REGULATORY T CELLS IN ALLERGIC ASTHMA

THE BIOLOGY OF REGULATORY T CELLS IN HUMAN ALLERGEN-INDUCED ASTHMA

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ABSTRACT

Regulatory T cells (Treg) are essential for the induction and maintenance of immunological tolerance to self and foreign antigens. The development of allergic asthma is mediated by T helper cell type-2 (Th2) inflammatory mechanisms and may also involve, based on murine and human studies of allergic asthma, compromised Treg immune regulation. Our overall objective was to more thoroughly elucidate the biology of Treg in allergic asthma, and to better understand their potential as a treatment for the disease.

Initially, we characterized three different Treg phenotypes based on frequency and functional capacity. We showed both quantitative and functional heterogeneity in circulating Treg. Quantitative variability was also observed in circulating, but not airway, Treg when comparisons were made between healthy controls and asthmatic subjects. These findings emphasize the need for clear definitions of Treg phenotypes, and that interpretation of their frequency and function in health and disease needs to be phenotype-specific.

Next, we assessed the Treg response in mild allergic asthmatic isolated early responders and dual responders after allergen inhalation challenge. We observed a reduced frequency of airway Treg after allergen challenge in DR, but not IER, associated with a smaller ratio of

Treg to $CD4^+$ cells. These data suggest that Treg to T effector cell (Teff) balance is important in the regulation of late asthmatic responses.

Lastly, we evaluated the effects of two novel monoclonal anti-asthma therapies on circulating Treg after allergen inhalation challenge. We demonstrated that neither anti-OX40L nor anti-TSLP therapy altered circulating Treg frequency, while anti-TSLP, but not anti-OX40L, was effective in attenuating allergen-induced airway responses. These observations demonstrate the need for further investigation into the effects of anti-asthma therapies on Treg as well as the development of novel therapies aimed at manipulating Treg in order to better control immune responses.

The findings of this thesis enhance our understanding of Treg in allergic asthma. Treg, utilized as stand-alone or adjunct therapy, may provide a novel therapy in the treatment of allergic asthma.

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LIST OF ABBREVIATIONS AND SYMBOLS

AHR	Airway hyper-responsiveness
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APC	Allophycocyanin
APC	Antigen presenting cell
aTreg	Adaptive regulatory T cell
BAL	Bronchoalveolar lavage
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CTLA-4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DR	Dual responder
DTT	Dithiothreitol
EAR	Early asthmatic response
FACS	Fluorescence activated cell sorting
FEV_1	Forced expiratory volume in one second
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box protein-3

FSC	Forward scatter
Garp	Glycoprotein-A repetitions predominant
GINA	Global Initiative for Asthma
GITR	Glucocorticoid-induced TNFR family related gene
ICS	Inhaled corticosteroids
IDO	Indoleamine 2,3-dioxygenase
IER	Isolated early responder
IgE	Immunoglobulin E
IgG_4	Immunoglobulin G_4
IL	Interleukin
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
iTreg	Induced regulatory T cell
LAP	**Latency-associated peptide**
LAR	Late asthmatic response
MHC	Major histocompatibility complex
nTreg	Natural regulatory T cell
OX40L	OX40 ligand
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

MCh PC_{20}	Provocative concentration of methacholine causing a 20% fall in FEV_1
NSAIDS	Non-steroidal anti-inflammatory drugs
PD-1	Programmed cell death-1
PE	Phycoerythrin
RPM	Revolutions per minute
SSC	Side scatter
SEM	Standard error of the mean
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Th	T helper
TNF	Tumour necrosis factor
Tr1	Type 1 regulatory cell
Treg	Regulatory T cell
Tresp	Responder T cells
TSDR	Treg-specific demethylated region
TSLP	Thymic stromal lymphopoietin

DECLARATION OF ACADEMIC ACHIEVEMENT

The studies comprising this “sandwich” thesis are presented as independent, but related, pieces of work. All three manuscripts have been accepted for publication in peer-reviewed journals. While I have been the main contributor to the work contained herein, it has been a collaborative effort that required the assistance of many persons. Author contributions have been acknowledged at the beginning of each chapter. In addition, I would like to acknowledge the contributions of several others. Rick Watson, Karen Howie and Heather Campbell acted as study coordinators. Rick Watson and George Obminski performed allergen/diluent challenges. Dr. Kieran Killian performed physical examinations and assisted with statistical analyses. Tara Strinich and Catie Obminski provided technical assistance with sample collection and processing. Hong Liang provided technical expertise with flow cytometry and cell sorting.

Chapter 1 – Introduction

The prevalence of allergic disease, including allergic asthma, has been steadily increasing over the past several decades and today represents a significant health problem in terms of morbidity and associated healthcare costs. Consequently, research in this area, both basic and clinical, has focussed on understanding more completely the mechanisms underlying the initiation and progression of allergic disease with the goal of identifying potential preventative and curative therapeutic targets. Regulatory T cells (Treg) are important in the control of immune responses in both health and disease. Of interest to us was the contribution of Treg to the control of Th2 inflammation in allergic disease. Specifically, we propose to more clearly elucidate the role of Treg in human allergic asthma and to determine its therapeutic potential as cells with disease modifying capabilities. Of note, although multiple immunosuppressive Treg subsets exist, this thesis will focus on Foxp3$^+$ Treg that develop in both the thymus and the periphery as these represent the predominant Treg populations important for immune homeostasis.

1.1 Tolerance

Immune tolerance is a state of unresponsiveness of the immune system to substances or tissue that has the capacity to elicit an immune response (Murphy 2012). It is now well accepted that immunological

tolerance can be divided into two mechanistic categories – recessive and dominant (Xing and Hogquist 2012). Recessive tolerance includes cell-intrinsic mechanisms whereby self-reactive thymocytes or chronically stimulated T cell clones are eliminated via processes of apoptosis or anergy. Dominant tolerance involves active peripheral processes that aim to inhibit the activation and expansion of effector cells (Shimon Sakaguchi et al. 2006). It is mediated by a specialized subset of immune cells that suppress pathogenic immune responses. There are several cell types that exhibit suppressive and/or immunomodulatory properties, but Foxp3$^+$ Treg are the only known population of lymphocytes that solely function to uphold dominant tolerance (Rudensky 2005). Their suppressor function is essential for immune homeostasis.

1.2 Regulatory T Cells

Treg represent a developmentally and functionally distinct T cell sub-population. These cells, through their immunosuppressive and immunomodulatory properties, are responsible for the maintenance of dominant self-tolerance and immune homeostasis in states of health and disease (Shimon Sakaguchi et al. 2008). The Treg population is made up of numerous Treg phenotypes broadly categorized into natural Treg (nTreg) or adaptive/induced Treg (iTreg) (Chatenoud 2011; Yuan and Malek 2012).

1.2.1 Treg phenotype

nTreg are generally characterized as T helper cells ($CD4^+$) that express CD25 (the IL-2 receptor alpha chain) and the transcription factor Foxp3, giving rise to the $CD4^+CD25^+Foxp3^+$ nTreg phenotype (Fontenot, Gavin, and Rudensky 2003; Hori, Nomura, and Sakaguchi 2003). This is the most widely accepted and reliable phenotype for describing nTreg. Along with these, Treg constitutively express Treg signature genes, namely glucocorticoid-induced tumour necrosis factor receptor family related gene (GITR) and cytotoxic T lymphocyte antigen 4 (CTLA4) . iTreg, displaying the $CD4^+CD25^+Foxp3^+$ Treg phenotype, can be generated *in vivo* in the periphery and are indistinguishable from naturally occurring thymic-derived Treg (Apostolou and von Boehmer 2004). Both nTreg and iTreg express the classical Treg markers – CD25, GITR and CTLA4; however, nTreg exhibit a higher expression of programmed cell death-1 (PD-1), neuropilin 1, Helios, and CD73 as compared to iTreg (Yadav et al. 2012). iTreg are also comprised of populations of Tr1 helper cells that express IL-10 and Th3 helper cells that express TGF-β (Roncarolo et al. 2014; Weiner 2001). These regulatory T cells are Foxp3-negative (CD4+IL-10+ and CD4+TGF-β+) and functionally inhibit via the suppressive properties of IL-10 and TGF-β.

Treg can also be divided into functional subsets. These include a central memory Treg population, which has circulatory characteristics similar to naive conventional $CD4^+$ T cells, several effector Treg populations, which have enhanced function and markers of antigen encounter, and polarized tissue-resident Treg populations, which are present in most non-lymphoid organs (Liston and Gray 2014). $CCR7^+$ Treg have been described as a central memory population while $CCR7^-$ Treg were indicative of an effector memory population (Tosello et al. 2008). In addition, functionally heterogeneous and distinct subsets have been identified based on the intensity of expression of Foxp3 in conjunction with CD45RA expression (Miyara et al. 2009).

1.2.2 Treg Development

Natural Treg (nTreg) develop in the thymus, specifically the medullary compartment, during a late stage of thymopoiesis (Cowan et al. 2013). The process of forkhead box P3 (Foxp3)-induced Treg differentiation requires: 1) increased strength of T cell receptor (TCR) stimulation by high affinity MHC class II restricted self-peptides, 2) co-stimulation via CD28 signaling induced by CD80 and CD86 ligand expressed on antigen-presenting cells (APC), and 3) high affinity IL-2 receptor signaling (Hsieh, Lee, and Lio 2012; Josefowicz, Lu, and Rudensky 2012). Treg selection occurs via positive selection after $Foxp3^-$

$CD4^+$ thymocytes are presented with self-antigens. Lineage commitment into a unique T cell subset is predominantly determined by TCR specificity for self-antigens associated with strong TCR signal strength (Ohkura and Sakaguchi 2010). In addition to the TCR, Treg lineage commitment also requires expression of forkhead box P3 (Foxp3) which is induced after strong TCR-ligand signaling and co-stimulation (Josefowicz and Rudensky 2009).

Co-stimulation signaling via CD28 as well as IL-2 stimulation is required for Treg development and homeostasis. As evidence of this, knockout mice deficient in CD28 ($CD28^{-/-}$) or IL-2R ($IL\text{-}2^{-/-}$) do not produce nTreg and develop severe autoimmunity (Salomon et al. 2000; Cheng et al. 2013). However, there is some evidence to suggest that IL-2 signaling may be important but not necessary in Treg development as Treg cells could be generated in the absence of IL-2 signalling (D'Cruz and Klein 2005; Fontenot, Rasmussen, Gavin, et al. 2005). Also, CD28 co-stimulation was determined to be critical for Treg development as it directly signals developing thymocytes to express Foxp3 and to initiate the Treg cell differentiation program (Tai et al. 2005).

iTreg develop in the periphery in response to a low/sub-optimal dose of high affinity alloantigen (Apostolou and von Boehmer 2004). They also demonstrated the peripheral conversion of naive $CD4^+CD25^-$ T cells from TCR-transgenic $RAG^{-/-}$ mice into functional antigen-specific

$CD4^+CD25^+Foxp3^+$ Treg. Like nTreg, iTreg generation requires TCR stimulation, IL-2 signaling and co-stimulation (Bilate and Lafaille 2012). TGF-β is essential to the differentiation of iTreg. The addition of TGF-β induced Foxp3 expression and suppressive activity in TCR-stimulated naïve T cells *in vitro* (Chen et al. 2003). In support of this, *in vivo* TGF-β neutralization experiments demonstrated impairment in oral tolerance and the inhibition of iTreg differentiation (Mucida et al. 2005). While nTreg develop in the thymus, iTreg develop under more varied conditions and in different anatomical locations. They can localize to the lymph nodes (M.-T. Huang and Chiang 2012), continuously differentiate in the gut lamina propria (Sun et al. 2007), or be generated in tumours (V. C. Liu et al. 2007) or chronically inflamed tissues (Lafaille et al. 2008).

APC, the microenvironment, cytokine milieu, and co-stimulatory molecules are all factors that contribute to the development and maintenance of nTreg and iTreg. Their existence henceforth is much the same as that of a conventional T cell – naïve cells acquire effector function after antigen encounter, proliferate and migrate to the target tissue with a small proportion surviving to form a pool of memory Treg (Gratz, Rosenblum, and Abbas 2013).

1.2.2.1 Foxp3

Foxp3, a nuclear transcription factor, is considered the principle lineage–specific marker of Treg and essential for Treg development and function (Fontenot, Rasmussen, Williams, et al. 2005; Hori, Nomura, and Sakaguchi 2003; Yagi et al. 2004). It is encoded on the X chromosome and belongs to the family of forkhead box (Fox) transcription factors. Human Foxp3 expression has two isoforms – Foxp3a is the full length protein and Foxp3b is a splice variant (Mercer and Unutmaz 2009). Both isoforms have the ability to induce Treg and suppress T cell activation (Aarts-Riemens et al. 2008; Smith et al. 2006). Foxp3 expression maintains the Treg cell suppressive phenotype; the experimental ablation of Foxp3 in Treg results in cells with a $Foxp3^-$ phenotype now capable of generating cytokines causing inflammation (Williams and Rudensky 2007). However, there exists functional and phenotypic heterogeneity of $Foxp3^+$ Treg in humans as this population is comprised of both $Foxp3^{hi}$ cells that have potent suppressive capacity as well as $Foxp3^{lo}$ cells that are non-suppressive and also secrete pro-inflammatory cytokines (Miyara et al. 2009).

1.2.3 Distinguishing nTreg from iTreg

At present there are no known biomarkers that have the ability to unequivocally differentiate nTreg from iTreg. However, Helios, Garp/Lap and Neuropilin-1 show considerable promise.

