effects cumulatively culminate in the allergic reaction (Cohn and Ray 2000; Busse and Lemanske 2001; Lambrecht and Hammad 2003; Hawrylowicz and O'Garra 2005).

DCs are known as the most efficient APCs for initiation of adaptive immune responses. In allergic reactions, the availability of an early source of IL-4 is critical to driving the Th2-type immune response. However, since DCs do not produce IL-4 (Galli, Tsai et al. 2008), the mechanisms of Th2 responses primed by APCs have not been understood clearly. An accepted old model is that Th2 initiation is driven by DCs that present antigens to Th2 cells together with extrinsic initial provision of IL-4 by other

cells such as mast cells, basophils, eosinophils and NKT cells. Recently it was reported that DCs are not required for the development of Th2 responses to (papain) (Sokol, Chu et al. 2009), antigen-IgE complexes (Yoshimoto, Yasuda et al. 2009) or helminth-derived secreted proteins (Perrigoue, Saenz et al. 2009). Each study identified basophils as the referent professional APCs and IL-4 producers. Basophils can take up soluble antigens, migrate to LNs rapidly and present antigen to Th2 cells (Siracusa, Comeau et al. 2011). However, unlike DCs, they can not process particulate antigens (Sokol, Chu et al. 2009).

Basophils express the high affinity IgE Fc receptor (i.e. FcεRI). After allergens enter body of an allergic patient, the binding of allergen-IgE complexes to FcεRI on the surface of basophils leads to rapid cellular degranulation with secretion of IL-4 and other Th2 mediators. Moreover basophil stimulation with IL-3 plus LPS or peptidoglycan induces the production of IL-4 and IL-13, implying that basophils may also be involved in bacterial infections (Yoshimoto and Nakanishi 2006).

In summary, DCs and basophils possess the following common features that allow them to function as antigen presenting cells: (1) expression of pattern recognition receptors (PRR) (e.g. TLRs) to recognize various conserved molecules from microbes; (2) ability to take up and process engulfed antigens into peptides; (3) up-regulation of MHC class II and costimulatory molecules following cellular activation; (4) ability to migrate to lymphoid tissues to present peptide-MHC II complexes to CD4 T cells; and (5) release of relevant cytokines for Th cell differentiation. However, as APCs, DCs and basophils also have their own distinct features: (1) basophils use different pattern recognition receptors to sense various pathogen-associated signals, but also express FcεRI to capture IgE-antigen complexes; (2) DCs are able to take up both particulate and

soluble antigens, but basophils can only handle soluble ones; (3) basophils can be activated immediately upon encounter of Th2 antigens and thereby release stored mediators; (4) DCs produce cytokines such as IL-12, IL-6 and IL-23, but not IL-4, to favor Th1 and/or Th17 responses. In contrast, basophils secrete IL-4, the key driver of Th2 responses, and other Th2 polarized cytokines including IL-13 and TSLP; and (5) DCs also present peptides to CD8 T cells by the MHC class I pathway and thereby induce CD8 activation (Perrigoue, Saenz et al. 2009; Sokol, Chu et al. 2009; Yoshimoto, Yasuda et al. 2009; Siracusa, Comeau et al. 2011; Min, Brown et al. 2012).

Recent reports present evidence that basophils initiate Th2 responses to allergens and helminthes by acting as professional APCs and IL-4 producers. However, Th2 responses are vastly heterogeneous, such that a large number of studies demonstrated DC-mediated Th2 responses (Hammad, Plantinga et al. 2010; Lambrecht and Hammad 2012), while other studies reported that M2-polarized macrophages, eosinophils and mast cells can also act as APCs to induce Th2 differentiation. Thus it is likely that basophils are not the only APCs to induce Th2 responses to antigens. There would also be potential co-operation between basophils and DCs or other APCs in the initiation and maintenance of Th2 response, alternatively, the different types of APCs may play dominate roles under different situations.

1.1.2.3. T lymphocytes

1.1.2.3.1. T cells subsets

T lymphocytes or T cells consist of functionally and phenotypically distinct populations, such as $CD4^+$ T cells, $CD8^+$ T cells, NKT cells and γδ T cells. There are at least four subsets of $CD4^+$ T cells, including Th1, Th2, Th17 and Treg cells (Zhu, Yamane et al. 2010). Th1, Th2 and Th17 cells exert critical functions to mediate adaptive immunity to various pathogens. Th1 and Th17 cells also play important roles in bacterial and viral infection and autoimmunity (Dardalhon, Korn et al. 2008), whereas Th2 cells are associated with allergic responses (Kim, DeKruyff et al. 2010). Treg cells are essential for the maintenance of self-tolerance and the modulation of immune responses to infections (Lloyd and Hawrylowicz 2009).

Naïve CD4 cells are activated by their cognate antigen presented by antigen presenting (APC) such as DCs to further differentiate into a variety of lineages, as determined by the cytokines in the T cell's microenvironment (Zhu, Yamane et al. 2010). IL-12 and IFN-γ are essential for Th1 cell differentiation, IL-4 is a key cytokine for Th2 cell differentiation, TGF-β and IL-6 together stimulate Th17 cell differentiation. TGF-β, retinoic acid (RA) and IL-2 can induce Treg differentiation. Moreover, master transcription factors distinct for each Th subset control their differentiation programs, including T-bet for Th1 cells, GATA3 for Th2 cells, RORγt for Th17 cells and Foxp3 for Treg cells (Zhu, Yamane et al. 2010).

Th1 cells produce IFN-γ as signature cytokine and exclusively make lymphotoxin, Th2 cells make IL-4, IL-5, IL-13 as their signature cytokines, Th17 cells are characterized by their IL-17 and IL-22 signature cytokines, while Treg cells produce IL-

10 and play critical roles in the maintenance of immune homeostasis by regulating Th1, Th2 and Th17 responses and therefore in prevention of immunological damage.

There exists a crossregulation between Th1 cells and Th2 cells. Th1 cell cytokines suppress Th2 differentiation, while Th2 cytokines suppress IL-12 signalling and thus prevent Th1 cell differentiation. In addition, it has been reported that both Th1 and Th2 signals potently inhibit Th17 cell differentiation.

The Th17 cells and Treg subsets may switch to other Th cell subsets under the incorrect cytokine environment (Zhou, Chong et al. 2009). Th17 cells can convert into Th1 or Th2 cells under IL-12 and IL-4 stimulation, respectively. Treg cells can become Th17 cell under the influence of IL-6 and IL-21.

1.1.2.3.2 T cells in asthma

There are many T cell subsets involved in the immunopathology of asthma. Th2 cells are the most important cells to mediate asthma inflammation. Numerous studies demonstrated that asthma was characterized by Th2 type inflammation (Robinson 2010). Allergens activated airway epithelium through toll-like receptors (TLRs) (Barrett and Austen 2009; Robinson 2010; Holgate 2011). The activated epithelium can secrete the pro-Th2 cytokines IL-25, IL-33 and TLSP, which can directly act on both T cells and non-T cells to promote Th2 cytokine production, including IL-4, IL-5, IL-6, IL-9 and IL-13. Eosinophils also produce some of these cytokines and can be activated by these cytokines, such that eosinophils can play an important role in asthma exacerbations. Mast cells and basophils, and IL-4 and IL-13 induce B cells to isotype switch to IgE production. Mast cells can also interact with airway smooth muscle cells to induce AHR, while the

Th2 cytokine IL-4 can act on epithelium which enhance mucus production.

Asthma induced by the Th2 response is characterized by predominant eosinophil and basophil infiltration of the airway (eosinophilic asthma). Eosinophilic asthma can be efficiently treated with corticosteroids. By contrast, it was reported that Th1 and Th17 cells induce predominantly neutrophilic inflammation (neutrophilic asthma) (Cho, Stanciu et al. 2005; Alcorn, Crowe et al. 2010), which is generally more severe due to the involvement of TNF-α, IFN-γ, and IL-17, and is largely steroid-resistant.

Treg cells can suppress allergen-driven inflammation and maintain airway tolerance (Braga, Quecchia et al. 2011) through release of IL-10 and TGF-β, which controls several aspects of the asthma reaction. Treg cells inhibit allergen-associated T cell activation, and also inhibit eosinophils, basophils and mast cells to restrict allergic inflammation. Their IL-10 efficiently suppresses allergen-specific IgE production, and induces non-inflammatory phenotype IgG4 synthesis.

Th9 and Th22 were recently identified Th cell subsets which produce IL-9 and IL-22, respectively, which can induce tissue inflammation in autoimmune diseases or atopic dermatitis (Jutel and Akdis 2011). However the roles of Th9 and Th22 in allergic asthma remain unclear. CD8 T cells, NKT cells and γδT cells can produce Th2 cytokines but also inhibit Th2 responses under some conditions, such that their roles in asthma need to be clarified (Robinson 2010).

1.1.2.4. Eosinophils

Eosinophils have been considered as protective effector cells for helminth infections (Shin, Lee et al. 2009), but also as an important feature in allergic

inflammation and asthma. The type 2 cytokine IL-5 mediates eosinophil differentiation, survival and expansion, while the chemokines CCL11, CCL24, CCL26 (i.e. eotaxins) attract eosinophils into allergic tissue. Eosinophils produce an array of cytotoxic agents and chemokines. Cytotoxic granule contents include major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN), each of which damage tissue and compromise their functions (Rothenberg and Hogan 2006). High number of eosinophils can be observed in the bronchoalveolar lavage (BAL) of asthmatic people and mice.

Eosinophils develop in the bone marrow under the influence of IL-3, IL-5 and GM-CSF, which play dominant roles in their differentiation and expansion. IL-5 also regulates the migration of eosinophils from the bone marrow into the peripheral blood. A number of chemokines are eosinophil chemoattractants, including CCL3, CCL5 and eotaxin. Eotaxin, which interacts with CC chemokine receptor-3 (CCR3), primarily expressed on eosinophils, is the only chemokine characterized as a specific eosinophil chemoattractant. It functions synergistically with IL-5 to recruit eosinophils into the allergic lung (Pope, Brandt et al. 2001).

Eosinophil adhesion molecules promote the transit of eosinophils across epithelial and endothelial barriers. The interaction of the integrin very late antigen-4 (VLA-4) and its ligands vascular cell adhesion molecule-1 (VCAM-1), P-selectin glycoprotein ligand-1 (PSGL-1) and P-selectin promotes eosinophil recruitment into the lung compartment, while the interactions of integrin α4β7 and its ligand, mucosal addressin cell adhesion molecule (MAdCAM)-1, promotes eosinophil recruitment into the intestinal compartment. Eosinophils express Fc receptors for IgE, IgA and IgG, and cross-linking

of these receptors by allergen co-activate eosinophils. Activated eosinophils secrete cytokines (IL-2, 3, 4, 5), chemokines (eotaxin-1 and RANTES) and lipid mediators (leukotrienes), which promote recruitment of cells, mucus secretion and smooth muscle constriction (Rothenberg and Hogan 2006; Rosenberg, Phipps et al. 2007; Wegmann 2011).

1.1.2.5. Dendritic cells (DC)

1.1.2.5.1. Introduction to the dendritic cells

DCs are the most efficient professional antigen presenting cells (APCs) and have an important role in the control of the adaptive immune responses (Steinman 2012). After antigen is engulfed by APCs, it is digested into its constituent peptides, which bind in the grooves of major histocompatibility complex (MHC) class I and/or MHC class II molecules. The MHC I or II-peptide complex is then transported to the surface of the APC to provide an antigen specific signal to CD8 or CD4 T cells, respectively. APCs also provide costimulatory signals (e.g., CD40, CD80 and 86, CD54) and cytokine signals to activate T cells (Chen, Linsley et al. 1993) although if the T cell expresses the Fas (CD95) molecule on its surface and receives signals from APCs that express Fas ligand, the T cells then undergo apoptosis. Other types of inhibitory T cell:APC interaction also can occur, such as the binding of CD80 expressed on DCs to cytotoxic T-lymphocyte antigen-4 (CTLA-4, or CD152) on T cells (Steinman, Hawiger et al. 2003).

1.1.2.5.2. Subsets of dendritic cells

DCs arise from both of lymphoid and myeloid progenitors and are usually divided into two major subsets, conventional (previously known as myeloid) DCs (cDCs) and plasmacytoid DCs (pDCs). The many $CD11c^{hi}$ DC populations can be classified as cDCs, which can potently stimulate T cell proliferation. $CD11c^{low}$ DC precursors with the appearance of plasma cells have been identified in human blood and are identical to the natural interferon (IFN) α/β–producing cells which secrete high levels of IFN-α/β in response to virus challenge. These plasmacytoid cells can give rise to DCs *in vitro* in the presence of IL-3 or during viral infections (Kadowaki, Antonenko et al. 2000). The corresponding murine type of IFNα/β-producing cell, consists of a population of $Ly6C/Gr\text{-}1^{+}$, $B220^{+}$, $CD11b^{-}$ and $CD11c^{low}$ DC (Asselin-Paturel, Boonstra et al. 2001; Nakano, Yanagita et al. 2001), which show a homogeneous plasmacytoid structure (i.e., a round shape, a smooth surface, and an eccentric nucleus). These plasmacytoid DCs (pDCs) can have immunostimulatory (Shaw, Wang et al.) and also immunoinhibitory properties (Wang, Peters et al. 2006), depending on the stimulus. In addition, $CD103^{+}$ DCs, found in the gut, are found to be immunoinhibitory cells that can promote $Foxp3^{+}$ Treg differentiation via retinoic acid (RA) to maintain oral tolerance (Coombes, Siddiqui et al. 2007; Sun, Hall et al. 2007).

1.1.2.5.3. Dendritic cells and the polarization of the immune response

Immature DCs express low levels of costimulatory and MHC Class II molecules, but possess high levels of phagocytosis function. On encounter of PRL-expressing pathogens these DC then become mature. Mature DCs up-regulate expression of LN-

homing receptor CCR7; binding its ligands, CCL19 and CCL21, secreted by LN stromal cells, recruits those maturing DCs into the draining LNs (Sozzani, Allavena et al. 1998; Alvarez, Vollmann et al. 2008). In the draining LNs, the mature DCs express high levels of costimulatory and antigen-peptide loaded MHC Class II molecules and secrete IL-1, IL-6, IL-12, TNF-α and IFN-γ, and thereby activate cognate naïve T cells with which they interact.

Both $CD4^+$ T-helper cell (Th) and Treg are the central to the orchestration of asthmatic responses. After interaction of DCs and cognate naïve T cells in the draining LNs, the different cytokine predicts to induce different subsets of Th. IL-2, IL-12 and IFN-γ stimulate the expression of transcription factor T-bet (T-cell-specific T-box transcription factor 21) to induce Th1 cells for resistance to viruses, intracellular bacteria and tumors (Huang, Hao et al. 2007); IL-4 and IL-9 stimulate the expression of Gata-3 to induce Th2 cells for resistance to ectoparasites, but they are also associated with asthma; IL-6, IL-23 and TGF-β stimulate the expression of RORγt (retinoic-acid-related orphan receptor) to induce Th17 cells for resistance to extracellular bacteria; and IL-10 and TGF-β stimulate the expression of Foxp3 to induce $CD4^+CD25^+Foxp3^+$ Tregs for counter-regulation of adaptive immunity (Figure 1-1) (Reiner 2007; Yamazaki, Bonito et al. 2007; Dumitriu, Dunbar et al. 2009; Reiner 2009).

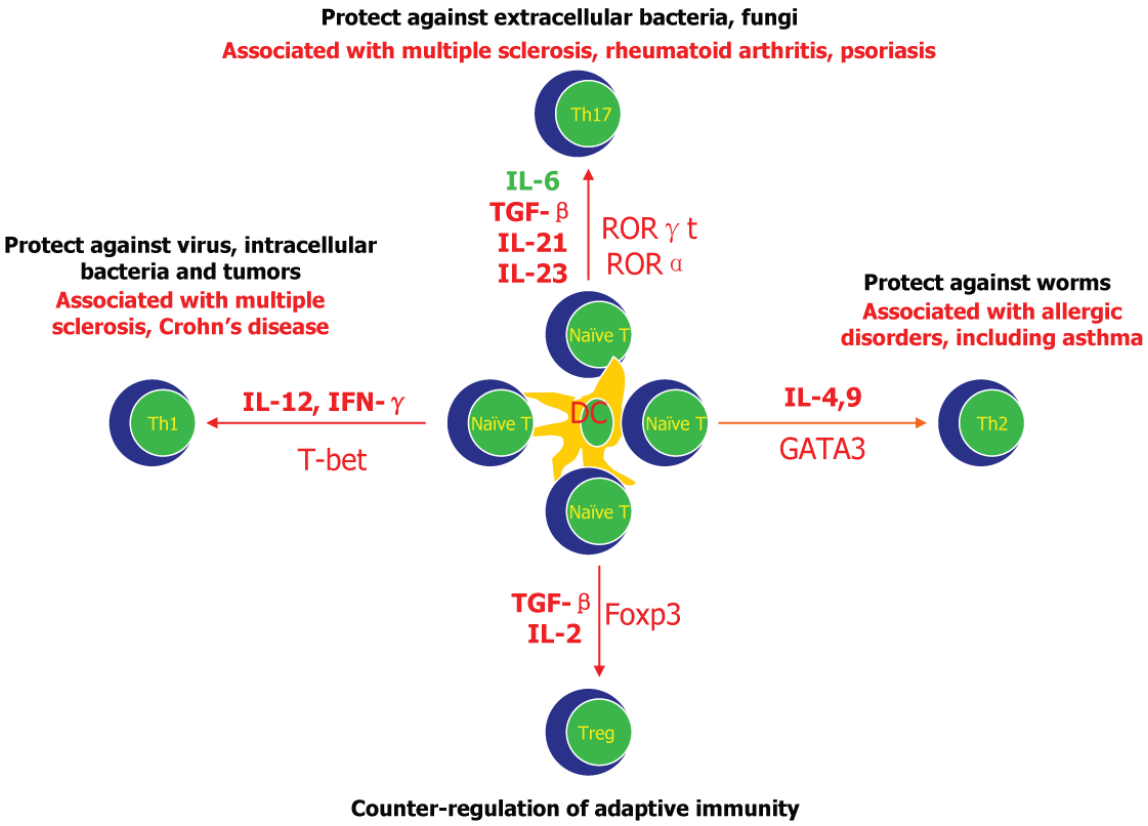

Figure 1-1. The Plasticity of CD4 T cells.

After interaction between antigen-presenting DC and cognate naïve T cells, different cytokines differentially induce CD4 T cell differentiation. For example, IL-4 and 9 induce Th2 cells which protect against parasites were associated with allergic disorders, including asthma. In another hand, TGF-β and IL-2 induce Treg cell which down-regulate adaptive immunity.

1.1.2.5.4. Role of dendritic cells in infection

During viral, bacterial, and parasitic infections, the induction of specific immunity related to these pathogens have been attributed to the relevant subsets of cDCs, including pulmonary interstitial DC, gut $CD11c^+$ DC (Qualls, Tuna et al. 2009), migratory epidermal Langerhans cells (LCs), dermal DCs, as well as resident $CD8^+$ and $CD8^-$ DCs found in the peripheral LNs and the spleen (Villadangos and Schnorrer 2007). Dramatic increases in the number of cDCs have been widely reported in both the infection sites, such as the skin, and the associated draining LNs or the spleen (Martin, Ruiz et al. 2002). In skin infections, dermal DCs can efficiently activate $CD4^+$ T cells within the draining LNs, whereas $CD8^+$ DCs are highly efficient in activating $CD8^+$ T cells and resident $CD8^-$ DCs preferentially activate $CD4^+$ T cells (Dudziak, Kamphorst et al. 2007). Other studies have suggested that $CD8^+$ and $CD8^-$ DCs induce Th1- and Th2-polarized responses, respectively (Maldonado-Lopez, Maliszewski et al. 2001). Th1 cells can also promote $CD8^+$ cytotoxic T lymphocytes (CTL) development for cytolysis of tumors and viruses, and cells infected with intracellular bacteria (Wong and Pamer 2003). Th2 cells are for resistance to parasitic worms (Reiner 2007; Yamazaki, Bonito et al. 2007; Dumitriu, Dunbar et al. 2009; Reiner 2009).

1.2. ASTHMA THERAPEUTICS

There are many effective inhaled and oral drugs for the treatment of asthma. Generally these are classified into two categories: quick-relief medications and long-term control medications. Quick-relief medications are used to treat acute symptoms. They include short-acting beta2-adrenoceptor agonists and anticholinergic medications. Long-

term control medications are often used daily to control asthma inflammations and symptoms thereby prevent acute episodes. They include glucocorticoids (GCs), long-acting beta2-adrenoceptor agonists (bronchodilators), leukotriene antagonists and theophylline. Asthma medications in different categories can be used alone or in combination to maintain control of symptoms and prevent further exacerbation. Anti-IgE therapy is a supplementary treatment and can be considered in patients who have poorly controlled and persistent asthma. Anti-cytokine therapy for asthma is being explored in clinical trails and has succeeded in some studies. In addition, cyclooxygenase is a key mediator of inflammation and cyclooxygenase (COX) antagonists may have the potential to become an attractive medicine for asthma therapeutics.

1.2.1. Steroids vs bronchodilators

Currently, the main drugs used to treat asthma include anti-inflammatory drugs (steroids) that suppress inflammation in the airway and bronchodilators that act by relaxing airway smooth muscle (Bateman, Hurd et al. 2008).

Glucocorticoids are the most effective treatment to control asthma. Inhaled corticosteroids (ICS) have become a major therapy for all asthma patients with long-term symptoms. There are several cellular and molecular mechanisms involved in the anti-inflammatory effects of glucocorticoids (Barnes 2011). Lipid-soluble glucocorticoids are able to easily pass through the cell membrane and bind to glucocorticoid receptors (GRs) in the cytoplasm. Once bound, GRs are activated and quickly translocate to the nucleus where they perform their molecular functions. GRs homodimerize and bind to glucocorticoid response elements within the promoters for target genes such as SLPI

(secretory leukoprotease inhibitor), MKP-1 (MAPK phosphatase-1) and GILZ (glucocorticoid-induced leucine zipper). The DNA-bound GRs also suppress transcription of multiple inflammatory genes by interacting with transcriptional activators such as CBP (cAMP-responsive element-binding protein-binding protein). The inflammatory genes switched off by glucocorticoids include cytokines, chemokines, adhesion molecules and inflammatory enzymes. In addition, glucocorticoids have post-transcriptional effects through destabilizing mRNAs of inflammatory proteins such as TNF-α, IL-6 and GM-CSF and thus reduce expression of these cytokines. For example, glucocorticoids are able to increase the expression of a zinc finger protein, tristetraprolin, which binds to the AU-rich untranslated region of mRNAs of inflammatory proteins, resulting in mRNA destabilization (Barnes 2011).

β2-adrenoceptor agonists are the most effective bronchodilators for asthma. They function as antagonists by reversing smooth muscle contraction in the airway. β2-adrenoceptor agonists bind to β2-adrenergic receptors and increase the formation of intracellular cAMP via coupling of β2-adrenergic receptors to a stimulatory G protein (Gs protein). cAMP activates PKA, which phosphorylates myosin light chain kinase, resulting in the relaxation of airway smooth muscle. Increased cAMP also inhibits the proliferation of airway smooth muscle cells. In addition, interactions between β2-adrenergic receptors and their agonists directly open large conductance Ca^{2+} channels, decreasing intracellular Ca^{2+} level, thereby inhibiting the activation of myosin light chain kinase and eventually relaxing airway smooth muscle cells (Barnes 2011).

There are important interactions between glucocorticoids and β2-adrenoceptor agonists (Newton, Leigh et al. 2010). They are often used in fixed-combination inhalers.

Glucocorticoids increase cell surface expression of the β2-adrenergic receptors, which compensate for the down-regulation of the β2-adrenergic receptors after long-term use of β2-adrenoceptor agonists. Glucocorticoids also enhance the activity of β2-adrenoceptor agonists by promoting the coupling of β2-adrenergic receptors to Gs protein. On the other hand, β2-adrenoceptor agonists increase the anti-inflammatory effects of glucocorticoids by affecting glucocorticoid receptor function. Long-acting β2-adrenoceptor agonists enhance the translocation of glucocorticoid receptor from the cytoplasm to nucleus following activation by glucocorticoids. Therefore, steroids and bronchodilators enhance each other's advantageous effects in asthma therapy.

1.2.2. Anti-IgE therapy

IgE is a crucial mediator in allergic asthma. IgE can be synthesized locally in airway mucosa. Antigen-specific IgE binds to high-affinity receptors (FcεRI) on mast cells, basophils and neutrophils. FcεRI-bound IgE enhances antigen presentation and promotes Th2 responses. When IgE encounters the same antigen again, it cross-links and activates mast cells and basophils, which triggers the degranulation and release of various inflammatory mediators. It has been shown that IgE levels correlate with the occurrence of asthma symptoms, AHR and severe exacerbations.

Because of the pivotal role of IgE in allergic asthma, targeting IgE and blocking its functions may bring significant clinical benefits for asthmatic patients (Kuhl and Hanania 2012). Xolair (Omalizumab) is the only available anti-IgE medicine approved by the FDA. It is a recombinant humanized IgG1 monoclonal antibody given by injection one to two times a month. Xolair attaches to circulating free IgE and prevents it from

binding with FcεRI on mast cells and basophils to block the downstream cascade of asthma responses.

Xolair is an add-on therapy to inhaled corticosteroids (ICS) and long acting β2-adrenoceptor agonists. It is used for patients over 12 years of age with poorly controlled moderate to severe persistent allergic asthma. Xolair's beneficial effects include reduction of free IgE levels in serum, downregulation of FcεRI expression on basophils, mast cells and DCs, and reduction of cell number in eosinophils, T cells and B cells in the airway of patients with allergic asthma (Holgate, Buhl et al. 2009; Holgate, Smith et al. 2009). Xolair has been demonstrated to decrease asthma episodes and has long-term beneficial efficacy in some patients. In addition, clinical data shows the long-term safety of Xolair adminstration. There are several rare, yet severe side-effects reported, including anaphylaxis and malignancy. Future research will need to evaluate the long-term effects of Xolair, particularly in children and elderly people.

1.2.3. Anti-cytokine therapy

The inflammatory processes in asthma are orchestrated by a cytokine network. Asthma is now recognized as a heterogeneous disorder. Th2 responses dominate in mild to moderate allergic asthma, whereas a mixed Th2/Th1 responses with a Th17 constituent involve in severe steroid-resistant asthma (Hansbro, Kaiko et al. 2011). Cytokine profiles produced by innate immune cells and structural cells in the airway are responsible for driving the differentiation of various Th cell subsets and sequentially presenting different pathogenetic features of asthma. IL-1, IL-6 and TGF-β drive Th17 cell development. IL-12 and IFN-γ promote Th1 responses. IL-4, TSLP, IL-25 and IL-33 induce Th2 responses,

which elicit predominately eosinophil, basophil and mast cell inflammation in the airway (allergic or eosinophilic asthma). Patients with eosinophilic asthma are generally sensitive to steroid treatment and have stable mild-to-moderate symptoms. Th1 and Th17 responses in the lung facilitate neutrophilic or neutrophilic with low level eosinophilic inflammation (neutrophilic asthma). Patients with neutrophilic asthma are mainly steroid-resistant and have severe asthma symptoms that involve TNF-α, IFN-γ and IL-17. Patients with both subtypes of asthma can have acute exacerbations triggered by various allergens and infections (Hansbro, Kaiko et al. 2011).

Glucocorticoids, either alone or in combination with long-acting β2-adrenoceptor agonists are the mainstay for asthma therapy, excluding that of steroid-resistant patients. Anti-cytokine therapy may become an alternative tool to treat refractory asthma. To date, there have been many clinical trials employing inhibitors of cytokines. These cytokines include IL-4, IL-5, TNF-α, IL-13, IL-9, GM-CSF, IFN-α, IFN-γ, IL-10 and IL-12 (Desai and Brightling 2009; Hansbro, Kaiko et al. 2011). Other potential cytokines such as IL-25, IL-33, TSLP, IL-17 and IL-27 that involve in severe asthma are being developed as new anti-inflammatory targets for asthma therapy (Hansbro, Kaiko et al. 2011).

Recently Corren *et al* conducted a clinical trial with a monoclonal antibody to IL-4Rα (AMG317) in patients with moderate-to-serve asthma (Corren, Busse et al. 2010). IL-4 and IL-13 are two key cytokines made by Th2 cells and thereby are essential for allergic inflammation. IL-4Rα is a common component of IL-4 and IL-13 receptors. Therefore AMG317 would block both IL-4 and IL-13 pathways. The results demonstrated that AM317 had beneficial effects on asthma symptoms and lung function in patients with uncontrolled and severe asthma, but not in all groups of patients.

Anti-IL-5 monoclonal antibody (mepolizumab) was evaluated in several clinical trials (Flood-Page, Swenson et al. 2007). The results showed that anti-IL-5 treatment was beneficial to patients with elevated numbers of eosinophils and severe exacerbations that are resistant to steroid treatment.

Anti-TNF-α monoclonal antibody (Infliximab) was tested in humans and shown to improve some lung functions and to decrease exacerbations in moderate asthma (Erin, Leaker et al. 2006; Morjaria, Chauhan et al. 2008). A soluble TNFα receptor-IgG_1Fc fusion protein (etanercept) that neutralize TNF-α was evaluated in a clinical trial with some promising results, including decreased airway histamine levels and AHR and improved lung function in refractory asthma (Berry, Hargadon et al. 2006).

Although various anti-cytokine clinical trials are being explored for asthma therapy, only a few of them have had success. This may reflect various treatment regimes and the patients selected in the different studies, but since many cytokines are involved in asthma, combination therapies that inhibit multiple cytokines may prove to be most effective.

1.2.4. Cyclooxygenase (COX) antagonists

The cyclooxygenase is the enzyme that promotes the conversion of arachidonic acid (AA) into prostaglandins (PGs), the key mediators of inflammation. PGs released by inflammatory cells amplify smooth muscle contraction and airway inflammation. Because of its crucial role in PG production, COX has the potential to be a target for asthma therapy (Malhotra, Deshmukh et al. 2012).

The COX enzyme exists as two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most normal tissues and has a role in regulating basal physiological processes. For example, PG regulated by COX-1 in the stomach lining prevents the stomach mucosa from being corroded by its own acid. COX-2 is normally expressed at low levels and is highly induced by pro-inflammatory cytokines such as IL-1 and TNF-α, which indicates that COX-2 may play a role in the pathophysiology of inflammatory conditions such as asthma. The discovery of COX-2 raised the idea that selective inhibition of COX-2 would result in only anti-inflammatory effects without interfering with the protective physiological processes of PGs.

To date, COX inhibitors, such as the non-steroidal anti-inflammatory drugs (NSAID) for pain and arthritis, have been extensively studied and widely used in the clinic, but data on COX inhibitors for respiratory disease is limited and conflicting.

Aspirin and other NSAIDS inhibit both COX-1 and COX-2. It is generally accepted that the beneficial anti-inflammatory effects of NSAIDS are due to inhibition of COX-2 and the harmful effects of NSAIDS are due to inhibition of COX-1. Aspirin can cause asthma to worsen in 3-5% of asthmatic people. Research has shown that when aspirin blocks PG formation in these asthma-sensitive people, the arachidonic acid cascade shifts entirely to leukotrienes, overproduction of which leads to the severe allergy-like effects. A recent study conducted by Ishiura *et al* demonstratrd that a potent, specific COX-2 inhibitor (etodolac) attenuated airway cough reflex sensitivity in asthma patients (Ishiura, Fujimura et al. 2009).

Studies using knockout (KO) mice showed that COX-1- and COX-2-KO mice had normal basal lung function and lung histology (Gavett, Madison et al. 1999).

Following allergen challenge, both COX-1- and COX-2-KO mice had more inflammatory cells and mediators in the lung than wild-type mice. Both COX-1- and COX-2-KO mice had increased brochoconstriction compared with wide-type mice (Zeldin, Wohlford-Lenane et al. 2001). There are several studies demonstrating that COX products reduce inflammation and AHR and that these effects are regulated through inhibition of Th2 responses. Therefore, although the current concepts favor COX inhibitors as potential drugs for airway diseases, these studies suggest that COX enzyme itself may serve as a drug for asthma. More research is required to clarify the mechanisms of action of COX metabolites.

1.3. TOLERANCE IN ALLERGIC DISEASE

There is a fine balance between immunity and tolerance in the stable state. In general, stimulatory DCs (e.g. mature $CD11c^{hi}$cDC) and Teff cells (e.g. Th1, Th2, Th17) mediate antigen-specific immunity, whereas, tolerogenic DCs (e.g. pDC, intestinal $CD103^{+}$DC, IL-10-secreting DC) and regulatory lymphocytes (e.g. Treg cells, $CD8^{+}$ T regulatory cells, Breg cells) mediate antigen specific tolerance. The imbalance between immunity and tolerance results in allergic decease, autoimmunity or cancer (Maldonado, von Andrian et al. 2010).

1.3.1. Regulatory lymphocytes

1.3.1.1. $CD4^+CD25^+$ T regulatory cells

$CD4^+CD25^+$ Tregs are characterized as having a powerful inhibitory capacity, consistent with their role in maintaining immunological self-tolerance in the periphery. Tregs not only control the development of autoimmune diseases, but also regulate or suppress allergy, transplant rejection, tumor immunity and responses to microbes.

1.3.1.1.1. The biology of $CD4^+CD25^+$ T regulatory cells

The IL-2 receptor α-chain (CD25) has been considered an activated T cell marker by immunologists for decades. In 1995, Sakaguchi published the seminal discovery that one subset of $CD4^+$ T cells very strongly expressed CD25 in naïve mice and these $CD4^+CD25^+$ T cells potently down-regulated immune responses to self and non-self antigens (Sakaguchi, Sakaguchi et al. 1995). Subsequently, numerous laboratories confirmed that $CD4^+CD25^+$ Treg cell expressed the forkhead box protein 3 (Foxp3). So far, Foxp3 has been found as the most important marker for Tregs (Randolph and Fathman 2006; Yamazaki, Bonito et al. 2007).

There are at least two main types of $Foxp3^+CD25^+CD4^+$Treg: natural Treg (nTreg), naturally occurring from thymus, and induced Treg (iTreg), derived from $Foxp3^-CD25^-CD4^+$ T cells in the periphery by stimulation with transforming growth factor (TGF)-β (Bilate and Lafaille 2012). The iTreg are further comprised of IL-10-producing regulatory type 1 T cells (Tr1), TGF-β producing T helper cells (Th3), and inducible $Foxp3^+$ T cells. At present, there are no markers to distinguish nTreg and iTreg. Both

types are $Foxp3^+$ and $CD4^+$ and the majority are $CD25^+$. Treg represent about 5% of peripheral blood $CD4^+$ T cells in normal rodents and humans. Treg cells can directly suppress responder $Foxp3^-$ T cells via the following mechanisms: (1) Treg cells may secrete suppressor cytokines (IL-10, TGF-β) to directly inhibit the function of responder T cells and myeloid cells; (2) Treg cells highly express CD25 to compete with effector T cells for IL-2. The effector cells undergo apoptosis without sufficient stimulation of IL-2 (Pandiyan, Zheng et al. 2007); and (3) some Treg cells can express granzyme A, B and perforin to kill target effector cells (Grossman, Verbsky et al. 2004; Gondek, Lu et al. 2005). Treg cells can also indirectly suppress responder $Foxp3^-$ T cells through suppression of antigen-presenting cell (APC) (Randolph and Fathman 2006).

Other markers which are thought to be associated with regulatory functions include inducible costimulatory molecule (ICOS), programmed cell death-1 (PD-1), glucocorticoid-induced TNF-receptor-related protein (GITR), lymphocyte activation gene 3 (LAG3) and CTLA-4 (Ohkura and Sakaguchi 2011). Treg cells substantially express CTLA-4 on their surface (Marta, Xuguang et al. 2008), which downregulates the costimulatory molecules (CD80 and CD86) on the APC (Wing, Onishi et al. 2008). LAG-3 on Treg cells interacts with the MHC class II molecule on DCs, resulting in an inhibitory signal that suppresses DC maturation (Liang, Workman et al. 2008). In general, immature DCs expressing low amounts of costimulatory molecules are immunoinhibitory (Perrot, Blanchard et al. 2007). It will be essential to understand how to control antigen-specific Treg so that these can specifically suppress a given clinical problem like autoimmunity, allergy and transplant rejection by Treg-based immune therapy, without blocking critical immune responses to tumor and microbes.

1.3.1.1.2. Therapeutic applications for $CD4^+CD25^+$ T regulatory cells

1.3.1.1.2.1. Preclinical evidence to support Treg cell adoptive therapy in asthma and allergy

Numerous laboratories have noted the importance of Treg cells in asthma, and suggested that asthma is due to an imbalance between allergen-specific Treg cells and Th2 cells (Stock, DeKruyff et al. 2006). A number of recent studies indicate that Treg cells play an important role in controlling allergic diseases and asthma that are caused by exaggerated Th2 -biased immune responses (Wing and Sakaguchi 2006). Strickland et al demonstrated that prevention of the aeroallergen-induced airway mucosal T cell activation and resultant airways hyperresponsiveness (AHR) in sensitized rats was mediated by $CD4^+CD25^+Foxp3^+LAG3^+$ CTLA-$4^+CD45RC^+$ T cells which appeared in the airway mucosa and regional LNs, and inhibited subsequent Th2-mediated upregulation of airway mucosal DCs function (Strickland, Stumbles et al. 2006). Lewkowich et al reported that depletion of Treg cells enhanced HDM-specific IgE synthesis and Th2 cytokine (IL-4, IL-5, IL-10 and IL-13) production (Lewkowich, Herman et al. 2005). Evidence from preclinical models demonstrates a central role for Tregs in controlling allergic disease. Adoptive transfer of Treg cells represents a potential therapeutic approach.

1.3.1.1.2.2. Clinical trials of adoptively transferred Treg cells and Treg cell large-scale manufacturing

When purifying Treg cells, it is difficult to avoid contamination with activated $CD4^+$ T cells, because, as an intracellular molecule, Foxp3 can not be used as a marker to purify Treg cells and CD25 is also expressed on activated T cells. Additionally, like conventional T cells (Tconv), Treg cells need T cell receptor (TCR) triggering and costimulation to become fully activated.

Large-scale ex vivo generation of Treg cells has been reported. Briefly, Treg cells are purified from adult peripheral blood apheresis preparations or umbilical cord blood. For peripheral blood apheresis, $CD4^+$ T cells are negatively selected by depletion of CD8, CD14, and CD19 with either magnetic beads or flow cytometry techniques (Godfrey, Ge et al. 2004). For umbilical cord blood, the negative selection of $CD4^+$ T cells is not required (Godfrey, Spoden et al. 2005), while $CD4^+CD25^{hi}$ cells are positively selected using sub-saturating concentration of CD25 antibody, after which the purified Treg cells are expanded by activating the cells with CD3 and CD28 mAb-coated microbeads. Exogenous IL-2, rapamycin and an irradiated feeder cell layer are also added to the cultures (Godfrey, Ge et al. 2004; Battaglia, Stabilini et al. 2005; Godfrey, Spoden et al. 2005; Hippen, Merkel et al. 2011). Recently, Wang J et al found that all-trans-retinoic acid (ATRA) and TGF-β consistently induces de novo generation of $CD4^+Foxp3^+$ Treg cells with potent and constant suppressive ability from $CD4^+CD25^-CD45RA^+$ naive T cells in adult human peripheral blood (Wang, Huizinga et al. 2009). Such *de novo* generation of Treg cells from non-Treg cells represents a promising strategy to obtain sufficient Treg cells for adoptive immunotherapy.

Although adoptive Treg cell therapy is a promising approach for autoimmune and allergic diseases, we still have a long way to go to cure these diseases. The real challenges are to identify Treg cell-specific surface markers to distinguish Treg cells from active T effector cells and to expand antigen-specific, highly-purified active Treg cells.