The expression of Helios was shown to differentiate thymus-derived from periphally-induced Foxp3$^+$ Treg (Thornton et al. 2010). Subsequently it was demonstrated that Helios alone could not completely distinguish nTreg from iTreg as Helios$^-$ cells were shown to make up a proportion of the nTreg population (Himmel et al. 2013). In addition, Helios$^+$ Treg cells were found in a population of iTreg and the expression of Helios was proposed to be associated with Treg activation/function and not origin (Gottschalk, Corse, and Allison 2012).

Garp (glycoprotein-A repetitions predominant) has been identified as a Treg-specific cell surface molecule that has suppressive function and the ability to induce Foxp3 expression (Wang et al. 2008; Probst-Kepper et al. 2009). Garp is the membrane anchoring molecule that binds to latent TGF-β within nTreg and facilitates its surface expression (Tran et al. 2009). Therefore, surface Lap on Treg is a complex of Garp, Lap, and active TGFβ and iTregs fail to express surface Lap or Garp. Therefore, the selection of Lap/Garp$^+$ Treg would differentiate thymic from peripherally-induced Treg.

Neuropilin-1 (CD304) was initially thought of as a cell surface marker for Treg (Bruder et al. 2004). Since then, it has been shown to be expressed on nTreg but not iTreg and provides a means of differentiating the two populations under certain physiological conditions (Weiss et al. 2012; Yadav et al. 2012). There is some debate as to the validity of neuropilin 1 as a discriminating marker as it has been shown that neuropilin 1 is not differentially expressed on human Treg from thymus, blood, lymph nodes, and tonsils (Milpied et al. 2009). There exists a discrepancy between the murine and human studies regarding Helios, Garp, and neuropilin-1 as markers that can differentiate nTreg from the other subsets of Treg.

More recently, the use of molecular markers has been proposed as a means of differentiating the two Treg subsets (X. Lin et al. 2013). Epigenetic changes in the Treg-specific demethylated region (TSDR) of the Foxp3 locus affects Foxp3 stability and such modifications have been used to distinguish nTreg from iTreg (Polansky et al. 2010; Kim et al. 2012). The differentiating factor lies in the relative degree of methylation at the TSDR – nTreg are mostly demethylated in the region whereas iTreg are prominently methylated (Toker et al. 2013; Lal et al. 2009).

1.2.4 Treg Mobilization and Trafficking

Treg are constitutively expressed in secondary lymphoid tissues but they can also be found in many non-lymphoid tissues, even in the absence of inflammation, and in tumours (Nishikawa and Sakaguchi 2010; Sather et al. 2007). Trafficking and migration to peripheral tissues and secondary lymphoid organs are required for Treg function *in vivo*. nTreg exit the thymus, circulate, and migrate to secondary lymphoid tissues and sites of inflammation to exert their suppressive function (Ding, Xu, and Bromberg 2012). Current data suggest that the migratory capacity of Treg cells is controlled by distinct signals from chemokines/chemokine receptors and integrins/integrin ligands (Wei, Kryczek, and Zou 2006). CD62L (L-selectin), an integrin, and CCR7, a chemokine receptor, are not only crucial lymphoid homing molecules for conventional T cells but have been shown to be essential for efficient Treg homing to draining lymph nodes (Ochando et al. 2005; Schneider et al. 2007). These two molecules are expressed on most of the Treg in circulation and lymphoid organs. Treg trafficking to sites of inflammation relies upon Treg with altered expression of migratory molecules. CD103 (α_E-integrin) has been used to distinguish Treg subsets that differentially traffic to lymphoid tissue versus peripheral tissue (Lehmann et al. 2002). Naive $CD103^-$ Treg express high levels of CD62L and CCR7 and re-circulate through lymphoid tissues. Effector/memory $CD103^+$ Treg express low levels of CD62L, high levels of

E- and P-selectin ligands and multiple adhesion molecules, including CD54, ICOS, β_1-integrin, and LFA-1 ($\alpha_L\beta_2$), as well as the chemokine receptors CCR2, CCR4, CCR6 and CXCR3, allowing efficient migration into inflamed tissues (Huehn et al. 2004; Sather et al. 2007; Brühl et al. 2004). Evidence from murine studies has brought some preliminary insight as to the kinetics of Treg mobilization in response to inflammation. It has been demonstrated that Treg follow a sequential and not a simultaneous migration pattern – Treg migrate from the circulation, into the tissue and then to the lymph nodes (Zhang et al. 2009). Treg use P/E-selectin ligands and CCR2, CCR4 and CCR5 to migrate first into the inflamed tissue. Then, Treg use CCR2, CCR5, and CCR7 to migrate from the inflamed tissue to the draining lymph nodes. This pattern allowed Treg to differentiate and display optimal suppressive function.

Together, these data suggest that Treg cells can be recruited into inflammatory tissues and distinct migratory molecular patterns are responsible for Treg migration.

1.2.5 Treg Function

Treg are an essential component of the peripheral dominant tolerance mechanism that maintains immune homeostasis (Shimon Sakaguchi et al. 2008). Treg possess the ability to suppress or modulate the activation, proliferation, differentiation, and effector function of a variety

of cell populations (including $CD4^+$ cells, NKT cells, DC, monocytes, B cells, and NK cells) in different anatomical locations/environments, and in many diseases (Tang and Bluestone 2008). They also possess a diverse suppressive arsenal utilizing both contact-dependent and independent mechanisms (Shevach 2009; Sakaguchi et al. 2009).

The mechanisms of action of Treg *in vivo* remain to be fully elucidated. *In vitro* model systems have been developed that identify molecules and processes that contribute to Treg suppressive function but it remains unclear as to how relevant these findings are to Treg function *in vivo*. Treg have the ability to suppress a large number of distinct cell types and the mechanisms by which this occurs can be generally divided into those that target T cells and those that target APC (Shevach 2009).

Our understanding of the cellular and molecular basis of Treg-mediated suppression stem from a number of key findings: 1) $Foxp3^+$ Treg can inhibit the development of autoimmune disease induced by Treg depletion (Sakaguchi et al. 1995), 2) Treg function to inhibit the proliferative response of antigen-stimulated naïve T cells *in vitro* (Thornton and Shevach 1998), and 3) forced expression of Foxp3 in normal naïve T cells confers *in vivo* and *in vitro* suppressive function (Fontenot, Gavin, and Rudensky 2003; Hori, Nomura, and Sakaguchi 2003).

Treg exert their suppressive/inhibitory effects on immune responses via a variety of mechanisms including cell to cell contact, competition for growth factors, cytotoxicity, and the secretion of inhibitory cytokines (Shimon Sakaguchi et al. 2009).

The cell contact-dependent mechanism was demonstrated utilizing *in vitro* suppression assays where a semi-permeable membrane was used to separate responder T cells from Treg. This resulted in the inability of Treg to suppress the proliferation of the responder T cells upon stimulation (A M Thornton and Shevach 1998). Subsequently, Treg may kill the responder cells through a granzyme and/or perforin-dependent mechanism (Gondek et al. 2005; Cao et al. 2007). Treg can be activated by a combination of antibodies to CD3 and CD46 to express granzyme A, resulting in cytolysis of activated effector T cells in a perforin-dependent manner (Grossman et al. 2004). Alternatively, a negative signal can be sent to responder T cells through the up-regulation of cyclic AMP, which leads to the inhibition of proliferation and IL-2 production (by inducing mRNA instability) or, via an interaction with B7 that disrupts co-stimulatory signaling (Bopp et al. 2007; Paust et al. 2004).

Competition for growth factors is mainly centred around the availability of IL-2. It has been demonstrated, in a host of *in vitro* studies, that Treg suppression is mediated by inhibiting the induction of IL-2 (A M Thornton and Shevach 1998). IL-2 is an important cytokine that promotes

T cell proliferation and survival. Another suppressive mechanism related to IL-2 involves Treg as a consumer of IL-2. Treg may in fact compete with effector T cells for IL-2, due to the high expression of CD25 (IL-2R), consume the IL-2, and consequently inhibit effector T cells through apoptosis (Pandiyan et al. 2007). The relative contribution of IL-2 inhibition/consumption as a Treg suppressive mechanism *in vivo* remains a contentious issue.

The modification of APC function is another proposed mechanism of Treg suppression. Activated Treg may modulate the expression of CD80 and CD86 on APC, or cause dendritic cells (DC) to express the enzyme indoleamine 2,3-dioxygenase (IDO) (Oderup et al. 2006; Grohmann et al. 2002). IDO catabolizes tryotophan to kynurenine, which is toxic to T cells in close proximity of the DC. Activated Treg have also been shown to induce apoptosis of APC, including B cells, or inhibit their activation and function (Zhao et al. 2006; André et al. 2009).

Actions of Treg are also mediated by soluble factors (IL-10, TGF-β) and cell-associated molecules (CTLA4). IL-10, produced predominantly by monocytes, macrophages and T cells, inhibits T cell proliferation and cytokine production by acting directly on $CD4^+$ cells and down-regulating production of IL-2 and IFN-γ from Th1 cells and IL-4 and IL-5 from Th2 cells (Del Prete et al. 1993). Treg are a major source of TGF-β and this suppressive cytokine has been shown to induce $Foxp3^+$ Treg conversion

from naïve T cells (Chen et al. 2003). TGB-β can inhibit Th1 differentiation by reducing IL-12R and T-Bet expression and can inhibit Th2 differentiation by suppressing GATA-3 expression and IL-4-mediated STAT6 activity (L Gorelik, Fields, and Flavell 2000; Leonid Gorelik, Constant, and Flavell 2002). CTLA4 competitively inhibits the binding of CD28 to its ligands CD80 and CD86 and thus inhibits co-stimulation of effector T cells. CTLA4, together with the adhesion molecule LFA-1, also down-regulates the expression of CD80 and CD86 on APC (Wing et al. 2008; Qureshi et al. 2011).

Multiple mechanisms exist in Treg-mediated suppression and a number of different molecules may be secreted or expressed on Treg that directly impact the importance/contribution of each mechanism, the number of mechanisms required for Treg to be functionally effective, and the effect of the environment on Treg function (Vignali 2012).

1.2.6 Treg Stability and Plasticity

Treg are a specialized T cell lineage with the ability to suppress a variety of immune responses and, as such, the predominant stability of these cells is essential for their robust suppressor function. However, the stability and plasticity of these cells remains a contentious issue (Shimon Sakaguchi et al. 2013; Hori 2014). nTreg are a stable cell population under most physiological and inflammatory conditions (Rubtsov et al.

2010). The stability/plasticity of Treg is influenced by: 1) their intrinsic Foxp3 expression, 2) stabilizing signals, and 3) de-stabilizing signals and together these are dependent on the type of inflammatory challenge (infection, allergy) and the kinetic aspect of inflammation (initiation versus resolution) (Sawant and Vignali 2014). The regulation of Foxp3 greatly influences Treg stability and plasticity. Indeed it has been shown that inflammatory cytokines such as IL-4, IL-6 and IL-12, and IFN-γ can down-regulate Foxp3 expression *in vitro* and may contribute to Treg reprogramming *in vivo* (Zheng, Wang, and Horwitz 2008; Caretto et al. 2010; Veldhoen et al. 2008; Dardalhon et al. 2008). These data show that Treg do retain some measure of functional plasticity.

1.2.7 Treg in Disease

The critical importance of Foxp3$^+$ Treg in immune regulation in mouse and human is best signified by observations related to mutations in the Foxp3 gene that results in the spontaneous scurfy mutation in mice and the syndrome in humans known as Immune dysregulation Polyendocrinopathy Enteropathy, X-Linked (IPEX) (Bennett et al. 2001; Brunkow et al. 2001). These mutations cause the loss/disruption of Treg function and result in severe autoimmune disease and death. A wide range of pathologies have been associated with altered Treg function. An impairment in Treg function along with an imbalance of Treg homeostasis

have been implicated in the development of common autoimmune and inflammatory diseases, including allergy, type-1 diabetes, rheumatoid arthritis, multiple sclerosis, and systemic lupus erythematosus (Tan et al. 2014; H. Zhang et al. 2014a; Cooles, Isaacs, and Anderson 2013; Kleinewietfeld and Hafler 2014; Ohl and Tenbrock 2015).

1.3 Asthma

Asthma, a chronic disease of the airways, affects 1-18% of the global population (varying by country) and is defined as follows:

> "Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation." (GINA 2015)

Asthma is heterogeneous in nature as its pathogenesis is associated with multiple biological mechanisms that lead to a spectrum of both clinical and emerging, more refined molecular phenotypes (Ray, Oriss, and Wenzel 2015; Wenzel 2012; Busse and Lemanske 2001). The predominant features that result in clinical symptoms are smooth muscle contraction and inflammation that lead to airway narrowing and obstruction (Holgate et al. 2010; Holgate 2008). Numerous triggers can induce broncho-constriction including environmental allergen, respiratory infections, exercise, irritants, and non-steroidal anti-inflammatory drugs (NSAIDs) in select patients (McCarty and Ferguson 2014). Persistent

inflammation in the airway may lead to re-modeling resulting in structural changes, mucus hypersecretion, smooth muscle hyperplasia, sub-epithelial fibrosis, blood vessel proliferation, and infiltration of inflammatory cells (Murphy and O'Byrne 2010; Al-Muhsen, Johnson, and Hamid 2011). The treatment of asthma involves the use of several different drugs (many reviewed in *Global Strategy for Asthma Management and Prevention,* (GINA) 2015) in order to achieve control of symptoms. These include the canonical β2-agonists, corticosteroids and leukotriene receptor antagonists (Zdanowicz 2007; Fanta 2009). Despite the general success of currently available asthma drugs, there is no known cure. With the prevalence of asthma on the rise, resulting in an increased burden on society, there exists the need for the development of novel asthma therapies with disease-modifying capabilities so as to alter the natural course of the disease (Olin and Wechsler 2014; Fajt and Wenzel 2015).