1.3.1.2. $CD8^+$ T regulatory cells

$CD4^+$ $Foxp3^+$ Treg cells have been extensively studied over the past ten years and shown to play critical roles in preventing inflammatory damage in various disease models. Although $CD8^+$ Tregs were shown in early studies to suppress $CD4^+$ T cells (Noble, Zhao et al. 1998), their exact function and mechanisms of action were mostly unknown. Recent evidence has revealed that $CD8^+$ Treg can play an important role in maintaining self-tolerance (Kim and Cantor 2011). The regulation of self-tolerance was mediated by Qa-1-restricted $CD8^+$ T regulatory cells. Qa-1 is a non-classical MHC class I molecule that is expressed mainly on activated T cells, B cells and DCs. Qa-1 interacts with two distinct receptors that have opposing functions. Qa-1 engages with the TCR of $CD8^+$ Treg, which results in the activation and proliferation of $CD8^+$ Treg, but Qa-1 engages with CD94/NKG2A receptors, which results in the inhibition of $CD8^+$ Treg. Kim et al generated Qa-1-mutant mice (Qa-1 D227K) in which the binding of Qa-1 to TCR on $CD8^+$ Treg was interrupted but the interaction between Qa-1 and the Inhibitory NKG2A receptor on $CD8^+$ Treg was intact (Kim, Verbinnen et al. 2010). Therefore these Qa-1-mutant mice lack $CD8^+$ Treg activity. At about six months of age, the Qa-1-mutant mice develop a severe lupus-like autoimmune disorder characterized by generation of

autoantibodies to multiple tissues (Kim, Verbinnen et al. 2010). Follicular T helper cells (T_{FH}) are a subset of Th cells (~5% CD4 cells) that control antigen-specific B cell immunity (Crotty 2011). T_{FH} highly express Qa-1 in the normal state and are the preferential target of Qa-1-restricted $CD8^+$ Treg cells. Since Qa-1-restricted $CD8^+$ Treg cells from Qa-1 mutant mice are unable to be activated through Qa-1-and TCR receptor interaction, the lack of suppressive effects of Qa-1-restricted $CD8^+$ Treg cell on T_{FH} leads to production of autoantibodies.

Qa-1-restricted $CD8^+$ Treg cells can be identified by a triad of surface markers: CD44, CD122 and Ly49 (Kim, Wang et al. 2011). Ly49 is a MHC class I inhibitory receptor. $Ly49^+CD8$ cells effectively inhibit $CD4^+$ T cells from wild-type, but not from Qa-1 mutant, mice indicating that $Ly49^+$ $CD8^+$ T cells possess Qa-1-dependent inhibitory activity. However $Ly49^-$ $CD8^+$ T cells do not inhibit CD4 responses (Kim, Wang et al. 2011). $CD44^+CD122^+Ly49^+$ $CD8^+$ T cells comprise 3-5% of all $CD8^+$ T cells.

The suppressive activity exerted by Qa-1-restricted $CD8^+$ Treg cells is mediated by perforin, which results in elimination of target T_{FH} cells (Kim, Verbinnen et al. 2010). Such elimination of target T_{FH} cells, which may include self-reactive $CD4^+$ T cell clones, may represent a very efficient suppressive mechanism compared with induction of anergy. In addition, Qa-1-restricted $CD8^+$ T reg cells are dependant on IL-15 for development and activity (Kim, Verbinnen et al. 2010).

Further studies demonstrated that T_{FH} cells from Qa-1 mutant mice but not from wild-type mice promote potent antibody responses in syngeneic mice with a complete supplement of $CD4^+$ Treg cells, indicating that this immune response was controlled via host Qa-1-restricted $CD8^+$ Treg cell-dependant suppression on donor T_{FH} cells (Kim,

Verbinnen et al. 2010). Therefore, Qa-1-restricted $CD8^+$ Treg cells are essential to maintaining self-tolerance and preventing autoimmune.

1.3.1.3. Regulatory B cells

B cells have been mainly considered to positively regulate immune responses through their production of antibodies and by acting as APCs to promote T cell activation. However, recent findings revealed that B cells also include a regulatory subset (regulatory B cell, Breg) that can suppress immune responses in both mice and humans (Mauri and Bosma 2012). The key trait of Breg is production of IL-10, which suppresses proinflammatory cytokine generation and facilitates Treg differentiation. Emerging data have demonstrated that IL-10-producing Bregs inhibit excessive inflammatory responses that are present in autoimmune diseases or that can arise during unresolved infections. It is reported that mice lacking endogenous IL-10-producing Bregs develop exacerbated disease and present with an increased frequency of Th1/Th17 cells and reduced numbers of Tregs (Carter, Vasconcellos et al. 2011).

Since Bregs have much diversity in phenotype and no identified master regulator transcription factor, there has been no unique marker or set of markers to identify this population. It is generally accepted that Bregs may have a common progenitor: transitional 2-marginal zone precursor B cells (T2-MZP B cells) that are $CD19^+Cd21^{hi}$ $CD23^{hi}$ $CD24^{hi}$ IgD^{hi} IgM^{hi} $CD1d^{hi}$. T2-MZP B cells are immature B cells and able to differentiate into various phenotypes of Bregs after exposure to different environmental triggers.

A number of molecules are involved in Breg cell function and differentiation. Toll-like receptors (TLRs) are a family of pattern recognition receptor (PRR) that recognize structurally-conserved molecules derived from pathogens. TLR engagement on DCs drives proinflammatory responses, but TLR engagement on B cells initiates a negative-regulatory effect that restrains excessive inflammatory responses. It is reported that the stimulation of marginal zone (MZ) B cells with TLR2 and TLR4 agonists induces IL-10 and IL-6 production (Lampropoulou, Hoehlig et al. 2008).

Interaction between CD40 on B cells and CD40L (CD154) on activated T cells generally induces B cell maturation and antibody production, but prolonged strong stimulation via CD40 has a suppressive effect on antibody production (Miyashita, McIlraith et al. 1997). Several studies have showed that CD40-CD40L interactions are an essential signal for Breg activation. In addition, the IL-10 induced by TLR engagement on marginal zone (MZ) B cells is further increased after CD40 engagement. These results suggest that the optimal production of IL-10 takes place when B cells receive signals via both TLR and CD40.

In addition to TLR and CD40 stimulation, several studies have shown that the activation of Bregs also requires B cell receptor (BCR) signalling or antigen recognition by Bregs (Yanaba, Bouaziz et al. 2008), such that a two-step pathway for Breg development has been proposed. In step 1, common progenitor T2-MZP Bregs are initially activated by TLR stimulation and release the first wave of IL-10. In step 2, as the inflammatory response ensues and intensifies, Bregs are further activated via CD40 and BCR pathways and release an optimal amount of IL-10. The release of IL-10 is the main suppressive mechanism for Bregs (Mauri and Bosma 2012). IL-10 production by Bregs

results in the suppression of Th1, Th17, and cytotoxic $CD8^+$ T cells and the differentiation of Tregs. Several studies have demonstrated that IL-10 produced by Bregs is pivotal for the differentiation and maintenance of the pool of $Foxp3^+$ Tregs (Sun, Flach et al. 2008; Sun, Czerkinsky et al. 2012) and Tr1 cells (Blair, Chavez-Rueda et al. 2009). It was also reported that Bregs induced the proliferation of Tregs in an IL-10-independent manner (Ray, Basu et al. 2012). In addition, activated Bregs can express Fas ligand (FasL) to mediate apoptosis of the target T cells and secrete TGF-β to induce immune suppression (Mauri and Bosma 2012).

1.3.2. Regulatory DC

Besides being inducers of immune reactivity, DCs are also critical regulators in the induction and maintenance of immune tolerance. Regulatory DCs comprise a heterogenous population that shows phenotypical and functional plasticity. Regulatory DCs induce immune tolerance by a variety of mechanisms, including the generation of anergy, deletion of effector T cells, stimulation of Treg cells and production of inhibitory molecules such as IL-10 or IDO.

1.3.2.1. $CD103^+$ DC

Numerous research groups found that a functionally specialized population of intestinal $CD103^+$ DC induces $Foxp3^+$ Tregs via a TGF-β- and retinoic acid (RA)-dependent mechanism. $CD103^+$ DCs used not only endogenous TGF-β to induce $Foxp3^+$ Treg, but also RA, which acts as a co-factor with TGF-β to induce $Foxp3^+$ Treg

(Coombes, Siddiqui et al. 2007; Sun, Hall et al. 2007; McDole, Wheeler et al. 2012). RA alone cannot induce Foxp3$^+$ Treg from Foxp3$^-$ cells, but the Treg induced by TGF-β plus RA are more stable and more efficient than the Treg induced by TGF-β alone (Sun, Hall et al. 2007). It is well recognized that intestinal CD103$^+$ DCs present intestinal antigens to induce Foxp3$^+$ Treg maintains oral tolerance (Scott, Aumeunier et al. 2011).

The function of pulmonary CD103$^+$ DCs are still controversial. Furuhashi K et al reported that CD103$^+$ DCs predominantly induce Th1, Th17 immune responses, while CD11b^{high} DCs provoke Th2 responses under steady state conditions in the lung, whereas Nakano H et al reported that pulmonary CD103$^+$ DCs prime Th2 responses to inhaled allergens (Furuhashi, Suda et al. 2012; Nakano, Free et al. 2012). More work is required to determine the exact function of CD103$^+$ DC in the lung, but current evidence suggests that, unlike their intestinal counterparts, they can promote Teff cell responses.

1.3.2.2. CD8$^+$ DEC-205$^+$ DC

Conventional DCs can be divided into two major subsets in the mouse spleen. One is CD8$^+$ DEC-205$^+$ and the other is CD8$^-$ DCIR-2$^+$ (dendritic cell inhibitory receptor-2) (Dudziak, Kamphorst et al. 2007). CD8$^+$ DEC-205$^+$ DCs can induce Foxp3$^+$ Treg from Foxp3$^-$ DO11.10 RAG-2 deficient CD4$^+$ T cells with antigen but without any exogenous source of TGF-β (Yamazaki, Dudziak et al. 2008). The induction of Foxp3$^+$ Treg by CD8$^+$ DEC-205$^+$ DCs was blocked by neutralizing anti-TGF-β mAb, indicating an endogenous source of TGF-β. In contrast, CD8$^-$ DCIR-2$^+$ DCs from the spleen require TGF-β as well as antigen in the culture to induce Foxp3$^+$ Treg in vitro. The induced Foxp3$^+$ Treg in these experiments showed suppressive activity *in vitro*. It is reported that

$CD8^+$ splenic DCs preferentially express Programmed death 1 ligand (PDL-1) and that this is essential to induce $Foxp3^+$ Treg in the presence of exogenous TGF-β (Wang, Pino-Lagos et al. 2008). $CD8^+$ $DEC\text{-}205^+$ DCs might contribute to systemic tolerance in the periphery by inducing tissue-antigen specific $Foxp3^+$ Treg. In order to investigate the importance of these two DC subsets in inducing $Foxp3^+$ Treg *in vivo*, OVA was engineered into anti-DEC-205 mAb (DEC-OVA) or anti-DCIR-2 33D1 mAb (33D1-OVA). *In vivo* injection of these chimera Abs delivers antigen to each DC subset selectively. The results showed that antigen targeting via DEC-205 led to the induction of $Foxp3^+$ Treg from $Foxp3^-$ precursors, whereas antigen targeting via 33D1 mAb expanded natural $Foxp3^+$ Treg *in vivo*(Yamazaki, Dudziak et al. 2008). These data suggest that $CD8^+$ $DEC\text{-}205^+$ DCs and $CD8^-$ $DCIR\text{-}2^+$ DCs may have different roles in inducing $Foxp3^+$ Treg from $Foxp3^-$ precursors and maintaining natural $Foxp3^+$ Treg in the periphery.

The functions of $CD8\alpha^+$ and $CD8\alpha^-$ DCs were also explored in our previous study (Gordon, Li et al. 2005). We reported that $CD8\alpha^+$, but not $CD8\alpha^-$ DCs from the spleens of normal mice used multiple mechanisms to inhibit the Th2 responses of cells from OVA induced asthmatic mice. This inhibition could largely be reversed by anti-IL-10 or anti-TGF-β Ab treatment. In addition, loss of direct DC-T cell contact also reduced inhibition, although to a lesser extent. Adoptive transfer of OVA-pulsed $CD8\alpha^+$, but not $CD8\alpha^-$ DCs was able to reverse AHR, Th2, and eosinophil responses in mice with well-established asthma.

1.3.2.3. Plasmacytoid DC

The conventional DCs (cDC) cells enter LNs through the afferent lymphatics under the influence of CCR7 binding to its ligands, CCL19 and CCL21, which are secreted by LN stromal cells. In contrast, pDC enter LNs through high endothelial venules (HEVs) via utilization of L-selectin (CD62L) and E-selectin (CD62E), CXCR3 play a critical role in the pDC migration (Randolph, Ochando et al. 2008).

The pDCs produce large amounts of type I interferons (IFNs) following Toll-like receptor (TLR) recognition of nucleic acids. Numerous experiments have demonstrated that pDCs play an important role in the induction of tolerance within the airway mucosal system (Castellaneta, Abe et al. 2004; Goubier, Dubois et al. 2008). In contrast to cDCs, pDCs present the antigen in an immunosuppressive manner to $CD4^+$ T cells (de Heer, Hammad et al. 2004). That pDCs stimulate the induction of Treg cells has been found in both *in vitro* and *in vivo* studies, possibly in an ICOSL-dependent manner (Ito, Yang et al. 2007). The induced Treg cells also can give feedback to pDC to inhibit Th2 response by inducing the production of the tryptophan catabolizing enzyme indoleamine 2, 3-dioxygenase (IDO) by the pDCs. Passive transfer of PD-L1-deficient ($CD274^{-/-}$) pDCs failed to transfer this regulatory effect in allergic airway inflammation, indicating that pDC PD-L1 has a prominent role in dampening lung immune responses (Kool, van Nimwegen et al. 2009).

Many laboratories have reported that pDCs mediate the induction of tolerance and the differentiation of Tregs in the lung. The deletion of pDCs turns inhalation of harmless antigen from a tolerance to a full immune response (de Heer, Hammad et al. 2004; Lambrecht and Hammad 2012).

1.3.2.4. The function of IL-10-treated DC

IL-10 has been identified as a key immunomodulatory cytokine that is capable of mediating immunosuppressive effects in the immune systems. It has long been implicated in the induction and maintenance of T cell tolerance (Moore, de Waal Malefyt et al. 2001; Ye, Huang et al. 2007). It reduces expression of MHC class II, ICAM-1, CD80 and CD86 on DCs, and this in effect prevents DCs from activating naïve T cells into Teff. IL-10 also acts directly on $CD4^+$ T cells to decrease their cytokine production and proliferation. As suggested above, antigen presentation by such 'immature' DC results in the induction of T cell anergy (Taga, Mostowski et al. 1993) or upregulation of Treg cell functions, leading to suppression of antigen-specific T cell responses (Steinbrink, Graulich et al. 2002). Many studies have demonstrated that IL-10-treated tolerogenic DCs (DC10) have powerful tolerogenic function in both mouse models and humans (*ex vivo*) (Steinbrink, Wolfl et al. 1997; Rutella, Danese et al. 2006; Koya, Matsuda et al. 2007). Administration of allergen-presenting DC10 to 'asthmatic' mice restores their AHR, eosinophilia and Th2 responses to near normal levels (Shigeharu, Naomi et al. 2008; Nayyar, Dawicki et al. 2012).

In vitro, culture of bone marrow cells in the presence of IL-10 induces the differentiation of a distinct subset of DCs that express CD45RB (DC10). These DC10 display an immature-like phenotype and secrete elevated levels of IL-10 after activation. The observation that DC10 secrete IL-10 may be linked to the stability of their immature-like phenotype. As noted, IL-10 inhibits MHC class II and costimulatory molecules on APC, and this is thought to account for their reduced ability to productively present

protein antigens to T cells. Immature DCs induce T cell anergy or Tregs, whereas mature DCs are potent inducers of effector T cells (Steinman, Hawiger et al. 2003).

Both mouse and human IL-10-treated tolerogenic DCs have been used as powerful tools to abrogate asthmatic responses (Steinbrink, Wolfl et al. 1997; Bellinghausen, Brand et al. 2001; Muller, Muller et al. 2002; Wakkach, Fournier et al. 2003; Bellinghausen, Konig et al. 2006; Koya, Matsuda et al. 2007; Perona-Wright, Anderton et al. 2007; Lau, Biester et al. 2008). Numerous laboratories, including ours (Li, Yang et al. 2010; Lu, Dawicki et al. 2011; Nayyar, Dawicki et al. 2012), have reported that administration of allergen-presenting bone marrow-derived DC10 to 'asthmatic' mice powerfully dampens their AHR, eosinophilia and Th2 responses. However, the mechanisms regulating this asthma tolerization by DC10 remain largely unknown. We previously reported that DC10 up-regulated the activity of $CD4^+CD25^+$ Treg (Li, Yang et al. 2010; Lu, Dawicki et al. 2011) and reversed the asthmatic Th2 phenotype via IDO (Nayyar, Dawicki et al. 2012). In this study, we further explored the differentiation of Treg cells in induction of tolerance by DC10.

1.3.3. The interaction between DC and $CD4^+CD25^+$ T regulatory cells

A large number of studies have reported the various mechanisms by which Treg can be induced. Overall, APCs, particularly DCs, are central to orchestration of the induction of Treg cells. Here, the role of DCs in inducing antigen-specific $Foxp3^+$ Treg in the experimental settings is discussed.

1.3.3.1. DCs can expand antigen-specific natural Treg

Antigen-presenting DCs can stimulate the natural Treg cell proliferation. This nTreg expansion is partly dependent on CD80/CD86 on the DCs and IL-2. IL-2 is an imperative factor for the survival and homeostasis of Treg *in vivo*. Furthermore, the expanded Treg suppress graft versus host disease (GVHD) in an alloantigen-specific manner. Therefore, DCs are important APCs to expand antigen-specific natural Treg (Sayuri, Kayo et al. 2006).

1.3.3.2. DCs can induce Foxp3$^+$ Treg from Foxp3$^-$ precursors in the presence of TGF-β and IL-10.

Numerous in vitro and in vivo studies have shown that co-delivery of TCR signaling, suboptimal costimulatory signals and immunosuppressive cytokines (IL-10 and TGF-β) facilitates the differentiation of Foxp3$^+$Treg cells from Foxp3$^-$ precursor cells (Josefowicz, Lu et al. 2012).

It has been reported that Foxp3$^+$Treg cells can differentiate from CD25$^-$CD4$^+$ T cells under the influence of anti-CD3 and -CD28 antibodies and TGF-β (Chen, Jin et al. 2003). DCs along with TGF-β can efficiently induce Foxp3$^+$ Treg from CD25$^-$CD4$^+$ T cells, and these Treg induced have suppressive activity (Luo, Tarbell et al. 2007). One study by Yamazaki et al. has showed that both immature and mature populations of DCs induce antigen-specific Foxp3$^+$Tregs from Foxp3$^-$ precursors with TGF-β. Using endogenous TGF-β, spleen DCs are 100-fold more potent than DC-depleted APCs for the induction of Tregs and require 10-fold lower doses of peptide antigen. IL-2 is essential,

and can be provided endogenously by T cells stimulated by DCs, but not by other APCs. The induced Foxp3^{+}Tregs exert suppression in vitro and block tumor immunity in vivo. These results indicate that DCs are more efficient in inducing Foxp3^{+} Treg from Foxp3^{-} precursors than other APCs. Moreover, the induced Foxp3^{+} Tregs suppress immune responses in an antigen-specific manner (Yamazaki, Bonito et al. 2007).

1.3.3.3. TGF-β- or IL-10-treated bone marrow-derived DCs induce Foxp3^{+} Treg from Foxp3^{-} precursors.

TGF-β treated tolerogenic DCs also have been reported to reverse or prevent asthmatic responses (Kosiewicz and Alard 2004; Zhang-Hoover, Finn et al. 2005). Many studies have reported that DC10 have powerful tolerogenic functions in both mouse models and humans (Steinbrink, Wolfl et al. 1997; Bellinghausen, Brand et al. 2001; Muller, Muller et al. 2002; Wakkach, Fournier et al. 2003; Bellinghausen, Konig et al. 2006; Koya, Matsuda et al. 2007; Perona-Wright, Anderton et al. 2007; Lau, Biester et al. 2008). Increasing evidence shows that DC10 can up-regulate the function and frequency of Treg cells (Bellinghausen, Konig et al. 2006; Li, Yang et al. 2010; Lu, Dawicki et al. 2011). DC10 express low levels of costimulatory molecules (CD40, CD80, CD86) and MHC II, but secrete inhibitory cytokines (IL-10) and express inhibitory receptors or molecules [Ig-like transcript-2 (ILT2)]. Thus DC10 have a potential capacity to induce Treg cell. Our lab have reported that activated CD4^{+}CD25^{+}Foxp3^{+} Treg cells are induced by co-culture of specific allergen-presenting DC10 from atopic patients with autologous peripheral blood Th2 phenotype cells (Li, Yang et al. 2010).

CHAPTER 2: HYPOTHESIS AND OBJECTIVES

Hypothesis:

Tolerogenic DCs require at least three T cell-DC signals to induce $CD4^+CD25^+Foxp3^+$ T regulatory cells in a mouse model of asthma. The induced Tregs are distinctly superior to natural regulatory T cells of the same specificity of allergen in their abilities to induce tolerance.

Objectives:

1. To demonstrate activities of tolerogenic DC10 that are affected by the expression of MHC II and costimulatory molecules as well as cytokine.

2. To assess the role of three signals in Treg-associated marker expression of $CD4^+$ $Foxp3^+$ Treg cells by DC10.

3. To demonstrate that tolerogenic DCs abrogate asthmatic response via induction of $CD4^+CD25^+Foxp3^+$ T regulatory cells.

4. To clarify whether iTreg or nTreg express higher Treg-associated markers in the induction of tolerance by DC10.

5. To clarify whether iTreg or nTreg from DC10-treated mice have greater inhibitory function for Th2 cells.

6. To compare the *in vivo* regulatory activities of iTreg and nTreg in a mouse model of asthma.

Chapter 3: REGULATORY DENDRITIC CELL EXPRESSION OF MHCII AND IL-10 ARE JOINTLY REQUISITE FOR INDUCTION OF TOLERANCE IN A MURINE MODEL OF ASTHMA[1]

Running title: Dendritic cell IL-10 and MHCII in tolerance

Key Words: asthma; CD80/CD86; IL-10; MHCII; tolerogenic dendritic cells

[1]This Chapter was has been invited for revision by Allergy for publication.

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Contribution: J.R.G. and H.H. designed the research; M.L. did preliminary experiments for Figure 3-1; X.Zh. set up the asthma mouse model, harvested BAL and did cytospin; H.H performed all other experiments; J.R.G. and H.H. analyzed the results; J.R.G., H.H. and W.D. contributed to the writing of the manuscript; H.H wrote the first draft.

3.1. Abstract

Allergen-presenting dendritic cells differentiated with IL-10 (DC10) reverse the asthma phenotype in mice by converting their Th2 cells to regulatory T cells (Treg). DC10 express elevated levels of IL-10 but substantially reduced levels of MHCII and co-stimulatory molecules, so the relationships of these factors with each other and tolerogenicity have not been clearly elucidated. We assessed the roles of these inputs in DC10-driven reversal of asthma by treating affected mice with DC10 generated from wild-type or IL-10-sufficient MHC-II$^{-/-}$ or CD80/CD86$^{-/-}$ mice, or with MHCII-intact IL-10-silenced DC10. IL-10 silencing did not discernibly affect the cells' immunobiology (e.g., costimulatory molecules, chemokines), but it eliminated IL-10 secretion and the cell's abilities to induce tolerance, as determined by assessments of airway hyperresponsiveness, eosinophilia and Th2 responses to recall allergen challenge. MHC-II$^{-/-}$ DC10 expressed normal levels of IL-10, but nevertheless were unable to induce allergen tolerance in asthmatic mice, while tolerance induced by CD80/CD86$^{-/-}$ DC10 was attenuated but not eliminated. We also assessed the induction of multiple Treg markers (e.g., ICOS, PD-1, GITR) on pulmonary CD25^{+}Foxp3^{+} cells in the treated mice. Wild-type DC10 treatments up-regulated expression of each marker, while neither IL-10-silenced or MHC-II$^{-/-}$ DC10 did so, and the CD80/86$^{-/-}$ DC10 induced an intermediate Treg activation phenotype. Both IL-10 and MCHII expression by DC10 are requisite, but not sufficient for tolerance induction, suggesting that DC10 and Th2 effector T cells must be brought together in a cognate fashion in order for their IL-10 to induce tolerance.

3.2. Introduction

As professional antigen presenting cells (APCs), dendritic cells have an important role in the control of the adaptive immune responses. Activation of T cells by APCs requires three signaling events, one elicited by T cell receptor (TCR) recognition of the major histocompatibility complex (MHC)/peptide complex presented by the APC, another by the APCs' co-stimulatory molecules (e.g., CD80, CD54) and a third T cell-polarizing signal that emanates from the stimulated APC (e.g., IL-12 or IL-10). Depending on the nature of these three signals the dendritic cells can induce either T effector cell (Teff cell) or regulatory T cell ($T_{reg\ cell}$) responses (Enk, Jonuleit et al. 1997; Steinbrink, Wolfl et al. 1997). It is thought that dendritic cells that express reduced levels of MHCII and costimulatory molecules thereby present antigen in an inherently inefficient manner, leading to T cell unresponsiveness or anergy (Taga, Mostowski et al. 1993), but tolerogenic dendritic cells can also upregulate the activity of $CD4^+CD25^+Foxp3^+$ Treg cells (Sakaguchi, Yamaguchi et al. 2008). IL-10 has been implicated in the induction and maintenance of T cell tolerance (Moore, de Waal Malefyt et al. 2001; Ye, Huang et al. 2007). It can reduce expression of MHC class II and co-stimulatory molecules on dendritic cells and thereby induce anergy (Taga, Mostowski et al. 1993), but IL-10-exposed dendritic cells can express IL-10 themselves and thereby augment Treg cell responses (Steinbrink, Graulich et al. 2002).

There have been numerous reports of IL-10-treated or -exposed dendritic cells influencing tolerance in mouse models and in people (Enk, Jonuleit et al. 1997; Steinbrink, Wolfl et al. 1997; Rutella, Danese et al. 2006). Instillation of allergen-presenting DC10 into the airways of systemically-sensitized mice reduces the

establishment of the asthma phenotype following airway allergen challenge and that is at least in part dependent on the dendritic cell's IL-10 expression (Koya, Matsuda et al. 2007). However, this leaves open the question of whether the IL-10 that is secreted by dendritic cells operates in the context of cognate dendritic cell-Th2 Teff cell interactions or whether it may exert its tolerogenic influence through by-stander effects. For example, infusion of IL-10 into the airway (van Scott, Justice et al. 2000) or airway epithelial transfection with an IL-10-expressing adenovirus (Fu, Chuang et al. 2006) both reportedly can dampen asthmatic Th2 responses. There are multiple reports showing that treatment of asthmatic mice with DC10 (Huang, Dawicki et al. 2010; Li, Yang et al. 2010; Lu, Dawicki et al. 2011) or with IL-10 lentivirus-transfected dendritic cells (Henry, Desmet et al. 2008) can fully reverse even well-entrenched asthma. Similarly, DC10 generated from asthmatic individuals can induce autologous Th2 cell tolerance ex vivo, and they also do so by fostering the outgrowth of $CD25^{+}Foxp3^{+}$ Treg from within the donor's Th2 Teff cell populations. These Treg express the activation markers lymphocyte activation gene 3 (LAG3) and cytotoxic T-lymphocyte antigen-4 (CTLA4) (Li, Yang et al. 2010). In mouse models DC10 induce Th2 Teff cells to trans-differentiate into Treg, and these activated Treg also express an array of activation markers, including LAG3, CTLA4, inducible costimulatory molecule (ICOS), programmed cell death-1 (PD-1), and glucocorticoid-induced TNF-receptor-related protein (GITR) (Huang, Dawicki et al. 2010). Despite our knowledge regarding the capacity of DC10 to induce such tolerance, the contributions to tolerance outcomes of each of the three antigen-presentation signals offered by these cells, and their relationship(s) to one another have not been rigorously investigated. Herein we critically assessed the roles that the MHC-Ag-TCR complex,

CD80/CD86 (i.e., costimulatory molecules) and IL-10 have in DC10-induced asthma tolerance. We generated DC10 from the bone marrow of H-2Ia$^{b-/-}$ (i.e., MHC-II$^{-/-}$) or CD80/86$^{-/-}$ mice and silenced IL-10 expression in wild-type DC10 using IL-10 small interfering RNA (siRNA), used these cells to treat asthmatic mice, and then characterized their impact on the asthma phenotype and on pulmonary Treg activation.

3.3. Materials and methods

3.3.1. Antibodies, cytokines and animals

FITC-conjugated antimouse CD11c, CD40, CD54, CD80, CD86 and MHC class II (Ia^{d}) antibodies (Abs) as well as their isotype controls were purchased from BD Pharmingen Inc. (Mississauga, Ontario, Canada). PE-conjugated anti-mouse ICOS-1, PD-1, GITR, LAG-3 and CTLA-4 Abs as well as mouse regulatory T cell staining kit were purchased from eBioscience, Inc. (San Diego, CA). The recombinant mouse granulocyte macrophage colony stimulating factor (GM-CSF) and IL-10 were obtained from R & D Systems (Minneapolis, MN). Wild-type CD45.2^{+} and congenic CD45.1^{+} female C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA), while CD80/86 and H-2Ia^{b} gene KO mice on C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, Maine). All mice were maintained in the animal facility at Animal Care Unit and treated according to Animal Care Committee guidelines of University of Saskatchewan.

3.3.2. Establishment of asthma mouse model and DC10 treatments

The generation of DC10 and establishment of ASTHMA mouse were described previously (Gordon, Li et al. 2005; Huang, Dawicki et al. 2010). Briefly, DC10 were differentiated from bone marrow of wild type or CD80/86 and H-2Ia^b gene KO mice. To establish ASTHMA mice, C57BL/6 mice are given 2μg of ovalbumin (OVA)/alum by *i.p.* injection on day 0, 14, and on day 28, 30, 32, they were exposed to 1% OVA aerosols for 20 min/day. This protocol reliably induces a severe asthma-like phenotype, characterized by high-level pulmonary Th2 cytokine (IL-4, -5, -9, and -13) and antibody (IgE and IgG1) responses to OVA, ≈40-60% airway eosinophilia and AHR to methacholine challenge. After a period of two wks, during which the pulmonary inflammatory responses (but not AHR or Th2 reactivity) subside, the mice are injected *i.p.* with 1×10^6/mouse DC10 (Schneider, Li et al. 2001).

3.3.3. Tracking of DC10 *in vivo*

Allergen-loaded CD45.1$^+$ congenic B6 mouse DC10 were injected *i.p.* into asthmatic CD45.2$^+$ congenic B6 mice (5×10^6 cells/mouse). After 2, 7, 14, 21 d, we collected the lungs, spleens, mesenteric and mediastinal (i.e., lung-draining) LN from each animal and generated single cell suspensions either by mechanical (spleens, LNs) or enzymatic (lungs) dispersal of the tissues (Schneider, Li et al. 2001). In addition, we performed bronchoalveolar (BAL) and peritoneal lavages on the recipients to recover the cells from these compartments. Each cell population was analysed by flow cytometry (Beckman Coulter Epics XL, analysis by Flowjo v.8 software).

3.3.4. Silencing of IL-10 expression by DC10

The IL-10 siRNA sequences were designed using online software (Qiagen, Valencia, CA); BLAST searches were carried out to ensure that the sequences would not target other transcripts. The target sequence of the IL-10siRNA was 5'-CAGGGATCTTAGCTAACGGAA-3'. The sense and antisense alignment for the siRNA were 5'-GGGAUCUUAGCUAACGGAAtt-3' and 3'-gtCCCUAGAAUCGAUUGCCUU-5', respectively. The siRNA's were transfected into DC10 using a commercial kit (RNAi human/mouse starter kit, Qiagen), according to the supplier's protocol. A non-silencing random nucleotide control siRNA of no known specificity was transfected into wild-type DC10 as an irrelevant siRNA control. All ribonucleotides were synthesized and annealed by the supplier (Qiagen).

3.3.5. ELISA

Our type 2 cytokine ELISA protocols have been reported in detail previously(Schneider, Li et al. 2001; Gordon, Li et al. 2005). The culture supernatants of the DCs or BAL fluids were collected and analysed with respect to IL-4, 5, 9, 13 production with a sandwich ELISA using corresponding specific capture and detection antibodies. Cytokine levels were calculated using standard curves constructed by recombinant murine cytokines (R&D).

3.3.6. Quantitative RT-PCR

Total RNA was obtained from DC cells by RNeasy Mini Kit (Qiagen, Valencia, CA). 1 μg total RNA was used for RT-PCR by RT-PCR ThermoScript One-Step System (Invitrogen) according to the manufacturer's instructions. The specific PCR IL-10 primers are the sense (5'-AAGCCTTATCGGAAATGATCCA-3') and antisense (5'-GCTCCACTGCCTTGCTCTTATT-3') In addition, another one set of primers was also used for control home keeping gene β-actin, including the sense (5'-AGAGGGAAATCGTGCGTGAC-3') and antisense (5'-CAATAGTGATGACCTGGCCGT-3'). About RT-PCR conditions, cDNA synthesis is at 50℃ (30 min), PCR is 1 cycle at 95℃ (5 min) and 35 cycles at 95℃ (15 sec), 60 ℃ (1 min). All PCR reaction products were resolved on 1% agarose gels with ethidium bromide staining.

3.3.7. Chemotaxis assay

The chemotactic responses of DC to MIP-1 and MIP-3 were examined using modified Boyden microchemotaxis chambers (Neuroprobe, Gaithersburg, MD) and polyvinyl pyrrolidone-free 5 μm pore size polycarbonate membranes, essentially as described previously(Gordon, Li et al. 2005). Recombinant MIP-1 and MIP-3, diluted in DMEM-0.1% BSA to 100 ng/ml, was added to triplicate lower chambers of the wells, and 10^5 DC in DMEM-BSA were added to the upper chambers. After incubation for 2 h at 37°C, adherent cells were stained using Diff-Quik staining kit (EM Science, Inc. Cat.

No. 65044/93). Mount the membrane shiny side down on microscope slides with Permount and Xylene. Count 5 fields per well at 40X power.

3.3.8. Measurement of AHR

Measurement of AHR was detailed in our previous paper (Gordon, Li et al. 2005). Briefly, AHR was assessed by head-out, whole-body plethysmography in conscious animals. Each mouse was sequentially exposed to aerosols of methacholine (0.5-20 mg/ml saline). AHR was monitored by sensor linked to a computer data collector system.

3.3.9. Activation markers of Treg cells

DC10 were generated from wild type, CD80/86 KO and Ia^b KO mice. DC10, DC10($CD80^{-/-}86^{-/-}$), DC10($Ia^{b-/-}$) and DC10/$siRNA_{IL-10}$ [DC10($IL\text{-}10^{-/-}$)] as well as saline were injected to asthmatic mice respectively. Then at 3 wk post-treatment we purified T cells from the lungs and draining LNs and stained them with FITC-CD4, PE-cy5-Foxp3 and PE-labeled antibodies against the Treg cell markers CD25, ICOS, PD-1, GITR, LAG3 and CTLA-4. The $CD4^+$ cells were gated then $Foxp3^+PE^+$ cells were assessed by cytometry.

3.3.10. Statistical analysis

All data were analyzed with the aid of a software program (GraphPad Prism 5.0). Multigroup comparisons were assessed by one-way ANOVA with Tukey post test.

whereas AHR to methacholine was assessed by linear regression analyses. Values of $p < 0.05$ were considered significant. All results are expressed as the mean ± SEM.

3.4. Results

3.4.1. DC10 delivered i.p. migrate to the lungs and lung draining LNs

We have reported previously that the onset of tolerance in DC10-treated asthmatic mice is progressive, such that AHR is moderately diminished in mice at 2 wk after treatment and disappears entirely within 3 wk of treatment, while the airway Th2 responses to recall allergen challenge (i.e., eosinophilia, Th2 cytokines) lags behind somewhat (Lu, Dawicki et al. 2011; Nayyar, Dawicki et al. 2012). We questioned whether there were temporal relationships between the onset of this tolerance and trafficking of the DC10 within the recipients. Others have reported that leukocytes within the peritoneum traffic via the diaphragmatic lymphatics to the thoracic duct and thereby into the peripheral circulation, although materials in the pleural cavity can be tracked to the mediastinal LN (Yoffey and Courtice 1970). To track our treatment DC10 we injected asthmatic $CD45.2^+$ B6 mice i.p. with DC10 generated from the bone marrow of $CD45.1^+$ congenic B6 mice and used FACS to detect the $CD45.1^+$ cells in an array of organs across 3 weeks (Figure 3-1 and Table 3-1). At 2 d post-transplant a substantial proportion of the cells recovered from the airways (BAL) and lung parenchyma (Lung par.) were $CD45.1^+$ (i.e., the injected DC10), with modest numbers also appearing in the lung-draining mediastinal LNs (Med. LN), while few DC10 were detectable in the spleens or cervical LNs (Cer. LN). At 1 wk the numbers of DC10 peaked in the lungs and

mediastinal LNs, and some DC10 were evident at this time in the spleens, but not cervical LNs. The numbers of DC10 were markedly reduced within each site at 2 wk post-treatment, and by 3 wk DC10 were no longer discernible in either the peripheral organs or LNs. We found negligible numbers of marker-positive cells in the blood, bone marrow or liver at any time (data not shown). These data indicate that DC10 that are delivered intraperitoneally increasingly accumulate in the asthma target-organs, the lungs and lung-draining LNs, across one week after implantation, and that they continue to have a presence at two weeks after treatment. We have reported previously that DC10 treatment is associated with activation of pulmonary Tregs beginning at 1 wk after treatment (Huang, Dawicki et al. 2010), although significant effects of DC10 on AHR are first discernible in asthmatic mice at 2 wk (Nayyar, Dawicki et al. 2012). This suggests that the treatment DC10 may well engage their target Th2 effector T cells *in situ* in the lungs or lung-draining LNs.

Figure 3-1. IL-10-differentiated dendritic cells that are delivered intraperitoneally in asthmatic mice migrate to the lungs and draining LNs.

IL-10-differentiated dendritic cells (DC10) were generated from the bone marrow of $CD45.1^+$ B6 mice and injected into asthmatic congenic $CD45.2^+$ B6 recipient mice. After 2, 7, 14 or 21 d the mice were sacrificed and the proportions of $CD45.1^+$ cells among total cells recovered from their airways (BAL), lung parenchyma (Par.), mediastinal (Med LN) and mesenteric (Mes. LN) LNs, and spleens, were assessed by flow cytometry, as noted in the materials and methods section.

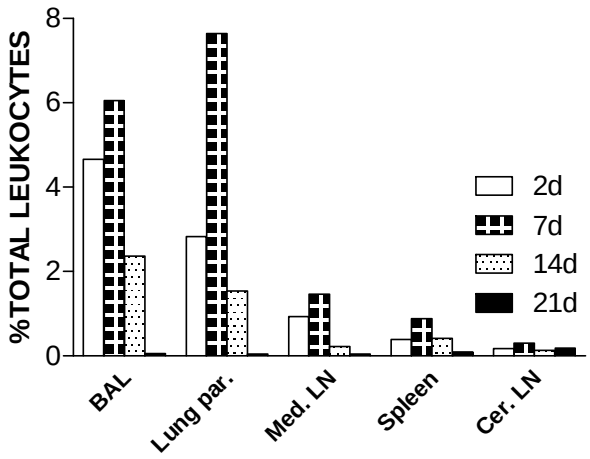

Figure 3-1. DC10 delivered i.p. migrate to the lungs and draining lymph nodes.

Table 3-1. Anatomical distribution of DC10 over three wks following intraperitoneal delivery to asthmatic mice.

Time (d)	Peri.	BAL	Lung par.	Med. LN	Spleen	Mes. LN	Cer. LN
2	2.63	4.66	2.83	0.93	0.39	0.80	0.17
7	0.72	6.05	7.64	1.46	0.89	2.23	0.31
14	0.61	2.37	1.54	0.23	0.42	0.00	0.14
21	0.00	0.06	0.00	0.00	0.10	0.06	0.18

In vitro-differentiated DC10 cells from CD45.1 congenic mice were injected i.p. into asthmatic B6 mice. After 2, 7, 14 and 21 d, BAL, lung parenchyma (Par.), mediastinal (Med) and mesenteric (Mes.) LN, spleen, and peritoneal (Peri.) wash cells from recipients were recovered and stained using a PE-anti-CD45.1 antibody. CD45.1-positive cells were assessed by flow cytometry. Values are %.

3.4.2. IL-10 secretion is essential to DC10-induced tolerance in established asthma

As noted, DC10 can prevent localization of allergic disease to the lungs in mice, and IL-10 plays a role in that process (Koya, Matsuda et al. 2007), but we wished to confirm whether IL-10 produced by DC10 also plays a role in the reversal of the asthma phenotype in our model. To test this we first generated DC10 in which IL-10 expression had been silenced using siRNA approaches, and then used these cells to treat asthmatic mice. We assessed the efficiency of our DC10 transfection protocols using a fluorescent control siRNA construct, and found that at 48 h after transfection essentially all DC10 in our cultures were siRNA-positive (Figure 3-2A). We then optimized the dose of IL-10-specific siRNA required to negate IL-10 secretion. Transfection of DC10 with 40 nmol siRNA essentially eliminated IL-10 secretion by our DC10, even after they had been stimulated overnight with LPS (1 μg/ml; Figure 3-2B), and brought DC10 IL-10 mRNA levels to background (Figure 3-2C), while transfection with 40 nmol scrambled nonsense siRNA had no discernible impact on IL-10 expression (Figure 3-2B). We also evaluated the potential toxicity of the siRNA and transfection reagents for our DC10 by measuring their viability at 24 h after transfection, as determined using annexin V and propidium iodine (PI) staining to assess apoptosis and necrosis, respectively (Figure 3-2D). The results confirmed that neither reagent affected DC10 viability. We also assessed the abilities of the control siRNA and IL-10 siRNA transfected cells to phagocytose FITC-dextran, as a surrogate antigen, and to respond to CCL3 and CCL19, as ligands for the tissue inflammatory chemokine receptor CCR5 and the lymph node-homing chemokine receptor CCR7, respectively. IL-10-silencing had no impact on the DC10's phagocytic

Figure 3-2. Silencing of IL-10 expression in the tolerogenic DC by transfection with $siRNA_{IL-10}$.

(A) IL-10 siRNA efficiently transfected DC10. DC10 cells were transfected with Alexa Fluor 488-labeled IL-10 siRNA and the efficiency of transfection was evaluated by flow cytometry and fluorescent microcope 48 hours later. (B) IL-10 secretion was assessed in tolerogenic DC transfected with different concentrations of $siRNA_{IL-10}$ using ELISA after 48 hour transfection. 40nmol $siRNA_{IL-10}$ was observed to be optimal for silencing IL-10 secretion by using RNAi Human/Mouse Starter Kit. (C) IL-10 mRNA expression level of DC10/$siRNA_{IL-10}$ was measured by RT-PCR. (D) Apoptosis and necrosis of DC10/$siRNA_{IL-10}$ was assessed using annexin V and propidium iodine (PI) staining.

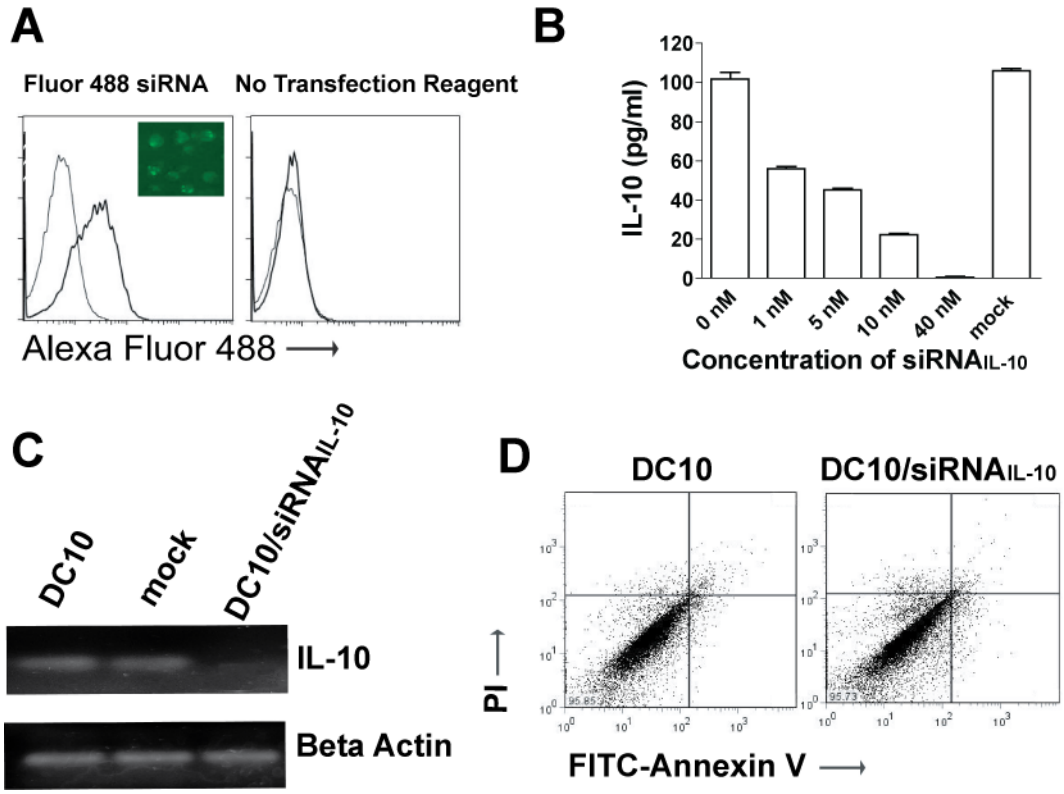

Figure 3-2. Silencing of IL-10 expression in the tolerogenic DC by transfection with siRNA$_{IL-10}$.

Figure 3-3. Characterization of IL-10-silenced DC10 (DC10/SiRNA$_{IL-10}$) in terms of surface markers, phagocytosis and migratory abilities.

(A) DC10/SiRNA$_{IL-10}$ or DC10 cells were incubated for 30 min with FITC-dextran (100μg/ml; 40 kD MW), and then analyzed by FACS. (B) The impact of the IL-10 silencing on chemokine receptor (CCR5 and CCR 7) expression was determined by modified Boyden chamber micro chemotaxis assays. (C) Surface markers [Ia^d, CD11c (HL3), CD40 (3/23), CD54 (3E2), CD80 (16-10A) and CD86] of DC10/SiRNA$_{IL-10}$ or DC10 cells were detected by flow cytometry.

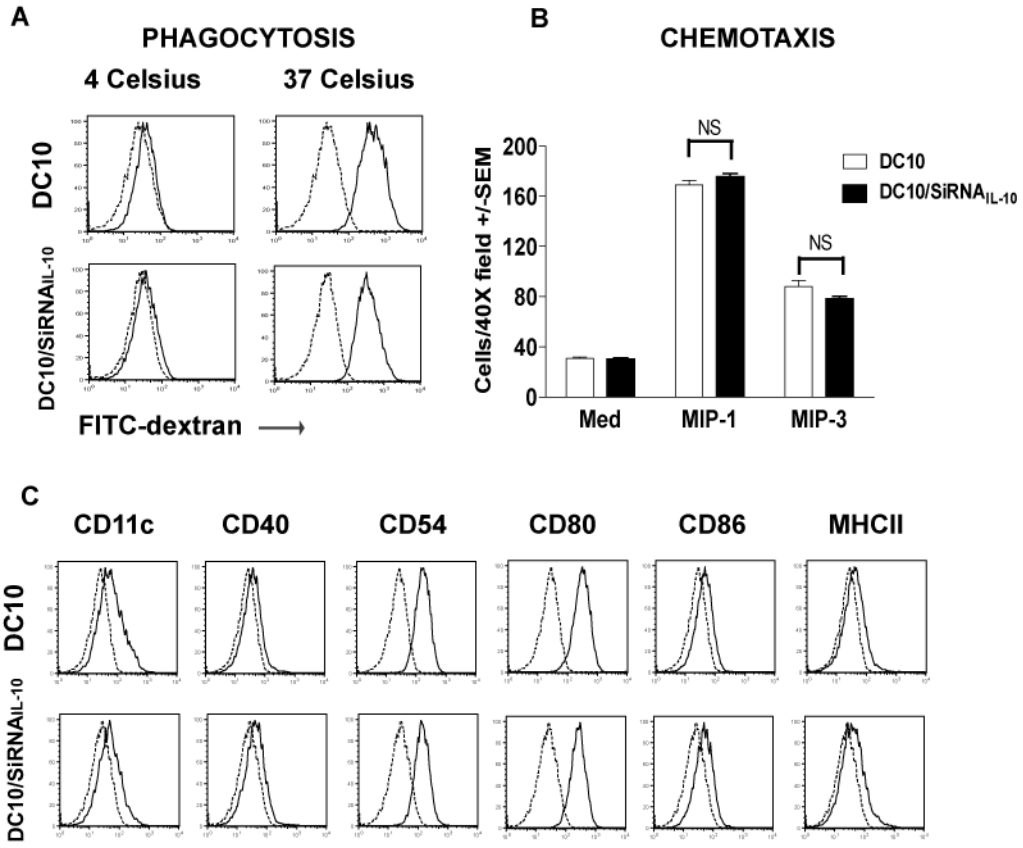

Figure 3-3. Characterization of the IL-10-silenced DC10 (DC10/SiRNA$_{IL-10}$) in terms of surface markers, phagocytosis and migratory abilities.

activities or chemotactic responses to dendritic cell-relevant chemokines (Figure 3-3A, B), and had no discernible impact on the cell's expression of MHCII, CD11c, CD40, CD54, CD80 or CD86, as determined by flow cytometry (Figure 3-3C). This data confirmed that silencing IL-10 expression by DC10 did not significantly alter their expression of these antigen presentation-associated markers or parameters. We next evaluated the impact of IL-10 silencing on the therapeutic effectiveness of DC10 in a mouse model of asthma. We treated asthmatic B6 mice with saline or 1×10^6 wild-type, IL-10-silenced, or scrambled nonsense siRNA-transfected DC10 and 25 days later assessed the treated animal's AHR to methacholine, as well as that of normal healthy control mice (Figure 3-4A). The airways of the saline-treated asthmatic animals were fully hyperresponsive to methacholine, while those of wild-type DC10- and negative control siRNA-transfected DC10-treated animals were normoresponsive. On the other hand, the IL-10-silenced DC10 were completely ineffective in reducing AHR in these animals. One day after assessing their AHR, we challenged the animals with aerosolized allergen (1 mg/ml OVA, 20 min) and two days later we sacrificed the animals and examined their airways for evidence of Th2 recall responses to the allergen challenge, looking at BAL fluid eosinophils and Th2 cytokines (IL-4, -5, -9 and -13). The wild-type and scrambled nonsense siRNA-transfected DC10 treatments brought each of these parameters to near baseline, while IL-10 silencing again negated the tolerogenic activities of the DC10 (Figure 3-4B, C). Taken together, this data indicates that IL-10 expression by DC10 is essential to their tolerogenic activities in our model.

3.4.3. DC10-induced asthma tolerance is also critically dependent on MHCII-TCR interactions

As suggested above, in principle the IL-10 that is secreted by DC10 could affect tolerance through by-stander effects. Infusion of recombinant IL-10 into the airway is known to reduce Th2 immunoinflammatory responses in experimental asthma(van Scott, Justice et al. 2000). Thus, we next assessed whether DC10 that do not express a MHCII molecule (i.e., cannot engage T cells through their TCR) or CD80 and CD86 are compromised in their abilities to induce tolerance. For this and the next experiment we generated DC10 from the bone marrow of $MHCII^{-/-}$ and CD80/CD86-double knockout mice, incubated them with specific allergen in vitro, and then used them to treat OVA-asthmatic mice, as above. We assessed whether these DC10 secreted IL-10 at levels observed in wildtype cells, and found no differences in IL-10 expression between the three populations of cells. The wild-type DC10 released 347+/-138 pg/ml over 48 hr, while the $MHCII^{-/-}$ DC10 secreted 241+/-44 pg/ml IL-10 and the $CD80/CD86^{-/-}$ DC10 secreted 249+/-55 pg/ml of IL-10 (for both knockout DC10 populations, $p \geqslant 0.05$ versus wild-type DC10).

When we assessed the AHR of the mice at $\approx$4 wk post-treatment, we found that the recipients of the $MHCII^{-/-}$ DC10 still presented with a fully developed AHR ($p \geqslant 0.05$ versus saline-treated asthmatic mice), while the animals that had been treated with wild-type DC10 were normoresponsive (Figure 3-5A). This loss of the ability to affect AHR applied also to the airway eosinophil and IL-4, -5, -9, and -13 responses to recall allergen challenge (Figure 3-5B and 5C). In each case there were no statistically significant

Figure 3-4. IL-10-silenced DC10 lose their therapeutic effectiveness in a mouse model of asthma.

Asthma mice were given 1×10^6 DC10 or DC10/siRNA$_{IL\text{-}10}$ or DC10/siRNAcontrol via *ip*. (A) 25 d later, their AHR to aerosolized methacholine was assessed ($p<0.05$, DC10/siRNA$_{IL\text{-}10}$ -treated group *vs.* the DC10 -treated group). The following day, we challenged the animals for 20 min with nebulized aerosols of 1% OVA, and 48 h later, assessed their pulmonary immunoinflammatory responses. Eosinophil (B) and Th2 (C) cytokine (IL-4, IL-5, IL-9 and IL-13) responses to the recall allergen challenge were assessed. One representative experiment of three is shown. *, ***, $p<0.05$ or 0.001 versus the DC10-treated group animals (n=5 mice/group).

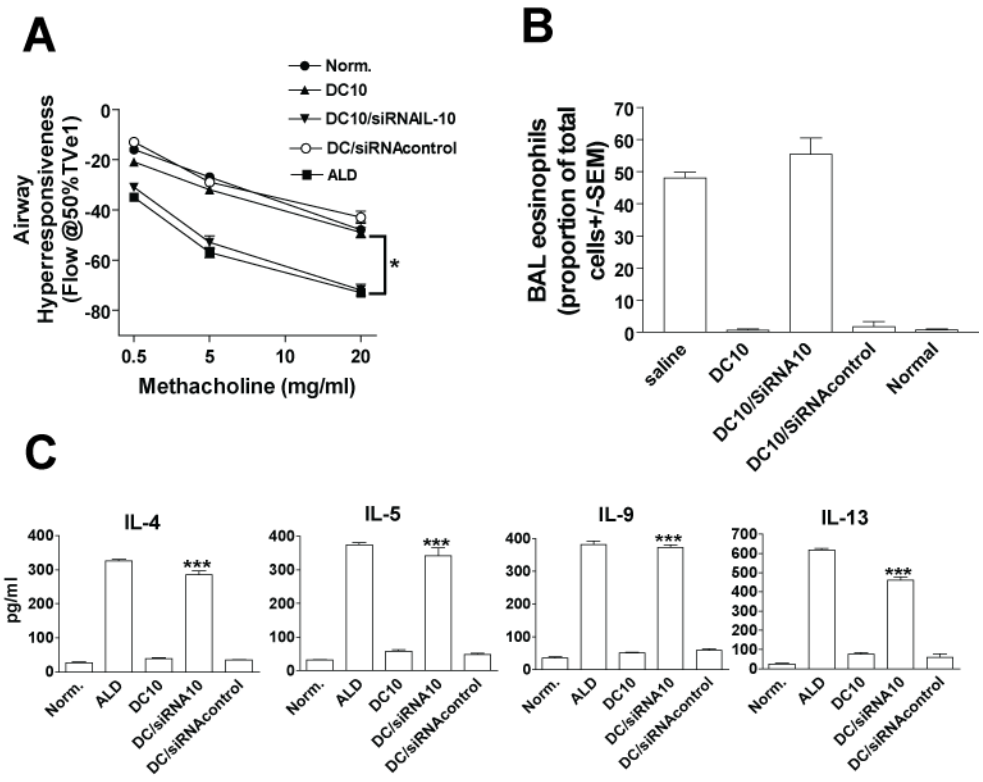

Figure 3-4. IL-10-silenced DC10 lose their therapeutic effectiveness in a mouse model of asthma.

differences in the responses of the saline- and MHCII$^{-/-}$ DC10-treated asthmatic mice ($p \geqslant 0.05$), while asthmatic mice that had been treated with wild-type OVA-presenting DC10 displayed a tolerant phenotype.

Interestingly, the AHR of the mice treated with the CD80/86$^{-/-}$ DC10 was partially reversed (Figure 3-5A), while their recall airway eosinophil and Th2 cytokine responses to OVA aerosol challenge were unaffected by the CD80/86$^{-/-}$ DC10 treatment (Figure 3-5B, 5C). Indeed, these costimulation-attenuated DC10 were no different than MHCII$^{-/-}$ DC10 in terms of their abilities to affect the airway Th2 cytokine response (Figure 3-5C). This data suggests that IL-10 expression alone is insufficient to render DC10 tolerogenic, and that they must engage their target Th2 Teff cells in a cognate (i.e., MHCII-dependent) fashion in order for their IL-10 to induce a Th2 to Treg phenotype switch. Moreover, optimal phenotype switching also required participation of CD80 and/or CD86, likely because of their contributions to high avidity immunological synapse formation.

3.4.4. IL-10 and MHCII expression by DC10 are essential for activation of Treg in DC10-treated asthmatic mice

We recently have reported that DC10 treatments lead to conversion of Th2 Teff cells into CD4^{+}CD25^{+}Foxp3^{+} Treg in the lungs of treated mice (Huang, Dawicki et al. 2010), and that these Treg display augmented levels of the activation markers ICOS, PD-1, GITR, LAG-3 and CTLA-4 (Huang, Dawicki et al. 2010). We thus sought to determine whether the altered antigen presentation skills of our various compromised DC10 also translated into a reduced ability to activate pulmonary Treg in our treated mice. We treated the asthmatic animals with saline, or with wild-type, IL-10-silenced, MHCII$^{-/-}$

Figure 3-5. CD80/86- or MHC II- knockout DC10 had lost their therapeutic effectiveness in a mouse model of asthma.

ASTHMA mice were given 1×10^6 DC10 or DC10($CD80^{-/-}$) or DC10($MHCII^{-/-}$) via *ip*. (A) 25 d later, their AHR to aerosolized methacholine was assessed [$p<0.05$, DC10($CD80^{-/-}$) or DC10($MHCII^{-/-}$) -treated group *vs* the DC10 -treated group]. The following day, we challenged the animals for 20 min with nebulized aerosols of 1% OVA, and 48 h later, assessed their pulmonary immunoinflammatory responses. Eosinophil (B) and Th2 (C) cytokine (IL-4, IL-5, IL-9 and IL-13) responses to the recall allergen challenge were assessed [$p<0.001$, DC10($CD80^{-/-}$) or DC10($MHCII^{-/-}$) -treated group *vs* the DC10 -treated group]. One representative experiment of three is shown. *, ***, $p<0.05$ or 0.001 versus the DC10-treated group animals (n=5 mice/group).

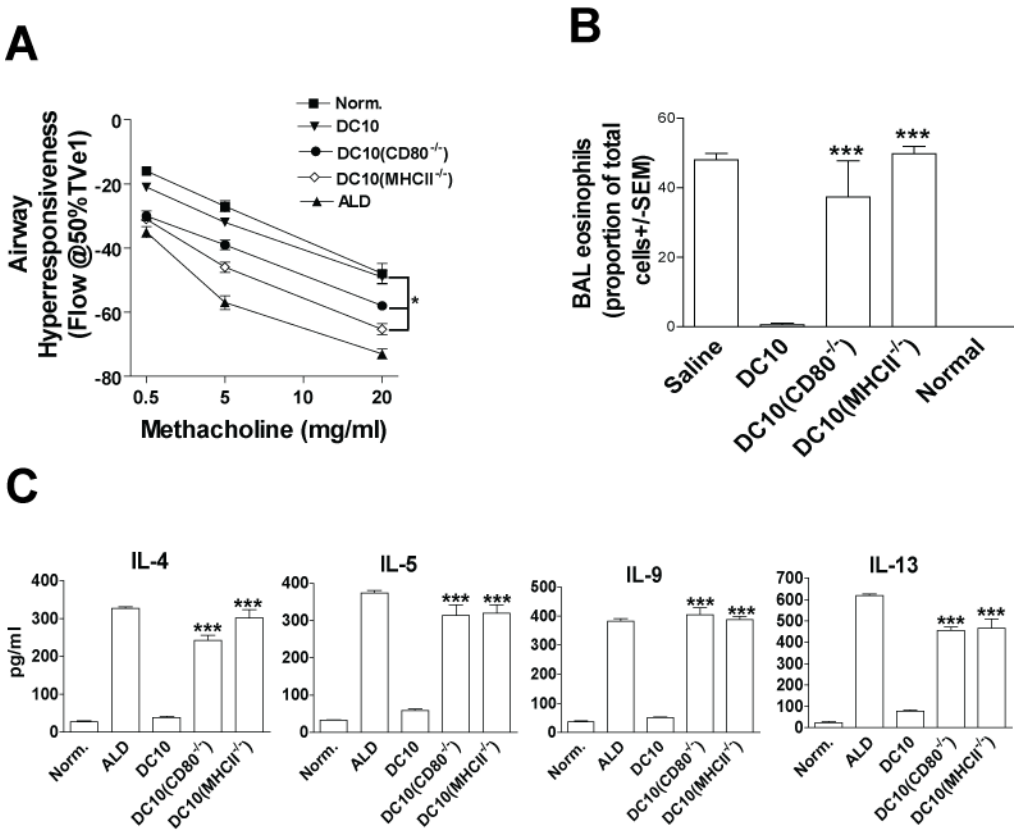

Figure 3-5. CD80/86- or MHC II- knockout DC10 had lost their therapeutic effectiveness in a mouse model of asthma.

or CD80/CD86$^{-/-}$ DC10, and 3 wk later we purified T cells from the lungs of these mice using nylon wool columns and assessed their expression of each of these markers by FACS. It was readily apparent that the OVA-presenting DC10 up-regulated expression of each marker as we had observed previously, while the pulmonary CD4^{+} T cells of the IL-10-silenced or MHCII$^{-/-}$ DC10-treated mice did not up-regulate these markers above the levels seen in asthmatic mice (Figure 3-6). Interestingly, the CD80/CD86$^{-/-}$ DC10 induced an intermediate level of Treg activation relative to that seen with wild-type, IL-10-silenced or MHCII$^{-/-}$ DC10 (Figure 3-6). In keeping with our previous observations of no differences in the numbers of CD4^{+}CD25^{+} cells in normal, asthmatic or tolerant mice (Huang, Dawicki et al. ; Li, Yang et al. 2010; Lu, Dawicki et al. 2011), we did not observe marked differences in the proportions of CD25^{+}Foxp3^{+} T cells in the lungs of our mice. This experiment confirmed that both IL-10 expression and an ability to engage target Th2 cells in a cognate fashion are both critical to DC10-induced asthma tolerance, but also that the costimulatory molecules CD80 and/or CD86 are also involved in this process.

Figure 3-6. Roles of by IL-10, MHC II and CD80/86 costimulatory molecule expression by DC10 in Treg-associated marker expression on $CD4^+Foxp3^+$ Treg cells.

DC10 were generated from wild type, CD80/86 knock-out and Ia^b knock-out mice. DC10, DC10($CD80^{-/-}86^{-/-}$), DC10($Ia^{b-/-}$) and DC10/$siRNA_{IL\text{-}10}$ [DC10($IL\text{-}10^{-/-}$)] as well as saline were injected to asthmatic mice. Then at 3 wk post-treatment we purified T cells from the lungs and draining LNs and stained them with FITC-CD4, PE-cy5-Foxp3 and PE-labeled antibodies against the Treg cell markers CD25, ICOS, PD-1, GITR, LAG3 and CTLA-4. The $CD4^+$ cells were gated then $Foxp3^+PE^+$ cells were assessed by cytometry. One representative experiment of two is shown.

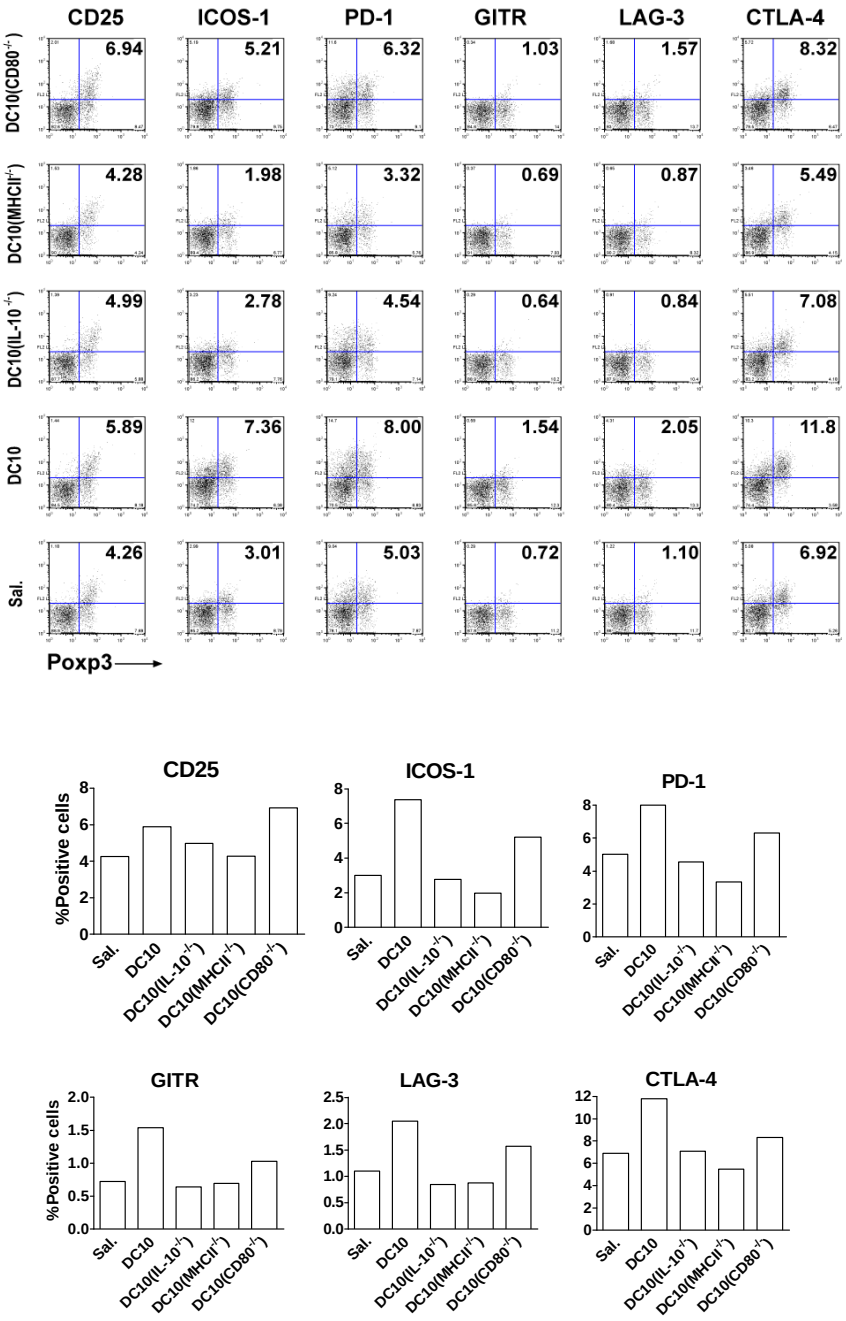

Figure 3-6. Roles of by IL-10, MHC II and CD80/86 costimulatory molecule expression by DC10 in Treg-associated marker expression on $CD4^{+}Foxp3^{+}$ Treg cells.

3.5. DISCUSSION

In this study we assessed the importance of IL-10 and MHCII expression by cognate allergen-presenting DC10 on their induction of immunologic tolerance in asthmatic mice, but also the role(s) of costimulatory molecule expression by these cells. Specifically, we used DC10 that were deficient in MHCII or CD80 and CD86, or IL-10-silenced DC10, and found in each case that the deficiency reduced the tolerogenic activity of these cells as determined by assessments of AHR and Th2 immunoinflammatory responses to recall allergen challenge. IL-10 silencing did not discernibly affect the viability of the DC10, their expression of MHCII or co-stimulatory molecules, or their phagocytic or chemotactic activities, indicating that it was indeed their IL-10 that was the critical effector molecule for these DC10. On the other hand, $MHCII^{-/-}$ DC10, which expressed wild-type DC10 levels of IL-10, were also incapable of inducing tolerance, while IL-10-sufficient $CD80/CD86^{-/-}$ DC10 were decidedly suboptimal in their abilities to induce tolerance.

IL-10 is a key immunomodulatory cytokine, with both immunosuppressive and immunostimulatory effects on T cells having been reported (Mocellin, Panelli et al. 2003; Mocellin, Marincola et al. 2004). It can directly inhibit T cell growth (Taga, Mostowski et al. 1993) and induce long-lasting antigen-specific T cell anergy (Groux, Bigler et al. 1996). IL-10 knock-out mice do not develop AHR after allergen sensitization and challenge despite a significant pulmonary inflammatory response that includes increased airway eosinophilia (Makela, Kanehiro et al. 2002), while IL-10 infusion into the airways of asthmatic mice dampens their responses to recall allergen challenge (van Scott, Justice et al. 2000). It had been reported that, unlike wild-type DC10, DC10 that are generated from the bone marrow of IL-10-knockout mice are unable to prevent the development of

asthma in a mouse model (Koya, Matsuda et al. 2007), Whether the IL-10 released by these DC is critical for reversal of the asthma phenotype in animals with well-established disease (Huang, Dawicki et al. 2010; Lu, Dawicki et al. 2011; Nayyar, Dawicki et al. 2012) had not been assessed, nor had it been determined whether cognate T cell engagement by the IL-10-expressing DC10 is critical to that tolerance. Our data indicates that the dendritic cell indeed must engage target Th2 Teff cells in a cognate manner in order for the IL-10 they secrete to productively affect the latter cells. It has been reported that expression of IL-10 by the host's cells, presumptively their Treg cells, is also critical to the induction of tolerance in asthmatic mice, even when the treatment dendritic cells have been engineered to express exceptional levels of IL-10 (Henry, Desmet et al. 2008). We have shown previously that DC10 induce the host's effector Th2 cells to transdifferentiate to a $CD25^{+}Foxp3^{+}$ Treg cell phenotype and that IL-10 expression by these Treg drives their suppression of Th2 responses, at least in vitro (Huang, Dawicki et al. 2010). IL-27 can promote the development of IL-10-secreting T cells (Awasthi, Carrier et al. 2007) and IL-27 produced by galectin-1-differentiated tolerogenic dendritic cells has been shown to induce IL-10 production by T cells with which these cells interact (Ilarregui, Croci et al. 2009). IL-10-silenced DC10 did not induce tolerance despite the fact that the recipient's Treg would be fully capable of secreting IL-10, suggesting that the lack of tolerance was attributable to a failure to induce or activate Treg by the IL-10-compromised DC10. In keeping with this, our data confirms that while wild-type DC10 treatments led to robust activation of the host's Treg, as determined by expression of ICOS-1, PD-1, GITR, LAG-3 and CTLA4, IL-10-silenced DC10 induced no such Treg activation. We had documented previously that DC10-induced Treg up-

regulate each of these activation markers (Huang, Dawicki et al. 2010; Li, Yang et al. 2010; Lu, Dawicki et al. 2011; Nayyar, Dawicki et al. 2012). Thus, our data confirms in another way the importance of IL-10 expression by DC10 in the development of Treg cells.

As suggested above, the fact that IL-10-intact, but MHCII-/- DC10 were also unable to induce tolerance clearly indicates that DC10 must be engaged in cognate interactions with T cells in order for their IL-10 to have a significant impact. This is of substantial significance in the therapeutic setting, because it indicates that tolerance induced by DC10 will be exquisitely antigen-specific and therefore not lead to broad immunosuppression. Our previous observations that DC10 must present cognate allergen in order to intimately engage Th2 cells (as determined by fluorescence resonance energy transfer [FRET] assays) from asthmatic people or mice and induce tolerance therein(Li, Yang et al. 2010; Lu, Dawicki et al. 2011; Nayyar, Dawicki et al. 2012) fit well with our present data. Our data also has implications for therapeutic use of these cells in the context of poly-allergic individuals. In principle, each allergen to which an individual is sensitive would need to be individually targeted by DC10, although it is open to speculation as to whether one could treat sensitivity to two independent allergens by loading dendritic cells with both. Others have reported that human $CD14^{+}$ monocyte-derived dendritic cells can present multiple antigens (Garritsen, Macke et al. 2010).

The dendritic cell's costimulatory molecules provide important signals that can independently influence the outcomes of antigen presentation, such that these cells can have either immunosuppressive or immunostimulatory effects on T cells (Hawiger, Inaba et al. 2001; Steinman 2003; Steinman, Hawiger et al. 2003). It is well recognized that

dendritic cells require both MHC-peptide-TCR complex and co-stimulatory molecule engagement in order to optimally activate T cells(Golovina, Mikheeva et al. 2008; Weaver, Charafeddine et al. 2008), but there is increasing evidence that co-stimulatory molecules are integral to the induction of tolerance by regulatory dendritic cells. Thus, CD40L/CD154 blockade can engender long-lived, antigen-specific tolerance (Quezada, Jarvinen et al. 2004), while the CD80/CD86 ligand CTLA-4 attenuates T-cell activation and fosters peripheral tolerance (Scalapino and Daikh 2008). The late-acting co-stimulatory molecules CD80 and CD86 are important to full induction of Teff cell activation (Hochweller and Anderton 2005), and our data from the present study suggests that these molecules are also required for successful activation of Treg cells. Dendritic cells have the capacity to acquire, and present, antigens from dying cells (Steinman, Turley et al. 2000) such that it might be argued that in our model DC10 or parts thereof (e.g., exosomes) are taken up by the recipient's APC, and that it is those endogenous APC that induce tolerance. The observed requirement for MHC II, CD80/86 and IL-10 expression by the treatment DC10 indicates that the these cells must directly interact with T cells to promote tolerance and that their therapeutic effects are not realized by transfer of their ingested allergens to the recipient's dendritic cells or other APC. An unanswered question in these studies is the impact on the tolerance outcomes of varying the intensity of the antigen presentation signaling between the DC10 and the Teff cells. For example, would the levels of IL-10 that are expressed by wild-type DC10 still induce tolerance if the DC10 expressed high levels of MHCII and co-stimulatory molecules (e.g., equivalent to that of endotoxin-activated dendritic cells) and thereby provided otherwise strong activation signals to the Teff cells? Or, would increasing the expression levels of IL-10

by DC10 induce a distinct form of tolerance? It has been reported that dendritic cells that IL-10-lentivirus-transfected dendritic cells, which express IL-10 at levels that are orders of magnitude greater than our DC10, induce a very robust tolerance (Henry, Desmet et al. 2008). The answers to these types of questions may allow us in the future to optimize such cell-based immunotherapies.

Taken together our observations confirm that DC10 engage intimately with pulmonary Teff cells to promote their conversion to Treg and show that MHCII and CD80/86 engagement, combined with the DC10's secretion of IL-10, are each integral to operationalization of DC10-induced asthma tolerance. Thus, DC10-induced tolerance would not arise because of a lack of Teff cell stimulation, but rather because these cells are engaged and activated by the tolerogenic APC in precisely the manner that induces Treg differentiation.

ACKNOWLEDGMENTS

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CHAPTER 4: TOLEROGENIC DENDRITIC CELLS INDUCE $CD4^+CD25^{HI}FOXP3^+$ REGULATORY T CELL DIFFERENTIATION FROM $CD4^+CD25^{-/LO}FOXP3^-$ EFFECTOR T CELLS[1]

Running title: Conversion of Teffs to Tregs

Key words: $CD4^+CD25^+$ T regulatory cell, tolerogenic dendritic cell, asthma, IL-10

[1]This Chapter was published in The Journal of Immunology. It is reproduced here with permission of the copyright owner (The American Association of Immunologists, Inc.).

Huang, H., W. Dawicki, X. Zhang, J. Town and J. R. Gordon. 2010. Tolerogenic Dendritic Cells Induce $CD4^+CD25^{hi}Foxp3^+$ Regulatory T Cell Differentiation from $CD4^+CD25^{-/Lo}Foxp3^-$ Effector T Cells. The Journal of Immunology, 2010, 185:5003-5010.

Contribution: J.R.G. and H.H. designed the research; X.Zh. and J.T. did animal model establishment, specific tissue harvest and cytospin; H.H performed all other experiments; J.R.G. and H.H. analyzed the results; J.R.G., H.H. and W.D. contributed to the writing of the manuscript; H.H wrote the first draft.

4.1. Abstract

IL-10-differentiated dendritic cells (DC10) induce allergen tolerance in asthmatic mice, during which their lung Th2 T effector cells (Teff) are displaced by activated $CD4^+CD25^{hi}Foxp3^+$ T cells (Treg). Intestinal DCs promote oral tolerance by inducing antigen-naïve T cells to differentiate into $CD4^+CD25^+Foxp3^+$ T cells (Treg), but whether DCs can induce Teff to differentiate into Treg remains uncertain. Herein we addressed this question in OVA-asthmatic mice that were treated with DC10. OVA-presenting DC10 treatment maximally activated lung Treg in these animals at 3 wk post-treatment, as determined by upregulation of Treg-associated markers (ICOS, PD-1, GITR, LAG3 and CTLA-4) and in functional assays. This *in vitro* regulatory activity was ≥90% reduced by treatment with anti-IL-10 but not anti-TGF-β antibodies. In parallel cultures OVA-, but not house dust mite (HDM)-, presenting DC10 induced ≈43% of CFSE-labeled $CD25^{-/lo}Foxp3^-$ Teff cells from asthmatic OVA-TCR transgenic mice to differentiate into tolerogenic $CD25^{hi}Foxp3^+$ Treg. We recapitulated this *in vivo* using OVA-asthmatic mice that were co-injected with OVA- or HDM-presenting DC10 (i.p.) and CFSE-labeled $CD4^+CD25^{-/lo}Foxp3^-$ Teff cells (i.v.) from the lungs of asthmatic DO11.10 mice. From ≈7 to 21% of the activated (i.e., dividing) DO11.10 Teff that were recovered from the lungs, lung-draining LNs or spleens of the OVA-DC10 recipients had differentiated into $CD4^+CD25^{hi}Foxp3^+$ Treg, while no CFSE-positive Treg were recovered from the HDM-DC10-treated animals. These data indicate that DC10 treatments induce tolerance at least in part by inducing Teff to differentiate into $CD4^+CD25^{hi}Foxp3^+$ Treg.

4.2. Introduction

Multiple laboratories have reported on the tolerogenic activities of IL-10-differentiated DCs (DC10) in mouse models or *ex vivo* with human T cells (Steinbrink, Wolfl et al. 1997; Bellinghausen, Brand et al. 2001; Muller, Muller et al. 2002; Wakkach, Fournier et al. 2003; Koya, Matsuda et al. 2007; Perona-Wright, Anderton et al. 2007; Lau, Biester et al. 2008; Li, Yang et al. 2010). Thus, DC10 can protect against the development of OVA-induced asthma (Koya, Matsuda et al. 2007) or reverse the asthmatic phenotype in OVA (Nayyar, Zhang et al. 2004) or house dust mite allergen-(HDM) (Lu, Dawicki et al. 2011) sensitized mice, reducing AHR to methacholine, eosinophilia and Th2 responses to allergen challenge, and circulating levels of allergen-specific IgE and IgG1. DC10-mediated asthma tolerance is allergen-specific (Koya, Matsuda et al. 2007; Li, Yang et al. 2010) and IL-10-dependent (Koya, Matsuda et al. 2007). In our hands, tolerance is first discernible at 2 wk following i.p. delivery of allergen-presenting DC10 and by 3 wk the asthmatic animal's AHR disappears entirely. The Th2 reactivity of pulmonary T cells wanes progressively from 2 wk forward, such that by 8 months their responsiveness to recall allergen challenge *in vivo* is near background (Nayyar 2009). Four DC10 treatments at two week intervals bring the asthma phenotype to near background within 8 weeks (Nayyar 2009). Studies employing DCs that have been transfected with an IL-10-expressing lentivirus, and which thus express exceptionally high levels of IL-10, indicated that endogenous IL-10 expression (e.g., by T cells) is also critical to asthma tolerance in that model (Henry, Desmet et al. 2008). In inducing a robust asthma tolerance, treatments with these virally-transfected DC increase the numbers of $CD4^+CD25^+Foxp3^+$ cells present in the lung-draining (mediastinal) LNs (Henry, Desmet et al. 2008). This occurs also in the lungs and mediastinal LNs of DC10-

treated HDM-asthmatic mice, and adoptive transfer of pulmonary $CD4^+$ T cells from these mice into asthmatic recipients induces full HDM tolerance in the recipients (Lu, Dawicki et al. 2011).