1.3.1 Allergic Asthma

Most, but not all, asthma is associated with atopy (the inherited predisposition to generate IgE against common environmental allergens) leading asthma to be regarded largely as an allergic disorder. Allergic asthma is an inflammatory, Th2-mediated respiratory disease caused by an exaggerated and inappropriate immune response to inhaled allergen that results in reversible airflow obstruction, airway hyper-responsiveness

and airway inflammation . The type and magnitude of the immune response depends on a host of factors including genetic susceptibility, route of entry and dose of allergen and co-exposure with other immune–stimulating antigens (Kay 2001). In sensitized asthmatic individuals, allergen exposure results in the cross-linking of allergen specific IgE, activating mast cells and basophils, with the release of preformed mediators such as histamine and cysteinyl leukotrienes. This triggers acute symptoms and results in early phase broncho-constriction, termed the early asthmatic response (EAR), as it occurs within 10 minutes and reaches a maximum after 30 minutes. Late phase broncho-constriction, termed the late asthmatic response (LAR), develops in a proportion of the individuals who develop an early response and occurs after three hours and reaches a maximum from six to 24 hours after allergen exposure (O'Byrne, Dolovich, and Hargreave 1987; O'Byrne 1998). It is characterized by the infiltration of effector cells into the airway and the release of inflammatory cytokines (Ishmael 2011).

In order to better understand the biology of the allergic asthmatic response, and for evaluating the efficacy of existing and novel therapies, models of allergic asthma have been developed. Murine models of acute and chronic allergen challenge exist and are constantly being refined in order to better mimic the features associated with human asthma (Nials and Uddin 2008). Human allergen challenge models have been

developed utilizing whole allergen via inhalation (Gauvreau and Evans 2007). This is the current gold standard laboratory method for the controlled mimicking of an asthma exacerbation as it reproduces the cardinal features of allergic asthma – reversible airflow obstruction, airway hyper-responsiveness and airway inflammation (Cockcroft et al. 2007).

1.3.2 Treg in Asthma

It is essential for the lungs to maintain tolerance while continually exposed to environmental allergens. Studies have shown that antigens entering through the respiratory route normally result in tolerance and weak Th2 cell responses, thereby maintaining immune homeostasis in the airway (Holt et al. 2008). In the majority of healthy individuals, the allergen-specific T cell proliferative response ranges from undetectable to low, in PBMC cultures stimulated with allergen, owing to active suppression by Treg (Ling et al. 2004). This suppressive Treg response appears to be essential for maintenance of immune tolerance by preventing Th2 induction and Th2 cytokine release (Cottrez et al. 2000).

Foxp3 deregulation has been implicated in the development of allergy. Evidence from three geographically and ethnically distinct populations have indicated that Foxp3 polymorphisms along with impaired Treg function have been associated with the development of allergy (Zhang et al. 2009; Bottema et al. 2010; Fodor et al. 2011). In a study of a

monozygotic twin cohort of adult twin pairs, one of which had allergic asthma, differential methylation at the Foxp3 locus was observed along with reduced Foxp3 expression and impaired function in Treg isolated from the asthmatic twin (Runyon et al. 2012). *In vitro* function of Foxp3 Treg has been shown to be reduced in subjects with allergic rhinitis and refractory asthma, and Treg from asthmatic subjects showed deregulation in chemokine signaling pathway (Grindebacke et al. 2004; Lee et al. 2007; Nguyen et al. 2009).

Animal models have been utilized to determine the importance of Treg in the development and regulation of allergy and allergic disease. Murine studies have shown that tolerance induction in the airways specifically correlated with the induction of Treg. Mice that were repeatedly exposed to low-dose allergen induced the development of a Treg population that expressed both TGF-β and Foxp3. These cells prevented the onset of allergic sensitization when adoptively transferred to naïve T cells (Ostroukhova et al. 2004). In a mouse model of allergic airway disease, adoptive transfer of antigen-specific $CD4^+CD25^+$ Treg was able to suppress both airway inflammation and airway hyper-reactivity in an IL-10-dependent manner (Kearley et al. 2005). When Treg were administered after the onset of disease, they were able to attenuate inflammation and prevent features of airway remodeling including mucus hypersecretion, smooth muscle hypertrophy and collagen synthesis

(Kearley, Robinson, and Lloyd 2008). However, the same study showed that Treg were unable to reverse established remodeling. Conversely, depletion of $CD4^+CD25^+$ Treg prior to sensitization resulted in augmented inflammation and AHR in the lung of mice relatively resistant to the development of AHR (Lewkowich et al. 2005). It was also determined that the *in vivo* resolution of allergic airway inflammation did not require Treg to be antigen specific (Leech et al. 2007).

In vitro studies have been carried out aiming to delineate the role of Treg in asthma. It was demonstrated using PB from non-atopic subjects that PB $CD4^+$ cells, isolated from a population of $CD25^+$ cells, were able to proliferate and release cytokines after allergen stimulation (Ling et al. 2004). These responses were abolished upon addition of $CD4^+CD25^+$ cells. The same study demonstrated that $CD4^+CD25^+$ cells from atopic individuals were less effective in suppressing allergen-stimulated effector T cells.

There have also been studies in humans assessing Treg in allergic and asthmatic disease (Ryanna et al. 2009). In a pediatric asthma population, airway, but not circulating, $CD4^+CD25^{hi}$ Treg number and function were demonstrated to be impaired as compared to children with cough but no asthma (Hartl et al. 2007). Another study utilizing a pediatric asthma population reported a lower frequency of circulating $CD4^+CD25^+$ cells in the asthmatics compared to healthy controls (Lee et al. 2007). Of

interest in this study was the observation that Treg from severe asthmatic patients displayed normal suppressive function indicating that the inability of Treg to effectively control the asthmatic response may relate more to Treg number than to Treg functional capacity.

It was demonstrated that adult asthmatics have normal circulating levels of $CD4^{+}CD25^{hi}$ Treg and $CD4^{+}CD25^{hi}Foxp3^{+}$ Treg compared with healthy controls but Foxp3 protein expression was decreased (Provoost et al. 2009). $CD4^{+}CD25^{hi}$ Treg, although displaying a higher frequency in asthmatics as compared to controls, had reduced expression of Foxp3 as well as reduced suppressive capacity in asthmatics (Lin, Shieh, and Wang 2008). Indeed the literature reveals discrepancies in Treg results from tissue compared to blood. Mild asthmatics exhibit higher numbers of circulating Treg as compared to those with moderate-to-severe disease and healthy controls (Abdulamir et al. 2009). In contrast, an increase in BAL $CD4^{+}Foxp3^{+}$ Treg was observed in patients with moderate-to-severe asthma as compared to those with mild disease and healthy controls (Smyth et al. 2010). In a model of disease exacerbation, allergic asthmatics were shown to have increased airway Treg after allergen provocation that was associated with enhanced expression of Th2 cytokines (Thunberg et al. 2010). Another study utilizing an acute house dust mite (HDM) allergen challenge model demonstrated a decrease in circulating Treg in a group of HDM-sensitive non-responders after allergen

challenge (Moniuszko et al. 2008). Both healthy and allergic individuals possess allergen-specific Th1, Th2 and Tr1 cells that recognize the same antigenic epitopes. It has been shown that in healthy, non-allergic individuals, Tr1 adaptive Treg cells represent the dominant T cell subset (Akdis et al. 2004). The balance between allergen-specific Treg and Th2 cells is important for the development of a healthy, normal immune response as compared to an allergic response.

1.4 Summary

Treg play an important role in the regulation of immune responses in a variety of different diseases, including allergic asthma. Treg are potent anti-inflammatory and immunosuppressive cells and alterations in Treg number and/or function can lead to inappropriate immune responses resulting in allergic diseases such as asthma. Although significant advancements have been made in recent years in the field of Treg biology, there remains an open question as to the role these cells play in allergic asthma. The work described in the following chapters attempts to better characterize the role of Treg using a human model of allergic asthma.

1.5 Hypotheses and Aims

General Hypothesis:

The development of allergen-induced airway responses is associated with an imbalance in T-regulatory and T-effector cells.

Specific Hypotheses:

1. Phenotypically different Treg are quantitatively and functionally different and are differentially expressed in asthmatics compared to healthy controls.

Aim 1 – To determine the frequency of three different Treg phenotypes from the peripheral blood of healthy control subjects.

Aim 2 – To determine the functional suppressive capacity of three different Treg phenotypes from the peripheral blood of healthy control subjects.

Aim 3 – To determine the frequency of three different Treg phenotypes from the peripheral blood and BAL from healthy control subjects compared to asthmatic subjects.

2. Allergic asthmatics who develop dual asthmatic responses exhibit an attenuated Treg response after allergen inhalation challenge.

Aim 1 - To determine Treg and Teff absolute number and frequency in the sputum and peripheral blood of isolated early versus dual asthmatic

responders before and after allergen challenge, and examine the ratio of Treg to Teff after allergen challenge.

3. **The anti-inflammatory effects of anti-OX40L or anti-TSLP monoclonal antibody therapy will result in an increase in circulating Treg.**

Aim 1 – To determine peripheral blood Treg frequency in mild allergic asthmatic subjects before and after allergen inhalation challenge and before and after treatment with an anti-OX40L monoclonal antibody.

Aim 2 – To determine peripheral blood Treg frequency in mild allergic asthmatic subjects before and after allergen inhalation challenge and before and after treatment with an anti-TSLP monoclonal antibody.

Taken together, these studies aim to further our understanding of the role of Treg in allergic asthma and to provide insight into ways and means of manipulating these cells for therapeutic use.

CHAPTER 2

Title

T-regulatory cell phenotypes in peripheral blood and bronchoalveolar lavage from non-asthmatic and asthmatic subjects

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Statement of Contribution

I developed this study in consultation with Drs. Paul O'Byrne, Mark Larché, Judah Denburg, Mark Inman, and Desmond Murphy. Subject recruitment was performed by Rick Watson and Karen Howie. I carried out the majority of the experimental work with assistance from Dr. Steven Smith. I prepared the manuscript in collaboration with Dr. Paul O'Byrne.

T-regulatory cell phenotypes in peripheral blood and bronchoalveolar lavage from non-asthmatic and asthmatic subjects

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Running head: T-regulatory cells in asthma

Key Words: asthma, T regulatory cell, Foxp3, peripheral blood, BAL, flow cytometry, suppression assay

Abstract

Background An unresolved issue in T regulatory (Treg) cell biology is the lack of consensus on phenotypic markers that accurately define the natural Treg (nTreg) population.

Objectives To examine nTreg frequency and functional capacity in healthy controls, and their frequency in asthmatic subjects using three different phenotypic strategies. We hypothesized that phenotypically different nTreg are quantitatively and functionally different.

Methods 34 healthy, non-asthmatic and 17 asthmatic subjects were studied. Three nTreg phenotypes were defined: nTreg1 ($CD4^+CD25^+Foxp3^+$), nTreg2 ($CD4^+CD25^+CD127^{low}Foxp3^+$), and nTreg3 ($CD4^+CD25^{high}Foxp3^+$). The flow cytometric determination of nTreg frequency in peripheral blood (PB) and bronchoalveolar lavage (BAL) was performed using fluorescently labelled antibodies. PB nTreg functional capacity was assessed using a CFSE-based suppression assay.

Results There was a significantly lower frequency of peripheral blood nTreg3 compared to nTreg2 and nTreg1 ($p<0.05$). Both nTreg2 and nTreg3 had a significantly greater suppressive capacity than nTreg1 at T responder (Tresp) to nTreg ratios of 16:1 up to 1:1 ($p<0.01$). Asthmatics exhibited a significantly lower peripheral blood nTreg3 and nTreg1 frequency than healthy controls ($p<0.05$). There were no differences

between healthy controls and asthmatic subjects when comparing BAL nTreg frequency.

Conclusions and Clinical Relevance Phenotypically different nTreg subsets are quantitatively and functionally different and are variably observed in asthma. The $CD4^{+}CD25^{high}Foxp3^{+}$ phenotype was the least frequent, but demonstrated the greatest suppression, and was significantly lower in peripheral blood of asthmatic subjects. Consequently, it is imperative that nTreg phenotypes be clearly defined and that the interpretation of their frequency and function be phenotype-specific.

Introduction

T regulatory cells (Treg), originally characterized as $CD4^+CD25^+$, are an independent, specialized subset of T cells that serve to regulate immune responses, thereby maintaining homeostasis and self-tolerance [1,2]. Since their initial identification, Foxp3, a forkhead family nuclear transcription factor, has been identified as a crucial molecule in the development and function of Treg [3,4]. $Foxp3^+$ Treg can be divided into two distinct groups, natural Treg (nTreg) derived from the thymus, or induced Treg (iTreg) that develop in the periphery [5-7]. Each are defined phenotypically by the expression of particular cell surface and intracellular markers. Functionally, Treg exhibit potent suppressive capacity that is manifest through several different mechanisms [8,9].

Over the last decade, extensive work in the field of Treg biology has resulted in the description of a number of molecules used to characterize Treg subsets (i.e. Neuropilin-1, CTLA-4, GITR) [10], as well as those used to distinguish nTreg from induced Treg (iTreg)(i.e. Helios)[11]. However, an important unresolved issue in Treg cell biology is the lack of consensus on phenotypic markers that uniquely define the Treg population. With the invention of new abbreviations and terminology, the Treg nomenclature has become complex and in need of simplification [12]. The Treg population is also becoming increasingly diverse, and is now composed of

several phenotypically and functionally distinct subsets [13], resulting in a confusing interpretation of the role of these cells in disease.