Intestinal DCs that present innocuous environmental (e.g., commensal bacterial) antigens to naïve T cells in the mesenteric LNs induce their differentiation to $CD4^+CD25^+Foxp3^+$ Treg, and evidence indicates that expression of TGF-β is central to this process (Mowat 2003). Naïve T cells can be readily converted to a Treg phenotype by culture with either CTLA-4-Ig (Razmara, Hilliard et al. 2008) or TGF-β (Chen, Jin et al. 2003; Pyzik and Piccirillo 2007; Siewert, Lauer et al. 2008), but there has been no hard data to date regarding the conversion of antigen-experienced (i.e., effector) T cells (Teff). It has been reported that tuberculin purified protein derivative (PPD)-specific IFNγ-producing $CD4^+$ cell lines from PPD-sensitive donors become anergized after stimulation with immobilized anti-CD3 and begin to express high levels of Foxp3 mRNA (Vukmanovic-Stejic, Agius et al. 2008), but Foxp3 expression by itself does not confer activated regulatory cell status on $CD4^+CD25^+$ T cells (Strickland, Stumbles et al. 2006; Lu, Dawicki et al. 2011). Co-culture of specific allergen-presenting DC10 from atopic asthmatic donors with autologous peripheral blood Th2 phenotype cells also induces allergen-tolerance associated with the outgrowth of activated $CD4^+CD25^+Foxp3^+$ Treg (Li, Yang et al. 2010). Nevertheless, while DC10 induce asthma tolerance through activation of Treg, it is not known whether activated Teff cells differentiate into Treg in treated subjects. Herein we examined the mechanisms by which DC10 induce Tregs in asthmatic mice. Our data indicate that DC10 do activate Treg in the lungs of asthmatic mice and that these cells do differentiate from $CD4^+CD25^-Foxp3^-$ Teff.

4.3. Methods

4.3.1. Reagents and mice

Phycoerythrin-conjugated anti-mouse CD25, ICOS, PD-1, GITR, LAG3 and CTLA-4 Ab and a mouse regulatory T cell staining kit were purchased from eBioscience Inc (San Diego, CA). Mouse rGM-CSF and IL-10, and matched capture and detection Ab pairs and protein standards for our ELISAs were obtained from R & D Systems (Minneapolis, MN). Anti-mouse CD4 MACS beads and mouse $CD4^+CD25^+$ regulatory T cell isolation kits were purchased from Miltenyi Biotec (Auburn, CA). The lipid dye DiI was purchased from Molecular Probes (Carlsbad, CA). The sources of all other reagents have been reported previously (Gordon, Li et al. 2005; Li, Yang et al. 2010). Female BALB/c and C57BL/6 mice (6~8 week old) were purchased from Charles River Laboratories (Charles River, Maine). DO11.10 OVA-specific TCR-transgenic mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). C57BL/6 mice that expressed GFP under the control of the Foxp3 promoter were kindly provided through Dr. S. Rudensky (University of Washington) and bred in our institutional animal care unit. All mice were treated in accord with the guidelines of the Canadian Council for Animal Care.

4.3.2. Generation of tolerogenic dendritic cells

Bone marrow DCs were generated largely as reported previously (Inaba, Inaba et al. 1992; Manfred B. Lutz 2000), and differentiated to tolerogenic or immunostimulatory

phenotypes by further culture with 50 ng/ml of rmIL-10 (DC10) or 1 μg/ml E. coli serotype 0127:B8 LPS (DC-LPS), respectively. The cells were pulsed with 50 μg/ml OVA or irrelevant control house dust mite allergen (HDM) for 2 hr, then washed extensively before use. In our hands DC10 express low levels of costimulatory molecules (e.g., CD40, CD80) and MHC II compared to mature DCs, and secrete significantly higher levels of IL-10, but little TGF-β, relative to immature DCs (Nayyar 2009; Li, Yang et al. 2010).

4.3.3. Establishment of the asthma mouse model and DC10 treatments

OVA-specific asthma was induced in BALB/c, C57BL/6 or OVA-TCR-transgenic DO11.10 or GFP-Foxp3 transgenic mice by sensitization with OVA-alum and airway exposure to nebulized aerosols of 1% OVA, as noted in detail previously (Schneider, Li et al. 2001; Gordon, Li et al. 2005). The asthmatic mice were treated *i.p.* with 10^6 allergen-presenting DC10 two weeks after their last airway exposure to allergen, also as noted previously (Nayyar 2009).

4.3.4. Assessments of T cell regulatory activities

We used *in vitro* and *in vivo* approaches to assess the regulatory activities of $CD4^+CD25^+$ T cells recovered from the enzymatically-dispersed lung tissues (Schneider, Li et al. 2001) of asthmatic mice that had been treated with OVA-presenting DC10 1, 2, 3, or 4 week earlier. The putative Tregs were purified by positive-selection magnetic sorting from these or untreated normal mice and assessed in functional assays for regulatory activities.

Flow cytometry for expression of activation markers. We purified T cells from the lungs of asthmatic GFP-Foxp3 transgenic mice that had been treated 3 week earlier with 1×10^6 OVA- or HDM-presenting DC10 (*i.p.*). The cells were stained with PE-cy5-CD4 and PE-labeled specific Ab against CD25, ICOS, PD-1, GITR, LAG3 or CTLA-4, then the $CD4^+$ cells were gated and GFP^+ (i.e., $Foxp3^+$) cells were analyzed by FACS.

In vitro assessments. $CD4^+$ Th2 Teff for the assays were magnetically sorted from the lungs of untreated asthmatic donor mice. In preliminary experiments done in 96-well plates we titrated the numbers of Th2 cells and irradiated (3000 rads) OVA-pulsed DC-LPS required to optimally stimulated Th2 cell proliferation and Th2 cytokine secretion, and the impact of similarly irradiated putative Tregs on this response. Thus, for the experiments reported herein, we co-cultured Th2 cells (10^5 cells/well) with half-maximal numbers of OVA-presenting DC-LPS (3.7×10^3 cells/well) and putative Tregs (10^5 cells/well). In some assays, we added neutralizing anti-IL-10 (IL-10) or anti-TGF-β Ab (each, 10 μg/ml) to the cultures. After 2 day the $CD4^+$ Th2 cells' proliferative and cytokine (IL-4, -5, -9, and -13, and IFN-γ) responses were assessed using standard ^3H-thymidine uptake and ELISA assays, respectively.

In vivo assessments. We injected 10^6, 5×10^5, 2.5×10^5 or 1.25×10^5 Treg *i.v.* into untreated asthmatic recipient mice and four weeks later assessed the AHR of the mice. The following day we challenged them for 20 minute with aerosols of 1% nebulized OVA and 2 day later we euthanized them to assess their pulmonary immunoinflammatory responses as noted in detail (Schneider, Li et al. 2001). We did differential cell counts on their BAL leukocytes and assessed the BAL fluid levels of IL-4, -5, -9 and -13.

4.3.5. Measurement of AHR

AHR was assessed in conscious animals by head-out, whole body plethysmography, as noted in detail (Schneider, Li et al. 2001; Gordon, Li et al. 2005). Briefly, air was supplied to the body compartment of a plethysmograph via a small animal ventilator and changes in the airflow through the body compartment were monitored. Doubling doses of nebulized methacholine (0.75–25 mg/ml) were delivered to the head compartment and bronchoconstriction data were gathered as running 1-s means of the air-flow at the 50% point in the expiratory cycle (Flow@50%TVe1). This parameter accurately reflects bronchiolar constriction, as opposed to alveolar constriction or airway occlusion (Vijayaraghavan, Schaper et al. 1993; Vijayaraghavan, Schaper et al. 1994).

4.3.6. ELISA for airway Th2 cytokines

Our capture cytokine ELISA protocols have been reported in detail previously (Schneider, Li et al. 2001; Gordon, Li et al. 2005). BAL fluids were not diluted for the assays. Cytokine levels are presented as pg/ml based on recombinant protein standard curves; all assays were sensitive to 5-10 pg/ml.

4.3.7. Assays for conversion of $CD4^+CD25^-Foxp3^-$ T effector cells to $CD4^+CD25^+$ T cells

We assessed whether Treg could differentiate from $CD4^+CD25^-Foxp3^-$ Teff both *in vitro* and *in vivo*, using $CD25^-Foxp3^-$ cells purified by negative selection from the

pulmonary $CD4^+$ cells of asthmatic OVA-specific TCR-transgenic DO11.10 mice. These cells were stained with CSFE (2 μM; 20 minute at 37˚C).

In vitro assay. We co-cultured Teff (10^5/well) with OVA- or HDM (i.e., nonspecific allergen)-presenting DC10 or OVA-presenting immunostimulatory DC-LPS (3×10^4/well) in culture medium that was supplemented with IL-2 (10 U/ml) as a growth factor. After 5 days, cells were fixed and permeabilized, then stained with PE-Cy5-labeled anti-Foxp3 antibody. For the FACS analysis we gated on the CFSE-positive cells and assessed both proliferation and Foxp3 expression. We also assessed the *in vitro* regulatory activities of the cells arising in these cultures as noted above.

In vivo assay. We injected 5×10^6 CFSE-stained Teff from asthmatic DO11.10 donors *i.v*. into asthmatic BALB/c recipient mice and at the same time treated the recipients with 1×10^6 OVA- or HDM-presenting DC10 *i.p.*. Two weeks later, single cell suspensions generated from the lungs, mediastinal LNs (MLNs) and spleens of the recipients were stained with PE-Cy5 Foxp3 and PE-CD25 mAbs and analyzed by FACS, wherein we gated on the activated (i.e., dividing) $CSFE^+$ cells.

4.3.8. Statistics

All data are expressed as the mean ± SEM. Multi-group comparisons were assessed by ANOVA with *post-hoc* Fisher's LSD testing, while AHR to methacholine was assessed by linear regression analyses. Significance was established at $p\leq0.05$.

4.4. RESULTS

4.4.1. Pulmonary $CD4^+CD25^+Foxp3^+$ cells become activated following tolerogenic dendritic cell treatment of asthmatic mice.

Treatment of asthmatic mice with DC10 broadly ameliorates airway Th2 and eosinophil responses to allergen challenge within 4 week of treatment (Nayyar 2009). In both OVA and HDM models tolerogenic DC treatments augment the regulatory activities of $CD4^+$ T cells at this time (Henry, Desmet et al. 2008; Lu, Dawicki et al. 2011). However, when we assessed the numbers of $CD4^+CD25^+Foxp3^+$ cells in the lungs of saline or DC10-treated OVA-asthmatic mice 4 week following DC10 delivery, we found no differences in the proportions of these cells in the two groups (Figure 4-1A). Thus, $CD4^+CD25^+Foxp3^+$ cells comprised a mean of 7.07+/-0.88% of the pulmonary $CD4^+$ T cells in the saline-treated animals, and 8.24+/-1.41% in the DC10-treated animals. This agrees with findings reported in other models of tolerance (Lu, Dawicki et al. 2011). We further analyzed the $CD25^+Foxp3^+$ Treg cells in the MLNs (lung draining LN) from saline or DC10-treated OVA-asthmatic mice 4 week following DC10 delivery, and found no differences in the proportions of these cells between the two groups (data not shown).

Figure 4-1. DC10 treatment of asthmatic mice increases the regulatory activity of their pulmonary $CD4^+CD25^+$ cells.

Asthmatic BALB/c mice were treated with 1×10^6 specific allergen-presenting DC10 and, at varying times thereafter, we MACS-sorted $CD4^+CD25^+$ from their lungs. We assessed the abilities of these cells (10^5 cells/well) to regulate proliferation and cytokine expression by allergen-presenting immunostimulatory DC (DC-LPS)-activated Th2 effector (Teff) cells (3.7×10^4 and 10^5 cells/well, respectively) from asthmatic donor mice. (A) The lung mononuclear cells were analyzed by FACS for expression of CD25 and Foxp3, after gating on $CD4^+$ T cells, from DC10 treatment and saline control group. (B) FACS analysis of the freshly purified $CD4^+CD25^+$ and $CD4^+CD25^-$ cells after co-staining for Foxp3. (C) $CD4^+$ Th2 cells from untreated asthmatic mice and irradiated DC-LPS and were cultured alone (medium) or with irradiated pulmonary $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from asthmatic mice that had been treated with DC10 four weeks earlier. After 48 hour we assessed the Teff cell proliferative responses by ^{3}H-thymidine uptake assays as well as (D) the levels of IL-4, -5, -9, and -13 in the culture supernatants by ELISA (*$p<0.05$, n=3, comparing with Teff group). (E) We also added 10 μg/ml of neutralizing anti-IL-10 or -TGF-β antibodies to some wells in order to assess the contributions of these mediators to the putative $CD4^+CD25^+$ T cell regulatory functions (*$p<0.05$, n=3, comparing with Treg group). (F) To determine whether the kinetics of Treg activation in the lungs of the DC10-treated mice matched the *in vivo* observation of greater tolerance at 4 week (versus 2 or 3 week) post-treatment, we harvested $CD4^+CD25^+$ cells from mice at 1, 2, 3 or 4 weeks after DC10 treatment and assessed their regulatory activities. The putative regulatory cells we isolated from DC10-treated

asthmatic mice were near 70% $CD25^{+}Foxp3^{+}$, while the $CD4^{+}CD25^{-}$ cells were <5% positive for these markers. These cells displayed significant regulatory activity, inasmuch as they, but not $CD4^{+}CD25^{-}$ cells, reduced expression of each Th2 cytokine in the cultures. Anti-IL-10 antibodies eliminated the regulatory activity of the $CD25^{+}Foxp3^{+}$ cells, while the anti-TGF-β antibodies had no impact. Peak activation of these regulatory cells in DC10-treated mice occurred at 3 week post-treatment. One representative experiment of two is shown.

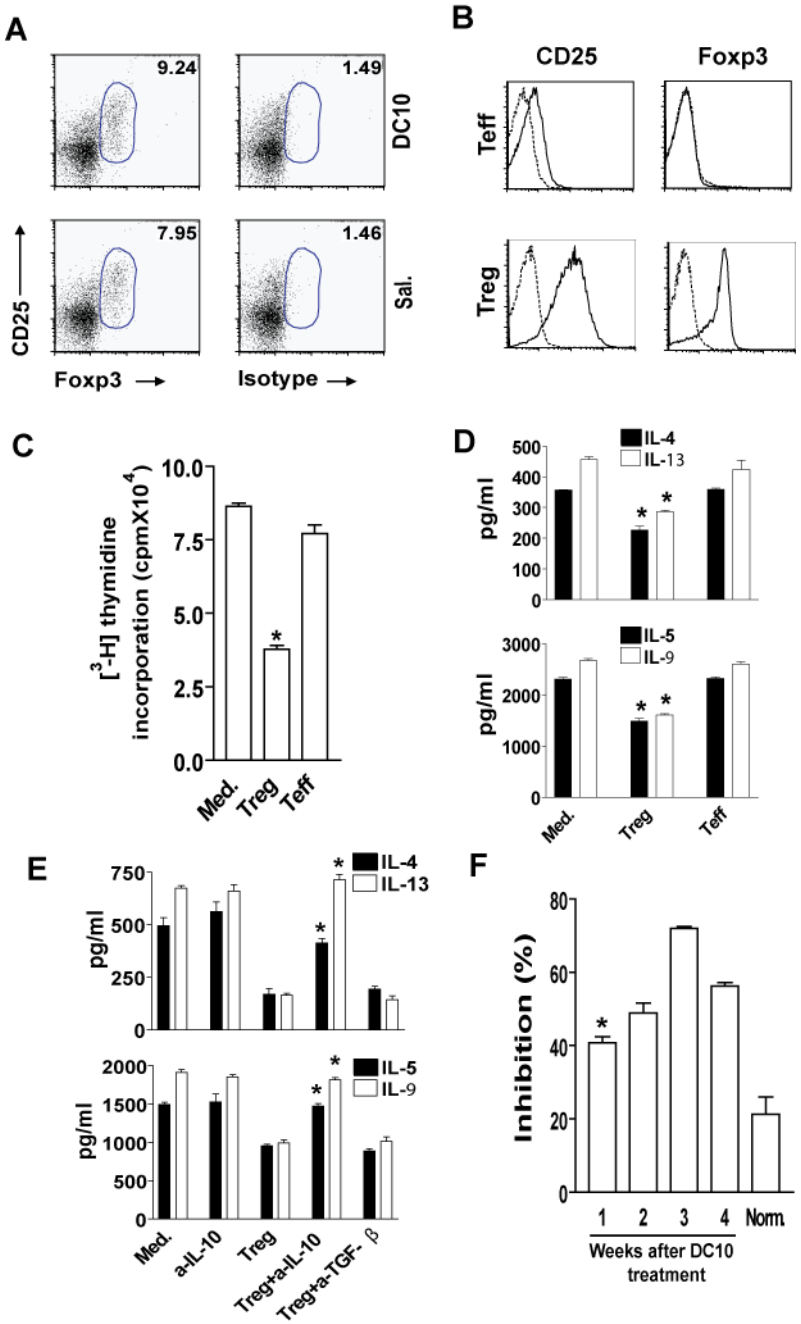

Figure 4-1. DC10 treatment of asthmatic mice increases the regulatory activity of their pulmonary $CD4^{+}CD25^{+}$ cells.

We MACS-sorted $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^-$ T cells from the lungs of asthmatic mice that had been treated with OVA-presenting DC10 4 wk earlier (Figure 4-1B) and assessed their abilities to suppress the activation of pulmonary Teff from asthmatic donors by OVA-presenting immunostimulatory DCs (DC-LPS). The $CD4^+CD25^+$ T cells from the lungs of the DC10-treated mice reduced proliferation of these Teff by 53.8+/-11.8% (Figure 4-1C) and reduced their expression of Th2 cytokines by 35-40% (Figure 4-1D). In order to determine whether the regulatory activity of these cells was dependent on their expression of IL-10 or TGF-β, we also tested the impact on this Th2 proliferative response of neutralizing antibodies. Anti-TGF-β antibody had no effect on T cell proliferation, but the anti-IL-10 antibodies eliminated the tolerogenic activities of the $CD4^+CD25^+Foxp3^+$ cells from the DC10-treated asthmatic mice (Figure 4-1E).

We next examined the kinetics of pulmonary Treg induction after DC10 treatment, purifying $CD4^+CD25^+Foxp3^+$ cells from the lungs of normal mice or asthmatic mice that had been treated with DC10 1-4 week earlier. We had previously found that DiI-stained DC10 traffic from the peritoneal cavities of recipient mice to the airways and lung-draining (mediastinal) LNs (Lu, Dawicki et al. 2011). Maximal accumulation of these cells in the mediastinal nodes occurs at 3 wk post-implantation, when up to 15% of the cells recovered from the nodes were signal-positive (Huang and Gordon, unpublished observation). In the present study we found that $CD4^+CD25^+$ T cells from the lungs of normal mice had modest regulatory activity in our *in vitro* assay, while pulmonary $CD4^+CD25^+$ T cells from the DC10-treated asthma phenotype mice were significantly more active (Figure 4-1F). At 1 week after DC10 delivery there was an ≈2-fold increase

in regulatory activity relative to the $CD4^+CD25^+$ T cells from normal mice and this activity continued to increase to a maximum (≈72% inhibition) at 3 week. Interestingly, despite the fact that tolerance in asthmatic mice is progressive well beyond 4 weeks (Nayyar and Gordon, unpublished), the activity of the purified 4-week Treg was diminished relative to that of 3-week Treg (Figure 4-1F).

To further assess the activation of Treg in our model, we used asthmatic mice that expressed green fluorescent protein (GFP) under the control of the Foxp3 promoter as a means of easily tracking Treg. Three weeks after treating these mice with OVA- or irrelevant allergen (i.e., HDM)-presenting DC10 and DC-OVA we examined the expression of a panel of regulatory cell surface markers (i.e., ICOS, PD-1, GITR, LAG3 and CTLA-4) on Treg recovered from their lungs and found that the OVA-pulsed DC10 treatments led to increased expression of a number of these (Figure 4-2). Thus, the proportions of $Foxp3^+$ cells expressing ICOS, PD-1, GITR and LAG3 were increased about 2 to 10 folds relative to the analogous cells from HDM-presenting DC10-treated and DC-OVA treated (i.e., non-tolerant) asthmatic mice. In keeping with our observation of no differences in the numbers of $CD4^+CD25^+$ cells in normal, asthmatic or tolerant mice (Figure 4-2), we did not observe any differences in CD25 expression in these mice.

Figure 4-2. $CD4^{+}CD25^{hi}$ cells from the lungs of DC10-treated asthmatic animals express augmented levels of regulatory T cell activation markers.

OVA-asthma was induced in GFP-Foxp3 transgenic mice as noted above for BALB/c mice. These mice were then treated with either OVA- or irrelevant allergen (house dust mite; HDM)-presenting DC10 (DC10-OVA and DC10-HDM, respectively) and non-tolerance DC-OVA in order to induce tolerance, then at 3 week post-treatment we purified T cells from the lungs of each mouse and stained them with PE-cy5-CD4 and PE-labeled antibodies against the Treg activation markers ICOS, PD-1, GITR, LAG3 or CTLA-4. It is readily apparent that the OVA-presenting DC10 upregulated expression of ICOS, PD1, GITR, and LAG3 on pulmonary $CD4^{+}CD25^{+}$ cells relative to their expression on $CD4^{+}CD25^{+}$ cells from the lungs of HDM-presenting DC10-treated and DC-OVA-treated mice. One representative experiment of three is shown.

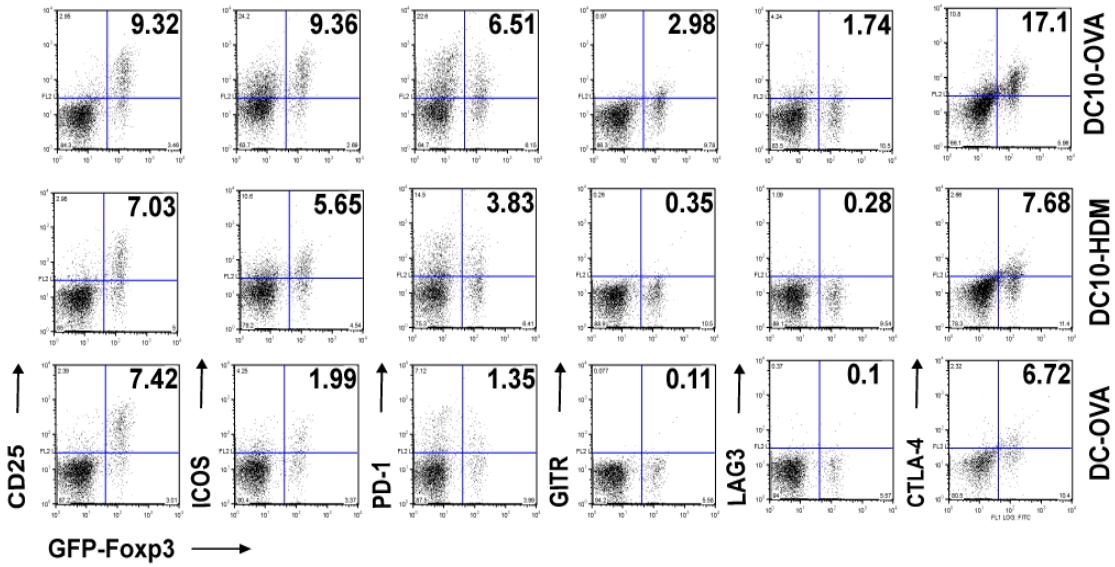

Figure 4-2. CD4^{+}CD25^{hi} cells from the lungs of DC10-treated asthmatic animals express augmented levels of regulatory T cell activation markers.

4.4.2. Passive transfer of tolerance with DC10-induced pulmonary $CD4^+CD25^+Foxp3^+$ cells.

We next sought confirmation that the $CD4^+CD25^+Foxp3^+$ cells from the lungs of DC10-treated mice could operate as regulatory cells *in vivo*. We titrated the numbers of Treg required to transfer tolerance, giving asthmatic mice either saline or 1.25 - 10×10^5 $CD4^+CD25^+$ cells (i.v.) from mice treated 3 week earlier with DC10. We assessed AHR in the recipients weekly thereafter, then administered a recall allergen challenge (20 min of nebulized 1% OVA aerosol) on day 28 and sacrificed the animals two days later. We assessed airway eosinophilic inflammation and Th2 cytokine responses, as determined by BAL fluid IL-4, -5, -9, and -13 levels. The AHR of the animals given 10^6 DC10-induced Treg was fully normalized by 21 days post-transfer (Figure 4-6) and remained so at 4 week (Figure 4-3A). When we transferred 5×10^5 3-week Treg, AHR was not discernibly affected at 2 or 3 week (Figure 4-6) but by 4 week it was significantly reduced ($p<0.05$ versus saline-treated asthmatic mice). Lower numbers of Treg were without effect on AHR, at least as of 4 week post-transplant (Figure 4-3A). Passive transfer of Treg also reduced airway eosinophilia and Th2 cytokine levels in a dose-dependent manner. Specifically, either 5×10^5 or 10^6 cells reduced the eosinophil responses to background, while 2.5×10^5 or fewer cells were without effect (Figure 4-3B; $p>0.05$ versus saline-treated asthmatic mice). In accord with this, Th2 responses were also amenable to Treg tolerance, such that delivery of 10^6 cells reduced the Th2 response to near background and 5×10^5 Treg reduced them very markedly ($p<0.05$, versus saline-treated asthmatic animals), whereas transfer of fewer cells was without discernible effect (Figure 4-3C).

For a more finely tuned *in vivo* confirmation of the kinetics for DC10-driven pulmonary Treg induction, we transferred a limiting number (5×10^5) of pulmonary Treg from mice treated 1-4 week earlier with DC10 and again assessed their impact on AHR and on Th2 cytokine and eosinophilic inflammatory responses to airway recall allergen challenge (Figure 4-7). When used at these limiting numbers, only the cells from animals treated 3 week earlier with DC10 were sufficiently activated to alter AHR or eosinophilia (for both, $p<0.05$ versus saline-treated asthmatic recipients) in the asthmatic recipients. The airway Th2 responses were slightly more amenable to regulation, such that 5×10^5 Treg from mice treated with DC10 either 3- or 4-week earlier significantly affected airway cytokine levels ($p<0.05$ for each cytokine versus saline-treated asthmatic recipients). The 1- and 2-week cells were ineffective in reducing airway Th2 cytokine expression ($p>0.05$ for each cytokine).

Figure 4-3. Passive transfer of $CD4^{+}CD25^{hi}$ Treg from DC10-treated animals reverses the asthma phenotype in asthmatic recipient mice.

$CD4^{+}CD25^{hi}$ cells were purified from the lungs of asthmatic mice treated 3 week earlier with DC10, as in Figure 4-1. We passively transferred 10^6, 5×10^5, 2.5×10^5 or 1.2×10^5 of these Treg into asthmatic recipients (i.v.), and 25 day later (A) assessed their AHR to aerosolized methacholine. The following day we challenged the animals for 20 min with nebulized aerosols of 1% OVA and 48 h later assessed their airway. The 10×10^5 Treg treatments nearly normalized AHR (relative to the normal saline controls) at 4 wk post-treatment. The AHR in the 5×10^5 Treg-treated mice was significantly different relative to the asthma phenotype mice (*$P<0.05$, n=5). (B) eosinophil and (C) Th2 cytokine responses to the recall allergen challenge. We observed reductions in each asthma parameter assessed, based on the numbers of Treg passively transferred. We found that Treg inhibited Th2 cytokine secretion in a dose-dependent manner and 5×10^5 Treg still significantly inhibited Th2 cytokine secretion in terms of IL-4, -5, -9 and -13 compared with the saline control group (*$p<0.05$, n=5). But transfer of $\leq2.5\times10^5$ Treg had no discernible impact on the asthma phenotype of the recipients. One representative experiment of two is shown.

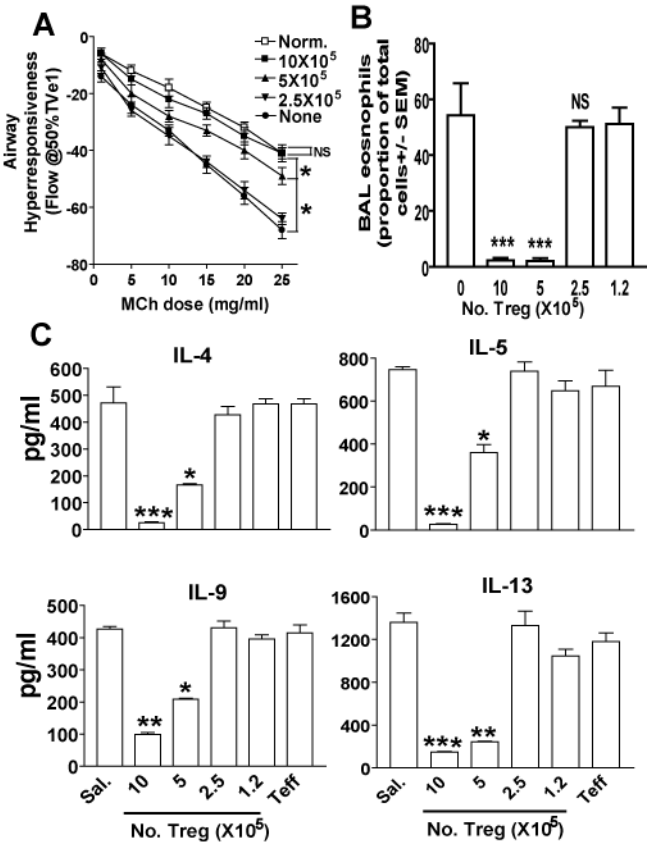

Figure 4-3. Passive transfer of $CD4^{+}CD25^{hi}$ Treg from DC10-treated animals reverses the asthma phenotype in asthmatic recipient mice.

4.4.3. DC10 induce the differentiation of $CD4^+CD25^-Foxp3^-$ Teff cells into $CD4^+$ $Foxp3^+$ Treg.

It has been reported that naïve T cells are readily converted to a regulatory phenotype when co-stimulated with CTLA-4-Ig (Razmara, Hilliard et al. 2008) or TGF-β (Chen, Jin et al. 2003; Pyzik and Piccirillo 2007; Siewert, Lauer et al. 2008). Our DC10 do not express significant levels of TGF-β relative to either immature or TNF-treated *in vitro*-differentiated DCs (Nayyar 2009), but our data indicates that they are clearly tolerogenic in asthmatic animals, wherein they appear to fully reverse Th2 Teff cell responses. Thus, we questioned whether they induce $CD25^-Foxp3^-$ Teff cells to differentiate into Treg. It has been shown that Teff cell lines generated from tuberculin PPD-challenge skin sites can be anergized *ex vivo* and that Foxp3 expression is upregulated in concert with this induction of anergy (Vukmanovic-Stejic, Agius et al. 2008) but, as far as we are aware, there have been no reports demonstrating that Treg can differentiate from antigen-experienced Teff cells. To test this directly, we set up an *in vitro* culture system in which $CD4^+CD25^-Foxp3^-$ Teff cells purified from the lungs of asthmatic OVA-TCR transgenic DO11.10 mice were stained with CSFE and co-cultured with specific (OVA) or irrelevant allergen (HDM)-presenting DC10 or OVA-presenting immunostimulatory cells (DC-LPS). After 5 day we analyzed the $CFSE^+$ cells from these cultures to assess their proliferation (CFSE dilution) and expression of Foxp3 (Figure 4-4A), but we also used magnetic sorting to purify the $CD25^+$ cells that were induced in these cultures and titrated their regulatory activities (Figure 4-4B). In the cultures containing irrelevant allergen-presenting DC10 we observed little if any proliferation of

the Teff cells and no expression of Foxp3 by the CFSE-labeled cells, and this makes sense based on the known allergen-specificity of DC10-induced tolerance (Koya, Matsuda et al. 2007; Li, Yang et al. 2010). The DC-LPS strongly induced Teff cell proliferation but not Foxp3 expression, but the bulk of the $CD4^+CD25^-Foxp3^-$ cells in the OVA-presenting DC10 co-cultures had proliferated and ≈45% of them expressed Foxp3 at high levels (Figure 4-4A). When we MACS-purified the induced $CD25^+$ cells back out of these cultures and titrated their activity *in vitro* we found them to be highly effective in dampening DC-LPS-induced Teff cell proliferation (Figure 4-4B; $p \leq 0.05$ versus $CD25^+$ T cells MACS purified from DC-LPS/ Teff cell cultures). These data confirmed that DC10 are fully capable of efficiently inducing $CD4^+CD25^-Foxp3^-$ Teff cells from asthmatic mice to differentiate into functional regulatory cells *in vitro* and that this response is fully dependent on cognate allergen presentation.

In order to confirm the *in vivo* relevance of this observation we also assessed whether delivery of DC10 to asthmatic mice would similarly induce Teff cells to differentiate into $CD25^+Foxp3^+$ Treg. We MACS-purified $CD4^+CD25^-Foxp3^-$ Teff cells from OVA-asthmatic DO11.10 mice, labeled them with CFSE, and injected them i.v. into asthmatic recipients. At the same time we injected the recipients ***i.p.*** with either OVA- or HDM-presenting wild-type DC10. Two weeks later we generated single cell suspensions from the lungs, mediastinal LNs and spleens of the treated mice, stained the cells with anti-Foxp3 and anti-CD25 antibodies and analyzed the cells by FACS, gating on the dividing (i.e., activated) $CFSE^+$ cells (Figure 4-5). We found negligible numbers of $CFSE^+CD25^+Foxp3^+$ T cells in the lungs (0.21%), lung-draining LNs (0.64%) or spleens (0.43%) of the animals we had treated with HDM-presenting DC10, but there were

Figure 4-4. Specific, but not irrelevant, allergen-presenting DC10 induce the differentiation of $CD4^+CD25^{-/lo}Foxp3^-$ Teff cells from asthmatic donors into $CD4^+Foxp3^+$ Treg *in vitro.*

OVA-asthma was induced in OVA TCR transgenic DO11.10 mice as noted above, then we purified $CD4^+CD25^{-/lo}Foxp3^-$ Teff cells from the lungs of these mice. (A) Teff from asthmatic DO11.10 mice were stained with CSFE and co-cultured with specific (OVA)- or irrelevant (HDM) allergen-presenting DC10 or OVA-presenting DC-LPS (10^5 Teff + 3×10^4 DC/well); 10 μg/ml IL-2 was added to the cultures as a growth factor. Five day later the cells in the cultures were analyzed by FACS for CFSE-dilution (Teff cell proliferation) and Foxp3 expression. (B) To confirm that the induced $CD4^+CD25^+Foxp3^+$ were functional as regulatory cells, we used magnetic sorting to purify the $CD25^+$ T cells from 5 day co-cultures of asthmatic DO11.10 mouse pulmonary Teff and OVA-DC10 or OVA-DC-LPS. These induced OTII $CD4^+CD25^+$ Treg (or control Teffs) were then irradiated (3000 rad) and added to cultures of OVA-presenting DC-LPS + Th2 Teff cells from asthmatic C57BL/6 mice. Th2 Teff cell proliferative responses were assessed as in Figure 4-1C. *$p<0.05$, n=4, comparison of proliferation between 10×10^4/well Treg and Teff group. One representative experiment of two is shown.

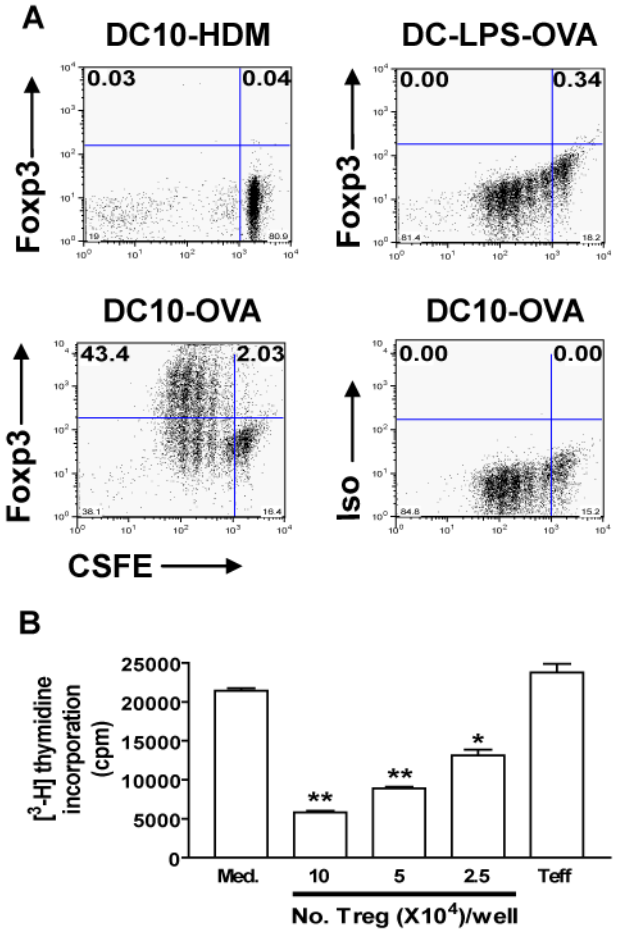

Figure 4-4. Specific, but not irrelevant, allergen-presenting DC10 induce the differentiation of $CD4^+CD25^{-/lo}Foxp3^-$ Teff cells from asthmatic donors into $CD4^+Foxp3^+$ Treg in vitro.

Figure 4-5. DC10-induced differentiation of $CD4^+CD25^{hi}Foxp3^+$ Treg from $CD4^+CD25^{-/lo}Foxp3^-$ T effector cells *in vivo*.

In order to confirm the *in vivo* relevance of DC10-driven conversion of $CD4^+CD25^{-/lo}Foxp3^-$ Teff cells into regulatory cells, we injected (*i.v.*) 5×10^6 CFSE-labeled $CD4^+CD25^-Foxp3^-$ T cells from OVA-asthmatic DO11.10 mice and 10^6 OVA- or HDM-presenting DC10 from wild-type BALB/c mice (*i.p.*) into asthmatic BALB/c recipients. After 2 week we prepared single cell suspensions from the lungs (upper panels), mediastinal LNs (middle panels) and spleens (lower panels), stained the cells for Foxp3 and CD25, and gated on the activated (i.e., dividing) $CFSE^+$ cells (left panels, boxed in cells) to assess the expression of these markers by FACS. There was no conversion of CFSE-labeled $CD4^+CD25^-Foxp3^-$ Teff cells into $CD25^+Foxp3^+$ regulatory cells in the mice treated with HDM-presenting DC10, while there was substantial differentiation of regulatory cells from the Teff cells in the OVA-presenting DC10-treated animals. One representative experiment of two is shown.

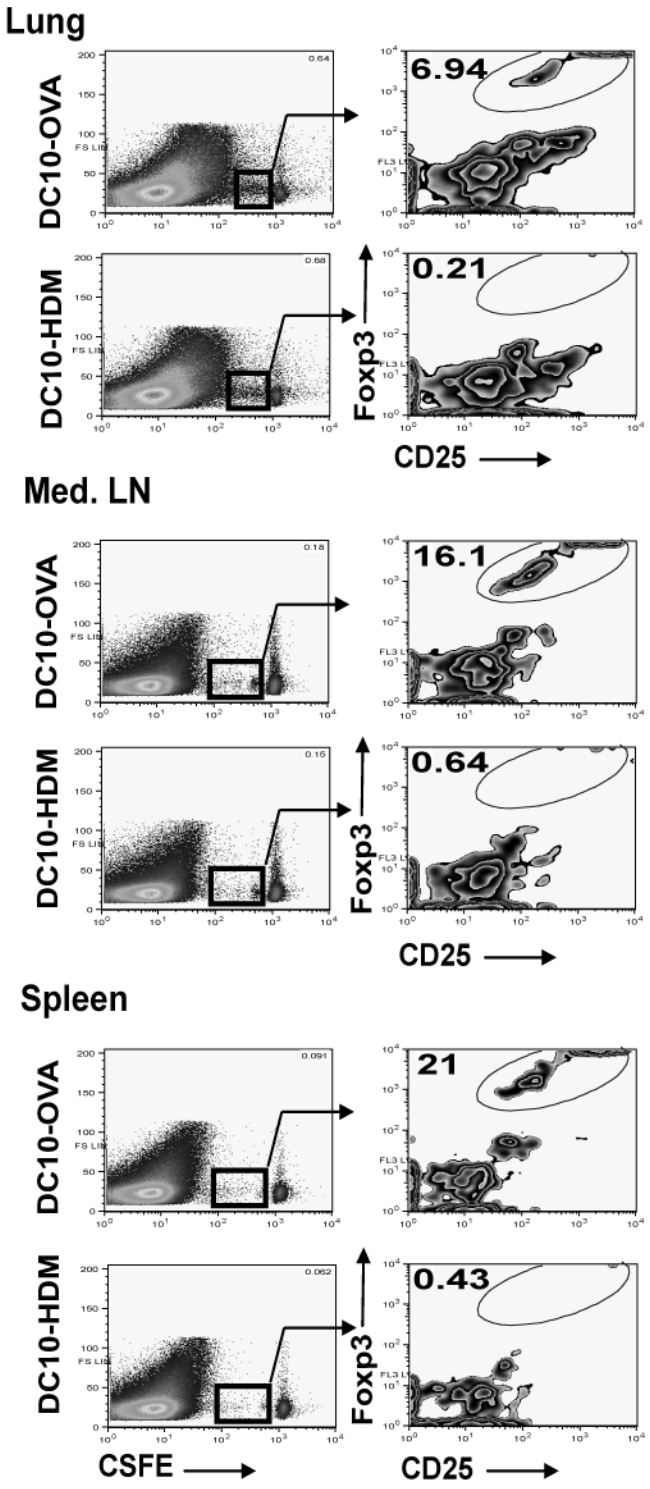

Figure 4-5. DC10-induced differentiation of $CD4^{+}CD25^{hi}Foxp3^{+}$ Treg from $CD4^{+}CD25^{-/lo}Foxp3^{-}$ T effector cells *in vivo*.

Figure 4-6. Kinetics with which airway hyperresponsiveness is reduced in asthmatic animals given varying numbers of $CD4^+CD25^+$ Treg from DC10-treated animals.

Varying numbers of $CD4^+CD25^+$ cells from the lungs of asthmatic mice treated 3 week earlier with DC10 were passively transferred into asthmatic recipients as in Figure 4-3. At 1, 2, and 3 wk post-transfer, the animals' AHR to aerosolized methacholine was assessed by head-out whole-body plethysmography. Transfer of 10^6 Treg treatments nearly normalized AHR (relative to the normal saline controls) at 2 wk post-treatment, while transfer of $\leq 5\times10^5$ Treg had no significant impact on the AHR of the recipients at 1, 2 or 3 wk. One representative experiment of two is shown.

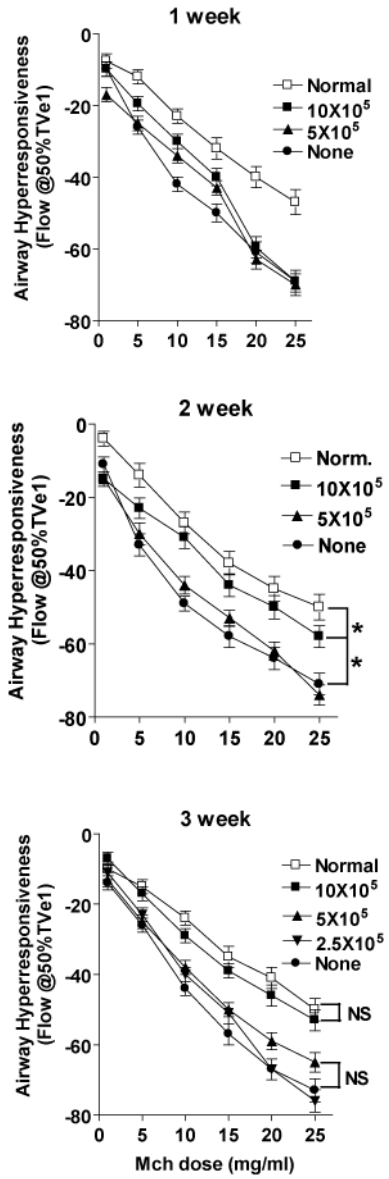

Figure 4-6. Kinetics with which airway hyperresponsiveness is reduced in asthmatic animals given varying numbers of $CD4^+CD25^+$ Treg from DC10-treated animals.

Figure 4-7. *In vivo* confirmation of the induction kinetics for the regulatory activities of pulmonary Treg from DC10-treated asthmatic mice.

In order to confirm *in vivo* the activation of the $CD4^+CD25^+Foxp3^+$ cells induced by DC10 treatment of asthmatic mice, we passively transferred limiting numbers of these (3 week) Treg into asthmatic recipients (5×10^5 per mouse), then assessed their asthma phenotype as in Figure 4-3. (A) AHR of the recipients to methacholine as determined at day 25 after treatment. At 48 hour after recall allergen challenge we assessed (B) eosinophilic airway inflammation and (C) BAL IL-4, -5, -9 and -13 levels. When used at limiting numbers, it was readily apparent that the pulmonary Treg were maximally activated at 3-week post DC10-treatment. (*$P<0.05$ *vs.* saline group, n=5). One representative experiment of two is shown.

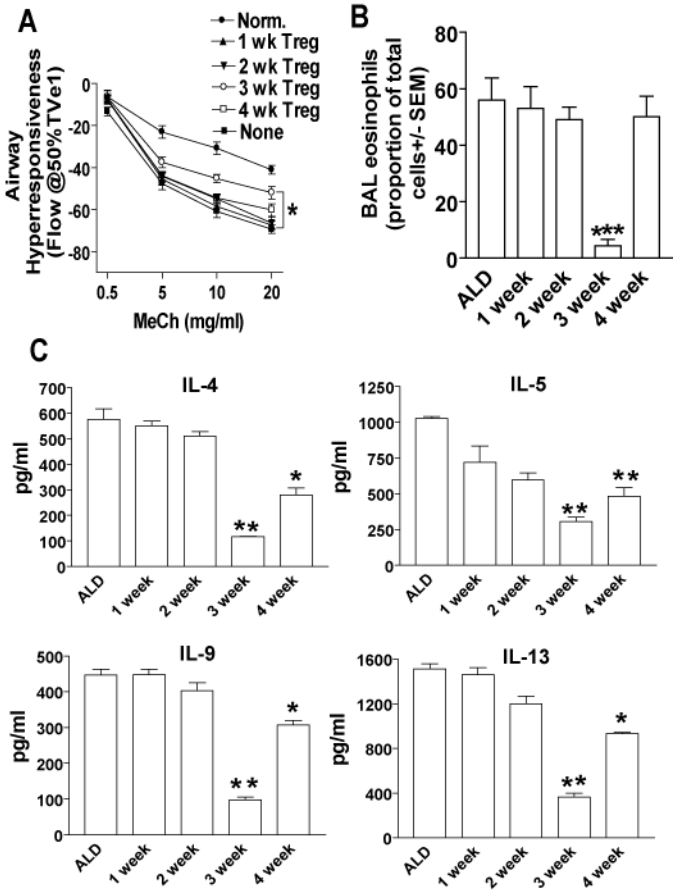

Figure 4-7. *In vivo* confirmation of the induction kinetics for the regulatory activities of pulmonary Treg from DC10-treated asthmatic mice.

significant numbers of activated $CFSE^{+}CD25^{+}Foxp3^{+}$ cells in the asthmatic mice that had been treated with specific allergen-presenting DC10. We found that 7, 16, and 21% of the proliferating $CFSE^{+}$ cells recovered from the lungs, mediastinal LNs, and spleens, respectively, were now $CD25^{+}Foxp3^{+}$ (Figure 4-5). Taken together, our *in vitro* and *in vivo* data indicated that during DC10-mediated induction of allergen-tolerance in asthmatic mice, $CD4^{+}CD25^{-}Foxp3^{-}$ Teff cells do differentiate into $CD4^{+}CD25^{+}Foxp3^{+}$ Treg.

4.5. DISCUSSION

Multiple labs have reported that $CD4^+$ cells take on regulatory activities with tolerance induction in asthma (Strickland, Stumbles et al. 2006; Henry, Desmet et al. 2008; Li, Yang et al. 2010; Lu, Dawicki et al. 2011), but the mechanisms by which this occurs had not been defined. We documented herein that in inducing tolerance in asthmatic mice, DC10 also induce $CD4^+CD25^-Foxp3^-$ Th2 effector cells (Teff) to differentiate into $CD4^+CD25^+Foxp3^+$ Treg. The kinetics with which these cells were activated in the lung correlated very well with the acquisition of asthma tolerance in mice model (Nayyar 2009; Lu, Dawicki et al. 2011). Nevertheless, while peak Treg activation occurred at 3 week after the DC10 treatment, we have found that tolerance induced by a single DC10 treatment is progressive over many months in mice model (Nayyar 2009). This suggests that alternate mechanisms supplant the pulmonary Treg-dependent tolerance that sets in over the first few weeks following DC10 treatment, perhaps in the context of infectious processes that incorporate regulatory DCs and/or alternate regulatory T cell populations (Mahnke, Johnson et al. 2007). For example, Treg can induce myeloid DCs to adopt a regulatory phenotype (Misra, Bayry et al. 2004; Houot, Perrot et al. 2006), while DCs can reciprocally express substantial control over Treg populations (Darrasse-Jeze, Deroubaix et al. 2009).

It had been shown previously that gut lamina propria or mesenteric LN, but not splenic, DCs can induce naïve T cells to differentiate to a regulatory phenotype without need for exogenous input (i.e., TGF-β) (Yamazaki, Bonito et al. 2007) and this would be appropriate in a compartment routinely presented with commensal (i.e., non-pathogenic) bacteria (Coombes and Powrie 2008). In the lungs, which are also under constant exposure to innocuous foreign antigens (e.g., pollens), pulmonary DCs that present such

antigens to naïve T cells express a semi-mature phenotype and express IL-10 and thereby induce regulatory T cell responses that prevent development of pathogenic (e.g., allergic) responses (Akbari, DeKruyff et al. 2001). The fact that we generated Teff cell pools from the lungs of fully symptomatic asthmatic mice indicates that these cells would have been educated Teff cells, as opposed to naïve T cells. Thus, our data showing that DC10 induced the differentiation of these Teff cells into $CD4^+CD25^+Fopxp3^+$ Treg provides our first clear documentation that Teff cells are amenable to such a phenotypic changes. We have shown previously that specific allergen-presenting DC10 generated from $CD14^+$ monocytes of asthmatic individuals similarly induce the outgrowth of $CD25^+Foxp3^+LAG3^+$ CTLA-4^+ Treg from autologous Th2 Teff cell populations. Those Treg subsequently suppressed autologous Teff responses in a contact-dependent manner (Li, Yang et al. 2010), as reportedly occurs with Treg in other model systems (Stephens, Andersson et al. 2007; Akdis and Akdis 2009). However, in the mouse model we employed herein our DC10-induced regulatory activity was dependent on expression of IL-10 by the T cells, a characteristic consistent with a Tr1-like phenotype regulatory cell. Others have reported that IL-10-treated DCs induce anergy among antigen-specific T cells in part via CTLA-4 (i.e., contact-dependent regulation)(Steinbrink, Graulich et al. 2002). Nevertheless, IL-10 expression by both the treatment DC10 (Koya, Matsuda et al. 2007) and endogenous host cells(Henry, Desmet et al. 2008) has been implicated in tolerance induction in asthmatic mice.

Interestingly, while ectopic expression of Foxp3 alone is reportedly sufficient to turn Teff cells into Treg (Hawrylowicz and O'Garra 2005; Randolph and Fathman 2006; Stock, DeKruyff et al. 2006; Toda and Piccirillo 2006), there were equivalent numbers of

$CD4^+CD25^+Foxp3^+$ cells in the lungs of asthmatic mice irrespective of whether they were fully asthmatic or their pulmonary $CD4^+CD25^+Foxp3^+$ cells were activated and expressed a regulatory phenotype. And, while DC10 induce Th2 Teff from atopic asthmatic individuals to take on an activated Treg phenotype (i.e., $CD4^+CD25^+Foxp3^+LAG3^+CTLA4^+$ IL-10-secreting T cells), the cells in these cultures do not express increased levels of Foxp3 relative to those in cultures containing immunostimulatory DC-activated Teff cells (Li, Yang et al. 2010). A similar observation has been made with $CD4^+CD25^+Foxp3^+$ cells from asthmatic rats versus those rendered allergen-tolerant by chronic airway exposure to allergen (Strickland, Stumbles et al. 2006). This provides further evidence that Foxp3 expression by itself is not sufficient for optimal induction of a regulatory phenotype in $CD4^+CD25^+$ cells. The fact that the numbers of pulmonary $CD4^+CD25^+Foxp3^+$ cells were equivalent in our asthmatic and tolerant animals despite the observation that sizable numbers of Teff had ostensibly converted to activated Treg in the latter group, suggests that Treg homeostatic control mechanisms (Almeida, Rocha et al. 2005) were operative in their lungs. It is recognized that DCs and Treg populations control one another in a reciprocal homeostatic fashion (Darrasse-Jeze, Deroubaix et al. 2009) and this raises the question of whether the tolerogenic DCs we introduced into asthmatic animals may exercise homeostatic control over lung Treg numbers. Perhaps the $CD4^+CD25^+Foxp3^+$ cells that were present in the asthmatic lung prior to tolerance induction represent a subpopulation of cells that are uniquely susceptible to apoptotic (Yolcu, Ash et al. 2008) or other control mechanisms.

Regulatory T cells play important roles in maintaining the balance between protective and pathogenic immune responses (Lewkowich, Herman et al. 2005;

Strickland, Stumbles et al. 2006). To date, both naturally-occurring thymic $CD4^+CD25^+$ Treg (nTreg) and inducible regulatory T cells have been recognized, with the latter cells including IL-10- and TGF-β-secreting Tr1 and Th3 cells, respectively (Xystrakis, Urry et al. 2007). Naturally-occurring Treg constitute 1-5% of the $CD4^+$ T cells in healthy adult mice and humans, but regulatory cells with similar surface markers and functions can also be induced in the periphery. These cells can be isolated from mice and humans based on their high-level expression of CD25 (the IL-2 receptor α-chain). Other markers that were originally thought to be specific for nTreg include CTLA-4 and GITR but, like CD25, they are also expressed by activated T cells. Our data indicated that DC10 increased the expression of ICOS, PD-1, GITR, LAG3 and CTLA-4 with the acquisition of the regulatory phenotype, and this fits well with observations by others (Piccirillo and Thornton 2004).

In summary, our data supports the novel observation that these cells induced $CD4^+CD25^-Foxp3^-$ Teff cells to differentiate into $CD4^+CD25^+Foxp3^+$ Treg cells. We did not assess whether they also directly activated or induced proliferation of pre-existing $CD4^+CD25^+Foxp3^+$ T cells, but these cells can indeed proliferate strongly and particularly so under the influence of IL-2 (Curotto de Lafaille and Lafaille 2009). We also did not rigorously investigate the cellular interactions between the DC10 and Teff cells in asthmatic animals, but we know that these populations do engage one another intimately and in an antigen-specific fashion (Li, Yang et al. 2010; Lu, Dawicki et al. 2011). These will be important issues to address in the future.

CHAPTER 5: COMPARISON OF INDUCED VERSUS NATURAL REGULATORY T CELLS OF THE SAME TCR SPECIFICITY FOR INDUCTION OF TOLERANCE TO AN ENVIRONMENTAL ANTIGEN[1]

Running title: Induced versus natural Treg

Key words: IL-10-differentiated dendritic cells; regulatory T cell; induced $CD25^{+}Foxp3^{+}$ regulatory T cells; naturally-occurring $CD25^{+}Foxp3^{+}$ regulatory T cells; asthma

[1]This Chapter has been invited for revision by The Journal of Immunology for publication.

Huang, H., W. Dawicki, X. Zhang, and J. R. Gordon. 2012. Comparison of Induced versus Natural Regulatory T Cells of the Same TCR Specificity for Induction of Tolerance to an Environmental Antigen. Submitted.

Contribution: J.R.G. and H.H. designed the research; X.Zh. did animal model establishment, cytospin, antibody ELISA and partial selection of double or triple positive transgenic mice; H.H performed all other experiments; J.R.G. and H.H. analyzed the results; J.R.G., H.H. and W.D. contributed to the writing of the manuscript; H.H wrote the first draft.

5.1. Abstract

Recent evidence shows that natural regulatory T cells (nTreg) and induced Treg (iTreg) cooperate in mediating tolerance in colitis models, but they do so also in models of asthma. However, there is little evidence regarding their relative contributions to such tolerance. We compared the abilities of nTreg versus iTreg, both from OVA-TCR transgenic OTII mice, to mediate tolerance in OVA-asthmatic C57BL/6 mice. The iTreg were differentiated from Th2 effector T cells (Teff) by exposure to IL-10-differentiated dendritic cells (DC10) *in vitro* or *in vivo*, while we purified nTreg from allergen-naïve mice, but also exposed them to DC10 before use. Each Treg population was subsequently re-purified and tested for its therapeutic efficacy *in vitro* and *in vivo*. DC10 engaged the nTreg in a cognate fashion in FRET assays, and these nTreg reduced *in vitro* OVA-asthmatic Th2 Teff responses by 41-56%, while the comparator iTreg reduced these responses by 72-86%. Neutralization of IL-10, but not TGFβ, eliminated the suppressive activities of iTreg, but not the nTreg. Delivery of $5x10^5$ purified nTreg reduced allergen challenge-induced airway IL-4 ($p \leq 0.03$) and IL-5 ($p \leq 0.001$) responses of asthmatic recipients by ≤23%, but did not affect airway hyperresponsiveness (AHR) or IgE levels, while equal numbers of iTreg of the same TCR specificity reduced all airway responses to allergen challenge by 82-96% ($p \leq 0.001$) and normalized AHR. These data confirm that allergen-specific iTreg and nTreg have active roles in asthma tolerance, but also that iTreg are substantially more tolerogenic in this setting.

5.2. Introduction

The extent to which naturally-occurring regulatory T cells (nTreg) and induced Treg (iTreg) have non-redundant versus complementary roles in immune tolerance is not clear (Bilate and Lafaille 2011). For example, iTreg alone may be sufficient to induce tolerance in a chronic model of pulmonary inflammation (Curotto de Lafaille, Kutchukhidze et al. 2008), but in a mouse model of colitis both nTreg and iTreg are required to induce full disease tolerance (Haribhai, Williams et al. 2011). The observations regarding largely disparate TCR beta chain specificities within these two populations notwithstanding, it is clear that at least in some settings deployment of both nTreg and iTreg is required for the full expression of tolerance (Haribhai, Williams et al. 2011). This contrasts with the popular notion that the *raison d'etre* for nTreg is to prevent the development of autoimmune disease (Takahashi, Tagami et al. 2000) while that of iTreg is to reduce the activation of T cells directed against innocuous environmental antigens (Bilate and Lafaille 2011). Additional observations that suggest a greater degree of complexity in the roles of nTreg and iTreg, at least in the context of environmental antigens, include the report that depletion of $CD25^+$ T cells in naive wild-type mice leads to augmented asthma severity following subsequent allergen sensitization (Lewkowich, Herman et al. 2005; Joetham, Takeda et al. 2007). Moreover, passive transfer of $CD25^+Foxp3^+$ T cells from naïve mice into asthmatic recipient animals reduces their asthma severity if the transfers are performed within 3-4 weeks of asthma induction (Kearley, Robinson et al. 2008).

The recognition that Treg are potently immunosuppressive has galvanized efforts to use these cells therapeutically (Horwitz, Zheng et al. 2004; Riley, June et al. 2009). Passive transfer of Treg that populate the periphery has been shown to suppress pathology

in, for example, experimental models of autoimmune disease (Mottet, Uhlig et al. 2003; Wright, Ehrenstein et al. 2011). One unanswered question regarding clinical application of this approach is which population of Treg (i.e., nTreg versus iTreg) would be best employed in any specific setting (Roncarolo and Battaglia 2007; Newell, Phippard et al. 2011). There has been speculation that, overall, iTreg would be therapeutically superior to nTreg in settings other than autoimmunity because iTreg would be expected to target antigen-specific T cells with greater efficiency, but there is a lack of compelling evidence to support this proposal. It was recently reported that, in mouse models of human skin transplantation, iTreg but not nTreg treatment led to modestly reduced expression of two of three inflammatory markers in one model, although no such effect was observed in the second model and transplant rejection was not assessed (Sagoo, Ali et al. 2011). This leaves open the question of just how comparable nTreg and iTreg truly are in terms of their abilities to ameliorate pathology.

Herein we compared the abilities of nTreg and iTreg of the same antigen specificity to suppress allergic Th2 responses *in vitro* and to alter the asthma phenotype in a mouse model of firmly-entrenched OVA-asthma. In both cases the Treg were derived from OVA TCR transgenic OT2 mice and were exposed either *in vitro* or *in vivo* to OVA-presenting IL-10-differentiated DCs (DC10). We have shown previously that such DC10 can efficiently induce Teff cells to differentiate into $CD4^+CD25^+Foxp3^+$ iTreg, but also that 5×10^5 unfractionated Treg (i.e., iTreg plus nTreg) recovered from the lungs of asthmatic mice rendered allergen-tolerant by treatment with DC10 can in turn reduce the asthma phenotype by about ≈50% in asthmatic passive transfer recipients (Huang, Dawicki et al. 2010). Herein we show that passive transfer of 5×10^5 iTreg from DC10-

treated asthmatic OT2 mice can suppress all aspects of the asthma phenotype, while equal numbers of DC10-stimulated OT2 mouse nTreg that are recovered from asthmatic recipient mice have only a modest impact in this model.

5.3. Materials and methods

5.3.1. Mice and the asthma model.

Female 6-8 week old C57BL/6 (B6) mice were purchased from Charles River Laboratories (Charles River, ME). B6.CD45.1 congenic and OT2 OVA-specific TCR-transgenic mice were obtained from the Jackson Laboratory (Bar Harbor, ME. Foxp3-GFP knock-in mice were kindly provided through Dr. S. Rudensky (University of Washington). Double-positive GFP-Foxp3/B6.CD45.1 mice were generated by crossing GFP-Foxp3 and B6.CD45.1 mice. Triple-positive GFP-Foxp3/B6.CD45.1/OT2 mice were generated by crossing double-positive GFP-Foxp3/B6.CD45.1 and OT2 mice. The GFP-Foxp3/B6.CD45.2/OT2 mice were generated by crossing GFP-Foxp3 and OT2 mice. The B6.CD45.1/OT2 mice were generated by crossing B6.CD45.1 and OT2 mice. The phenotypes of offspring mice were confirmed by flow cytometry analyses in which peripheral blood leukocytes from all mice were stained with anti-mouse CD45.1 and/or Vβ5.1,5.2 TCR antibodies (Appendix-1).

Asthma was induced in the mice by two i.p. injections (days 0 and 14) of 2 mg OVA/alum, followed by 20-min exposures (day 28, 30, and 32) to nebulized aerosols of 1% OVA in saline (Nayyar, Dawicki et al. 2012). DC10 and Treg treatments were initiated two weeks later (dy 46). All mice were bred and housed in our institutional animal care unit and used in accord with the guidelines of the Canadian Council for Animal Care.

5.3.2. Reagents.

PE-conjugated anti-mouse CD25, ICOS, PD-1, GITR, CTLA-4 and LAG3 antibodies (Abs) and mouse Treg cell staining kits were purchased from eBioscience, Inc (San Diego, CA). Recombinant mouse IL-4 and IL-5 and matched capture and detection antibody pairs and protein standards for our ELISAs and neutralizing anti-TGF-β Ab were obtained from R & D Systems (Minneapolis, MN). The neutralizing anti-IL-10 Ab was gift from Sigma-Aldrich (Mississauga, ON). Anti-mouse CD4 MACS beads and mouse $CD4^+CD25^+$ Treg cell isolation kits were purchased from Miltenyi Biotec (Auburn, CA). Bead-free mouse $CD4^+CD25^+$ regulatory T cell isolation kits were purchased from Invitrogen (Burlington, ON). The sources of all other reagents have been reported previously (McLellan, Kapp et al. ; Gordon, Li et al. 2005; Huang, Dawicki et al. 2010).

5.3.3. Generation and comparison of *in vitro*-induced iTreg and nTreg cells.

$CD4^+$ $CD25^-$ $Foxp3^-$ Teff cells and $CD4^+$ $CD25^+$ $Foxp3^+$ Treg were purified from asthmatic and naïve OT2 mice by negative- and positive-selection magnet sorting, respectively; these Teff cells have been previously reported as $CD4^+CD25^{lo}CD44^{hi}CD69^+CD62L^{lo}Foxp3^-$ (Huang, Dawicki et al. 2010; Schallenberg, Tsai et al. 2010). The purified Teff and Treg cells (10^5 cells/well) were co-cultured with DC10 (3×10^4 cells/well) in U-bottom 96-well plates for five days, then the $CD4^+CD25^+$ iTreg and DC10-exposed $CD4^+CD25^+$ nTreg were positively selected from their respective cultures by magnetic sorting. Both populations of cells were stained with PE-labelled antibodies against the Treg markers ICOS, PD-1, GITR, LAG3 and CTLA-4 to determine their relative expression. The 5-day-cultured iTreg and nTreg (10^5 cells/well)

cells were also co-cultured for 48 h with LPS-activated immunostimulatory OVA-presenting DCs (DC-LPS; 3.7×10^3 cells/well) and effector Th2 cells (10^5 cells/well) from asthmatic mice (Huang, Dawicki et al. 2010). The cell cultures were pulsed with ^{3}H-thymidine during the last 18 hr and thymidine incorporation was determined by liquid scintillation counting. Type 2 cytokines (IL-4, -5) were assessed in the cultural supernatant by ELISA. In some assays, we added neutralizing anti-IL-10 or anti-TGF-β Ab (each, 10 μg/ml) to the cultures.

5.3.4. Generation and comparison of *in vivo*-induced Treg and nTreg.

FACS Analysis. $CD4^+CD25^-Foxp3^-$ Teff and $CD4^+CD25^+Foxp3^+$ Treg were purified from asthmatic GFP-Foxp3/B6.CD45.1/OT2 and naïve GFP-Foxp3/B6.CD45.2/OT2 mice, respectively. Both Teff and Treg (5×10^6 cell each) were injected *i.v.* into asthmatic B6.CD45.2 recipient mice, which were also treated at the time of passive transfer with $1x10^6$ B6.$CD45.2^+$ DC10 (*i.p.*). CD45.1 and GFP were used as markers for transplanted $CD45.1^+$ Teff that had converted to an iTreg phenotype (i.e., $CD45.1^+GFP^+$ cells), and as markers for the transplanted nTreg or their progeny (i.e., $CD45.1^-GFP^+$ cells). One wk after the injection, $CD4^+$ T cells were purified from the spleens of the DC10-treated recipients and stained with PE-cy5-CD45.1 and PE-labeled antibodies against the indicated Treg markers and analyzed by flow cytometry. $CD45.1^+GFP^+$ (iTreg) and $CD45.1^-GFP^+$ (nTreg) cells were gated and evaluated for the expression of ICOS, PD-1, GITR and LAG3 (Figure 5-1).

Figure 5-1. Protocol to compare Treg-associated marker expression on iTreg and nTreg cells in DC10-treated asthmatic mice.

$CD4^+CD25^-Foxp3^-$Teff and $CD4^+CD25^+Foxp3^+$Treg cells were purified from asthmatic GFP-Foxp3/B6.CD45.1/OT2 and naïve GFP-Foxp3/B6.CD45.2/OT2 mice, respectively. Both Teff and Treg cells were injected *i.v.* into asthmatic B6.CD45.2 mice along with DC10 (i.p.). One week after the injections, $CD4^+$ T cells were purified from the recipients'spleens and stained with PE-cy5-CD45.1 and PE-labeled antibodies against the Treg cell markers ICOS, PD-1, GITR, LAG3 and CTLA-4. $CD45.1^+GFP^+$ (iTreg) and $CD45.1^-GFP^+$ (nTreg) cells were gated and the expression of ICOS, PD-1, GITR, LAG3 and CTLA-4 was analyzed by FACS.

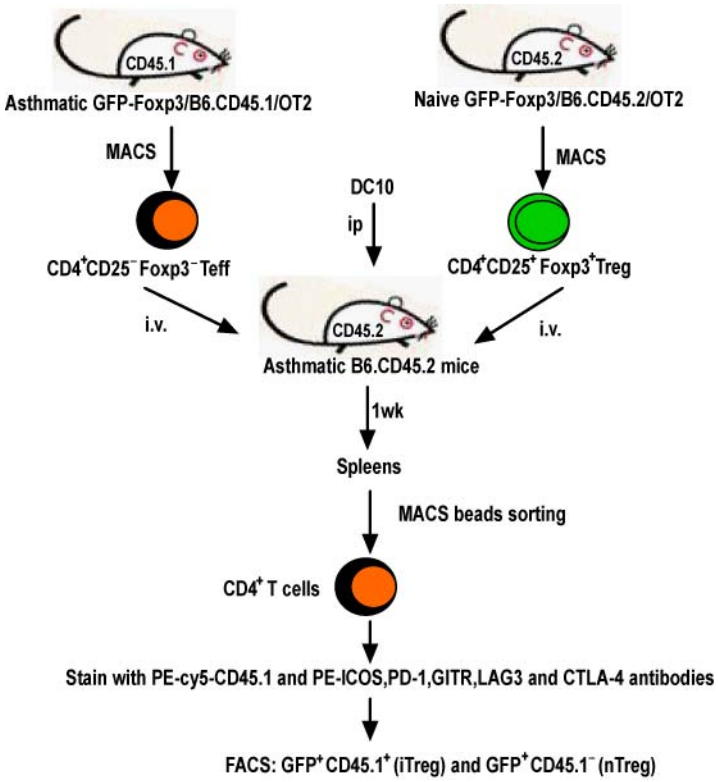

Figure 5-1. Protocol to compare Treg-associated marker expression on iTreg and nTreg cells in DC10-treated asthmatic mice.

Assessment of T cell regulatory activities. $CD4^+CD25^-Foxp3^-$ Teff and $CD4^+CD25^+Foxp3^+$ nTreg were selected from asthmatic and naïve B6.CD45.1/OT2 mice, respectively, using magnetic sorting, then injected i.v. into different groups of asthmatic B6.CD45.2 recipient mice (5×10^6 cell/mouse) that were simultaneously treated i.p. with 10^6 OVA-presenting $CD45.2^+$ DC10. CD45 was used as a marker for the donor-origin (i.e., $CD45.1^+$) Teff and nTreg cells, versus the endogenous $CD45.1^-$ Treg of the recipients. One week later, $CD4^+CD25^+$ Treg cells were purified from each group of mice by magnetic sorting, then labelled for further sorting with PE-CD45.1 antibody and anti-PE-magnet beads. The iTreg and nTreg cells were purified from their respective populations of mice and their abilities to inhibit Th2 cell activation were assayed as above.

In vivo comparison of iTreg and nTreg cells. We injected 5×10^5 *in vitro*-induced iTreg and nTreg cells, generated as noted (Huang, Dawicki et al. 2010), into otherwise untreated asthmatic recipient mice (i.v.).

Airway hyperresponsiveness (AHR). Four weeks later we assessed the airway responses of the mice to doubling doses of nebulized methacholine (0.5-20 mg/ml) using by head-out, whole body plethysmography (Schneider, Li et al. 2001; Lu, Dawicki et al. 2011). This parameter, gathered as running 1-s means of the air-flow at the 50% point in the expiratory cycle (Flow@50%TVe1), accurately reflects bronchiolar versus alveolar constriction or airway occlusion (Vijayaraghavan, Schaper et al. 1993; Vijayaraghavan, Schaper et al. 1994) and accurately correlates with invasive measurements of AHR (Glaab, Daser et al. 2001; Glaab, Ziegert et al. 2005).

Airway immunoinflammatory response. The day after AHR was assessed the mice were given a recall allergen challenge (1% nebulized OVA for 20 min) and 2 d later we

euthanized them to assess airway eosinophil numbers and IL-4 and IL-5 levels, as well as their relative levels of serum OVA-specific IgE and IgG1, as noted (Schneider, Li et al. 2001).

5.3.5. Statistical analysis.

All data are presented as means ± standard error of the mean (SEM). Multi-group comparisons were assessed by one-way ANOVA with Tukey *post-hoc* testing, whereas AHR to methacholine was assessed by linear regression analyses. P values of <0.05 were considered significant.

5.4. Results

5.4.1. T_{reg} cells induced by co-culture with DC10 are better than DC-10-stimulated nT_{reg} in inhibiting Th2 cell responses *in vitro*.

We have previously shown by fluorescence resonance energy transfer (FRET) analysis and other approaches that, in both mouse and human systems, DC10 efficiently engage Teff cells in an allergen-specific fashion (Li, Yang et al. 2010; Lu, Dawicki et al. 2011) and promote their conversion into allergen-specific iTreg cells both *in vitro* and *in vivo* (Huang, Dawicki et al. 2010). We took advantage of this latter observation to generate OVA-specific iTreg for the present study. As nTreg require cognate activation via their TCR for induction of their suppressive effects (Thornton and Shevach 2000), we previous assessed whether OVA-presenting DC10 engage freshly purified nTreg in a productive manner, as determined by FRET analysis. We labeled irrelevant allergen (house dust mite; HDM)- and OVA-presenting DC10 or LPS-activated OVA-pulsed (^{OVA}DC-LPS) DCs with the lipophylic FRET donor dye DiI, and stained pulmonary $CD4^+CD25^+Foxp3^+$ nTreg cells from healthy OVA TCR-transgenic OT2 mice with the FRET partner dye DiO. The labeled DCs were cocultured with the stained nTreg cells, while negative controls included DiO-stained T cells or DiI-stained DCs alone. We found that the OVA-presenting DC10 and DC-LPS both intimately engaged the OT2 nTreg over a number of hours, albeit with somewhat different kinetics, while no FRET signals above background were detected in the HDM-DC10/nTreg cultures (Appendix-2). We had reported previously that human DC10, which express reduced levels of MHCII and costimulatory molecules relative to immunostimulatory DCs, induce long-lasting immunological synapse formation with autologous Teff cells, although less intense FRET

Figure 5-2. OVA TCR-transgenic Treg cells induced *in vitro* by DC10 treatment more efficiently inhibit asthmatic T effector cell activation than do nTreg of identical antigen specificity.

$CD4^+CD25^{lo}Foxp3^-$ T effector (Teff) cells were purified from the lungs of asthmatic OVA TCR-transgenic OT2 mice, while $CD4^+CD25^+Foxp3^+$ nTreg were purified from allergen-naïve OT2 mice. OVA-presenting DC10 were cocultured for 5 dy with these Teff or nTreg cells, then the DC10-induced Treg (iTreg) and nTreg were positively selected from their respective cultures by magnetic sorting. The abilities of each of these Treg populations (1×10^5 cells/well) to inhibit **(A)** proliferation and **(B)** Th2 cytokine (IL-4, -5) secretion by DC-LPS-activated Teff (3×10^4 and 1×10^5 cells/well, respectively) freshly purified from the lungs of OVA-asthmatic C57BL/6 (B6) mice were assessed *in vitro* as noted in the Materials and Methods. In order to assess the contributions of IL-10 and TGF-β to the suppressive activities of these Treg we also assessed the impact of neutralizing antibodies (each, 10 μg/ml) on DC-LPS-induced **(C)** proliferation and **(D)** Th2 cytokine secretion. ** or ***, $p<0.01$ or 0.001, respectively, versus the indicated group(s). One representative experiment of three is shown.

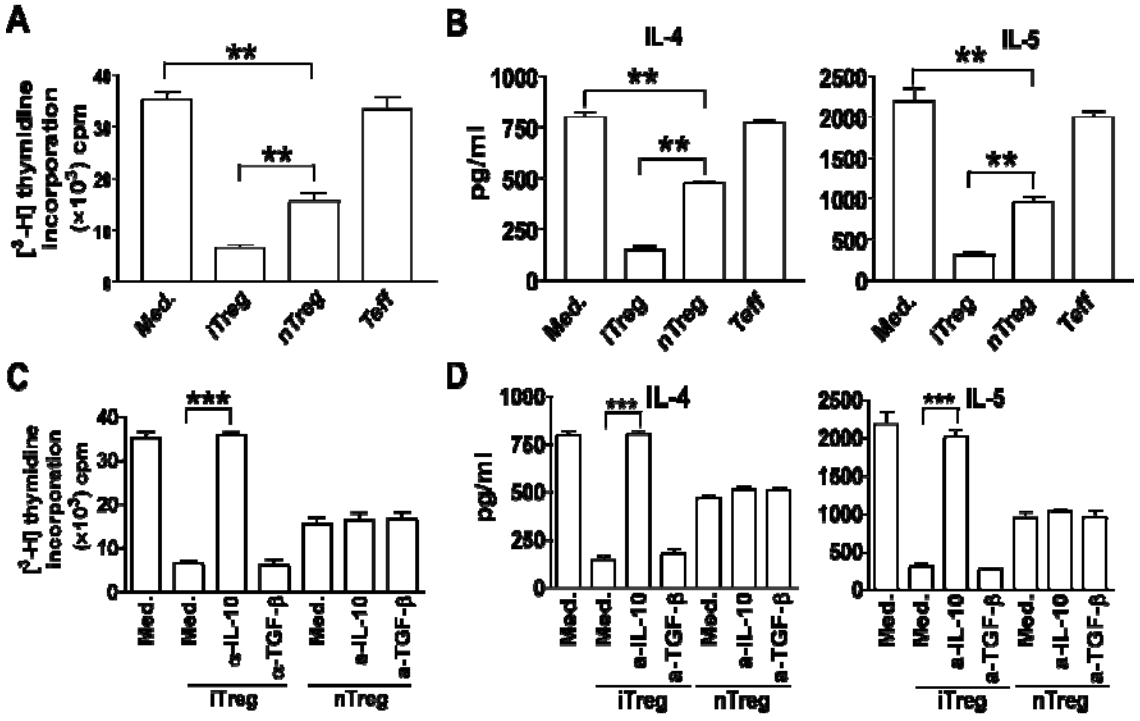

Figure 5-2. OVA TCR-transgenic T$_{reg}$ cells induced *in vitro* by DC10 treatment more efficiently inhibit asthmatic T effector cell activation than do nT$_{reg}$ of identical antigen specificity.

signals than do immunostimulatory DCs (Li, Yang et al. 2010). This data thus indicated that OT2 nTreg do engage in a cognate fashion with OVA-presenting DC10.

To directly compare the suppressive activities of iTreg and nTreg we purified $CD4^{+}CD25^{lo}Foxp3^{-}$ Teff cells from the lungs of asthmatic OT2 mice and purified nTreg from allergen-naïve OT2 mice. We co-cultured each of these T cell populations with OVA-presenting DC10 for 5 d, isolated the iTreg and DC10-exposed nTreg from their respective cultures, and then assessed their relative abilities to suppress proliferation and Th2 cytokine expression in co-cultures of OVA-presenting DC-LPS and Th2 Teff cells from OVA-asthmatic mice. The Th2 Teff cells proliferated strongly and secreted IL-4 and IL-5 in response to the DC-LPS triggering (Figure 5-2 A, B; Med. group), and adding further Teff cells to these cultures did not alter that response. On the other hand, adding DC10-exposed nTreg to the cultures reduced each of these Th2 responses by ≈41-56%, while adding iTreg had a substantially greater suppressive effect (81-86%) on all three parameters. As IL-10 and TGF-β are two important inhibitory cytokines secreted by Treg cells (Josefowicz, Lu et al. 2012), we also assessed their relevance to the suppressive abilities of our iTreg and nTreg cells, adding specific neutralizing antibodies to the co-cultures to determine their impact on Th2 cell proliferation and Th2 cytokine secretion (Figure 5-2 C, D). Neither anti-IL-10 nor anti-TGFβ antibodies appeared to discernibly affect the suppression associated with the nTreg, while neutralization of IL-10 in the iTreg co-cultures abrogated their suppressive effects in these cultures. The anti-TGFβ had no impact on the iTreg activities. This data indicated that DC10-induced iTreg cells had a substantially greater contribution to regulation of Th2 responses *in vitro* than do nTreg that are of identical antigen specificity and which have seen similar exposure to allergen-

presenting DC10. It also indicated that these iTreg employed IL-10 as their primary tolerogenic mediator, as has been reported previously for peripherally-induced $CD4^+CD25^+Foxp3^+$ Treg (Akbari, Freeman et al. 2002; Cobbold, Nolan et al. 2003).

5.4.2. DC10-induced activation of nTreg and iTreg.

As noted, nTreg induce tolerance in a contact-dependent fashion, employing cell surface molecules, as opposed to secreted mediators, as primary tolerogenic effectors (Takahashi, Tagami et al. 2000; Thornton and Shevach 2000). To determine whether our DC10-induced iTreg and nTreg differentially expressed Treg-associated markers, we assessed their expression of ICOS, PD-1, GITR, LAG3 and CTLA-4 by FACS. We found that our *in vitro*-induced Treg expressed modestly to markedly higher levels of ICOS and CTLA-4 than the nTreg, but largely equivalent levels of PD-1, GITR and LAG3 (Figure 5-3 A). As an assessment of the relevance of these *in vitro* findings to Treg that might mediate tolerance responses *in vivo*, we also assessed expression of these markers on iTreg and nTreg that were induced or stimulated *in vivo*. To generate the iTreg, we transferred $CD4^+CD25^{lo}Foxp3^-$ Teff cells from asthmatic $CD45.1^+$ GFP-Foxp3 OT2 donor mice (Figure 5-3 B) into asthmatic B6 ($CD45.2^+$) mice, which we simultaneously treated i.p. with 1×10^6 OVA-presenting DC10. To obtain the nTreg, we purified $CD4^+CD25^+Foxp3^+$ nTreg from allergen-naïve $CD45.2^+$ GFP-Foxp3 OT2 donors (Figure 5-3 B) and co-transferred these into the DC10-treated Teff cell recipient mice. One wk after the DC10 treatment, the animals' $CD4^+$ T cells were magnetically sorted and submitted for FACS analysis, gating on the $CD45.1^+GFP^+$ (iTreg) and $CD45.1^-GFP^+$ (nTreg) $CD4^+$ T cells (Figure 5-3 C). It was readily apparent that *in vivo*-induced iTreg

Figure 5-3. DC10-induced Treg cells express substantially higher levels of PD-1, LAG3 and CTLA-4 than do nTreg cells.