Treg have been implicated in immuno-regulatory roles in allergic diseases such as asthma [14,15], where it has been shown that a deficiency in the both the quantity and function of Treg cells play a role in the pathogenesis and propagation of the disease [16]. However, such studies have not adequately addressed Treg heterogeneity, which could impact study conclusions. The aim of the current study was to compare three different nTreg phenotypes with respect to frequency and function. We chose our nTreg phenotypes based on the expression of CD4, CD25, CD127 and Foxp3 as these markers were the most comprehensive and would serve to detect the vast majority of the human nTreg population [17]. We hypothesized that nTreg subsets would not only be expressed at different cellular frequencies, but would also exhibit functional heterogeneity. To examine this, we first used multi-parameter flow cytometry to assess the frequency of the three different nTreg phenotypes in the peripheral blood of healthy, non-asthmatic subjects. Subsequently, we isolated each nTreg phenotype from the peripheral blood of healthy control subjects and measured the functional capacity using an *in vitro* suppression assay. Finally, we measured the nTreg phenotypes in a population of asthmatic subjects. We hypothesized that any difference in

nTreg frequency observed between healthy and asthmatic subjects would be a function of nTreg phenotype.

Materials and Methods

Subjects

Thirty four healthy, non-asthmatic and 17 asthmatic subjects between the ages of 18 and 65 years of age were studied (Table 1). Healthy control subjects had no medical history of respiratory symptoms or asthma diagnosis, a negative skin prick test to common aero-allergens, and a PC_{20} > 32mg/ml. All asthmatic subjects had both a history of asthma and a methacholine $PC_{20} \leq 16$mg/ml. All subjects had no other chronic illness and no lower respiratory tract infection at the time of their study visits. Subjects refrained from using β_2-agonists or caffeinated beverages for a minimum of eight hours before laboratory visits. Exclusion criteria included those subjects that were pregnant, current smokers, or ex-smokers with a more than 10-pack-year history. This study was approved by the Hamilton Integrated Research Ethics Board and written informed consent was obtained from all study participants.

Study Design

This study was comprised of three phases: Phase 1, we assessed PB nTreg frequency in 14 healthy control subjects; Phase 2, we determined PB nTreg functional capacity using eight healthy control subjects; and Phase 3, we compared PB and BAL nTreg frequency in 12 healthy control subjects versus 17 asthmatic subjects. Healthy control

subjects were selected to avoid the confounding issue of chronic disease on the initial quantification of the different phenotypes. For phases 1 and 2, a single laboratory visit was required for a medical history, physical examination, skin prick test, methacholine inhalation challenge, performed as described by Cockcroft *et* al. [18], and blood collection. For phase 3, subjects were required to attend the laboratory on two non-consecutive days separated by at least one week. On day one, a medical history, physical examination, and methacholine inhalation challenge was performed along with blood collection. On day two, a bronchoscopy was performed with the collection of BAL fluid.

Blood collection and processing

Venous blood was drawn into Vacutainer® blood collection tubes (BD Biosciences, Mississauga, Ontario, CA) containing lithium heparin. For nTreg phenotyping, blood was diluted 1:1 with McCoy's 5A (Gibco, Carlsbad, California, USA) and layered over Lymphoprep density gradient medium (STEMCELL Technologies Inc., Vancouver, British Columbia, CA). Density gradient separation was performed via centrifugation of layered blood at 800g for 20 minutes at room temperature. Peripheral blood mononuclear cells (PBMC) were removed, washed twice with PBS (centrifugation at 250g for 10 minutes at 4°C), re-suspended in FACS

buffer (PBS supplemented with 0.1% sodium azide and 0.5% BSA), and subjected to the nTreg phenotyping flow cytometric staining protocol.

For nTreg function, PBMC were separated as described above and used as responder T cell (Tresp) in the suppression assay. In addition, CD4+ T cells were indirectly isolated from whole blood by rosetting unwanted cells using a RosetteSep™ $CD4^+$ T cell enrichment cocktail (STEMCELL Technologies Inc.) prior to RosetteSep™DM-L (STEMCELL Technologies Inc.) density gradient centrifugation. Isolated $CD4^+$ T cells were then subjected to the nTreg flow cytometric staining protocol for sorting of nTreg used in the suppression assay.

BAL fluid collection and processing

Fibreoptic bronchial bronchoscopies were performed using an Olympus flexible bronchoscope (Tokyo, Japan). The bronchoscope was inserted into the right middle lobe and 3 x 50ml aliquots of room temperature sterile, normal saline were instilled and then aspirated with suction. Pooled BAL fluid was kept on ice and processed within one hour. BAL fluid was then passed through a nylon mesh filter and centrifuged at 250g for 10 minutes at 4°C. The supernatant was removed and the cell pellet washed twice with PBS. BAL cells were re-suspended in FACS buffer and subjected to the nTreg cell phenotyping flow cytometric staining protocol.

Flow cytometric staining

n*Treg phenotyping*. Freshly processed PBMC or BAL cells in FACS buffer were divided into tubes at 0.5-1x10^6 cells per tube. Cells were stained first for surface markers using the following fluorescently labelled antibodies: CD3-AmCyan (Clone SK7; BD Biosciences), CD4-eFluor®450 (Clone OKT4; eBioscience, San Diego, California, USA), CD25-PE (Clone M-A251; BD Biosciences), CD127-FITC (Clone eBioRDR5; eBioscience), and relevant isotype controls. Cells were incubated with antibodies for 30 minutes at 4°C in the dark. Cells were washed with FACS buffer (centrifugation at 250g for 10 minutes at 4°C), re-suspended in one mL/tube of 1x fixation/permeabilization solution (eBioscience), and incubated for 30 minutes at 4°C in the dark. Two washes with Permeabilization buffer (eBioscience) were performed after which cells were re-suspended in 100µL of 5% normal rat block and incubated for 15 minutes at 4°C in the dark. This was followed by intracellular staining using Foxp3-APC (Clone 236A/E7; eBioscience), with relevant isotype control, and incubated for 30 minutes at 4°C in the dark. Cells were washed twice with Permeabilization buffer, re-suspended in 400µL of Cytofix (1% paraformaldehyde; BD Biosciences), and stored at 4°C in the dark until flow cytometric acquisition.

nTreg sorting. Enriched CD4+ T cells, at $20x10^6$/ml, were stained as per the method outlined in the human regulatory T cell sorting kit (BD Biosciences). Briefly, cells were stained for surface markers using the following fluorescently labelled antibody cocktail: CD4-PerCP-Cy™5.5 (Clone L200; BD Biosciences), CD25-PE (Clone 2A3; BD Biosciences), CD127-Alexa Fluor®647 (Clone 40131.111, BD Biosciences), and relevant isotype controls (Note: Foxp3 was omitted as intracellular staining would render the cells non-functional). The antibody cocktail was added at $2\mu L/10^6$cells and incubated for 30 minutes at room temperature in the dark. Cells were washed with PBS containing 1% human AB serum (Life Technologies Inc., Burlington, Ontario, Canada), re-suspended at $10x10^6$/mL in RPMI complete medium, and stored on ice in the dark until flow sorting.

Flow cytometric sorting

Three different PB nTreg phenotypes, not stained for Foxp3 (Treg1 – $CD4^+CD25^+$, Treg2 – $CD4^+CD25^+CD127^{low}$, and Treg3 – $CD4^+CD25^{high}$), were sorted from stained $CD4^+$ enriched cells using a FACS Vantage SE, three laser, eight colour capable digital sorter (Becton Dickinson, Mississauga, ON, Canada) in the McMaster University Flow Cytometry Facility. Using a 70 micron nozzle, nTreg were sorted into human AB serum coated tubes containing RPMI complete medium to a

purity routinely >95% with a minimum yield of 100,000 cells per phenotype. The sorted nTreg were then washed using complete RPMI medium and re-suspended to $1x10^6$cells/ml for use in the T cell suppression assay.

CFSE labelling

A 5mM stock solution of CellTrace™ CFSE (Life Technologies) was prepared using DMSO. Isolated PBMC were re-suspended to 5-10 x 10^6 cells/mL in sterile PBS and 1ml was added to a 14mL conical tube. 110µL of sterile PBS was placed near the top of the tube. To this droplet 1.1µL of 5mM CFSE stock was added, the tube was inverted and immediately vortexed for two seconds. Cells and CFSE were incubated for seven minutes at room temperature in the dark. CFSE labelled PBMC were then washed twice with cold RPMI complete medium and re-suspended to $1x10^6$cells/ml. Cells were kept on ice, protected from light, and used as Tresp in the suppression assay.

Suppression assay

The assay was performed as outlined previously [19,20]. Briefly, CFSE-labelled autologous PBMC were used as Tresp and cultured at 50,000 cells per well in 96 well, round bottom plates (Sigma Aldrich, Oakville, Ontario, Canada). Tresp were then co-cultured with FACS

sorted nTreg1, nTreg2, or nTreg3 at Tresp to nTreg ratios of 32:1, 16:1, 8:1, 4:1, 2:1, and 1:1, and relevant controls. Cells were stimulated with plate-bound, purified, functional grade anti-CD3 (eBiosciences) at a concentration of 125ng/ml for five days in a humidified incubator (37°C and 5% CO_2). After five days, cells were harvested, stained for CD4 and then analyzed by flow cytometry. The suppressive capacity of Treg versus Tresp proliferation in co-culture was expressed using the conventional gating method (analyzing only proliferated cells)[21]. The calculation of per cent suppression was as follows: % Suppression = 100 – (Tresp:Treg/Tresp)*100.

Flow cytometry

nTreg phenotyping. Data was acquired using a 15-colour, three laser, LSRII flow cytometer equipped with FACSDiva acquisition software (BD Biosciences). At least 10,000 CD4+ events were collected from PBMC whereas as many CD4+ events as possible were collected from BAL. Collected data was analyzed using FlowJo analysis software (FlowJo LLC, Ashland, Oregon, USA). Our nTreg gating strategy, as applied to both blood and BAL, is outlined in Fig 1A and Fig 1B. Briefly, a CD3 versus CD4 plot was generated from the lymphocyte population and a gate placed around the $CD3^+/CD4^+$ double-positive population. For the determination of nTreg1 a plot of CD4 vs CD25 was used and a gate

placed around cells expressing CD25 above that of the isotype control. These cells were used to plot CD25 vs CD127 with a gate then placed around those cells expressing CD25 and low expression of CD127. These cells defined the nTreg2 phenotype. nTreg3 were identified using $CD25^+$ cells on a CD4 versus CD25 plot. A gate was placed on those cells expressing the highest levels of CD25. Foxp3 expression was determined by plotting cells from each respective nTreg phenotype on a plot of CD4 versus Foxp3. Cells above that of the isotype control were deemed positive for Foxp3 (Fig 1B).

Treg sorting. The three nTreg phenotypes were sorted from enriched $CD4^+$ cells as per the above gating strategies with the exception that cells were not stained for CD3.

Suppression assay. As many $CD4^+$ events as possible were collected. Briefly, a dot plot of CD4 vs SSC was generated and a gate placed on the $CD4^+$ population. These single positive, $CD4^+$ cells were used to construct a histogram of CFSE expression. Proliferated cells were deemed to be those cells to the left of the unstimulated cells and to the right of those cells not stained with CFSE (Fig 3a).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, California, USA). Data normally distributed were expressed as mean ± SEM. Between phenotype comparisons were performed using unpaired t tests. Repeated measures analysis of variance (ANOVA) was used to compare the between phenotype suppressive capacity.

Results

We assessed the frequency and function of three different nTreg phenotypes using surface markers to CD3, CD4, CD25, CD127 and an intracellular marker to Foxp3. We defined our nTreg as follows: nTreg1 – $CD4^+CD25^+Foxp3^+$, nTreg2 – $CD4^+CD25^+CD127^{low}Foxp3^+$, and nTreg3 – $CD4^+CD25^{high}Foxp3^+$ (Fig 1A, 1B).

Healthy control subjects had normal lung function, a normal methacholine PC_{20}, defined as >32 mg/ml (Table I) and were non-atopic. The healthy control and asthmatic subjects were matched for age, but were significantly different with respect to FEV_1 (% predicted) and methacholine PC_{20} (Table 1).

The frequency of peripheral blood nTreg3 in healthy control subjects, as a percentage of $CD4^+$ cells (1.8 ± 0.10%), was significantly lower when compared to nTreg1 (3.49 ± 0.28%) or nTreg2 (3.14 ± 0.22%, $p<0.05$; Fig 2). This difference was also seen when the frequency of peripheral blood nTreg3, without Foxp3 expression (2.20±0.14%), was compared to nTreg1 (18.51 ± 1.34%) or nTreg2 (4.20 ± 0.24%, $p<0.05$). When viewed as the percentage of cells expressing Foxp3, nTreg1 (21.12 ± 3.24%) was significantly lower compared to nTreg2 (74.62 ± 2.46%) or nTreg3 (82.40 ± 1.56%) and nTreg2 was significantly lower than nTreg3 ($p<0.05$). The nTreg populations are phenotypically different but not

distinct from one another – nTreg1 subset encompasses both nTreg2 and nTreg3 subsets while nTreg2 also encompasses nTreg3.

All three nTreg phenotypes functionally suppressed Tresp cells in a concentration dependent manner (Fig 3A and Fig 3B). However, for nTreg3 and nTreg2, there were no significant differences in functional capacity, but both demonstrated significantly greater suppression than nTreg1 for all Tresp to nTreg ratios except for 32:1 ($p<0.05$, Fig 3B).

In the asthmatic subjects, as in healthy control subjects, the frequency of peripheral blood nTreg3 (0.45 ± 0.11%) was significantly lower when compared to nTreg1 (2.71 ± 0.34%) or nTreg2 (2.21 ± 0.38%, $p<0.05$; Table 2). In addition, asthmatics exhibited a significantly lower peripheral blood nTreg1 and nTreg3 frequency than healthy control subjects ($p<0.05$, Fig 4A).