In vitro (A) and *in vivo* (B)-induced iTreg and nTreg were assessed for expression of the Treg-associated markers ICOS, PD-1, GITR, LAG3 and CTLA-4, as noted in the Materials and Methods. **(A)** iTreg and nTreg were generated *in vitro* and sorted from their respective cultures as in Figure 5-2, then submitted for FACS analysis. **(B)** Analogous populations of Treg were also generated *in vivo* by transferring (i.v.) 5×10^6 $CD4^+CD25^{lo}Foxp3^-$ Teff cells that had been purified from asthmatic $CD45.1^+$ GFP-Foxp3 OT2 donor mice (left panel) and $CD4^+CD25^+Foxp3^+$ nTreg purified from allergen-naïve $CD45.2^+$ GFP-Foxp3 OT2 donors (right panel) into the same DC10-treated asthmatic $CD45.2^+$ B6 recipients. **(C)** One week after the DC10 treatment, the animal's $CD4^+$ T cells were magnetically sorted and stained with specific or isotype control antibodies for CD45.1 (upper two panels), as well as the markers indicated in panel A, with gating on GFP and CD45 to discern the iTreg and nTreg populations. For each panel one representative experiment of three is depicted.

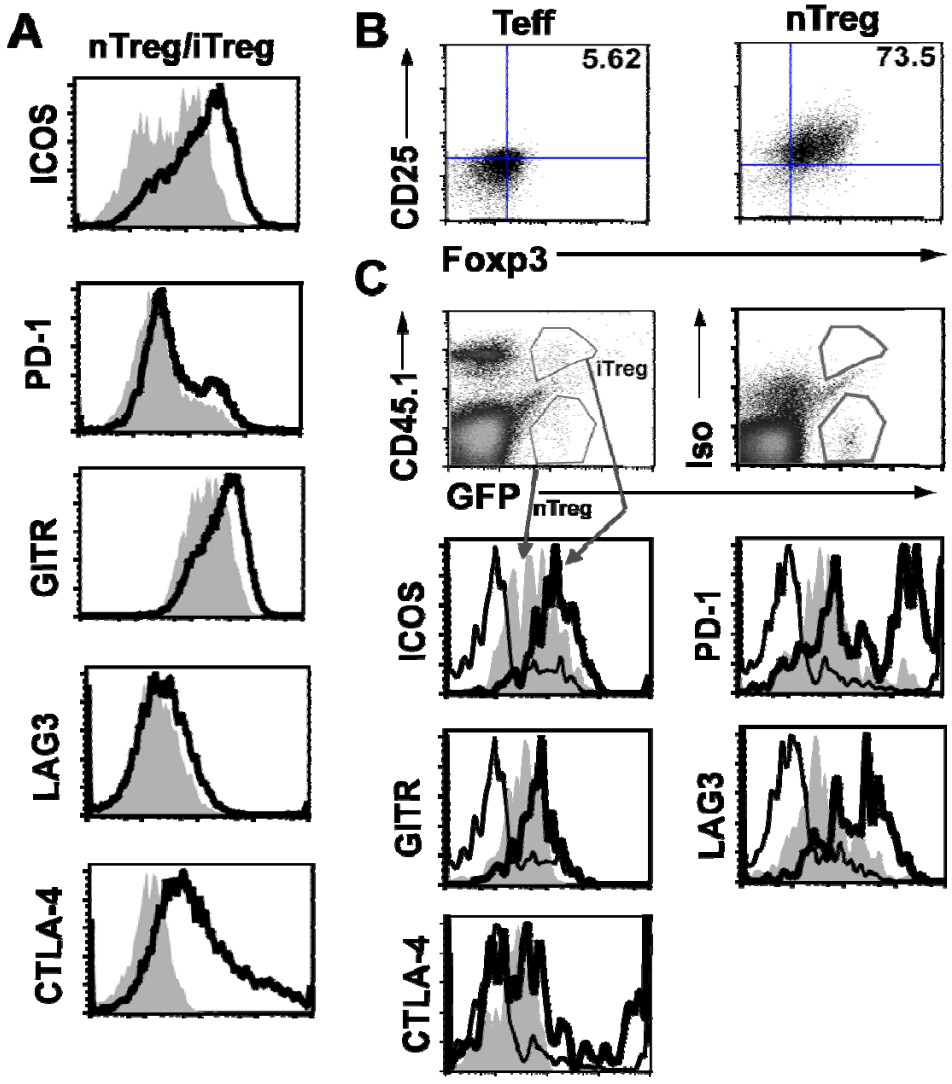

Figure 5-3. DC10-induced Treg cells express substantially higher levels of PD-1, LAG3 and CTLA-4 than do nTreg cells.

cells expressed substantially higher levels of PD-1, LAG3 and CTLA-4 than the nTreg cells, and modestly elevated levels of ICOS and GITR. Nevertheless, the mean fluorescence intensity for each of these markers on the nTreg was significantly elevated relative to isotype control-stained cells. Similarly, both the nTreg and the iTreg expressed higher levels of each marker than did transferred Teff cells from asthmatic mice (Figure 5-6).

Having seen differences in expression of regulatory markers by *in vitro*- and *in vivo*-differentiated Treg, we next compared their activities. We generated cells for these assays *in vivo,* as above, with the exception that the Teff and nTreg were originally derived from asthmatic and naïve CD45.1$^+$ OT2 donor mice, respectively. Each Treg population was transferred into its own group of DC10-co-treated asthmatic B6 recipient mice (5×10^6 T cells/mouse) and 1 wk later the CD4$^+$CD25$^+$CD45.1$^+$ iTreg and CD4$^+$CD25$^+$CD45.1$^+$ nTreg cells were purified from the respective recipients by magnet sorting (Figure 5-4 A). We assessed the activities of these Treg by adding them into co-cultures of Teff cells from OVA-asthmatic B6 mice and OVA-presenting DC-LPS (Figure 5-4 B, C). Control cultures were either supplemented with no Treg (Med.) or with additional CD4$^+$CD25$^-$Foxp3$^-$ Teff cells purified from asthmatic donor B6 mice. We found that the *in vivo*-stimulated nTreg reduced DC-LPS-induced Teff cell proliferation in this assay by 45.7+/-7,1%, while the same numbers of iTreg of the same TCR specificity reduced proliferation by 73+/-5.5% ($p\leq0.01$). When we measured the levels of IL-4 and IL-5 in these cultures we observed a similar outcome, with the nTreg reducing the levels of these two Th2 mediators by 40.5+/-6.5 and 52.2+/-13.6%, respectively, while the iTreg reduced their expression by 72.7+/-8.5 and 82.7+/-10.3%, respectively (for both

cytokines, $p \leq 0.01$). Thus, consistent with the data from our *in vitro*-induced iTreg, *in vivo*-induced iTreg were substantially more effective than nTreg cells in blocking Th2 activation *in vitro*.

5.4.3. DC10-induced Treg more efficiently ameliorate the asthma phenotype than do DC10-stimulated nTreg of the same antigen-specificity.

We had previously reported that passive transfer of 5×10^5 total $CD4^+CD25^+Foxp3^+$ cells (i.e., unfractionated Treg) from DC10-tolerized asthmatic mice approximately 50% reverses the asthma phenotype of asthmatic recipients; 2.5×10^5 of these Treg are completely ineffective in this model, while 1×10^6 Treg fully corrects the phenotype (Huang, Dawicki et al. 2010). Herein we directly compared the *in vivo* regulatory activities of iTreg versus nTreg from OVA TCR-transgenic OT2 mice. We used 5×10^5 purified DC10-induced Treg or equal numbers of DC10-exposed nTreg, both generated *in vitro* as above, to treat asthmatic mice at dy 46 after initiation of sensitization, and 4 wk later examined the impact of the treatments on the animals' asthma phenotype. We first assessed their airway hyperresponsivenss (AHR) to methacholine, using head-out whole body plethysmography to determine the airflow rates at the 50% point in the expiratory cycle (Gordon, Li et al. 2005; Huang, Dawicki et al. 2010; Lu, Dawicki et al. 2011). The AHR of the animals given 5×10^5 DC10-induced iTreg cells was fully normalized ($p>0.05$ versus normal mice) whereas the same number of nTreg cells had no discernible effect on the asthmatic AHR (Figure 5-5A). We then administered a recall allergen challenge to the animals and two days later assessed their airway eosinophil and Th2 cytokine (IL-4 and IL-5) responses. Across three experiments,

Figure 5-4. T_{reg} cells induced *in vivo* by DC10 treatment of asthmatic mice have greater inhibitory activity *in vitro* than do nT_{reg} cells of equivalent antigen specificity.

(A) $CD4^+CD25^+Foxp3^+CD45.1^+$ iT_{reg} and nT_{reg} cells were induced *in vivo* and purified by CD45-based magnetic sorting as in Figure 5-3. These cells, together with control populations of T_{eff} from asthmatic B6 mice, were assessed for their abilities to antagonize **(B)** proliferation and **(C)** IL-4 and IL-5 secretion by DC-LPS-activated T_{eff} (3×10^4 and 1×10^5 cells/well, respectively) freshly purified from the lungs of OVA-asthmatic B6 recipient mice. (**$p<0.01$, vs. iT_{reg} values). One representative experiment of three is shown.

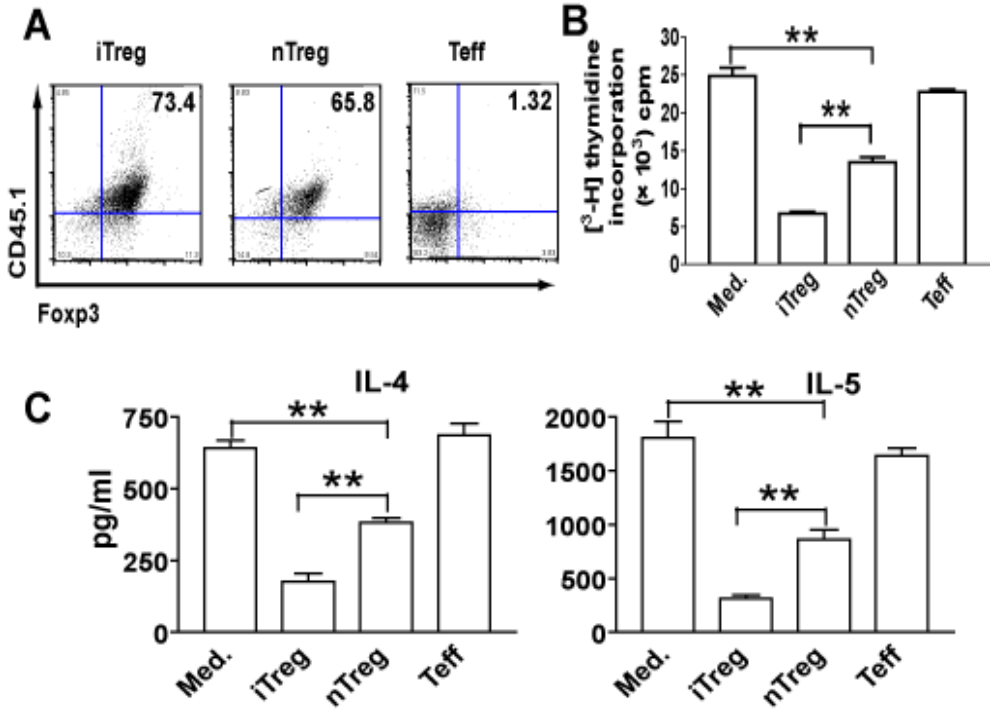

Figure 5-4. Treg cells induced *in vivo* by DC10 treatment of asthmatic mice have greater inhibitory activity *in vitro* than do nTreg cells of equivalent antigen specificity.

Figure 5-5. Passive transfer of DC10-induced OT2 Treg, but not nTreg of identical antigen specificity, efficiently ameliorates the asthma phenotype in recipient mice.

We injected 5×10^5 iTreg or nTreg cells (i.v.) generated *in vitro* as in Figure 5-2 into otherwise untreated asthmatic recipient mice (n=5) and four weeks later assessed the impact of the treatments on the animals' asthma status. **(A)** At 4 wk we assessed AHR to methacholine, as determined by the airflow rate at the 50% point in the expiratory cycle (Flow@50%TVe1). The following day we challenged them with an aerosol of 1% nebulized OVA and 2 d later assessed **(B)** the airway eosinophilia **(C)** and BAL IL-4 and IL-5 levels, and **(D)** the serum levels of OVA-specific IgE and IgG1. One representative experiment of three is shown.

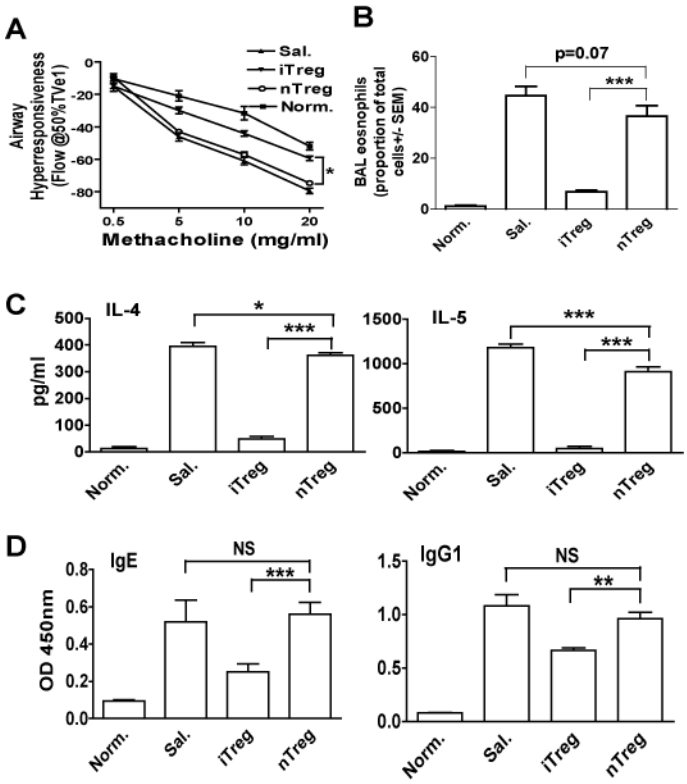

Figure 5-5. Passive transfer of DC10-induced OT2 Treg, but not nTreg of identical antigen specificity, efficiently ameliorates the asthma phenotype in recipient mice.

Figure 5-6. Comparison of the expression levels of ICOS, PD-1, GITR, LAG3 and CTLA-4 by Teff, iTreg and nTreg cells.

$CD4^+CD25^-Foxp3^-$Teff and $CD4^+CD25^+Foxp3^+$Treg cells were purified from asthmatic GFP-Foxp3/CD45.1 and naïve GFP-Foxp3/CD45.2 OT2 mice, respectively. Both T cell populations (5×10^6 cells/recipient) were injected *i.v.* into asthmatic B6 (CD45.2) mice which were then treated i.p. with 1×10^6 OVA-presenting B6 DC10. One wk later $CD4^+$ T cells were purified from the recipients' spleens, and the cells stained with PE-cy5-CD45.1 and PE-labeled antibodies against the indicated Treg cell markers. $CD45.1^+GFP^+$ (iTreg), $CD45.1^-GFP^+$ (nTreg) and control $CD45.1^+GFP^-$ (Teff) cells were gated and their marker expression was analyzed by flow cytometry. One representative experiment of two is shown.

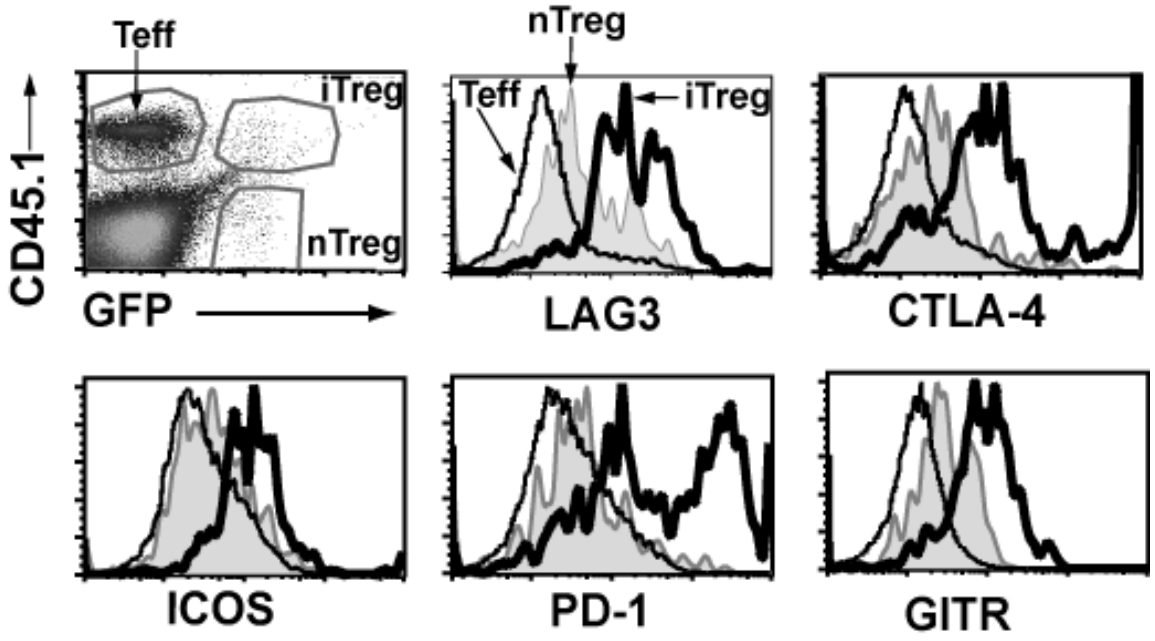

Figure 5-6. Comparison of the expression levels of ICOS, PD-1, GITR, LAG3 and CTLA-4 by Teff, iTreg and nTreg cells.

passive transfer of nTreg cells reduced IL-4 levels by ≈8.8 +/- 1.8 % ($p \leq 0.03$ versus saline-treated asthmatic mice), IL-5 levels by 22.9 +/- 6 % ($p \leq 0.001$ versus saline-treated asthmatic mice), and BAL eosinophil numbers by 16.2+/-5.1% ($p = 0.07$ versus saline-treated asthmatic mice)(Figure 5-5B and C). Transfer of iTreg cells reduced the numbers of airway eosinophils and IL-4 and IL-5 levels to near background ($p \leqslant 0.001$ versus nTreg-treated asthmatic mice). In addition, the iTreg treatment markedly reduced the levels of OVA-specific IgE and IgG1 Ab ($p \leqslant 0.001$ and 0.01, respectively, versus nTreg-treated asthmatic mice), while the nTreg had no discernible impact ($p > 0.05$) on the IgE or IgG1 Ab responses (Figure 5-5D).

5.5 DISCUSSION

We demonstrated in this report that allergen-presenting IL-10-differentiated DCs (DC10) engage nTreg in a cognate fashion, as determined using FRET assays. These DC10-stimulated nTreg 41-56% suppressed proliferation and Th2 cytokine expression by immunostimulatory DC-activated Th2 Teff cells *in vitro*, although DC10-induced iTreg were substantially more tolerogenic in this *in vitro* system. The regulatory activities of these iTreg, but not nTreg, were completely blocked by anti-IL10 antibodies, and they also expressed higher levels of immunoregulatory molecules (e.g., PD-1, CTLA-4). We further found that transferring 5×10^5 purified DC10-exposed nTreg only modestly reduced the asthma phenotype in recipient mice, while equal numbers of purified DC10-induced iTreg were highly effective in reducing asthmatic responses. As we have observed previously, asthmatic B cell (i.e., OVA-specific IgE and IgG1) responses take substantially longer to wane than do Th2 responses following tolerogenic treatments (Nayyar, Dawicki et al. 2012).

It has been proposed that nTreg and iTreg subserve distinct functions, such that nTreg primarily target autoimmune responses (Takahashi, Tagami et al. 2000) while iTreg modulate responses to exogenous antigens (Bilate and Lafaille 2011). One piece of evidence that supports this is that depletion of nTreg in naïve mice leads to an autoimmune syndrome (Sakaguchi 2005), but it has since been reported that similar depletion of $CD4^+CD25^+Foxp3^+$ T cells in naive mice also leads to augmented asthma severity following subsequent allergen sensitization (Lewkowich, Herman et al. 2005; Joetham, Takeda et al. 2007). As noted above, it has also been shown that passive transfer of nTreg cells from naïve mice into asthmatic recipient animals relatively early after asthma induction reduces their asthma phenotype (Kearley, Robinson et al. 2008).

This suggests that while there may well be some divergence of function between iTreg and nTreg, the story may well be more complex than a simple dichotomy between endogenous versus exogenous antigen dedication for the two cell types. For example, recent evidence indicates also that both iTreg and nTreg play requisite roles in establishment of full disease tolerance in a mouse model of colitis (Haribhai, Williams et al. 2011), although it is evident that there are situations wherein one Treg population alone can mediate tolerance. Thus, also as noted above, iTreg are sufficient to induce tolerance in a mouse model of pulmonary inflammation (Curotto de Lafaille, Kutchukhidze et al. 2008). It was reported that the TCR repertoires of the iTreg and nTreg that are effective in the above-noted colitis model overlap by only 3-15% (Haribhai, Williams et al. 2011), and that is taken as further evidence that these two cells target distinct sets of immunologic processes. However, many antigens feature multiple epitopes, some of which may be immunodominant and others less so, while many environmental challenges (e.g., microorganisms, allergens) are complex and present the immune system with numerous, likely multi-epitope, antigens, and each would require a unique TCR. Thus, the reported somewhat restricted overlap in TCR β chain specificities of iTreg and nTreg (Haribhai, Williams et al. 2011) notwithstanding, there could be ample room for iTreg and nTreg with disparate TCR specificities to target the same disease complex. The evidence we present herein confirms that iTreg and nTreg of identical TCR specificity were each able to target anti-OVA responses of Th2 cells from OVA-asthmatic wild-type mice both *in vitro* and *in vivo*, although they do so with substantially different efficacy. In our model system the iTreg were much more effective in suppressing

asthmatic Th2 responses than were nTreg of identical TCR specificity (i.e., $OVA_{323-339}$) (Robertson, Jensen et al. 2000).

Natural Treg require TCR signaling via APCs in order to be activated (Thornton and Shevach 2000), and our FRET data indicates that cognate, but not irrelevant, allergen-presenting tolerogenic DC10 did intimately engage the nTreg cells. An unanswered question however is whether these naturally tolerogenic DC10 would have activated the nTreg with which they were cultured in the same way as immunostimulatory DCs might have. Irrespective of whether the DC10 did so, in our *in vitro* study the nTreg were added into co-cultures of LPS-activated OVA-presenting DCs, and these would be expected to also activate the nTreg via TCR signaling (Thornton and Shevach 2000). In principle, that could explain our data that suggests that nTreg were apparently qualitatively better at inducing tolerance in our *in vitro* assays than they were *in vivo*. An alternate explanation could be that the nTreg we transferred do not as efficiently enter the lung tissues or lung-draining lymph node compartment as do iTreg, so did not have the same opportunity to suppress the pulmonary Th2 Teff cell responses in the time-frames of our experiments. In our model these iTreg would have recently differentiated from Teff cells under the influence of treatment DC10 and would have been highly activated at the time of passive transfer (Huang, Dawicki et al. 2010).

Our data indicated that DC10-induced iTreg cells employed IL-10 as their primary tolerogenic mediator, as has been reported previously for peripherally-induced $CD4^+CD25^+Foxp3^+$ Treg (Akbari, Freeman et al. 2002; Cobbold, Nolan et al. 2003), and this is consistent with our previous report that bulk $CD25^+Foxp3^+$ Treg from the lungs of DC10-treated asthmatic mice also employ IL-10 as their primary effector molecule

(Huang, Dawicki et al. 2010). Although iTreg can also include a population of $CD25^-$ cells that employ IL-10 to suppress T cell responses (Nicolson, O'Neill et al. 2006; Roncarolo, Gregori et al. 2006), based on our FACS analysis our magnetically-sorted iTreg were predominantly $CD25^+Foxp3^+$ cells (data not shown). FACS analysis of our Treg cells confirmed that the iTreg also strongly expressed the CD4-related MHCII-binding iTreg marker LAG3, which is associated with both cell-intrinsic and -extrinsic signaling (Okamura, Fujio et al. 2012), but also PD-1 and CTLA4, which provide inhibitory signals via the CD28/B7 pathway (Fife and Bluestone 2008). LAG3 on iTreg can provide inhibitory signals to immature DCs on engaging their MCHII molecules (Okamura, Fujio et al. 2012), and this fits well with other observations in our lab that the endogenous lung DCs of DC10-treated mice take on a regulatory phenotype several weeks after DC10 treatment of asthmatic mice (C. Li, H. Huang, W. Dawicki, and J.R. Gordon, unpublished), in concert with the appearance of highly activated Treg in the lungs (Huang, Dawicki et al. 2010). The augmented expression of PD-1 by the *in vivo*-induced iTreg is consistent with PD-1 playing an important role in the formation and activities of these cells (Francisco, Salinas et al. 2009). PD-1 expression by our iTreg also fits well with the fact that these cells were induced by exposure to DC10, which express low levels of MHCII, CD40, CD80 and CD86 (Li, Yang et al. 2010; Nayyar, Dawicki et al. 2012) leading to reduced TCR stimulation by these cells relative to mature allergen-presenting DCs (Li, Yang et al. 2010). It has been reported that low-level TCR stimulation augments PD-1-dependent negative signaling in T cells (Keir, Butte et al. 2008). Moreover, we know that our DC10 express high levels of PD-L1 (Y. Ma, C. Li, S.A. Gordon, W. Dawicki, and J.R. Gordon, unpublished observation), and PD-L1/PD-1

signaling is important in induction of iTreg (Francisco, Salinas et al. 2009). CTLA-4 can play a dual function in T cells, wherein it is inducible and controls Teff cell homeostasis (Jain, Nguyen et al. 2010), but also regulatory T cells within which it is constitutively expressed (Takahashi, Tagami et al. 2000). Others have reported upregulation of CTLA-4 and GITR expression on Treg, presumably iTreg, in the context of hookworm infections (Ricci, Fiuza et al. 2011), while vasoactive intestinal peptide (VIP)-induced Treg similarly express augmented levels of CTLA-4 (Pozo, Anderson et al. 2009).

It had been reported previously that passive transfer of 5×10^5 wild-type nTreg is sufficient to offset otherwise inevitable lethality in Foxp3-deficient mice (Haribhai, Williams et al. 2011). This number of nTreg was also sufficient to ameliorate the asthma phenotype in an OVA/alum model not unlike our own, but only if the cells were transferred early on in the induction protocol; nTreg transfer after entrenchment of the phenotype (i.e., dy 46) had no discernible impact on the disease (Kearley, Robinson et al. 2008). Interestingly, our lab routinely treats fully asthmatic mice with DC10 (Lu, Dawicki et al. 2011; Nayyar, Dawicki et al. 2012) or Treg (Huang, Dawicki et al. 2010) at precisely this time (i.e., dy 46), and we see substantial to full reversal of the phenotype within 3-4 weeks. The nTreg employed by Kearley et al were unmanipulated nTreg (Kearley, Robinson et al. 2008) whereas ours had been exposed to tolerogenic DC10, which might explain the differences in our dy 46 treatment outcomes. We did show herein that our OVA-presenting DC10 engaged OT2 mouse nTreg in a cognate fashion, and thus could have stimulated the regulatory activities of these cells. We had previously titrated the numbers of unfractionated $CD4^+CD25^+Foxp3^+$ Treg from the lungs of DC10-treated asthmatic mice that are required to passively transfer tolerance to asthmatic

recipients and found that 1×10^6 cells ablate the asthma phenotype in the recipients, while 5×10^5 or 2.5×10^5 Treg approximately 50% reduced or had little if any impact, respectively, on the phenotype (Huang, Dawicki et al. 2010). Thus it seems feasible that our transference of limiting numbers of (i.e., 5×10^5) Treg was important to distinguishing so clearly the disparate activities of iTreg versus nTreg in our asthma model. We have no evidence relating to whether the nTreg we transferred were able to successfully engage Teff cells within the lungs of the asthmatic recipient mice, but functional nTreg are recruited into and reside within the lungs of both healthy and asthmatic mice (McGee and Agrawal 2009). Nevertheless, we cannot rule out that the nTreg we transferred were simply less efficient in engaging allergen-specific Teff cells in the lungs than their comparator iTreg. These caveats notwithstanding, our data indicated that DC10-induced Treg were distinctly superior to nTreg of identical antigen specificity in their abilities to antagonize the asthma phenotype in a mouse model of fully entrenched disease.

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CHAPTER 6: GENERAL DISCUSSION

As APCs, DCs process allergens that enter the airway and present their peptide products to naïve Th cells. In individuals destined to develop allergic disease, allergen-specific activation of naïve Th cells induces their differentiation into Th2, but not Th1 cells. Subsequent allergen challenge induces Th2 cell proliferation and cytokine production, and thereby also eosinophilic inflammation and IgE production. With repeated allergen exposure these effects culminate in the AHR and eosinophilic airway inflammation and airway remodeling that typifies allergic asthma (Cohn and Ray 2000; Busse and Lemanske 2001; Lambrecht and Hammad 2003; Hawrylowicz and O'Garra 2005).

The initial interactions between APCs and the naïve T cell determines whether the T cell differentiates into an activated effector cell, an anergized cell or a regulatory cell. One factor that critically regulates this process is the maturation status of the DC, as judged by the levels at which they express MHCII and costimulatory molecules and the cytokines they secrete, such that allergen-presenting quiescent ($CD8\alpha^+$) splenic DCs are tolerogenic when delivered to asthmatic mice (Gordon, Li et al. 2005). Culture of immature DCs with IL-10 induces their differentiation into cells (DC10) that display a relatively immature phenotype, but secrete elevated levels of IL-10 after activation. IL-10 moderately dampens MHC class II and costimulatory molecule expression on DCs, and this was thought to account for their abilities to induce tolerance.

Multiple laboratories have reported on the tolerogenic activities of DC10 in mouse models and with human T cells (Steinbrink, Wolfl et al. 1997; Bellinghausen, Brand et al. 2001; Muller, Muller et al. 2002; Wakkach, Fournier et al. 2003; Koya,

Matsuda et al. 2007; Perona-Wright, Anderton et al. 2007; Lau, Biester et al. 2008; Li, Yang et al. 2010). For example, OVA-presenting DC10 can protect against the development of OVA-induced asthma (Bellinghausen, Brand et al. 2001; Koya, Matsuda et al. 2007; Henry, Desmet et al. 2008) or reverse the asthmatic phenotype (Lu, Dawicki et al. 2011; Nayyar, Dawicki et al. 2012) in allergen-sensitized mice, reducing AHR to methacholine, eosinophilia and Th2 responses to allergen challenge, although the mechanisms by which DC10 induce Th2 cell allergen tolerance have not been fully unexplored.

Numerous laboratories have noted the importance of $CD4^+CD25^+Foxp3^+$ Treg in protecting against the development of asthma (Stock, DeKruyff et al. 2006) and there is substantial evidence that tolerogenic DCs have effects on Treg (Zhang-Hoover, Finn et al. 2005; Adler and Steinbrink 2007; Koya, Matsuda et al. 2007; Henry, Desmet et al. 2008). For example, DC10 generated from atopic asthmatics are tolerogenic for autologous Th2 effector cells, fostering the outgrowth of $CD4^+CD25^+Foxp3^+LAG3^+CTLA\text{-}4^+$ regulatory T cells (Li, Yang et al. 2010). DC10 up-regulated expression of the activated $CD4^+CD25^+Foxp3^+$ Treg cell markers CTLA-4 and LAG3 in the lungs of the DC10-treated mice (Lu, Dawicki et al. 2011). While we have some insights into how DC10 affect Treg cell differentiation (Adler and Steinbrink 2007), there is much we do not know, particularly in the context of tolerance induction in allergic asthma.

In general, activation of T cells by APC requires at least three signalling events, one elicited by TCR recognition of the MHC/peptide complex on the APC and the second incorporates the interactions of costimulatory molecules on the APC with the T cells' counter-ligands (e.g., CD28 and LFA on the T cells with CD80 and CD54, respectively,

on the APC). The third is a polarizing cytokine signal (e.g. IL-4, IL-10 or IL-12). The initial interactions between antigen-presenting DCs and the immunologically naïve T cell determines the outcome of the T cells' differentiation into an activated effector cell, an anergized cell or a regulatory cell. One factor that critically regulates this process is the maturation status of the DC, as judged by the levels at which they express MHCII and costimulatory molecules and the cytokines they secrete. Roughly speaking, stable interactions between DCs and T cells during antigen presentation effectively prime productive immune responses, while brief DC-T cell interactions are associated with immune tolerance (Hugues, Boissonnas et al. 2006).

Both immunosuppressive and immunostimulatory effects of DC on T cells have been reported (Hawiger, Inaba et al. 2001; Steinman, Hawiger et al. 2003; Steinman, Hawiger et al. 2003). As noted large number of reports have shown that stimulatory DC cells activate naïve T cells by providing at least three signals (MHC-peptide-TCR complex and costimulatory molecules as well as cytokine) (Chen, Linsley et al. 1993; Golovina, Mikheeva et al. 2008; Weaver, Charafeddine et al. 2008). Costimulatory molecules can increase APC functions to activate both Th1 and Th2 cells, but there is increasing evidence that other "costimulatory" molecules can promote tolerance induction by DC. Sergio *et al.* reported that CD154 (CD40L) blockade can, in some instances, engender long-lived, antigen-specific tolerance (Quezada, Jarvinen et al. 2004). Scalapino reported that the cell surface CTLA-4 is a critical attenuator of T-cell activation and an essential component of the regulatory systems that serve to maintain peripheral tolerance (Scalapino and Daikh 2008). Interesting, DCs have been reported to

possess distinct immunoregulatory effects on anergy induction and activation of T cells (Hochweller and Anderton 2005; Hadeiba, Sato et al. 2008).

Small interfering RNA (siRNA) is part of a biological process that can be used experimentally to silence gene expression. The introduction of double-stranded RNA (dsRNA) into a cell results in targeted posttranscriptional gene silencing. Double-stranded RNA oligos of 23 nucleotide (nt) in length can be used to mediate gene silencing in mammalian cells, because they foster the degradation of mRNA having the siRNA target sequence. The application of siRNA technology to mammalian cells is advancing the field of functional genomics. It has been used to regulate immune function in DC by silencing specific genes (Hill, Ichim et al. 2003; Liu, Ng et al. 2004; Li, Zhang et al. 2007; Xiang, Gu et al. 2007). In 2006 the importance of siRNA was recognized when its discoverers (Andrew Z. Fire and Craig C. Mello) were awarded the Nobel Prize.

In the present study, we illustrated that IL-10, CD80/86 and MHCII play important roles in tolerance induction by DC10. Our data on IL-10-silenced DC10 is consistent with a previous study which results demonstrated that siRNA modulation of IL-10 in DC10 decreased tolerogenic capacity in delayed-type hypersensitivity (DTH) (Ruffner, Kim et al. 2009). Although the APC signals required for T effector cell activation have been studied extensively, herein we documented the importance of these three signals in the context of tolerogenic DC for the first time (Figure 6-1). Tolerogenic DCs take up and digest allergen into peptides which bind to groove of the MHC class II molecule. This MHC-peptide complex is transported to the surface of tolerogenic DC cell to provide allergen-specific signals to Th2 cells with an immunological synapse (Steinman, Hawiger et al. 2003). Some DC-T cell costimulatory molecule pairings (APC

CD40/T cell CD154, APC CD54/T cell LFA-1) increase the avidity of the synapse, thereby further engage tolerogenic DCs and Th2 cells, while others (APC CD80, 86/T cell CTLA-4, et al) can provide inhibitory signals. Some tolerogenic DCs secrete inhibitory cytokines such as IL-10 and TGF-β to inhibit T effector cell activation and promote Treg activity (Maldonado, von Andrian et al. 2010).

Increasingly, it has been reported that the differentiation of Treg cells is related to the strength of TCR and costimulatory signals. High doses of antigen have been shown in vitro and in vivo to prevent $Foxp3^+$ Treg differentiation (Molinero, Miller et al. 2011) while low doses of antigen promotes induction of Treg cells (Long, Rieck et al. 2011). Gabryšová et al reported that reducing the strength of TCR or costimulatory CD28 signals enhanced the differentiation of Treg cell (Gabryšová, Christensen et al. 2011). These observations are consistent with the findings in our study. DC10 generated in our lab express low level of MHCII molecules and costimulatory molecules. When pulsed with OVA peptide, DC10 were found using on OT2 transgenic mouse system to induce Treg differentiation from Foxp3- precursor. Low expression of MHC II and costimulatory molecules on DC10 provides appropriate signals to induce Treg differentiation by DC10 (Morelli and Thomson 2007). Recently we generated bone marrow-derived DCs in the presence of retinoic acid (DC_{RA}), and found that although these DC_{RA} highly expressed costimulatory molecules (i.e. CD40, CD54, CD80), they still efficiently inhibited both food allergy (Dawicki, Zhang and Gordon; Therapeutic treatment for food allergy with suppressive DCs, manuscript in preparation) and asthmatic responses (Li, Ma, Dawicki, Gordon, Zhang and Gordon; Therapeutic treatment for asthma with retinoic acid treated DCs, manuscript in preparation). This is

probably due to the presence of strong alternate regulatory signals expressed by DC_{RA} (i.e. RA and TGF-β).

Figure 6-1. Three APC signals required for induction of asthma tolerance by DC10

DC10 provides three signals to Treg cells and induces asthma tolerance. Signal 1 is elicited by TCR recognition of the major histocompatibility complex/peptide complex presented by DC10. Signal 2 is the stimulation of MHCII and costimulatory molecules (e.g., CD80, CD54) expressed on DC10. Signal 3 is immunosuppressive cytokine IL-10 produced by DC10 itself. These three signals facilitate the expression of Treg-associated markers on $Foxp3^{+}$Treg cells and thereby augment Treg cell responses. Consequently, DC10 activated Treg cells tolerize asthma via inhibiting Th2 responses in term of cytokine (e.g., IL-4,5,9,13) secretion, eosinophilia and AHR.