Similar to peripheral blood, the frequency of the nTreg phenotypes differed numerically in BAL, but was only significantly different in the asthmatic subjects (nTreg3 was significantly lower when compared to nTreg1 or nTreg2), being 3.98 ±1.57% for nTreg1, 2.77 ± 0.89% for nTreg2, and 1.48 ± 0.45% for nTreg3 ($p<0.05$, Table 2). There were no significant differences between asthmatic and healthy control subjects when comparing BAL nTreg frequency (Fig 4B).

Discussion

Our data highlight the heterogeneity that exists in the nTreg population with respect to phenotype and function. We have demonstrated, in a population of healthy, non-asthmatic individuals, that three phenotypically different peripheral blood nTreg subsets are expressed at different frequencies and possess different functional capacities. In addition, we have shown that there are differences in peripheral blood nTreg frequency, when comparing asthmatic to non-asthmatic subjects, is dependent on the phenotype studied.

The characterization of Treg has substantially evolved since Sakaguchi *et al*. proposed the $CD4^+CD25^+$ phenotype two decades ago [1]. As evidence of this, we chose to study the $CD4^+CD25^+Foxp3^+$ (nTreg1), $CD4^+CD25^+CD127^{low}Foxp3^+$ (nTreg2), and $CD4^+CD25^{high}$ $Foxp3^+$ (nTreg3) Treg subsets. We have shown that the frequency of peripheral blood nTreg3 is significantly lower than that of nTreg1 and nTreg2. nTreg1 has been shown to be the most inclusive phenotype, essentially encompassing all of the Foxp3+ Treg cells [17]. However, this comes with the notable drawback that this phenotype also includes activated T cells that transiently express Foxp3 [22]. The co-expression of CD127, the alpha chain of the IL-7R, and high levels of expression of CD25 has since been shown to discriminate bona fide Treg from activated T cells [23]. The low expression of CD127 on Treg inversely correlated

with the expression of Foxp3 [24]. Thus, the inclusion of CD127 in the $CD4^{+}CD25^{+}CD127^{low}Foxp3^{+}$ (nTreg2) phenotype allows for the study of a more pure nTreg population. The nTreg3 phenotype includes those Treg expressing the highest levels of CD25; on a spectrum of CD25 expression these cells possess the highest suppressive capacity as compared to the $CD4^{+}CD25^{low}$ population [25]. By definition, nTreg are inherently suppressive, and nTreg3 therefore allowed us to examine the population of nTreg with the greatest functional capacity. In addition, we have shown that these three phenotypes are functionally different as measured using a CFSE-based *in vitro* suppression assay. Miyara *et al*. [26] elegantly showed that distinct subsets of Treg exist when CD4+Foxp3+ cells are stratified based on the level of Foxp3 expression. In support of our data, they too concluded that different Treg subsets are functionally heterogeneous. However, our nTreg subsets, while phenotypically different, are not distinct populations. In addition, our results are in concert with those published by d'Hennezel *et al*. [27] who showed that three different Treg phenotypes (based on $CD25^{high}$, $CD25^{low}$ or $CD25^{neg}$ expression) expressed different levels of suppression using a tritiated thymidine based suppression assay. Even with the use of different functional assays, it is clear that Treg expressing high levels of CD25 possess the most robust suppressive capacity. Our data also highlight the relationship of Foxp3 and Treg suppressive capacity. We showed that the

three different nTreg phenotypes differentially expressed Foxp3 and that the highest Foxp3 expression was associated with the higher suppressive capacity. These data support the essential role of Foxp3 for Treg suppressor function [28,29].

Others have quantified circulating and airway nTreg in an asthmatic population. An increase in circulating $CD4^+CD25^+CD127^{low}$ nTreg frequency was observed in asthmatic children compared to healthy controls and was attributable to inhaled corticosteroid (ICS) use and not disease severity [30]. When the asthmatic group was stratified based on inhaled corticosteroid use, comparisons between controls and asthmatics not on ICS yielded no difference in Treg frequency. These data support our finding that there is no difference in nTreg2 frequency between asthmatics and controls. Provoost *et al*. [31] identified no difference in the frequency of peripheral blood $CD4^+CD25^{high}$ or $CD4^+CD25^{high}$ $Foxp3^+$ Treg between asthmatic and control subjects. This is in contrast to our data which showed a significant decrease in the frequency of nTreg3 ($CD4^+CD25^{high}$ $Foxp3^+$) in asthmatics compared to healthy controls. This discrepancy may be attributable to the method of calculating frequency. We expressed nTreg frequency as a percentage of $CD4^+$ cells whereas Provoost *et al.* expressed nTreg as a percentage of the lymphocyte gate. Smyth et al. [32] evaluated Treg in the airways of healthy, mild asthmatic, and moderate/severe asthmatic subjects and found that differences in BAL

Treg frequency between healthy and moderate/severe asthmatic subjects utilizing the $CD4^+CD25^+CD127^-$ phenotype were not present when using the $CD4^+CD25^{bright}$ phenotype. Thus, the conclusion that moderate/severe asthmatics have a higher frequency of BAL Treg is only true using the $CD4^+CD25^+CD127^-$ phenotype. While the phenotypes were not identical, these data support our findings that there may be differences in nTreg frequency depending on the phenotype studied. Of note in the Smyth study was that no differences were observed in Treg frequency, regardless of phenotype, when comparisons were made between healthy and mild asthmatic subjects. Although the numbers of BAL samples from the asthmatic subjects were few, no differences in nTreg frequency were observed comparing asthmatic and healthy control subjects. This may also be because the asthmatic subjects had very mild asthma. Thus, while the conclusions reached about airway nTreg must be very limited, the frequency of nTreg may depend not only on the compartment from which the cells are obtained, but also the phenotype of the subjects studied.

There are several limitations to our study. In the sorting of nTreg for use in the suppression assay, we were unable to use Foxp3 in our phenotypic definition. Foxp3 is an intracellular marker and, as such, staining for it would render nTreg non-functional. Due to insufficient volumes of peripheral blood (limited allowable volume of blood drawn) as

well as low cell yields in the BAL, we were unable to assess nTreg function in the comparison of asthmatic and healthy subjects. Also, with no reliable phenotypic or functional markers that make it possible to distinguish between natural and induced Treg in the periphery [33], we may have enhanced nTreg frequency.

In summary, phenotypically different nTreg cells are quantitatively and functionally different and are variably expressed in states of health and disease. Our results highlight the need for nTreg phenotypes to be clearly defined in studies and for interpretations to be made using consistent and specific phenotypes. Finally, we demonstrate that the $CD4^{+}CD25^{high}Foxp3^{+}$ phenotype exhibited the greatest suppressive capacity and is significantly less frequent in the peripheral blood of mild asthmatic subjects.

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Statement of Contribution

The study was developed by AJ Baatjes, PM O'Byrne, M Larche, MD Inman, JA Denburg, and D Murphy. Subject recruitment was performed by R Watson and K Howie. The experimental work was performed by AJ Baatjes and SG Smith. The paper was written by AJ Baatjes and PM O'Byrne. All authors reviewed and approved the manuscript before submission.

Statement of Conflict of Interest

None of the authors have any conflict of interest to disclose in relation to the study described in the manuscript.

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Table 1. Subject characteristics. Subjects were separated based on those used for assessment of PB nTreg frequency (n=14) or function (n=8) in healthy controls (Phases 1 and 2), and those used for comparison of PB and BAL nTreg frequency between asthmatics and healthy controls (Phase 3). Values expressed as mean ± SEM.

	Healthy Control Subjects	**Asthmatic Subjects**
Phases 1 and 2		
Subjects, n	22	NA
Male	10	NA
Female	12	NA
Age, years	34.27 ± 2.600	NA
FEV_1, % predicted	96.12 ± 2.060	NA
PC_{20}, mg/ml	>32	NA
Phase 3		
Subjects, n	12	17
Male	5	7
Female	7	10
Age, years	37.83 ± 3.988	38.59 ± 3.657
FEV_1, % predicted	100.8 ± 2.831	82.82 ± 4.437*
PC_{20}, mg/ml	>32	3.808 ± 1.586*

* - $p<0.05$ compared to healthy control subjects
NA – not applicable

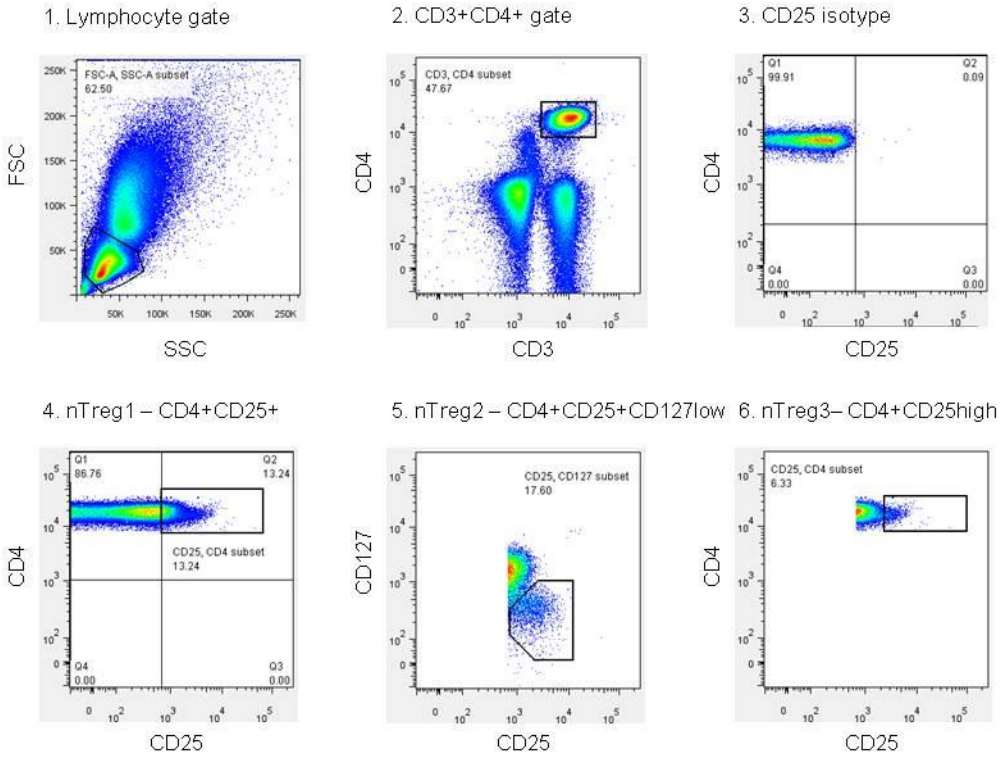
Table 2. Within group comparisons of peripheral blood or BAL nTreg frequency. Values expressed as mean ± SEM.

	nTreg1	nTreg2	nTreg3
Healthy Control Subjects			
Peripheral blood frequency (%CD4)	4.43 ± 0.52	2.45 ± 0.36#	1.12 ± 0.14*
BAL frequency (%CD4)	5.12 ± 0.78&	3.29 ± 0.66	2.63 ± 0.53
Asthmatic Subjects			
Peripheral blood frequency (%CD4)	2.71 ± 0.34	2.21 ± 0.38	0.45 ± 0.11*
BAL frequency (%CD4)	3.98 ± 1.57	2.77 ± 0.89	1.48 ± 0.45*

* - $p<0.05$ versus nTreg1 and nTreg2
# - $p<0.05$ versus nTreg1
& - $p<0.05$ versus nTreg2 and nTreg3

Fig 1. Flow cytometric dot plots, using a representative peripheral blood sample, outlining the gating strategy for the three nTreg phenotypes. **A.** 1, Lymphocyte gate; 2, $CD3^{+}CD4^{+}$ gate; 3, CD4 vs CD25 isotype control; 4, nTreg1 gate; 5, nTreg2 gate; 6, nTreg3 gate. **B.** Determination of Foxp3 expression in each nTreg phenotype – CD4 vs Foxp3 isotype control (left panel) and CD4 vs Foxp3 (right panel).

A.



B.

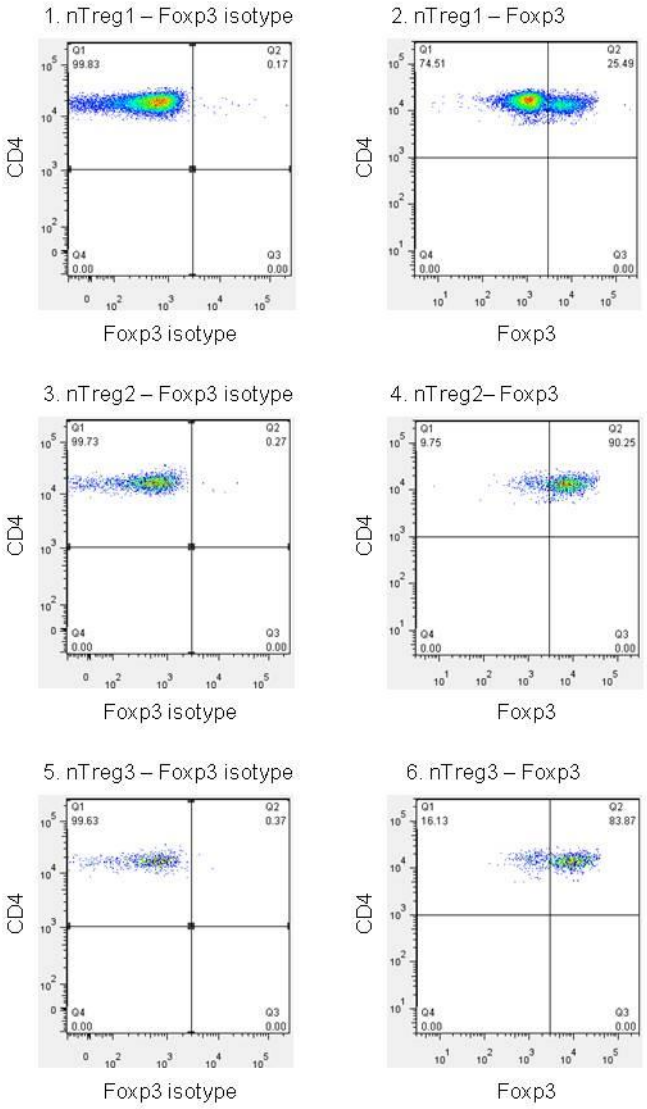

1. nTreg1 – Foxp3 isotype
Q1 99.83
Q2 0.17
Q4 0.00
Q3 0.00
CD4
Foxp3 isotype
2. nTreg1 – Foxp3
Q1 74.51
Q2 25.49
Q4 0.00
Q3 0.00
CD4
Foxp3
3. nTreg2 – Foxp3 isotype
Q1 99.73
Q2 0.27
Q4 0.00
Q3 0.00
CD4
Foxp3 isotype
4. nTreg2– Foxp3
Q1 9.75
Q2 90.25
Q4 0.00
Q3 0.00
CD4
Foxp3
5. nTreg3 – Foxp3 isotype
Q1 99.63
Q2 0.37
Q4 0.00
Q3 0.00
CD4
Foxp3 isotype
6. nTreg3 – Foxp3
Q1 16.13
Q2 83.87
Q4 0.00
Q3 0.00
CD4
Foxp3

Fig 2. Differences in peripheral blood nTreg frequency in healthy control subjects comparing three different nTreg phenotypes (n=14). Values expressed as individual data points with the bar denoting the mean percentage. * denotes $p<0.05$.

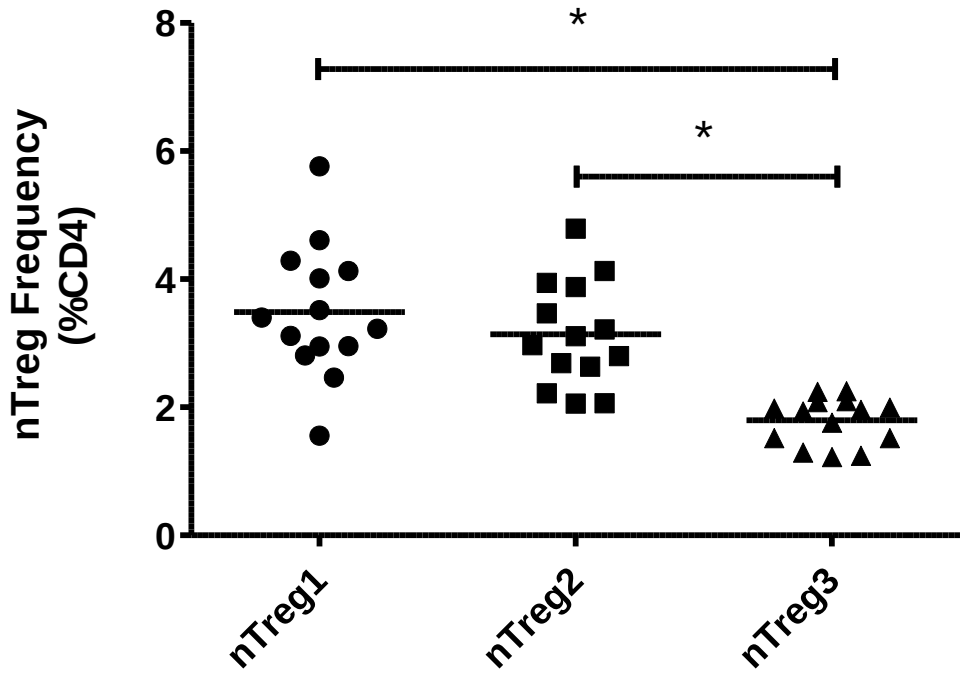
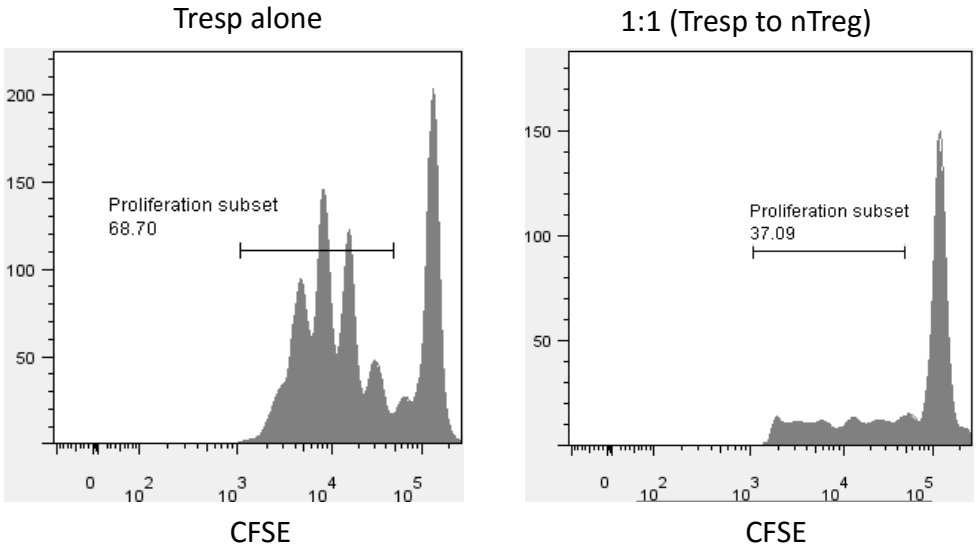


Fig 3. Assessment of peripheral blood nTreg function using a CFSE-based suppression assay. A. Representative flow cytometric CFSE histograms – left panel, proliferation profile of Tresp alone; right panel, proliferation profile of Tresp co-cultured with nTreg at a ratio of 1:1. B. Comparison of suppressive capacity of three nTreg phenotypes at six Tresp:nTreg ratios (n=8). Results are expressed as mean + SEM. * denotes $p<0.05$.

A.



B.

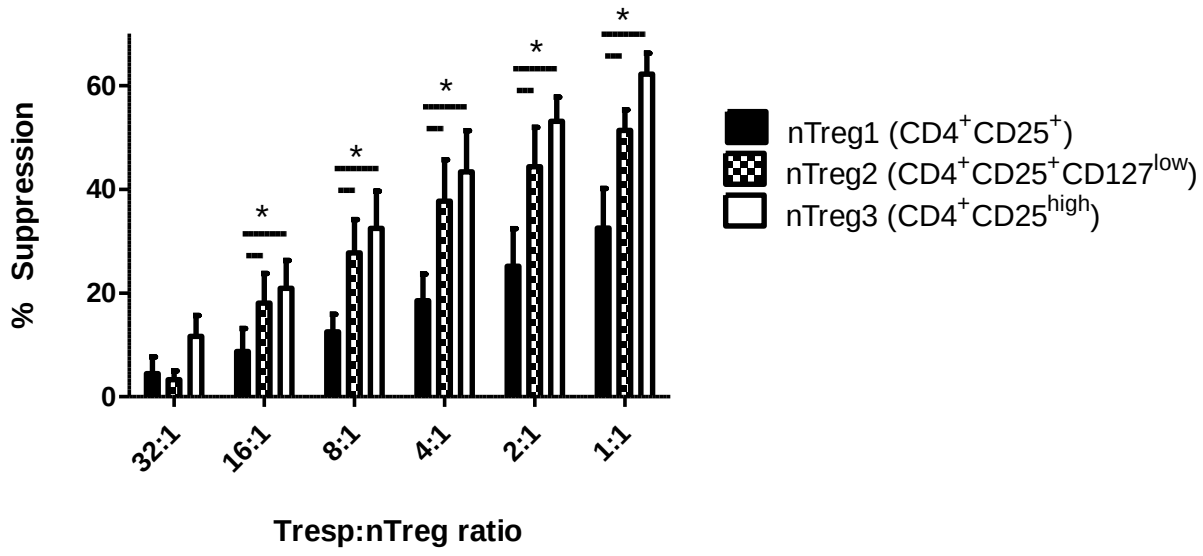
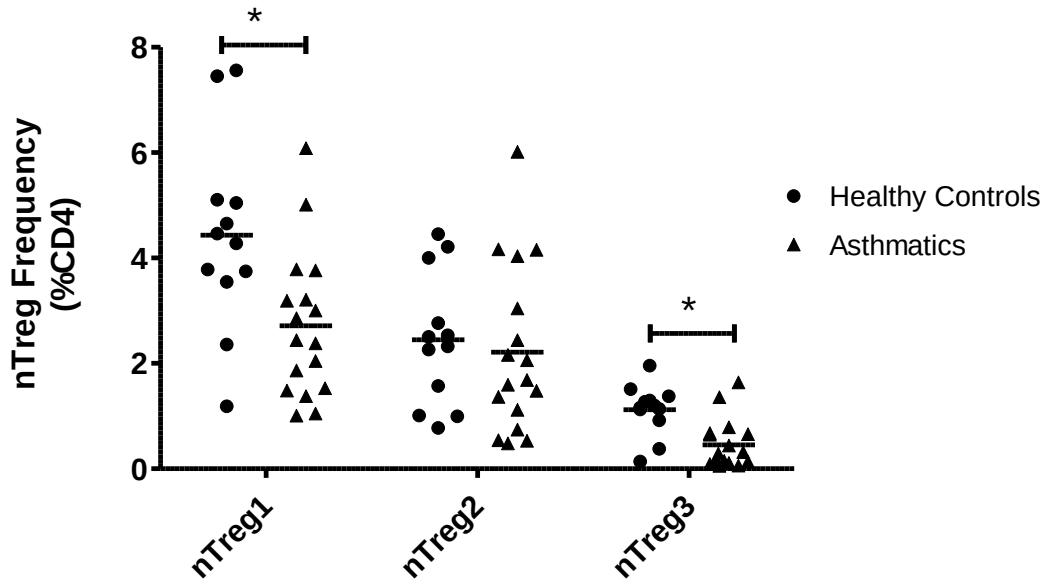
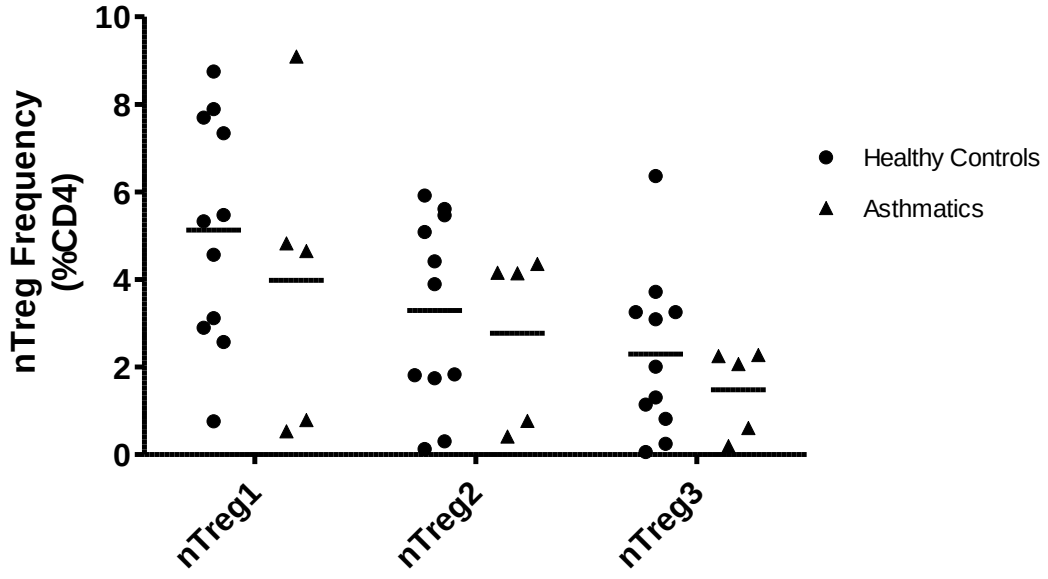

% Suppression
60
40
20
0
*
*
*
*
*
32:1
16:1
8:1
4:1
2:1
1:1
Tresp:nTreg ratio
nTreg1 (CD4+CD25+)
nTreg2 (CD4+CD25+CD127low)
nTreg3 (CD4+CD25high)

Fig 4. Comparison of nTreg frequency between asthmatic and healthy control subjects. A. Differences in peripheral blood nTreg frequency from asthmatic (n=17) versus healthy control subjects (n=12). B. Differences in BAL nTreg frequency from asthmatic (n=5) versus healthy control subjects (n=11). Values expressed as individual data points with the bar denoting mean percentage. * denotes $p<0.05$.

A.



B.


nTreg Frequency (%CD4)
10
8
6
4
2
0
nTreg1
nTreg2
nTreg3
Healthy Controls
Asthmatics

CHAPTER 3

Title

Natural regulatory T cells in isolated early responders compared with dual responders with allergic asthma.

Authors

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Statement of Contribution

Takashi Kinoshita and I developed this study in consultation with Drs. Paul O'Byrne, Gail Gauvreau and Tomotaka Kawayama. Subject recruitment was performed by Rick Watson. I, along with Dr. Takashi Kinoshita, carried out the majority of the experimental work with assistance from Drs. Steven Smith and Benny Dua. I, along with Dr. Takashi Kinoshita, prepared the manuscript in collaboration with Dr. Paul O'Byrne.

Natural regulatory T cells in isolated early responders compared with dual responders with allergic asthma.

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Abbreviations

Th – T helper

IL – Interleukin

Treg - Regulatory T cell

IER – Isolated early responder

DR – Dual responder

AHR – Airway hyper-responsiveness

PC_{20} – The provocative concentration of methacholine causing a 20% fall in FEV_1

FEV_1 – Forced expiratory volume in one second

Foxp3 – Forkhead box P3

CTLA-4 – Cytotoxic T-lymphocyte antigen 4

BALF - Bronchoalveolar lavage fluid

ABSTRACT

Background: Natural regulatory T (Treg) cells are implicated in the regulation of the inflammatory response in patients with allergic asthma.