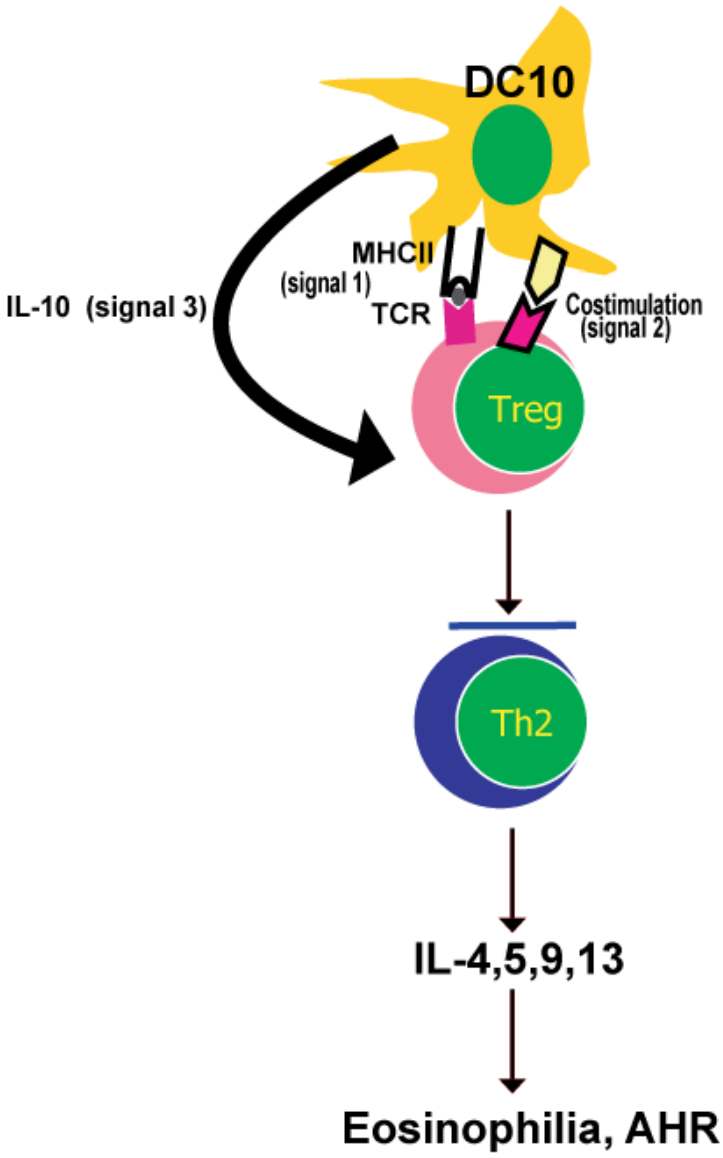


Figure 6-1. Three APC signals necessary for induction of tolerance by DC10

$CD4^+CD25^+$ Treg cell can be broadly divided into two subsets, natural $CD4^+CD25^+$ Treg (nTreg) cells and induced Treg (iTreg) cell based on where and how they develop. The nTreg cells, which constitute around 5% of peripheral blood $CD4^+$ T cells in healthy adult mice and humans, originate in the thymus, while iTreg cells develop in the periphery (Bilate and Lafaille 2012; Josefowicz, Lu et al. 2012). The forkhead (winged helix) transcription factor forkhead box P3 (Foxp3) is expressed by Treg cells, and is essential to their function (Curotto de Lafaille and Lafaille 2009). It is reported that the adoptive transfer of nTreg cells could prevent asthmatic response in term of AHR, eosinophilia and Th2 cytokines (IL-5, 13) in OVA-alum sensitized asthma mice (Kearley, Barker et al. 2005; Kearley, Robinson et al. 2008; Presser, Schwinge et al. 2008). In mice and humans, ectopic expression of Foxp3 within non-regulatory $CD4^+$ T cells has been shown to convert these cells into functional Treg cells. There is increasing evidence that iTreg cells can be differentiated in the periphery from $Foxp3^-$ non-regulatory $CD4^+$ T cells by APCs with IL-10 or TGF-β (Yamazaki, Dudziak et al. 2008; Huang, Dawicki et al. 2010). Treg cells play an important role in the homeostasis of T effector cell (Teff cell). Natural Treg cell inhibit Teff cell activity in a contact-dependent manner. Induced Treg cells, which secrete inhibitory cytokines (e.g. IL-10 [Tr1] or TGF-β [Th3]) share similarities with nTreg cell, but perform their inhibitory functions in a cytokine-dependent manner (Bluestone and Abbas 2003). In general nTreg cells are specific to self antigens for control of excessive autoimmune response, while iTregs cells are specific to environmental antigens, and inhibit immune response induced by commensal bacterium or allergens (Josefowicz, Niec et al. 2012). Recently it has been suggested that, *in vivo*, nTreg and iTreg cells are functionally similar and that the main function of iTreg cell is

to increase the diversity of the Treg cell pool (Haribhai, Williams et al. 2011). We have shown that DC10 secrete IL-10 and up-regulate the inhibitory function of Tregs and convert $CD4^{+}CD25^{-}Foxp3^{-}$ T precursor cells to $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs in the lung of asthmatic mice. These Tregs inhibit Th2 responses in mice model of asthma (Figure 6-2).

Figure 6-2. A simplistic view of induction of tolerance cascade by DC10 therapy.

^{OVA}DC10 are progressively recruited to the lungs and draining LNs in asthmatic mice after *i.p.* injection, where they are thought to secrete high levels of IL-10 and interact with lung-resident Tregs and Teffs. Furthermore, ^{OVA}DC10 up-regulate the inhibitory function of Tregs and convert $CD4^+CD25^-Foxp3^-$ T precursor cells to $CD4^+CD25^+Foxp3^+$ Tregs. Finally, nTregs and iTreg cells reverse Th2 response in airway.

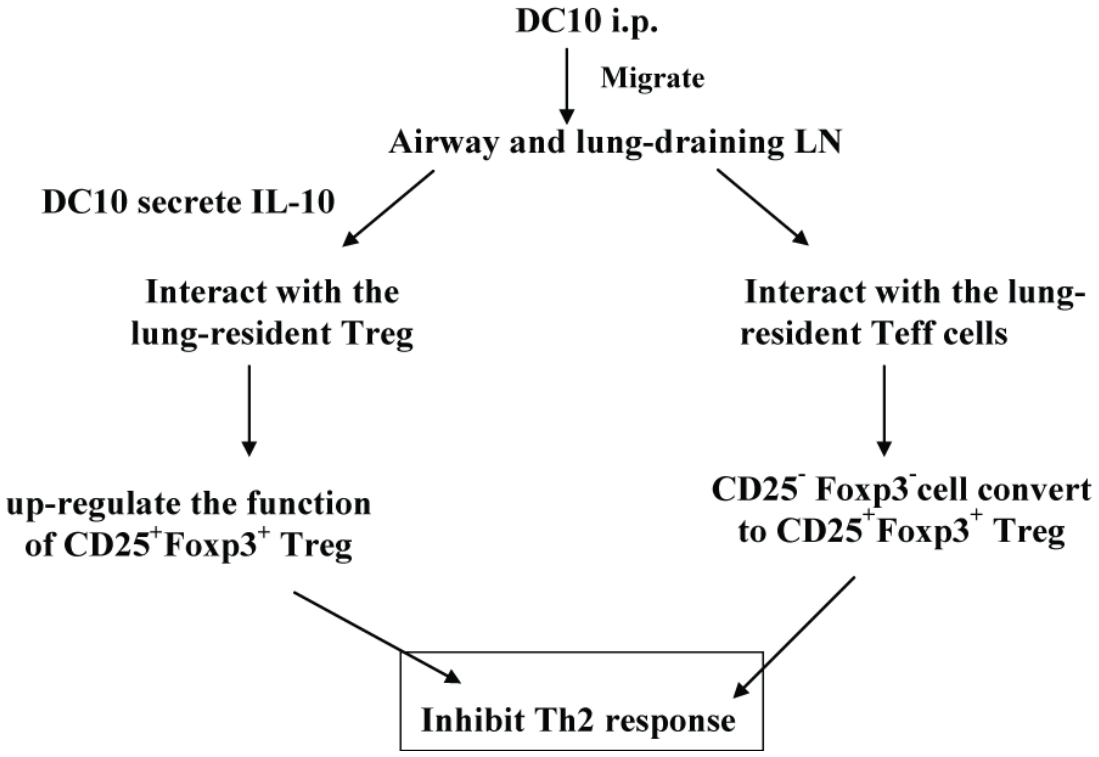


Figure 6-2. A simplistic view of induction of tolerance cascade by DC10 therapy.

Tregs can be isolated from mice and humans on the basis of their expression of high levels of CD25 (the α-chain of the IL-2 receptor), but it is critical to remember that CD25 is expressed by all activated T cells (ie, effector and regulatory cells alike). Other cell-surface markers that were originally thought to be specific for naturally-occurring Tregs and, in some cases, involved in their function, include CTLA-4 and GITR but, like CD25, these are also expressed by activated T cells. Therefore, to date, we have not yet identified any cell-surface markers that are specific for Tregs. Foxp3 was shown to be specifically expressed in the cytoplasmic compartment of naturally-occurring Tregs and related to the function of these Tregs cells (Fontenot, Rasmussen et al. 2005; Hawrylowicz and O'Garra 2005; Randolph and Fathman 2006; Stock, DeKruyff et al. 2006; Toda and Piccirillo 2006) although human Teff cells transiently express Foxp3. Foxp3 is also expressed by immunologically-induced Tregs (Toda and Piccirillo 2006). Since Foxp3 is an intracellular marker of Tregs, it can not be used to isolate these cells. For example, the $CD4^+CD25^+$ draining LN cells isolated by Strickland et al for adoptive transfer showed 73% $Foxp3^+$ cells as reported (Strickland, Stumbles et al. 2006), which means the transferred cells contained 27% $CD4^+CD25^+Foxp3^-$ activated T cells, which could have been activated Teff cells. Therefore the need for a good cell-surface marker to distinguish activated effector T cells from Tregs should be highlighted.

In this study, maximal pulmonary Treg activity was observed at 3 wk (versus 1, 2 or 4 wk) after DC10 treatment of asthmatic mice in both *in vitro* (inhibitory assay) and *in vivo* (adoptive therapy) experiments. While we successfully induced $CD4^+CD25^+Foxp3^+$ Treg differentiation from $CD4^+CD25^-Foxp3^-$ Teff both *in vitro* and *in vivo*, the Treg induce *in vivo* expressed high levels of the Treg-associated markers LAG3, CTLA-4,

ICOS, PD-1 and GITR. These results suggest that specific allergen-presenting DC10 initiated an infectious tolerance process, which includes the induction of Tregs from $CD4^+CD25^-Foxp3^-$ Teff cells and up-regulation of the inhibitory functions of lung-resident DC and Tregs, both of which are likely involved in the down-regulation of allergen- specific Th2 responses.

We isolated endogenous DCs from lung in the DC10-treated asthmatic mice and observed the increased abilities of these cells to inhibit Th2 response. (Li, Zhang, Dawicki and Gordon; The characterization and function of endogenous pulmonary DCs in regulatory DC-treated asthmatic mice; unpublished data). This indicated that DC10 transferred tolerance to endogenous DCs and induced an infectious tolerance.

Numerous groups have reported that iTreg cells can be differentiated from naive $CD4^+$ T cells (Coombes, Siddiqui et al. 2007; Yamazaki, Bonito et al. 2007; Yamazaki, Dudziak et al. 2008), but we and others have reported that $CD4^+CD25^+Foxp3^+$ iTreg cells can also be differentiated from $CD62L^{int}CD69^+$ antigen-experienced T cells (i.e. Teff cells) (Huang, Dawicki et al. 2010; Schallenberg, Tsai et al. 2010). We found that DC10 induced $CD4^+CD25^+Foxp3^+$ Treg cell differentiation in our mouse model of asthma (Huang, Dawicki et al. 2010), but we did not know to what extent nTreg versus iTreg contribute to the induction of tolerance. This was an important question in our exploration of the mechanisms of this tolerance induction.

Although two subsets of Treg cells, nTreg and iTreg cells, are relatively well defined by where and how they develop, so far no markers have been found to distinguish iTreg from nTreg cells (Murai, Krause et al. 2010). Thus, in this study we generated GFP-Foxp3/CD45.1/OT2 and GFP-Foxp3/CD45.2/OT2 mice and used CD45.1 and GFP-

Foxp3 as markers to trace the fate of allergen-specific iTreg and nTreg cell. Since both Treg cell types have the same specificity of TCR (OVA), we were able to directly compare their suppressive activity. Since the iTreg and nTreg cells were compared in the same asthmatic mouse, the experimental conditions in this study closely approximated natural physiological processes. We compared the suppressive properties of iTreg and nTreg that were induced in vitro and in vivo under the influence of DC10. All our results indicated that DC10-induced Treg cells are more effective than DC10-exposed nTreg cells in blocking Th2 effector cell responses in vitro and in asthmatic mice (Figure 6-3).

In a mouse colitis model, transfer of naïve $CD45RB^{high}CD4^{+}$ T cells into immunodeficient mice induces inflammatory bowel disease (IBD), while transfer of $CD4^{+}CD25^{+}$T cells cured intestinal inflammation and fostered the reappearance of normal intestinal tissue structure (Mottet, Uhlig et al. 2003). Further study conducted by Haribhai et al (Haribhai, Lin et al. 2009) showed that if naïve $CD45RB^{high}CD4^{+}$ T cells were derived from mice that were able to generate $Foxp3^{+}$ iTreg in vivo, treatment with nTreg cells could completely resolve the colitis because besides nTreg, iTreg generated in vivo from transferred naïve cells were also present. However, if the naïve cells were isolated from Foxp3 KO mice, thus, iTreg cells were not able to be converted in vivo, then complete protection could only be obtained when nTreg cells were co administered with in vitro-generated iTreg cells. These results indicated that both nTreg and iTreg were required for protection from the disease, supporting synergistic actions between nTreg and iTreg cells.

In a lymphoproliferative disease model (Haribhai, Williams et al. 2011), mice with Foxp3 deficiency developed autoimmune lymphoproliferative disease and did not

survive beyond day 32 of life. Adoptive transfer of only nTreg cells prevented disease lethality, but did not suppress chronic inflammation and autoimmunity. However, a combined transfer of nTreg cells with conventional T cells (Tconv) reconstituted the iTreg pool and established tolerance. Selective iTreg cell depletion in successfully treated mice resulted in weight loss and inflammation, but not lethality. This data demonstrated that both nTreg and iTreg cells played roles in rescuing mice from lymphproliferative disease caused by Foxp3 deficiency.

Although nTreg and iTreg cells have a distinct roles in immune tolerance to self and environmental antigens respectively, they seemingly have overlapping effects or act in concert to achieve immune tolerance as shown above. TCR repertoire studies may provide molecular mechanisms for these observations. It was found that the TCR repertoires of Tconv and Treg cells overlap by 4-40% (Hsieh, Liang et al. 2004; Pacholczyk, Ignatowicz et al. 2006; Wong, Obst et al. 2007; Lathrop, Santacruz et al. 2008) and nTreg and iTreg cells by 3-15% (Haribhai, Williams et al. 2011) depending what approach was used for the study. Therefore, it seems possible that, at least in part, iTreg and nTreg share TCR specificity and recognize overlapping sets of self or non-self antigens. In addition, the gene expression profiles of iTreg and nTreg cells from rescued mice with lymphoproliferative disease are remarkably similar to one another (Haribhai, Williams et al. 2011). This suggests that iTreg and nTreg cells may share inhibitory functions through overlapping effector mechanisms.

Previous studies reported that iTreg and nTreg cells performed synergic inhibitory functions, and *in vitro* and *in vivo* differentiated iTreg cells were functionally equivalent (Curotto de Lafaille and Lafaille 2009; Haribhai, Williams et al. 2011). Herein our data

suggest that allergen-specific iTreg cells play a more important role in DC10-induced asthma tolerance than do allergen-specific nTreg cells. While exploring this difference, we found that iTreg cells expressed higher levels of markers (i.e. ICOS, PD-1, GITR, LAG3 and CTLA-4) than nTreg cells and that iTreg cells employed IL-10 to inhibit Th2 responses, unlike nTreg cells. The broader mechanisms by which iTreg and nTreg cells mediate asthma tolerance are still largely unknown. To further elucidate these mechanisms, we would need determine the full array of inhibitory molecules and cytokines expressed by iTreg and nTreg cells. We recently reported that amelioration of Th2 response last at least 8 months (Nayyar, Dawicki et al. 2012). Thus another important issue for further research is to address the relative contributions of iTreg cells and nTreg cells in the late stage of the induction of tolerance. In addition, it has been reported that Breg cells suppress experimental autoimmune encephalomyelitis (EAE) through their impact on $Foxp3^+$ Treg cells (Ray, Basu et al. 2012; Sun, Czerkinsky et al. 2012). Exploring the roles of Breg in the induction of tolerance by tolerogenic DCs is another pertinently and potentially important question. Tracking the fate of iTreg cells and studying their memory in asthma tolerance are other promising avenues for future work.

Over the past 17 years Treg cells have become a increasingly promising avenues for clinical immunotherapy in transplant rejection, autoimmune and allergic diseases (Leslie 2011). Several clinical trials have been completed, and others are underway, in which the therapeutic cells came from freshly isolated $CD4^+CD25^+$ Treg cells that were ex vivo expanded by anti-CD3 and anti-CD28 antibodies or APC (Wang, Lu et al. 2011). Our study suggests that Treg cells differentiated from Teff cells may be more effective

than those from nTreg cells at suppressing ongoing Th2 responses. This is clinically important because it is the suppression of ongoing responses (i.e. wherein Teff cells are present and activated) that is the major challenge.

Herein we developed a unique protocol to address the relative roles of nTreg and iTreg in immune tolerance in our mouse model of asthma. This protocol has several advantages, and it overcomes some limitations posed in earlier studies (Huter, Stummvoll et al. 2008; Haribhai, Lin et al. 2009). First, the lack of phenotypical markers that distinguishes iTreg from nTreg cells has been a major obstacle to us gaining a complete understanding of the relative roles of these two subsets in overall Treg cell biology. Thus, we reported herein that we generated two strains of congenic mice, namely GFP-Foxp3/CD45.1/OT2 and GFP-Foxp3/CD45.2/OT2, and this allowed us to use GFP-Foxp3/CD45.1 to distinguish iTreg from nTreg cells. Second, while it is not difficult to obtain nTreg or iTreg using in vitro culture protocols, nTreg or iTreg cells generated in this way may not be representative of cells generated in vivo; in our protocols we generated both allergen-specific iTreg and nTreg in vivo. Third, since the nTreg and iTreg cells used in our experiments had the same OVA-specific TCR, we are able to evaluate their relative contributions to immune tolerance in the same subject. Finally, the congenic (CD45) and knock-in (GFP) markers between nTreg and iTreg allowed us to track the fate and assess their individual functions in the different organs (e.g. spleen, lung, gut) in vivo. This strategy would also be valuable as a tool to explore the relative roles of iTreg and nTreg cells in other disease models (e.g., autoimmune diseases and cancers). For example, because we know of no specific surface markers for nTreg and iTreg cells, their roles in tumour immunity and tolerance are controversial. Indeed, the

OT2 transgenic cross-bred mice we generated for our study would also lend themselves to exploration in tumour models. A case in point is the EG7 mouse lymphoma cell line, generated from EL4 cells that have been transfected to express OVA, or the MO4 mouse melanoma cell line generated by OVA-transgene tranfection of B10F10 cells. Clarification of the relative contributions of nTreg and iTreg cells to health and disease in asthma and other models is important from a fundamental immunology perspective, but it will also be potentially important for clinical applications.

Tolerogenic DCs (tDCs) can be experimentally generated in vitro from bone marrow-derived DCs (BMDC) in mice or peripheral blood monocyte-derived DCs in humans. To date there are three major ways to obtain experimentally-induced tDCs for potential immunotherapy. (1) Such DCs can be generated through exposure of immature DC to various inhibitory cytokines (e.g. IL-10 and/or TGFβ). The immunosuppressive efficacy of these cells has been tested in various disease models. For example, murine splenic or BMDCs, or human monocyte-derived DCs that have been generated through exposure to IL-10 are able to induce $CD4^{+}CD25^{+}$ Treg cells in asthma (Huang, Dawicki et al. 2010; Nayyar, Dawicki et al. 2012), EAE (Benkhoucha, Santiago-Raber et al. 2010), T1D (Tai, Yasuda et al. 2011), and GVHD (Sato, Eizumi et al. 2009). The IL-10 signalling pathway reportedly maintains immature APCs in an immature state even when they are exposed to maturation signals (Lang, Patel et al. 2002). (2) Tolerogenic DCs can also be generated by exposure to immunosuppressive or anti-inflammatory drugs (e.g. GC, rapamycin and cyclosporine), which block DC maturation or impair their ability to produce IL-12. (3) They can also be generated through genetically modification such that they express inhibitory molecules such as IL-10, TGFβ, IDO, or CTLA4-Ig which,

among other things, can block the expression of co-stimulation molecules. DCs transduced to express IDO can prevent the proliferation of allogenetic T cells (Morelli and Thomson 2007; Maldonado, von Andrian et al. 2010).

Therapeutic application of tDCs in the clinic is an attractive option that brings with it multiple advantages: (1) tolerance induced by tDCs would be antigen-specific (Huang, Dawicki et al. 2010; Lu, Dawicki et al. 2011) such that is would not impact unrelated required immune responses (e.g., against pathogens or tumours) and (2) tDCs can induce prolonged infectious tolerance (Huang, Dawicki et al. 2010; Nayyar, Dawicki et al. 2012). However therapeutic application of tDCs also brings its own challenges: (1) The numbers of DCs that can be obtained from each individual may be limiting (Morelli and Thomson 2007); (2) the delivery route, timing, dose and frequency of administration of the tDCs will need to be optimized for use in the clinic; and (3) some populations tDCs generated in vitro could potentially develop into stimulatory phenotype DCs under some (e.g., inflammatory) conditions in vivo. Taken together though, the prospect for clinical translation of tDC use is promising, despite such challenges. It appears imminent that tDC therapeutics will enter the clinic, just as immunostimulatory DCs have entered clinical trials in the context of cancer (Bauer, Dauer et al. 2011). Indeed, multiple laboratories have begun exploring the potential use of DC10 and similar tDC in the context of human disease.

Figure 6-3. Tolerogenic dendritic cell-induced iTreg cells are more effective than nTreg cells in blocking Th2 effector cell responses in asthmatic mice.

After *i.p.* DC10 treatment, DC10 quickly and continually are recruited to the airway (i.e. lung parenchyma and lung draining LN) from the peritoneal cavity. In the airway, DC10 are thought to secrete high levels of IL-10 and interact with lung-resident Tregs and Teffs. Furthermore, DC10 up-regulate the inhibitory function of Tregs and convert $CD4^+CD25^-Foxp3^-$ Teffs to $CD4^+CD25^+Foxp3^+$ Tregs. DC10-induced regulatory T cells (iTreg) are more effective than natural regulatory T cells (nTreg) in blocking Th2 T effector cell responses in asthmatic mice.

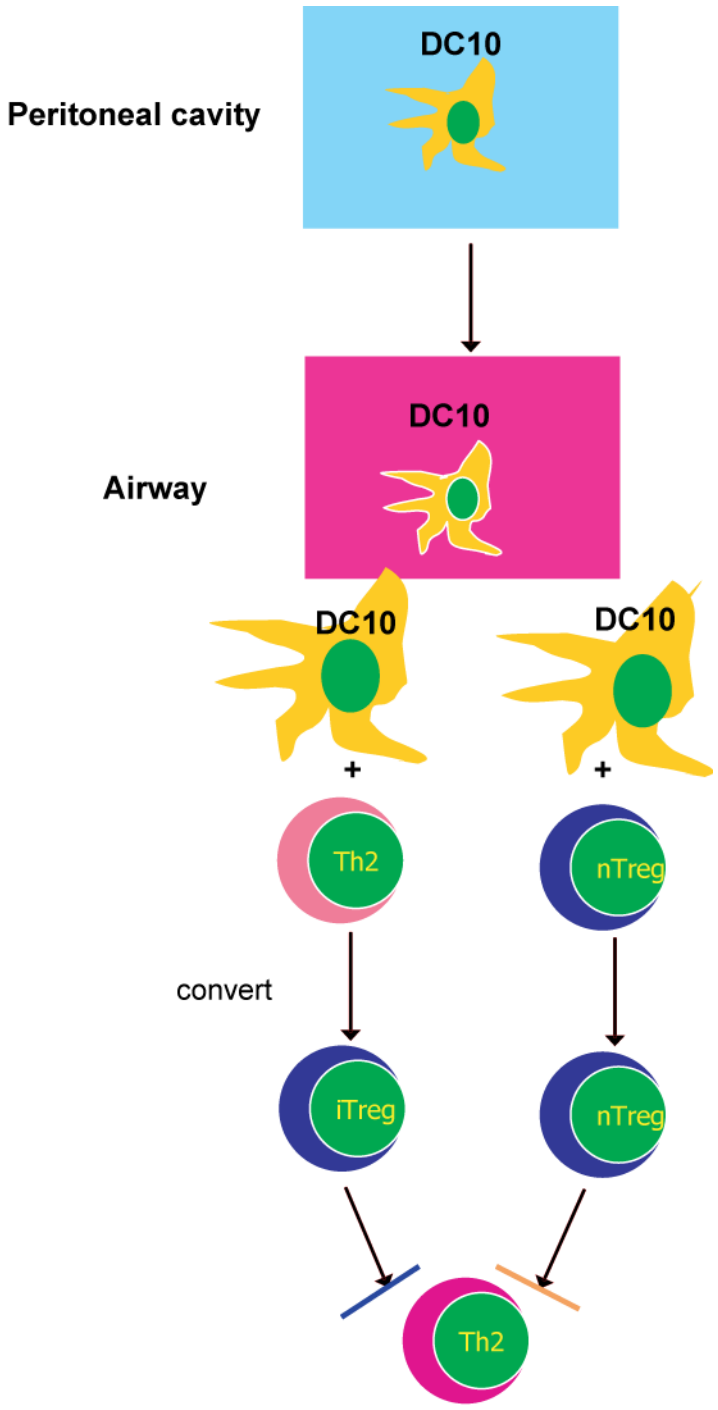


Figure 6-3. Tolerogenic dendritic cell-induced iTreg cells are more effective than nTreg cells in blocking Th2 effector cell responses in asthmatic mice.

Overall Summary:

IL-10 differentiated dendritic cells (DC10) are widely reported to abrogate asthmatic responses in both mice and humans. To explore the mechanisms of DC10-induced asthma tolerization we used DC10 that were deficient in MHCII, or CD80 and CD86, or DC10 that were unable to express IL-10 to assess the roles of these three distinct components of the antigen-presenting machinery of DC10. We found that in each case the deficiency significantly reduced the tolerogenicity of these cells as determined by assessment of AHR and Th2 immunoinflammatory responses to recall allergen challenge. The data demonstrate that the activities of DC10 are differentially affected by IL-10, MHC II and costimulatory molecules expression.

Next we monitored effects of DC10 on the Treg cells in well established OVA-asthmatic mice. We observed that adoptive transfer of DC10 activated lung Tregs in OVA-asthmatic mice via upregulating Treg-associated markers and enhancing inhibitory functions. More importantly, we showed that DC10 treatment induce tolerance at least in part by inducing Teff to differentiate into $CD4^{+}CD25^{hi}Foxp3^{+}$ Treg.

Natural Treg (nTreg) and induced Treg (iTreg) are the two main types of $CD4^{+}CD25^{hi}Foxp3^{+}$ Treg. We further assessed the relative contribution of nTreg and iTreg to the tolerization of asthma. We found that Treg cells induced by DC10 were better than nTreg in inhibiting Th2 cell responses in vitro. Induced Treg efficiently ameliorated the asthma phenotype in vivo, while equal numbers of nTreg of the same antigen-specificity displayed no remarkable activity. These data suggest that induced Treg are distinctly superior to nTreg in their abilities to induce asthma tolerance (Figure 6-3).

LIST OF REFERENCES

Adler, H. S. and K. Steinbrink (2007). "Tolerogenic dendritic cells in health and disease: friend and foe!" Eur J Dermatol **17**(6): 476-91.

Akbari, O., R. H. DeKruyff, et al. (2001). "Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen." Nat Immunol **2**(8): 725-31.

Akbari, O., G. J. Freeman, et al. (2002). "Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity." Nat Med **8**(9): 1024-32.

Akdis, C. A. and M. Akdis (2009). "Mechanisms and treatment of allergic disease in the big picture of regulatory T cells." J Allergy Clin Immunol **123**(4): 735-46; quiz 747-8.

Alcorn, J. F., C. R. Crowe, et al. (2010). "TH17 cells in asthma and COPD." Annu Rev Physiol **72**: 495-516.

Almeida, A. R., B. Rocha, et al. (2005). "Homeostasis of T cell numbers: from thymus production to peripheral compartmentalization and the indexation of regulatory T cells." Semin Immunol **17**(3): 239-49.

Alvarez, D., E. H. Vollmann, et al. (2008). "Mechanisms and consequences of dendritic cell migration." Immunity **29**(3): 325-42.

Andersson, C. K., E. Tufvesson, et al. (2011). "Alveolar mast cells shift to an FcεRI-expressing phenotype in mild atopic asthma: a novel feature in allergic asthma pathology." Allergy **66**(12): 1590-1597.

Asselin-Paturel, C., A. Boonstra, et al. (2001). "Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology." Nat Immunol **2**(12): 1144-50.

Awasthi, A., Y. Carrier, et al. (2007). "A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells." Nature immunology **8**(12): 1380-9.

Barnes, P. J. (2008). "Immunology of asthma and chronic obstructive pulmonary disease." Nat Rev Immunol **8**(3): 183-192.

Barnes, P. J. (2011). "Biochemical basis of asthma therapy." J Biol Chem **286**(38): 32899-905.

Barrett, N. A. and K. F. Austen (2009). "Innate Cells and T Helper 2 Cell Immunity in Airway Inflammation." Immunity **31**(3): 425-437.

Bateman, E. D., S. S. Hurd, et al. (2008). "Global strategy for asthma management and prevention: GINA executive summary." Eur Respir J **31**(1): 143-78.

Battaglia, M., A. Stabilini, et al. (2005). "Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells." Blood **105**(12): 4743-4748.

Bauer, C., M. Dauer, et al. (2011). "Dendritic cell-based vaccination of patients with advanced pancreatic carcinoma: results of a pilot study." Cancer Immunology, Immunotherapy **60**(8): 1097-1107.

Bellinghausen, I., U. Brand, et al. (2001). "Inhibition of human allergic T-cell responses by IL-10-treated dendritic cells: differences from hydrocortisone-treated dendritic cells." J Allergy Clin Immunol **108**(2): 242-9.

Bellinghausen, I., B. Konig, et al. (2006). "Inhibition of human allergic T-helper type 2 immune responses by induced regulatory T cells requires the combination of

interleukin-10-treated dendritic cells and transforming growth factor-beta for their induction." Clin Exp Allergy **36**(12): 1546-55.

Benkhoucha, M., M.-L. Santiago-Raber, et al. (2010). "Hepatocyte growth factor inhibits CNS autoimmunity by inducing tolerogenic dendritic cells and CD25+Foxp3+ regulatory T cells." Proceedings of the National Academy of Sciences **107**(14): 6424-6429.

Berry, M. A., B. Hargadon, et al. (2006). "Evidence of a Role of Tumor Necrosis Factor Î± in Refractory Asthma." New England Journal of Medicine **354**(7): 697-708.

Bilate, A. B. and J. J. Lafaille (2011). "It takes two to tango." Immunity **35**(1): 6-8.

Bilate, A. M. and J. J. Lafaille (2012). "Induced CD4+Foxp3+ Regulatory T Cells in Immune Tolerance." Annual Review of Immunology **30**(1): 733-758.

Blair, P. A., K. A. Chavez-Rueda, et al. (2009). "Selective targeting of B cells with agonistic anti-CD40 is an efficacious strategy for the generation of induced regulatory T2-like B cells and for the suppression of lupus in MRL/lpr mice." J Immunol **182**(6): 3492-502.

Bluestone, J. A. and A. K. Abbas (2003). "Natural versus adaptive regulatory T cells." Nat Rev Immunol **3**(3): 253.

Braga, M., C. Quecchia, et al. (2011). "T regulatory cells in allergy." Int J Immunopathol Pharmacol **24**(1 Suppl): 55S-64S.

Busse, W. W. and R. F. Lemanske, Jr. (2001). "Asthma." N Engl J Med **344**(5): 350-62.

Byrne, S. N., A. Y. Limon-Flores, et al. (2008). "Mast cell migration from the skin to the draining lymph nodes upon ultraviolet irradiation represents a key step in the induction of immune suppression." J Immunol **180**(7): 4648-55.

Carter, N. A., R. Vasconcellos, et al. (2011). "Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells." J Immunol **186**(10): 5569-79.

Castellaneta, A., M. Abe, et al. (2004). "Identification and characterization of intestinal peyer's patch interferon-[alpha] producing (plasmacytoid) dendritic cells." Human Immunology **65**(2): 104.

Chen, L., P. S. Linsley, et al. (1993). "Costimulation of T cells for tumor immunity." Immunol Today **14**(10): 483-6.

Chen, W., W. Jin, et al. (2003). "Conversion of Peripheral CD4+CD25- Naive T Cells to CD4+CD25+ Regulatory T Cells by TGF-{beta} Induction of Transcription Factor Foxp3." J. Exp. Med. **198**(12): 1875-1886.

Cho, S. H., L. A. Stanciu, et al. (2005). "Increased interleukin-4, interleukin-5, and interferon-gamma in airway CD4+ and CD8+ T cells in atopic asthma." Am J Respir Crit Care Med **171**(3): 224-30.

Cobbold, S. P., K. F. Nolan, et al. (2003). "Regulatory T cells and dendritic cells in transplantation tolerance: molecular markers and mechanisms." Immunol Rev **196**: 109-24.

Cohn, L. and A. Ray (2000). "T-helper type 2 cell-directed therapy for asthma." Pharmacol Ther **88**(2): 187-96.

Coombes, J. L. and F. Powrie (2008). "Dendritic cells in intestinal immune regulation." Nat Rev Immunol **8**(6): 435.

Coombes, J. L., K. R. R. Siddiqui, et al. (2007). "A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-{beta} and retinoic acid dependent mechanism." J. Exp. Med. **204**(8): 1757-1764.

Corren, J., W. Busse, et al. (2010). "A randomized, controlled, phase 2 study of AMG 317, an IL-4Ralpha antagonist, in patients with asthma." Am J Respir Crit Care Med **181**(8): 788-96.

Crotty, S. (2011). "Follicular helper CD4 T cells (TFH)." Annu Rev Immunol **29**: 621-63.

Curotto de Lafaille, M. A., N. Kutchukhidze, et al. (2008). "Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation." Immunity **29**(1): 114-26.

Curotto de Lafaille, M. A. and J. J. Lafaille (2009). "Natural and adaptive Foxp3+ regulatory T cells: more of the same or a division of labor?" Immunity **30**(5): 626.

Dardalhon, V., T. Korn, et al. (2008). "Role of Th1 and Th17 cells in organ-specific autoimmunity." J Autoimmun **31**(3): 252-6.

Darrasse-Jeze, G., S. Deroubaix, et al. (2009). "Feedback control of regulatory T cell homeostasis by dendritic cells in vivo." J Exp Med **206**(9): 1853-62.

de Heer, H. J., H. Hammad, et al. (2004). "Essential Role of Lung Plasmacytoid Dendritic Cells in Preventing Asthmatic Reactions to Harmless Inhaled Antigen." The Journal of Experimental Medicine **200**(1): 89-98.

Desai, D. and C. Brightling (2009). "Cytokine and anti-cytokine therapy in asthma: ready for the clinic?" Clinical & Experimental Immunology **158**(1): 10-19.

Dudziak, D., A. O. Kamphorst, et al. (2007). "Differential Antigen Processing by Dendritic Cell Subsets in Vivo." Science **315**(5808): 107-111.

Dumitriu, I. E., D. R. Dunbar, et al. (2009). "Human Dendritic Cells Produce TGF-{beta}1 under the Influence of Lung Carcinoma Cells and Prime the Differentiation of CD4+CD25+Foxp3+ Regulatory T Cells." J Immunol **182**(5): 2795-2807.

Enk, A. H., H. Jonuleit, et al. (1997). "Dendritic cells as mediators of tumor-induced tolerance in metastatic melanoma." Int J Cancer **73**(3): 309-16.

Erin, E. M., B. R. Leaker, et al. (2006). "The Effects of a Monoclonal Antibody Directed against Tumor Necrosis Factor-Î± in Asthma." American Journal of Respiratory and Critical Care Medicine **174**(7): 753-762.

Fife, B. T. and J. A. Bluestone (2008). "Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways." Immunol Rev **224**: 166-82.

Flood-Page, P., C. Swenson, et al. (2007). "A study to evaluate safety and efficacy of mepolizumab in patients with moderate persistent asthma." Am J Respir Crit Care Med **176**(11): 1062-71.

Fontenot, J. D., J. P. Rasmussen, et al. (2005). "Regulatory T Cell Lineage Specification by the Forkhead Transcription Factor Foxp3." Immunity **22**(3): 329.

Francisco, L. M., V. H. Salinas, et al. (2009). "PD-L1 regulates the development, maintenance, and function of induced regulatory T cells." Journal of Experimental Medicine **206**(13): 3015-3029.

Fu, C. L., Y. H. Chuang, et al. (2006). "Effects of adenovirus-expressing IL-10 in alleviating airway inflammation in asthma." J Gene Med **8**(12): 1393-9.

Furuhashi, K., T. Suda, et al. (2012). "Mouse Lung CD103+ and CD11bhigh Dendritic Cells Preferentially Induce Distinct CD4+ T-Cell Responses." American Journal of Respiratory Cell and Molecular Biology **46**(2): 165-172.

Gabryšová, L., J. R. Christensen, et al. (2011). "Integrated T-cell receptor and costimulatory signals determine TGF-β-dependent differentiation and maintenance of Foxp3+ regulatory T cells." European Journal of Immunology **41**(5): 1242-1248.

Galli, S. J., M. Tsai, et al. (2008). "The development of allergic inflammation." Nature **454**(7203): 445.

Garritsen, H. S. P., L. Macke, et al. (2010). "Efficient generation of clinical-grade genetically modified dendritic cells for presentation of multiple tumor-associated proteins." Transfusion **50**(4): 831-842.

Gauvreau, G. M., J. M. Lee, et al. (2000). "Increased Numbers of Both Airway Basophils and Mast Cells in Sputum after Allergen Inhalation Challenge of Atopic Asthmatics." Am. J. Respir. Crit. Care Med. **161**(5): 1473-1478.

Gavett, S. H., S. L. Madison, et al. (1999). "Allergic lung responses are increased in prostaglandin H synthase-deficient mice." J Clin Invest **104**(6): 721-32.

Glaab, T., A. Daser, et al. (2001). "Tidal midexpiratory flow as a measure of airway hyperresponsiveness in allergic mice." Am J Physiol Lung Cell Mol Physiol **280**(3): L565-73.

Glaab, T., M. Ziegert, et al. (2005). "Invasive versus noninvasive measurement of allergic and cholinergic airway responsiveness in mice." Respir Res **6**: 139.

Godfrey, W. R., Y. G. Ge, et al. (2004). "In vitro-expanded human CD4+CD25+ T-regulatory cells can markedly inhibit allogeneic dendritic cell-stimulated MLR cultures." Blood **104**(2): 453-461.

Godfrey, W. R., D. J. Spoden, et al. (2005). "Cord blood CD4+CD25+-derived T regulatory cell lines express FoxP3 protein and manifest potent suppressor function." Blood **105**(2): 750-758.

Golovina, T. N., T. Mikheeva, et al. (2008). "CD28 costimulation is essential for human T regulatory expansion and function." J Immunol **181**(4): 2855-68.

Gondek, D. C., L.-F. Lu, et al. (2005). "Cutting Edge: Contact-Mediated Suppression by CD4+CD25+ Regulatory Cells Involves a Granzyme B-Dependent, Perforin-Independent Mechanism." J Immunol **174**(4): 1783-1786.

Gordon, J. R., F. Li, et al. (2005). "CD8 alpha+, but not CD8 alpha-, dendritic cells tolerize Th2 responses via contact-dependent and -independent mechanisms, and reverse airway hyperresponsiveness, Th2, and eosinophil responses in a mouse model of asthma." J Immunol **175**(3): 1516-22.

Gordon, J. R., F. Li, et al. (2005). "The combined CXCR1/CXCR2 antagonist CXCL8(3-74)K11R/G31P blocks neutrophil infiltration, pyrexia, and pulmonary vascular pathology in endotoxemic animals." J Leukoc Biol **78**(6): 1265-72.

Goubier, A., B. Dubois, et al. (2008). "Plasmacytoid Dendritic Cells Mediate Oral Tolerance." Immunity **29**(3): 464.

Grossman, W. J., J. W. Verbsky, et al. (2004). "Human T Regulatory Cells Can Use the Perforin Pathway to Cause Autologous Target Cell Death." Immunity **21**(4): 589.

Groux, H., M. Bigler, et al. (1996). "Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells." J Exp Med **184**(1): 19-29.

Hadeiba, H., T. Sato, et al. (2008). "CCR9 expression defines tolerogenic plasmacytoid dendritic cells able to suppress acute graft-versus-host disease." Nat Immunol.

Hammad, H., M. Plantinga, et al. (2010). "Inflammatory dendritic cellsâ€"not basophilsâ€"are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen." The Journal of Experimental Medicine **207**(10): 2097-2111.

Hansbro, P. M., G. E. Kaiko, et al. (2011). "Cytokine/anti-cytokine therapy - novel treatments for asthma?" Br J Pharmacol **163**(1): 81-95.

Haribhai, D., W. Lin, et al. (2009). "A central role for induced regulatory T cells in tolerance induction in experimental colitis." J Immunol **182**(6): 3461-8.

Haribhai, D., J. B. Williams, et al. (2011). "A requisite role for induced regulatory T cells in tolerance based on expanding antigen receptor diversity." Immunity **35**(1): 109-22.

Hart, P. H., M. A. Grimbaldeston, et al. (1998). "Dermal mast cells determine susceptibility to ultraviolet B-induced systemic suppression of contact hypersensitivity responses in mice." J Exp Med **187**(12): 2045-53.

Hawiger, D., K. Inaba, et al. (2001). "Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo." J Exp Med **194**(6): 769-79.

Hawrylowicz, C. M. and A. O'Garra (2005). "Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma." Nat Rev Immunol **5**(4): 271-83.

Henry, E., C. J. Desmet, et al. (2008). "Dendritic cells genetically engineered to express IL-10 induce long-lasting antigen-specific tolerance in experimental asthma." J Immunol **181**(10): 7230-7242.

Hill, J. A., T. E. Ichim, et al. (2003). "Immune modulation by silencing IL-12 production in dendritic cells using small interfering RNA." J Immunol **171**(2): 691-6.