Objectives: We sought to determine changes in Treg cell numbers in the airways and peripheral blood of isolated early responder (IER) versus dual responder (DR) subjects with mild allergic asthma before and after allergen challenge.

Methods: Induced sputum was collected from 22 subjects with allergic asthma (10 IERs and 12 DRs) and peripheral blood collected from 8 DRs with allergic asthma at 0, 7, and 24 hours after allergen challenge. Treg cells were identified by using fluorescently labeled antibodies to CD4 and forkhead box protein 3 and enumerated by using flow cytometry.

Results: There was a significant increase in the percentage of sputum $CD4^+$ cells 24 hours after allergen challenge in both IERs and DRs. The percentage of sputum Treg cells significantly decreased 24 hours after challenge in DRs but not IERs. This change was significantly correlated with the magnitude of the late asthmatic response. There was also a significant increase in the absolute number of sputum $CD4^+$ cells and Treg cells at 24 hours in DRs only. The ratio of the number of Treg cells to $CD4^+$ cells at 24 hours was significantly smaller in DRs compared with that in IERs. None of the above changes were observed in peripheral blood.

Conclusions: DRs exhibit a diminished percentage of airway Treg cells after allergen challenge that is not observed in IERs and a significantly lower ratio of Treg cells to $CD4^+$ cells, which might contribute to the development of the late asthmatic response.

KEY MESSAGES

- This study demonstrated that CD4+Foxp3+Tregs can be measured in induced sputum, and this method can be used to follow the changes in Tregs over time after allergen inhalation
- The ratio of Tregs to CD4+ cells in induced sputum after inhaled allergen is lower in dual responders, and the changes in CD4+Foxp3+Tregs correlate with the magnitude of the allergen-induced late response.
- The results suggest that an imbalance in the numbers of inhibitory to effector T-cells is important in determining the development of allergen-induced late responses.

CAPSULE SUMMARY

CD4+Foxp3+Tregs can be measured in induced sputum. After allergen inhalation, the ratio of CD4+Foxp3+Tregs as a percentage of CD4+ cells declines in dual when compared to isolated early responders.

KEY WORDS

Allergic asthma, induced sputum, CD4+ T-cells, late asthmatic responses, T-regulatory cells.

INTRODUCTION

Allergic asthma occurs as a result of an inappropriate immune response to innocuous inhaled allergens. The immune and inflammatory processes of this response are complex and include evidence for impaired regulation by regulatory T cells (Tregs). Tregs, formerly known as suppressor T cells, are immunosuppressive and immunomodulatory T cells that play a role in the maintenance of self-tolerance and immune homeostasis, and which play a crucial regulatory role in human diseases, such as allergy, cancer and autoimmune disease [1-3].

They can be classified as natural Tregs (nTregs), which develop in the thymus, or induced Tregs (iTregs) which develop in the periphery upon antigen encounter [4]. Recently, lung tissue macrophages have been shown to have the ability to generate iTregs [5]. Human Tregs were initially defined as the CD4+CD25+ lymphocyte population [6;7]. Subsequently, Foxp3, a forkhead family transcription factor, was identified as a master control gene for Treg development, function and homeostasis [8;9]. Thus, nTregs are typically defined as T helper cells that express CD25 and Foxp3 (CD4+CD25+Foxp3+), and iTregs as Tr1 helper cells that express IL-10 (CD4+IL-10+) or Th3 helper cells that express TGF-β (CD4+TGF-β+). Tregs exert their suppressive/inhibitory effects via a variety of mechanisms including cell to cell contact, competition for growth factors, cytotoxicity, and the secretion of inhibitory cytokines.

Evidence exists for an immunoregulatory role for Tregs in animal models of allergic airway responses [10;11], and this is increasingly supported by clinical evidence. Tregs are deficient numerically and functionally in pediatric asthmatic compared to healthy individuals [12] and the failure to properly regulate immune responses in asthma may be attributable to an insufficiency in tolerance mechanisms. This has led to the hypothesis that in allergic asthma the Treg response is insufficient to control the T-cell effector (Th2-induced) inflammatory response. To examine this hypothesis we have evaluated the absolute number and percentage of Tregs, as a proportion of T-effector cells, in the airways and in peripheral blood before and after inhaled allergen challenge in atopic asthmatics. We reasoned that atopic asthmatic subjects, who develop a late phase asthmatic response and who develop greater allergen-induced airway inflammation [13], would have fewer number of airway Tregs following allergen inhalation challenge.

METHODS

Subjects

Thirty subjects with very mild allergic asthma (10 isolated early responders and 20 dual responders), between the ages of 18 and 60 years of age, were enrolled (Table I). All were atopic on the basis of one or more skin wheal and flare responses to common aeroallergens, had a forced expiratory volume in one second (FEV_1) ≥ 70% of predicted and the baseline methacholine PC_{20} (the provocative concentration of methacholine causing a 20% fall in FEV_1) ≤ 32 mg/ml. All subjects had no other lung disease or chronic illness, no lower respiratory tract infection or worsening of asthma for six weeks before screening. Subjects had not been using inhaled corticosteroids for at least 1 year, had infrequent use of β_2-agonists for treatment of asthma (less than daily), and refrained from β_2-agonist and caffeinated beverages at least eight hours before laboratory visits. Subjects were excluded if they were pregnant, current smokers, or ex-smokers with more than 10 pack-years. Isolated early responders were defined as those having only an early asthmatic response (EAR - an acute fall in FEV_1 ≥ 20% within two hours following allergen challenge). Dual responders were defined as those subjects having both an EAR and a late asthmatic response (LAR – a fall in FEV_1 ≥ 15% between three and seven hours following allergen challenge). Eight subjects (all dual responders), provided peripheral blood for Treg analysis.

The study was approved by the hospital's Research Ethics Review board and all subjects gave written informed consent.

Study design

Subjects attended the study period on three consecutive days. On day 1, a medical history and physical examination were performed and subjects underwent a skin prick test, spirometry, methacholine inhalation challenge, blood collection, and sputum induction. On day 2, subjects underwent an inhaled allergen challenge and spirometry was measured up to seven hours post-challenge. Sputum was induced and blood was collected at seven hours post-challenge. On day 3, 24 hours after allergen inhalation challenge subjects performed spirometry, methacholine inhalation challenge, blood collection and sputum induction.

Methacholine inhalation challenge

Methacholine inhalation challenge was performed as previously described by Cockcroft et al [14], by using tidal breathing from a Wright nebulizer. The test was terminated when a fall in FEV_1 of at least 20% of the baseline value occurred.

Allergen inhalation challenge

Allergen inhalation was performed as described by O'Byrne *et al* [15]. The concentration of allergen extract used for inhalation was determined from a formula described by Cockcroft and colleagues [16]. After allergen administration, the FEV_1 was then measured at regular intervals until seven hours after allergen inhalation. The maximum percent fall in FEV_1 was recorded for the EAR and LAR. The EAR was identified by a fall in $FEV_1 \geq 20\%$ from baseline within 2h after allergen and a LAR was identified by a fall in $FEV_1 \geq 15\%$ from baseline between 3h-7h after allergen.

Sputum induction and processing

Sputum was induced and processed according to the method described by Pizzichini et al [17]. The total cell count was determined using a Neubauer hemocytometer chamber (Hausser Scientific, Blue Bell, PA) and expressed as the number of cells per gram of sputum. Cells were prepared on two glass slides and subsequently stained with Diff Quik (American Scientific Products, McGaw Park, IL). Duplicate differential counts (400 cells/slide) were averaged. The remaining sputum cells were used for measurement of Treg.

Blood collection and processing

Venous blood was withdrawn into vacuum blood collection tubes containing lithium heparin. Blood was diluted 1:1 with McCoy's 5A (Gibco) and layered over Accuprep density gradient medium (Accurate Chemical and Scientific Corp., New York, USA). Layered blood was subjected to density gradient separation (centrifugation at 2000 rpm for 20 minutes at room temperature). Mononuclear cells were removed and washed with McCoy's 5A (centrifugation at 1500 rpm for 10 minutes at 4 ºC). Cells were re-suspended in fluorescence activated cell sorting (FACS) buffer (PBS supplemented with 0.1% sodium azide and 0.5% bovine serum albumin) and subjected to Treg staining protocol.

Staining

Freshly processed sputum cells were divided into 0.6 -1 x 10^6 cells per tube. Cells were washed with FACS buffer (centrifugation at 1500 rpm for 10 minutes at 4 ºC), re-suspended in 80µl of 5% normal mouse block and incubated on ice in the dark for 10 minutes. This was followed by surface staining with the following fluorescent antibodies: Pacific Blue-CD3 (eBioscience, San Diego, CA), PE-Cy7-CD4 (Beckman Coulter) and relevant isotype controls (BD Pharmingen). Cells and antibodies were incubated for 30 minutes on ice in the dark. Cells were then washed with FACS buffer and re-suspended in 1ml Fix/Perm solution (eBioscience,

San Diego, CA) for 30 minutes at 4 ºC in the dark. Cells were washed with Perm buffer (eBioscience, San Diego, CA), re-suspended in 100µl of 5% normal rat block and incubated for 15 minutes at 4 ºC in the dark. This was followed by intracellular staining with FITC-Foxp3 (eBioscience, San Diego, CA), and relevant isotype control, and incubated for 30 minutes at 4 ºC in the dark. Cells were then washed twice with Perm buffer, re-suspended in 500µl of Cytofix (1% paraformaldehyde, BD Biosciences, San Diego, CA) and kept at 4 ºC in the dark until flow cytometric acquisition.

Staining of peripheral blood cells was performed as per the sputum protocol using the following antibodies: surface antibodies – CD3-AmCyan, CD4-Pacific Blue, CD127-FITC and relevant isotype controls; intracellular antibody – FoxP3-APC and relevant isotype control (all from eBioscience, San Diego, CA).

Flow cytometry

Data were acquired with a 15-colour LSRII flow cytometer equipped with three lasers (Becton Dickinson Instrument Systems) using FACS Diva acquisition software (Becton Dickenson BioSciences, Mississauga, ON, Canada). For sputum, as many CD4+ events were collected as possible. For peripheral blood, at least 20,000 CD4+ events were collected. Collected data were analyzed using FlowJo flow cytometric analysis

software (Treestar Inc., Ashland, OR). Our Treg gating strategy is outlined in Figure 1. Briefly, a gate was placed on the CD3+ population. A CD3 vs CD4 dot plot was generated using only cells from the CD3+ gate. Double positive CD3+/CD4+ cells were analyzed on a dot plot of CD4 vs isotype control for Foxp3. A threshold was set at 2% and this was then applied to a dot plot of CD4 vs Foxp3. Cells to the right of the 2% threshold were deemed positive for Foxp3.

Determination of absolute number

It is difficult to obtain the absolute number of Treg in induced sputum, as there are so few T cells to enumerate on sputum slides. Thus, to quantify absolute numbers in sputum, the number of Treg was divided by the total number of cells in the total cell gate on the CD3 vs SSC plot (Figure 2). This percentage was then multiplied by the absolute cell number as determined by hemacytometer count after sputum processing. In the peripheral blood, the number of Tregs was divided by the number of lymphocytes in the lymphocyte gate generated on the FSC vs SSC plot. This percentage was then multiplied by the number of lymphocytes recorded on the complete blood count, with the absolute number of cells expressed as cells/ml of blood.

Statistics

Statistical analysis was done using GraphPad Prism5 software (GraphPad Software Inc., La Jolla, CA). Data with normal distribution were expressed as mean±SEM. Pre-allergen challenge versus post-allergen challenge comparisons at each time point were performed with unpaired t tests. Repeated measures ANOVA was used for comparisons of baseline, 7h and 24h post-allergen challenge time points. If the ANOVA was significant, then subsequent two-way comparisons were performed using the Tukey's multiple comparison tests. $P<0.05$ was considered significant for all analyses.

RESULTS

The mean maximal percent fall in FEV_1 during the EAR was 34.04%±2.46 in isolated early responders and 38.01%±2.24 in dual responders, while the mean maximal percent fall in FEV_1 during the LAR was 8.01%±1.23 in isolated early responders and 23.99%±2.59 in dual responders ($p<0.01$) (Figure 2)(Table 1). Baseline FEV_1 values were not different between the two groups.

There were no differences in the sputum total cell counts between isolated and dual responders at baseline or at 7h and 24h after allergen inhalation (Table 2); however, the absolute number of CD4+ cells in sputum significantly increased in dual responders, from 80.5±27.89 x 10^3 cells to 372.3±115.2 x 10^3 cells 24h after allergen ($p<0.05$), but not isolated early responders (Figure 3a). Also, the percentage of CD4+ cells of CD3+ cells significantly increased from 46.7%±3.88 at baseline to 67.0%±5.90 in dual responders ($p<0.05$), and from 43.7±3.94 % to 58.2±6.29% in isolated early responders ($p<0.05$) 24h after allergen (Figure 3b).

There were no significant differences in CD4+Foxp3+ Tregs in sputum between IER and DR at baseline; however the absolute number of CD4+Foxp3+ Tregs significantly increased 24h after allergen compared to baseline in induced sputum, but not peripheral blood, in dual responders only ($p<0.05$) (Figure 4a, Table 3). When expressed as a percentage of

total CD4+ cells CD4+Foxp3+ Tregs were significantly lower at 24h after allergen in dual responders, being 19.88% ± 3.56 at baseline and 8.10% ± 1.27 after allergen ($p<0.05$)(Figure 4b). The ratio of Tregs:CD4+ cells at 24h after allergen was 0.08 ± 0.01 in dual responders and 0.23±0.04 in isolated early responders ($p<0.05$) (Figure 5).