Hippen, K. L., S. C. Merkel, et al. (2011). "Massive ex Vivo Expansion of Human Natural Regulatory T Cells (Tregs) with Minimal Loss of in Vivo Functional Activity." Science Translational Medicine **3**(83): 83ra41.

Hochweller, K. and S. M. Anderton (2005). "Kinetics of costimulatory molecule expression by T cells and dendritic cells during the induction of tolerance versus immunity in vivo." Eur J Immunol **35**(4): 1086-96.

Holgate, S., R. Buhl, et al. (2009). "The use of omalizumab in the treatment of severe allergic asthma: A clinical experience update." Respir Med **103**(8): 1098-113.

Holgate, S., N. Smith, et al. (2009). "Effects of omalizumab on markers of inflammation in patients with allergic asthma." Allergy **64**(12): 1728-36.

Holgate, S. T. (2011). "The sentinel role of the airway epithelium in asthma pathogenesis." Immunological Reviews **242**(1): 205-219.

Horwitz, D. A., S. G. Zheng, et al. (2004). "Regulatory T cells generated ex vivo as an approach for the therapy of autoimmune disease." Semin Immunol **16**(2): 135-43.

Houot, R., I. Perrot, et al. (2006). "Human CD4+CD25high regulatory T cells modulate myeloid but not plasmacytoid dendritic cells activation." J Immunol **176**(9): 5293-8.

Hsieh, C. S., Y. Liang, et al. (2004). "Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors." Immunity **21**(2): 267-77.

Huang, H., W. Dawicki, et al. (2010). "Tolerogenic dendritic cells induce CD4+CD25hiFoxp3+ regulatory T cell differentiation from CD4+CD25-/loFoxp3- effector T cells." J Immunol **185**(9): 5003-10.

Huang, H., S. Hao, et al. (2007). "CD4+ Th1 cells promote CD8+ Tc1 cell survival, memory response, tumor localization and therapy by targeted delivery of interleukin 2 via acquired pMHC I complexes." Immunology **120**(2): 148-159.

Hugues, S., A. Boissonnas, et al. (2006). "The dynamics of dendritic cell-T cell interactions in priming and tolerance." Curr Opin Immunol **18**(4): 491-5.

Huter, E. N., G. H. Stummvoll, et al. (2008). "Cutting Edge: Antigen-Specific TGFÎ²-Induced Regulatory T Cells Suppress Th17-Mediated Autoimmune Disease." The Journal of Immunology **181**(12): 8209-8213.

Ilarregui, J. M., D. O. Croci, et al. (2009). "Tolerogenic signals delivered by dendritic cells to T cells through a galectin-1-driven immunoregulatory circuit involving interleukin 27 and interleukin 10." Nature immunology **10**(9): 981-91.

Inaba, K., M. Inaba, et al. (1992). "Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor." J Exp Med **176**(6): 1693-702.

Ishiura, Y., M. Fujimura, et al. (2009). "COX-2 inhibition attenuates cough reflex sensitivity to inhaled capsaicin in patients with asthma." J Investig Allergol Clin Immunol **19**(5): 370-4.

Ito, T., M. Yang, et al. (2007). "Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand." J. Exp. Med. **204**(1): 105-115.

Jain, N., H. Nguyen, et al. (2010). "Dual function of CTLA-4 in regulatory T cells and conventional T cells to prevent multiorgan autoimmunity." Proc Natl Acad Sci U S A **107**(4): 1524-8.

Joetham, A., K. Takeda, et al. (2007). "Naturally occurring lung CD4(+)CD25(+) T cell regulation of airway allergic responses depends on IL-10 induction of TGF-beta." J Immunol **178**(3): 1433-42.

Josefowicz, S. Z., L.-F. Lu, et al. (2012). "Regulatory T Cells: Mechanisms of Differentiation and Function." Annual Review of Immunology **30**(1): 531-564.

Josefowicz, S. Z., R. E. Niec, et al. (2012). "Extrathymically generated regulatory T cells control mucosal TH2 inflammation." Nature **482**(7385): 395-399.

Jutel, M. and C. A. Akdis (2011). "T-cell subset regulation in atopy." Curr Allergy Asthma Rep **11**(2): 139-45.

Kadowaki, N., S. Antonenko, et al. (2000). "Natural Interferon {alpha}/{beta}-Producing Cells Link Innate and Adaptive Immunity." J. Exp. Med. **192**(2): 219-226.

Kalesnikoff, J. and S. J. Galli (2008). "New developments in mast cell biology." Nat Immunol **9**(11): 1215-23.

Karasuyama, H., K. Mukai, et al. (2011). "Nonredundant Roles of Basophils in Immunity." Annual Review of Immunology **29**(1): 45-69.

Kearley, J., J. E. Barker, et al. (2005). "Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is

interleukin 10 dependent." The Journal of Experimental Medicine **202**(11): 1539-1547.

Kearley, J., D. S. Robinson, et al. (2008). "CD4+CD25+ regulatory T cells reverse established allergic airway inflammation and prevent airway remodeling." J Allergy Clin Immunol **122**(3): 617-24 e6.

Keir, M. E., M. J. Butte, et al. (2008). "PD-1 and its ligands in tolerance and immunity." Annu Rev Immunol **26**: 677-704.

Kim, H. J. and H. Cantor (2011). "Regulation of self-tolerance by Qa-1-restricted CD8(+) regulatory T cells." Semin Immunol **23**(6): 446-52.

Kim, H. J., B. Verbinnen, et al. (2010). "Inhibition of follicular T-helper cells by CD8(+) regulatory T cells is essential for self tolerance." Nature **467**(7313): 328-32.

Kim, H. J., X. Wang, et al. (2011). "CD8+ T regulatory cells express the Ly49 Class I MHC receptor and are defective in autoimmune prone B6-Yaa mice." Proc Natl Acad Sci U S A **108**(5): 2010-5.

Kim, H. Y., R. H. DeKruyff, et al. (2010). "The many paths to asthma: phenotype shaped by innate and adaptive immunity." Nat Immunol **11**(7): 577-84.

Kitawaki, T., N. Kadowaki, et al. (2006). "IgE-activated mast cells in combination with pro-inflammatory factors induce Th2-promoting dendritic cells." Int Immunol **18**(12): 1789-99.

Kool, M., M. van Nimwegen, et al. (2009). "An Anti-Inflammatory Role for Plasmacytoid Dendritic Cells in Allergic Airway Inflammation." J Immunol **183**(2): 1074-1082.

Kosiewicz, M. and P. Alard (2004). "Tolerogenic antigen-presenting cells." Immunologic Research **30**(2): 155-170.

Koya, T., H. Matsuda, et al. (2007). "IL-10-treated dendritic cells decrease airway hyperresponsiveness and airway inflammation in mice." J Allergy Clin Immunol **119**(5): 1241-50.

Kuhl, K. and N. A. Hanania (2012). "Targeting IgE in asthma." Curr Opin Pulm Med **18**(1): 1-5.

Lambrecht, B. N. and H. Hammad (2003). "Taking our breath away: dendritic cells in the pathogenesis of asthma." Nat Rev Immunol **3**(12): 994-1003.

Lambrecht, B. N. and H. Hammad (2012). "Lung Dendritic Cells in Respiratory Viral Infection and Asthma: From Protection to Immunopathology." Annual Review of Immunology **30**(1): 243-270.

Lampropoulou, V., K. Hoehlig, et al. (2008). "TLR-activated B cells suppress T cell-mediated autoimmunity." J Immunol **180**(7): 4763-73.

Lang, R., D. Patel, et al. (2002). "Shaping Gene Expression in Activated and Resting Primary Macrophages by IL-10." The Journal of Immunology **169**(5): 2253-2263.

Lathrop, S. K., N. A. Santacruz, et al. (2008). "Antigen-specific peripheral shaping of the natural regulatory T cell population." J Exp Med **205**(13): 3105-17.

Lau, A. W., S. Biester, et al. (2008). "Lipopolysaccharide-activated IL-10-secreting dendritic cells suppress experimental autoimmune uveoretinitis by MHCII-dependent activation of CD62L-expressing regulatory T cells." J Immunol **180**(6): 3889-99.

Leslie, M. (2011). "Regulatory T Cells Get Their Chance to Shine." Science **332**(6033): 1020-1021.

Lewkowich, I. P., N. S. Herman, et al. (2005). "CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function." J Exp Med **202**(11): 1549-61.

Li, M., X. Zhang, et al. (2007). "Immune modulation and tolerance induction by RelB-silenced dendritic cells through RNA interference." J Immunol **178**(9): 5480-7.

Li, X., A. Yang, et al. (2010). "Induction of type 2 T helper cell allergen tolerance by IL-10-differentiated regulatory dendritic cells." Am J Respir Cell Mol Biol **42**(2): 190-9.

Liang, B., C. Workman, et al. (2008). "Regulatory T Cells Inhibit Dendritic Cells by Lymphocyte Activation Gene-3 Engagement of MHC Class II." J Immunol **180**(9): 5916-5926.

Liu, G., H. Ng, et al. (2004). "Small interference RNA modulation of IL-10 in human monocyte-derived dendritic cells enhances the Th1 response." Eur J Immunol **34**(6): 1680-7.

Lloyd, C. M. and C. M. Hawrylowicz (2009). "Regulatory T Cells in Asthma." Immunity **31**(3): 438-449.

Locksley, R. M. (2010). "Asthma and Allergic Inflammation." Cell **140**(6): 777-783.

Long, S. A., M. Rieck, et al. (2011). "Low-Dose Antigen Promotes Induction of FOXP3 in Human CD4+ T Cells." The Journal of Immunology **187**(7): 3511-3520.

Lu, L. F., E. F. Lind, et al. (2006). "Mast cells are essential intermediaries in regulatory T-cell tolerance." Nature **442**(7106): 997-1002.

Lu, M., W. Dawicki, et al. (2011). "Therapeutic induction of tolerance by IL-10-differentiated dendritic cells in a mouse model of house dust mite-asthma." Allergy **66**(5): 612-20.

Luo, X., K. V. Tarbell, et al. (2007). "Dendritic cells with TGF-尾 1 differentiate na茂ve CD4+CD25 鲨?T cells into islet-protective Foxp3+ regulatory T cells." Proceedings of the National Academy of Sciences **104**(8): 2821-2826.

Macfarlane, A. J., O. M. Kon, et al. (2000). "Basophils, eosinophils, and mast cells in atopic and nonatopic asthma and in late-phase allergic reactions in the lung and skin." Journal of Allergy and Clinical Immunology **105**(1, Part 1): 99.

Mahnke, K., T. S. Johnson, et al. (2007). "Tolerogenic dendritic cells and regulatory T cells: a two-way relationship." J Dermatol Sci **46**(3): 159-67.

Makela, M. J., A. Kanehiro, et al. (2002). "The failure of interleukin-10-deficient mice to develop airway hyperresponsiveness is overcome by respiratory syncytial virus infection in allergen-sensitized/challenged mice." Am J Respir Crit Care Med **165**(6): 824-31.

Maldonado-Lopez, R., C. Maliszewski, et al. (2001). "Cytokines Regulate the Capacity of CD8{alpha}+ and CD8{alpha}- Dendritic Cells to Prime Th1/Th2 Cells In Vivo." J Immunol **167**(8): 4345-4350.

Maldonado, R. A., U. H. von Andrian, et al. (2010). Chapter 4 - How Tolerogenic Dendritic Cells Induce Regulatory T Cells. Advances in Immunology, Academic Press. **Volume 108:** 111-165.

Malhotra, S., S. S. Deshmukh, et al. (2012). "COX inhibitors for airway inflammation." Expert Opin Ther Targets **16**(2): 195-207.

Manfred B. Lutz, N. A. K. M. M. S. R. G. S. (2000). "Culture of bone marrow cells in GM-CSF plus high doses of lipopolysaccharide generates exclusively immature dendritic cells which induce alloantigen-specific CD4 T cell anergy <I>in vitro</I>." European Journal of Immunology **30**(4): 1048-1052.

Marta, C., T. Xuguang, et al. (2008). "TcR-induced regulated secretion leads to surface expression of CTLA-4 in CD4⁺CD25⁺ T cells." Immunology **125**(1): 70-79.

Martin, P., S. R. Ruiz, et al. (2002). "Dramatic increase in lymph node dendritic cell number during infection by the mouse mammary tumor virus occurs by a CD62L-dependent blood-borne DC recruitment." Blood **99**(4): 1282-1288.

Mauri, C. and A. Bosma (2012). "Immune regulatory function of B cells." Annu Rev Immunol **30**: 221-41.

Mazzoni, A., R. P. Siraganian, et al. (2006). "Dendritic cell modulation by mast cells controls the Th1/Th2 balance in responding T cells." J Immunol **177**(6): 3577-81.

McDole, J. R., L. W. Wheeler, et al. (2012). "Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine." Nature **483**(7389): 345-349.

McGee, H. S. and D. K. Agrawal (2009). "Naturally occurring and inducible T-regulatory cells modulating immune response in allergic asthma." Am J Respir Crit Care Med **180**(3): 211-25.

McLellan, A. D., M. Kapp, et al. (2002). "Anatomic location and T-cell stimulatory functions of mouse dendritic cell subsets defined by CD4 and CD8 expression." Blood **99**(6): 2084-93.

Min, B., M. A. Brown, et al. (2012). "Understanding the roles of basophils: breaking dawn." Immunology **135**(3): 192-197.

Misra, N., J. Bayry, et al. (2004). "Cutting Edge: human CD4+CD25+ T cells restrain the maturation and antigen-presenting function of dendritic cells." J Immunol **172**(8): 4676-4680.

Miyashita, T., M. J. McIlraith, et al. (1997). "Bidirectional regulation of human B cell responses by CD40-CD40 ligand interactions." J Immunol **158**(10): 4620-33.

Mocellin, S., F. Marincola, et al. (2004). "The multifaceted relationship between IL-10 and adaptive immunity: putting together the pieces of a puzzle." Cytokine Growth Factor Rev **15**(1): 61-76.

Mocellin, S., M. C. Panelli, et al. (2003). "The dual role of IL-10." Trends Immunol **24**(1): 36-43.

Molinero, L. L., M. L. Miller, et al. (2011). "High TCR Stimuli Prevent Induced Regulatory T Cell Differentiation in a NF-ÎºBâ€“Dependent Manner." The Journal of Immunology **186**(8): 4609-4617.

Moore, K. W., R. de Waal Malefyt, et al. (2001). "Interleukin-10 and the interleukin-10 receptor." Annu Rev Immunol **19**: 683-765.

Moore, M. L., D. C. Newcomb, et al. (2009). "STAT1 Negatively Regulates Lung Basophil IL-4 Expression Induced by Respiratory Syncytial Virus Infection." J Immunol **183**(3): 2016-2026.

Morelli, A. E. and A. W. Thomson (2007). "Tolerogenic dendritic cells and the quest for transplant tolerance." Nat Rev Immunol **7**(8): 610-621.

Morjaria, J. B., A. J. Chauhan, et al. (2008). "The role of a soluble TNFalpha receptor fusion protein (etanercept) in corticosteroid refractory asthma: a double blind, randomised, placebo controlled trial." Thorax **63**(7): 584-91.

Mottet, C., H. H. Uhlig, et al. (2003). "Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells." J Immunol **170**(8): 3939-43.

Mowat, A. M. (2003). "Anatomical basis of tolerance and immunity to intestinal antigens." Nat Rev Immunol **3**(4): 331-41.

Muller, G., A. Muller, et al. (2002). "Interleukin-10-treated dendritic cells modulate immune responses of naive and sensitized T cells in vivo." J Invest Dermatol **119**(4): 836-41.

Murai, M., P. Krause, et al. (2010). "Regulatory T-cell stability and plasticity in mucosal and systemic immune systems." Mucosal Immunol **3**(5): 443-449.

Nakano, H., M. E. Free, et al. (2012). "Pulmonary CD103+ dendritic cells prime Th2 responses to inhaled allergens." Mucosal Immunol **5**(1): 53-65.

Nakano, H., M. Yanagita, et al. (2001). "Cd11c+B220+Gr-1+ Cells in Mouse Lymph Nodes and Spleen Display Characteristics of Plasmacytoid Dendritic Cells." J. Exp. Med. **194**(8): 1171-1178.

Nayyar, A. (2009). "Tolerogenic dendritic cell-induced tolerance induction in a mouse model of asthma." Department of Veterinary Microbiology. Saskatoon, Canada, University of Saskatchewan. **PhD:** 184.

Nayyar, A., W. Dawicki, et al. (2012). "Induction of Prolonged Asthma Tolerance by IL-10-Differentiated Dendritic Cells: Differential Impact on Airway

Hyperresponsiveness and the Th2 Immunoinflammatory Response." J Immunol **189**(1): 72-9.

Nayyar, N., X. Zhang, et al. (2004). "Therapeutic modulation of severe allergic lung disease (ALD) using bone marrow-derived dendritic cells." Clinical and Investigative Medicine **27**: 28C.

Newell, K. A., D. Phippard, et al. (2011). "Regulatory cells and cell signatures in clinical transplantation tolerance." Curr Opin Immunol **23**(5): 655-9.

Newton, R., R. Leigh, et al. (2010). "Pharmacological strategies for improving the efficacy and therapeutic ratio of glucocorticoids in inflammatory lung diseases." Pharmacol Ther **125**(2): 286-327.

Nicolson, K. S., E. J. O'Neill, et al. (2006). "Antigen-induced IL-10+ regulatory T cells are independent of CD25+ regulatory cells for their growth, differentiation, and function." J Immunol **176**(9): 5329-37.

Noble, A., Z. S. Zhao, et al. (1998). "Suppression of immune responses by CD8 cells. II. Qa-1 on activated B cells stimulates CD8 cell suppression of T helper 2 responses." J Immunol **160**(2): 566-71.

Ohkura, N. and S. Sakaguchi (2011). "Maturation of effector regulatory T cells." Nat Immunol **12**(4): 283-284.

Okamura, T., K. Fujio, et al. (2012). "Roles of LAG3 and EGR2 in regulatory T cells." Ann Rheum Dis **71 Suppl 2**: i96-100.

Pacholczyk, R., H. Ignatowicz, et al. (2006). "Origin and T cell receptor diversity of Foxp3+CD4+CD25+ T cells." Immunity **25**(2): 249-59.

Pandiyan, P., L. Zheng, et al. (2007). "CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells." Nat Immunol **8**(12): 1353.

Perona-Wright, G., S. M. Anderton, et al. (2007). "IL-10 permits transient activation of dendritic cells to tolerize T cells and protect from central nervous system autoimmune disease." Int Immunol **19**(9): 1123-34.

Perrigoue, J. G., S. A. Saenz, et al. (2009). "MHC class II-dependent basophil-CD4+ T cell interactions promote TH2 cytokine-dependent immunity." Nat Immunol **10**(7): 697.

Perrot, I., D. Blanchard, et al. (2007). "Dendritic Cells Infiltrating Human Non-Small Cell Lung Cancer Are Blocked at Immature Stage." The Journal of Immunology **178**(5): 2763-2769.

Piccirillo, C. A. and A. M. Thornton (2004). "Cornerstone of peripheral tolerance: naturally occurring CD4+CD25+ regulatory T cells." Trends in Immunology **25**(7): 374.

Pope, S. M., E. B. Brandt, et al. (2001). "IL-13 induces eosinophil recruitment into the lung by an IL-5â€“ and eotaxin-dependent mechanism." The Journal of allergy and clinical immunology **108**(4): 594-601.

Pozo, D., P. Anderson, et al. (2009). "Induction of alloantigen-specific human T regulatory cells by vasoactive intestinal peptide." J Immunol **183**(7): 4346-59.

Presser, K., D. Schwinge, et al. (2008). "Coexpression of TGF-Î²1 and IL-10 Enables Regulatory T Cells to Completely Suppress Airway Hyperreactivity." The Journal of Immunology **181**(11): 7751-7758.

Pyzik, M. and C. A. Piccirillo (2007). "TGF-Î²1 modulates Foxp3 expression and regulatory activity in distinct CD4+ T cell subsets." Journal of Leukocyte Biology **82**(2): 335-346.

Qualls, J. E., H. Tuna, et al. (2009). "Suppression of experimental colitis in mice by CD11c+ dendritic cells." Inflammatory Bowel Diseases **15**(2): 236-247.

Quezada, S. A., L. Z. Jarvinen, et al. (2004). "CD40/CD154 interactions at the interface of tolerance and immunity." Annu Rev Immunol **22**: 307-28.

Randolph, D. A. and C. G. Fathman (2006). "Cd4+Cd25+ regulatory T cells and their therapeutic potential." Annu Rev Med **57**: 381-402.

Randolph, G. J., J. Ochando, et al. (2008). "Migration of Dendritic Cell Subsets and their Precursors." Annual Review of Immunology **26**(1): 293-316.

Rauter, I., M. T. Krauth, et al. (2008). "Mast cell-derived proteases control allergic inflammation through cleavage of IgE." J Allergy Clin Immunol **121**(1): 197-202.

Ray, A., S. Basu, et al. (2012). "A Novel IL-10â€“Independent Regulatory Role for B Cells in Suppressing Autoimmunity by Maintenance of Regulatory T Cells via GITR Ligand." The Journal of Immunology **188**(7): 3188-3198.

Razmara, M., B. Hilliard, et al. (2008). "CTLA-4 x Ig converts naive CD4+CD25- T cells into CD4+CD25+ regulatory T cells." Int Immunol **20**(4): 471-83.

Reiner, S. L. (2007). "Development in Motion: Helper T Cells at Work." Cell **129**(1): 33.

Reiner, S. L. (2009). "Decision making during the conception and career of CD4+ T cells." Nat Rev Immunol **9**(2): 81.

Ricci, N. D., J. A. Fiuza, et al. (2011). "Induction of CD4(+)CD25(+)FOXP3(+) regulatory T cells during human hookworm infection modulates antigen-mediated lymphocyte proliferation." PLoS Negl Trop Dis **5**(11): e1383.

Riley, J. L., C. H. June, et al. (2009). "Human T regulatory cell therapy: take a billion or so and call me in the morning." Immunity **30**(5): 656-65.

Robertson, J. M., P. E. Jensen, et al. (2000). "DO11.10 and OT-II T cells recognize a C-terminal ovalbumin 323-339 epitope." J Immunol **164**(9): 4706-12.

Robinson, D. S. (2010). "The role of the T cell in asthma." J Allergy Clin Immunol **126**(6): 1081-91; quiz 1092-3.

Roncarolo, M. G. and M. Battaglia (2007). "Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans." Nat Rev Immunol **7**(8): 585-98.

Roncarolo, M. G., S. Gregori, et al. (2006). "Interleukin-10-secreting type 1 regulatory T cells in rodents and humans." Immunol Rev **212**: 28-50.

Rosenberg, H. F., S. Phipps, et al. (2007). "Eosinophil trafficking in allergy and asthma." The Journal of allergy and clinical immunology **119**(6): 1303-1310.

Rothenberg, M. E. and S. P. Hogan (2006). "THE EOSINOPHIL." Annual Review of Immunology **24**(1): 147-174.

Ruffner, M. A., S. H. Kim, et al. (2009). "B7-1/2, but not PD-L1/2 molecules, are required on IL-10-treated tolerogenic DC and DC-derived exosomes for in vivo function." European Journal of Immunology **39**(11): 3084-3090.

Rutella, S., S. Danese, et al. (2006). "Tolerogenic dendritic cells: cytokine modulation comes of age." Blood **108**(5): 1435-40.

Rutella, S., S. Danese, et al. (2006). "Tolerogenic dendritic cells: cytokine modulation comes of age." Blood **108**(5): 1435-1440.

Sagoo, P., N. Ali, et al. (2011). "Human regulatory T cells with alloantigen specificity are more potent inhibitors of alloimmune skin graft damage than polyclonal regulatory T cells." Sci Transl Med **3**(83): 83ra42.

Sakaguchi, S. (2005). "Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self." Nat Immunol **6**(4): 345-52.

Sakaguchi, S., N. Sakaguchi, et al. (1995). "Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases." J Immunol **155**(3): 1151-1164.

Sakaguchi, S., T. Yamaguchi, et al. (2008). "Regulatory T cells and immune tolerance." Cell **133**(5): 775-87.

Sato, K., K. Eizumi, et al. (2009). "Naturally occurring regulatory dendritic cells regulate murine cutaneous chronic graft-versus-host disease." Blood **113**(19): 4780-4789.

Sayed, B. A., A. Christy, et al. (2008). "The master switch: the role of mast cells in autoimmunity and tolerance." Annu Rev Immunol **26**: 705-39.

Sayuri, Y., I. Kayo, et al. (2006). "Dendritic cells expand antigen-specific Foxp3⁺CD25⁺CD4⁺ regulatory T cells including suppressors of alloreactivity." Immunological Reviews **212**(1): 314-329.

Scalapino, K. J. and D. I. Daikh (2008). "CTLA-4: a key regulatory point in the control of autoimmune disease." Immunol Rev **223**: 143-55.

Schallenberg, S., P.-Y. Tsai, et al. (2010). "Identification of an immediate Foxp3âˆ’ precursor to Foxp3+ regulatory T cells in peripheral lymphoid organs of nonmanipulated mice." The Journal of Experimental Medicine **207**(7): 1393-1407.

Schneider, A. M., F. Li, et al. (2001). "Induction of pulmonary allergen-specific IgA responses or airway hyperresponsiveness in the absence of allergic lung disease following sensitization with limiting doses of ovalbumin-alum." Cell Immunol **212**(2): 101-9.

Scott, C. L., A. M. Aumeunier, et al. (2011). "Intestinal CD103+ dendritic cells: master regulators of tolerance?" Trends in Immunology **32**(9): 412-419.

Shaw, J., Y.-H. Wang, et al. "Plasmacytoid dendritic cells regulate B cell growth and differentiation via CD70." Blood: blood-2009-08-239145.

Shigeharu, F., Y. Naomi, et al. (2008). "Regulatory dendritic cells protect against allergic airway inflammation in a murine asthmatic model." The Journal of allergy and clinical immunology **121**(1): 95.

Shin, M. H., Y. A. Lee, et al. (2009). "Eosinophil-Mediated Tissue Inflammatory Responses in Helminth Infection." Korean J Parasitol **47**(Suppl): S125-S131.

Siewert, C., U. Lauer, et al. (2008). "Experience-Driven Development: Effector/Memory-Like Î±E+Foxp3+ Regulatory T Cells Originate from Both Naive T Cells and Naturally Occurring Naive-Like Regulatory T Cells." The Journal of Immunology **180**(1): 146-155.

Siracusa, M. C., M. R. Comeau, et al. (2011). "New insights into basophil biology: initiators, regulators, and effectors of type 2 inflammation." Annals of the New York Academy of Sciences **1217**(1): 166-177.

Sokol, C. L., N.-Q. Chu, et al. (2009). "Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response." Nat Immunol **10**(7): 713.

Sokol, C. L., N.-Q. Chu, et al. (2009). "Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response." Nat Immunol **10**(7): 713-720.

Sozzani, S., P. Allavena, et al. (1998). "Cutting Edge: Differential Regulation of Chemokine Receptors During Dendritic Cell Maturation: A Model for Their Trafficking Properties." J Immunol **161**(3): 1083-1086.

Steinbrink, K., E. Graulich, et al. (2002). "CD4(+) and CD8(+) anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity." Blood **99**(7): 2468-76.

Steinbrink, K., M. Wolfl, et al. (1997). "Induction of tolerance by IL-10-treated dendritic cells." J Immunol **159**(10): 4772-80.

Steinman, R. M. (2003). "The control of immunity and tolerance by dendritic cell." Pathol Biol (Paris) **51**(2): 59-60.

Steinman, R. M. (2012). "Decisions About Dendritic Cells: Past, Present, and Future." Annual Review of Immunology **30**(1): 1-22.

Steinman, R. M., D. Hawiger, et al. (2003). "Dendritic cell function in vivo during the steady state: a role in peripheral tolerance." Ann N Y Acad Sci **987**: 15-25.

Steinman, R. M., D. Hawiger, et al. (2003). "Tolerogenic dendritic cells." Annu Rev Immunol **21**: 685-711.

Steinman, R. M., S. Turley, et al. (2000). "The Induction of Tolerance by Dendritic Cells That Have Captured Apoptotic Cells." The Journal of Experimental Medicine **191**(3): 411-416.

Stephens, G. L., J. Andersson, et al. (2007). "Distinct subsets of FoxP3+ regulatory T cells participate in the control of immune responses." J Immunol **178**(11): 6901-11.

Stock, P., R. H. DeKruyff, et al. (2006). "Inhibition of the allergic response by regulatory T cells." Curr Opin Allergy Clin Immunol **6**(1): 12-6.

Strickland, D. H., P. A. Stumbles, et al. (2006). "Reversal of airway hyperresponsiveness by induction of airway mucosal CD4+CD25+ regulatory T cells." J. Exp. Med. **203**(12): 2649-2660.

Sun, C.-M., J. A. Hall, et al. (2007). "Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid." J. Exp. Med. **204**(8): 1775-1785.

Sun, J.-B., C. Czerkinsky, et al. (2012). "B Lymphocytes Treated In Vitro with Antigen Coupled to Cholera Toxin B Subunit Induce Antigen-Specific Foxp3+ Regulatory T Cells and Protect against Experimental Autoimmune Encephalomyelitis." The Journal of Immunology **188**(4): 1686-1697.

Sun, J. B., C. F. Flach, et al. (2008). "B lymphocytes promote expansion of regulatory T cells in oral tolerance: powerful induction by antigen coupled to cholera toxin B subunit." J Immunol **181**(12): 8278-87.

Taga, K., H. Mostowski, et al. (1993). "Human interleukin-10 can directly inhibit T-cell growth." Blood **81**(11): 2964-71.

Tai, N., H. Yasuda, et al. (2011). "IL-10-conditioned dendritic cells prevent autoimmune diabetes in NOD and humanized HLA-DQ8/RIP-B7.1 mice." Clinical Immunology **139**(3): 336-349.

Takahashi, T., T. Tagami, et al. (2000). "Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4." J Exp Med **192**(2): 303-10.

Thornton, A. M. and E. M. Shevach (2000). "Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific." J Immunol **164**(1): 183-90.

Toda, A. and C. A. Piccirillo (2006). "Development and function of naturally occurring CD4+CD25+ regulatory T cells." J Leukoc Biol **80**(3): 458-70.

van Scott, M. R., J. P. Justice, et al. (2000). "IL-10 reduces Th2 cytokine production and eosinophilia but augments airway reactivity in allergic mice." Am J Physiol Lung Cell Mol Physiol **278**(4): L667-74.

Vijayaraghavan, R., M. Schaper, et al. (1993). "Characteristic modifications of the breathing pattern of mice to evaluate the effects of airborne chemicals on the respiratory tract." Arch Toxicol **67**(7): 478-90.

Vijayaraghavan, R., M. Schaper, et al. (1994). "Computer assisted recognition and quantitation of the effects of airborne chemicals acting at different areas of the respiratory tract in mice." Arch Toxicol **68**(8): 490-9.

Villadangos, J. A. and P. Schnorrer (2007). "Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo." Nat Rev Immunol **7**(7): 543.

Vukmanovic-Stejic, M., E. Agius, et al. (2008). "The kinetics of CD4+Foxp3+ T cell accumulation during a human cutaneous antigen-specific memory response in vivo." J Clin Invest **118**(11): 3639-50.

Wakkach, A., N. Fournier, et al. (2003). "Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo." Immunity **18**(5): 605-17.

Wang, H., N. Peters, et al. (2006). "Plasmacytoid Dendritic Cells Limit Viral Replication, Pulmonary Inflammation, and Airway Hyperresponsiveness in Respiratory Syncytial Virus Infection." J Immunol **177**(9): 6263-6270.

Wang, J., T. W. J. Huizinga, et al. (2009). "De Novo Generation and Enhanced Suppression of Human CD4+CD25+ Regulatory T Cells by Retinoic Acid." J Immunol **183**(6): 4119-4126.

Wang, L., K. Pino-Lagos, et al. (2008). "Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells." Proceedings of the National Academy of Sciences **105**(27): 9331-9336.

Wang, X., L. Lu, et al. (2011). "Regulatory T Cells: Customizing for the Clinic." Science Translational Medicine **3**(83): 83ps19.

Weaver, T. A., A. H. Charafeddine, et al. (2008). "Costimulation blockade: towards clinical application." Front Biosci **13**: 2120-39.

Wegmann, M. (2011). "Targeting Eosinophil Biology in Asthma Therapy." American Journal of Respiratory Cell and Molecular Biology **45**(4): 667-674.

Wing, K., Y. Onishi, et al. (2008). "CTLA-4 Control over Foxp3+ Regulatory T Cell Function." Science **322**(5899): 271-275.

Wing, K. and S. Sakaguchi (2006). "Regulatory T cells as potential immunotherapy in allergy." Curr Opin Allergy Clin Immunol **6**(6): 482-8.

Wong, J., R. Obst, et al. (2007). "Adaptation of TCR repertoires to self-peptides in regulatory and nonregulatory CD4+ T cells." J Immunol **178**(11): 7032-41.

Wong, P. and E. G. Pamer (2003). "CD8 T CELL RESPONSES TO INFECTIOUS PATHOGENS." Annual Review of Immunology **21**(1): 29-70.

Wright, G. P., M. R. Ehrenstein, et al. (2011). "Regulatory T-cell adoptive immunotherapy: potential for treatment of autoimmunity." Expert Rev Clin Immunol **7**(2): 213-25.

Xiang, J., X. Gu, et al. (2007). "Administration of dendritic cells modified by RNA interference prolongs cardiac allograft survival." Microsurgery **27**(4): 320-3.

Xystrakis, E., Z. Urry, et al. (2007). "Regulatory T cell therapy as individualized medicine for asthma and allergy." Curr Opin Allergy Clin Immunol **7**(6): 535-41.

Yamazaki, S., A. J. Bonito, et al. (2007). "Dendritic cells are specialized accessory cells along with TGF- for the differentiation of Foxp3+ CD4+ regulatory T cells from peripheral Foxp3 precursors." Blood **110**(13): 4293-4302.

Yamazaki, S., D. Dudziak, et al. (2008). "CD8+CD205+ Splenic Dendritic Cells Are Specialized to Induce Foxp3+ Regulatory T Cells." J Immunol **181**(10): 6923-6933.

Yanaba, K., J. D. Bouaziz, et al. (2008). "A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses." Immunity **28**(5): 639-50.

Ye, Z., H. Huang, et al. (2007). "IL-10 has a distinct immunoregulatory effect on naive and active T cell subsets." J Interferon Cytokine Res **27**(12): 1031-8.

Yoffey, J. and F. Courtice (1970). Lymphatics, lymph and the lymphomyeloid complex. London, New York, Academic Press.

Yolcu, E. S., S. Ash, et al. (2008). "Apoptosis as a mechanism of T-regulatory cell homeostasis and suppression." Immunol Cell Biol **86**(8): 650-8.

Yoshimoto, T. and K. Nakanishi (2006). "Roles of IL-18 in basophils and mast cells." Allergol Int **55**(2): 105-13.

Yoshimoto, T., K. Yasuda, et al. (2009). "Basophils contribute to TH2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells." Nat Immunol **10**(7): 706.

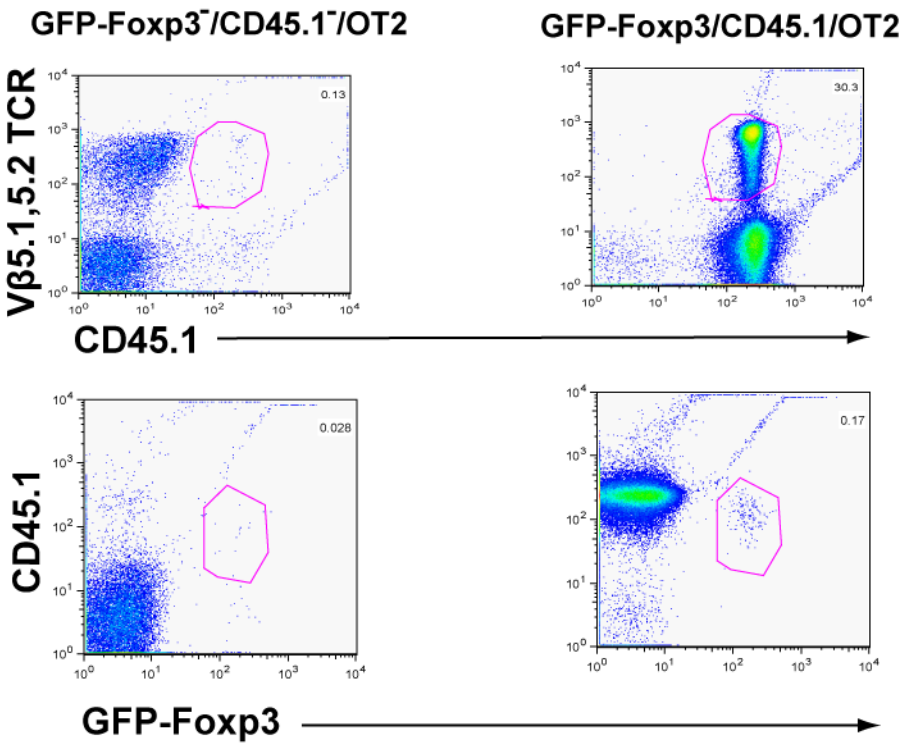
Zeldin, D. C., C. Wohlford-Lenane, et al. (2001). "Airway inflammation and responsiveness in prostaglandin H synthase-deficient mice exposed to bacterial lipopolysaccharide." Am J Respir Cell Mol Biol **25**(4): 457-65.

Zhang-Hoover, J., P. Finn, et al. (2005). "Modulation of Ovalbumin-Induced Airway Inflammation and Hyperreactivity by Tolerogenic APC." J Immunol **175**(11): 7117-7124.

Zhou, L., M. M. Chong, et al. (2009). "Plasticity of CD4+ T cell lineage differentiation." Immunity **30**(5): 646-55.

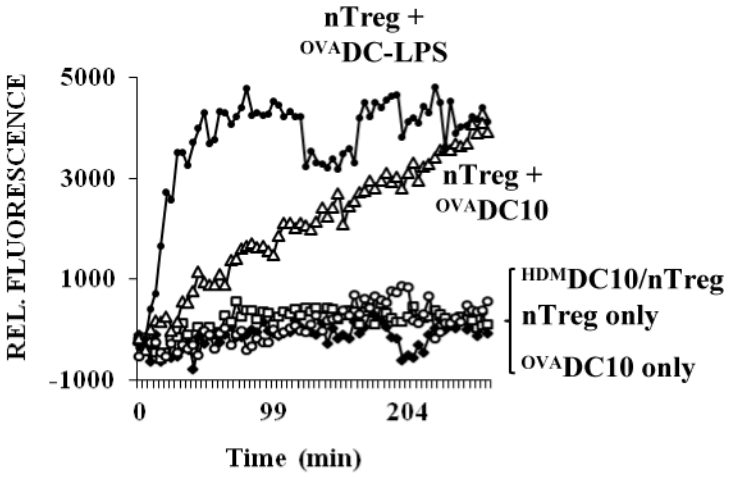
Zhu, J., H. Yamane, et al. (2010). "Differentiation of effector CD4 T cell populations (*)." Annu Rev Immunol **28**: 445-89.

Appendix-1. Selection of GFP-Foxp3/CD45.1/OT2 mice.



The periphery blood samples were harvested from tail vein of offspring mice. Then red blood cells were removed by cell lysis. The remaining leucocytes were stained with anti-mouse CD45.1 and Vβ5.1,5.2 TCR antibodies and analyzed by cytometry.

Appendix-2. IL-10-differentiated dendritic cells intimately engage naturally-occurring regulatory T cells in an allergen-specific fashion*.



Irrelevant allergen (house dust mite; HDM)- and OVA-pulsed IL-10-differentiated dendritic cells **(**DC10) or LPS-activated OVA-presenting dendritic cells (^{OVA}DC-LPS) were stained with the FRET donor dye DiO, while lung $CD4^+CD25^+Foxp3^+$ T cells purified from healthy OVA TCR-transgenic OT2 mice (nTreg) were stained with the lipophylic FRET partner dye DiI. The OVA- or HDM-presenting DC10 or DC-LPS were cocultured with the OT2 nTreg, while negative controls included DiO-stained T cells or DiI-stained DC10 alone. The cells were exposed to fluorescent light (488 nm; the DiO excitation wavelength) and assessed for FRET energy release at 565 nm wavelength (the DiI emission wavelength) over ≈5 h. The data are expressed as relative fluorescence units. OVA-presenting DC10 induced strong FRET responses among the OT2 nTreg, albeit with somewhat delayed kinetics relative to OVA-presenting DC-LPS, while HDM–presenting DC10 induced no significant FRET signals.

*Contributed by Xiaobei Zhang