Allergen challenge significantly increased the percentage of sputum eosinophils and macrophages at 7h and 24h after allergen challenge in both early and dual responders (Table 2). There was no significant difference in the percentage of eosinophils or macrophages between the groups at baseline or 7h and 24h after allergen (Table 2). Neutrophils significantly increased in DR only at 7h and 24h after allergen inhalation (Table 2). The change in the percentage of CD4+Foxp3+Tregs was significantly correlated with the LAR ($r=0.55$, $p=0.008$)(Figure 6).

DISCUSSION

For the first time, this study has demonstrated a decreased percentage of $CD4^{+}Foxp3^{+}$ Treg cells in induced sputum 24 hours after allergen inhalation in dual responders, but not in isolated early responders, in subjects with allergic asthma. In addition, the ratio of CD4+Foxp3+Tregs to total CD4+ cells was significantly lower in dual responders at 24h after allergen when compared to isolated early responders and the change in Treg percentage was correlated to the magnitude of the late asthmatic response.

Other studies have evaluated Treg numbers and function in peripheral blood and/or bronchoalveolar lavage fluid (BALF), but not sputum. Induced sputum has an advantage that the kinetics of change in airway cell numbers can be studied more readily than with BALF. Hartl et al [12] reported reduced CD4+CD25hi T cell numbers and function, as well as reduced Foxp3 mRNA in BALF, but not peripheral blood, from patients with pediatric asthma compared to children with cough, but no asthma. In addition, Thunberg et al [18] described increases in CD4+Foxp3+ cells in BALF with mild asthma patients, 24h after allergen inhalation compared with before allergen inhalation, but not in peripheral blood; however, this study did not compare subjects developing isolated early and dual asthmatic responses after inhaled allergen. Smyth et al [19] reported an increased number of CD4+Foxp3+Treg and CD4+CTLA-4+Tregs in BALF

of asthma subjects, with the highest numbers being observed in moderate to severe asthma. Also, Sjaheim et al [20] described higher numbers of airway Tregs, together with effector T cells, activated CD8+ and activated CD4+ T cells in patients with occupational asthma. Afshar et al [21] demonstrated that allergic asthmatic subjects had an increase in CCR4-expressing Tregs in BALF after segmental allergen challenge. Finally, Moniuszko et al [22] compared the number of circulating CD4+CD25+CD127low cells in peripheral blood, but not the airways, of atopic subjects after allergen inhalation, and compared isolated early responders, dual responders and non-responders. The investigators demonstrated a significant decrease in CD4+CD25+CD127low cells at 6h and 24h after allergen, but this was only consistently seen in non-responders. Thus, the numbers or percentage of Tregs in asthma depends not only on the compartment from which the cells are obtained, but also the phenotype of the subjects studied.

Another interesting approach to examine the ability of human T-regs to attenuate allergen-induced airway responses and inflammation was reported by Martin et al [23]. In this study, PBMCs from allergic or healthy donors were injected into NOD-severe combined immunodeficiency mice. This was followed by airway allergen challenges. The investigators activated Tregs by treatment with the CD4-binding, lck-activating recombinant HIV-1 surface protein, gp120. They concluded that

injection of PBMCs from allergic donors, but not healthy donors, allowed the development of allergen-induced airway inflammation and airway hyperresponsiveness, and that treatment with gp120 prior attenuated both responses. By contrast, gp120 had no effect in mice that received CD25-depleted PBMCs. These studies indicate that human Tregs can attenuate allergen-induced airway responses.

It was because of the differences in the results in studies of asthmatic subjects, that we assessed CD4+Foxp3+Tregs both in induced sputum and peripheral blood, and in allergic asthmatic subjects who developed isolated early or dual responses. We hypothesized those dual responders, who develop more allergen-induced airway inflammation [24;25], may have an attenuated Treg response associated with the enhanced inflammation and prolonged bronchoconstriction. The study demonstrated that both the percentage and absolute number of sputum CD4+ cells increased, while the percentage and absolute number of sputum CD4+Foxp3+Tregs decreased in dual responders at 24h after inhaled allergen. In addition, the ratio of allergen-induced changes in sputum CD4+Foxp3+Tregs to CD4+ cells was significantly lower in dual responders when compared to isolated early responders. This suggests that the balance between effector T-cells and regulatory T-cells may be important in the prolonged airway responses to inhaled allergens. An important caveat, however, is that the results do not identify whether the

reduced ratio in dual responders is a cause or an effect of the airway inflammatory response. The correlation identified between the percentage of sputum Tregs and the magnitude of the allergen-induced late asthmatic response is consistent with both explanations. None of the allergen-induced changes in CD4+ cells or CD4+Foxp3+Tregs were demonstrated in peripheral blood. This emphasizes the importance of examining airway cells to understand the immune responses after inhaled allergen.

There are a number of limitations with the study. Somewhat surprisingly, while allergen inhalation increased the numbers of sputum eosinophils, we did not demonstrate significant differences between isolated early and dual responders. This is different to other studies that we have conducted, using similar methodology, where sputum eosinophils are higher at 24h after allergen in dual responders [24;25]. The definition of a LAR being a 15% fall in FEV_1 between 3-7h after allergen is arbitrary, although widely used. In the current study, five of the 10 isolated early responders had a fall in FEV_1 of between 9-14%, but because of the *a priori* definition used in designing the protocol, they were included in the early responder group. The measurements of Tregs in sputum may not reflect the trafficking of these cells within the airway wall; however, the invasive nature of the methods needed to obtain airway tissue makes it very difficult to follow the temporal events occurring after allergen inhalation. Finally, measuring the absolute number of Tregs in sputum is

challenging because of the low frequency of cells. To do this, we used both data from the flow measurements and the absolute sputum cell numbers measured with a hemocytometer, which may be less precise that the calculations used to quantify other sputum cells.

In summary, these results demonstrated that CD4+Foxp3+Tregs can be measured in induced sputum, and this method can be used to follow the time course of events after allergen inhalation, which is not true for measurements in peripheral blood. Allergen inhalation increases CD4+ cells in dual responders, but the ratio of CD4+Foxp3+Tregs as a percentage of CD4+ cells declines in dual when compared to isolated early responders. The changes in CD4+Foxp3+Tregs correlate with the magnitude of the late response. The results are consistent with the hypothesis that an inability to mobilize sufficient Tregs into the airways in relation to the increased numbers of effector T cells may be responsible for the prolonged allergen-induced airway responses in dual responders.

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TABLE 1 – Subject characteristics

A) Sputum IER

Sex	Age(y)	FEV_1 % Predicted	$MchPC_{20}$ (mg/ml)	Ag Inhaled	EAR (% Fall in FEV_1)	LAR (% Fall in FEV_1)
F	22	82	1.87	Cat	41.5	9.4
F	19	77	0.52	Cat	40.5	4.7
M	50	106	10.08	HDM DP	27.5	10.7
M	19	86	21.6	HDM DP	24.5	9.9
F	22	91	4	Ragweed	38.3	3.3
M	60	103.6	7.78	Cat	24.0	13.7
F	52	86	10.56	HDM DP	35.1	5.4
M	60	75	15.45	HDM DP	36.0	7.0
F	32	91	2.07	Alternaria	46.0	3.0
F	27	78	32	Ragweed	27.0	13.0
Mean±SEM	36.3±5.44	87.5±3.36			34.0±2.46	8.01±1.23

B) Sputum DR

Sex	Age(y)	FEV_1 % Predicted	$MchPC_{20}$ (mg/ml)	Ag Inhaled	EAR (% Fall in FEV_1)	LAR (% Fall in FEV_1)
M	26	102	3.32	Ragweed	46.0	19.0
F	18	98	19.6	Ragweed	31.0	28.0
M	21	101	28.8	Grass	36.8	28.7
F	21	100	10.29	Cat	37.5	15.3
M	50	100	5.62	HDM DP	39.5	20.5
F	25	93	12.44	HDM DP	53.6	15.2
M	19	103	0.97	HDM DP	41.9	16.3
M	18	77	0.8	Cat	27.9	45.9
M	21	110	14.55	HDM DP	38.1	17.9
M	22	75	0.5	HDM DP	44.1	32.4
F	44	99	0.74	Cat	26.7	21.7
M	61	88	8	Cat	33.0	27.0
Mean±SEM	28.8±4.17	95.5±3.04			38.0±2.29	23.9±2.59

C) Peripheral Blood DR

Sex	Age(y)	FEV_1 % Predicted	$MchPC_{20}$ (mg/ml)	Ag Inhaled	EAR (% Fall in FEV_1)	LAR (% Fall in FEV_1)
M	20	94	32	Grass	44.0	22.0
M	19	70	0.11	Cat	28.0	27.0
F	20	107	3.6	Cat	35.0	29.0
F	22	93	0.56	Cat	40.0	17.0
F	24	89	11.62	HDM DP	54.0	25.0
M	41	79	1.18	Grass	40.0	23.0
F	46	89	0.55	Cat	45.0	17.0
F	20	93	0.14	Cat	40.0	18.0
Mean±SEM	26.5±3.78	89.2±3.88			40.8±2.68	22.2±1.63

TABLE 2 – Allergen-induced changes in sputum inflammatory cells

Cell Type	Group	Baseline	7h post allergen	24h post allergen
TCC (10^6/ml)	IER	4.80±1.22	6.97±1.65	7.37±0.71
	DR	4.59±0.75	9.95±2.25	10.3±2.42
	IER vs DR	p=0.88	p=0.31	p=0.29
Eosinophils (%)	IER	1.07±0.31	13.3±3.34**	10.3±1.88*
	DR	1.55±0.64	16.3±4.40*	13.2±3.85*
	IER vs DR	p=0.54	p=0.59	p=0.52
Neutrophils (%)	IER	49.0±9.38	64.1±8.3	67.2±5.54
	DR	36.1±5.38	64.4±6.86**	60.7±5.81*
	IER vs DR	p=0.22	p=0.98	p=0.43
Macrophages (%)	IER	49.8±9.35	22.1±6.55*	23.6±4.09*
	DR	62.3±5.32	19.2±4.65**	25.9±3.96**
	IER vs DR	p=0.23	p=0.71	p=0.68

*P<0.05 vs baseline, **P<0.01 vs baseline. All values expressed as mean±SEM.

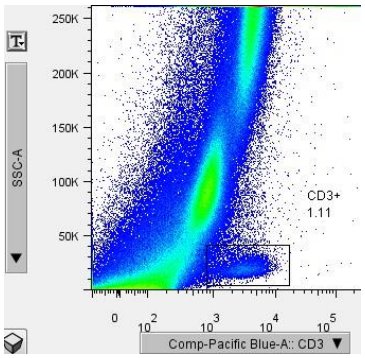
Lymphocytes were not detected, and therefore not enumerated, on the sputum differential slides.

Table 3 – Changes in peripheral blood CD4+ and CD4+Foxp3+ Treg absolute number and percentage after allergen challenge.

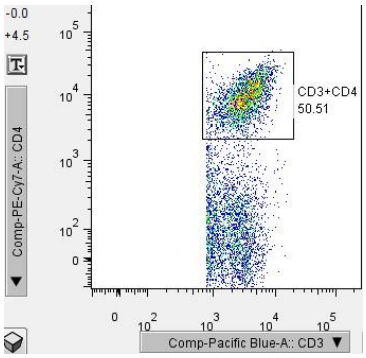
Cell Type	Group	Baseline	7h post allergen	24h post allergen
CD4+ # ($x10^3$/ml)	DR	866.4±182.0	1204±413.2	1201±343.4
CD4+ % (of CD3+)	DR	54.11±3.604	55.41±2.808	52.46±5.150
CD4+Foxp3+ # ($x10^3$/ml)	DR	52.81±21.76	93.36±52.95	86.48±40.22
CD4+Foxp3+ % (of CD4+)	DR	5.005±0.8439	5.700±1.043	5.476±1.040

Figure 1- Flow cytometric CD4+Foxp3+ Treg gating strategy and gating strategy for the determination of absolute number in sputum and peripheral blood.

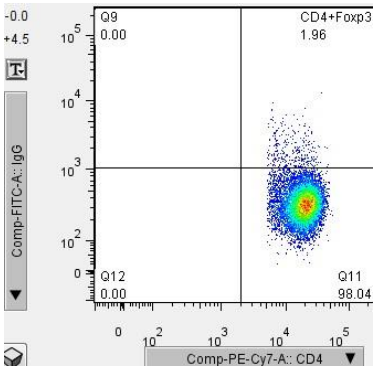
a) CD4+Foxp3+ Treg gating strategy (representative sputum sample)



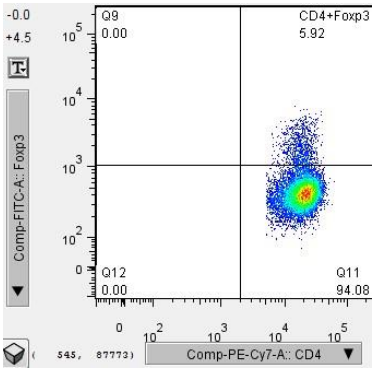
1. CD3+ gate



2. CD3+CD4+ gate



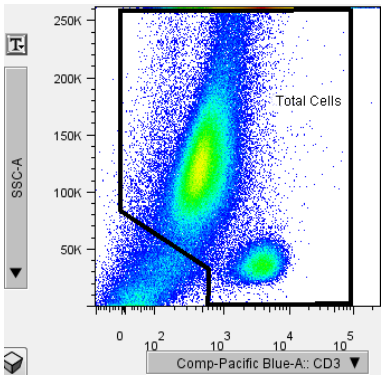
3. CD4 vs Foxp3 Isotype control



4. CD4 vs Foxp3

b) Flow cytometric gating for the determination of absolute number

i) Sputum



ii) Peripheral Blood

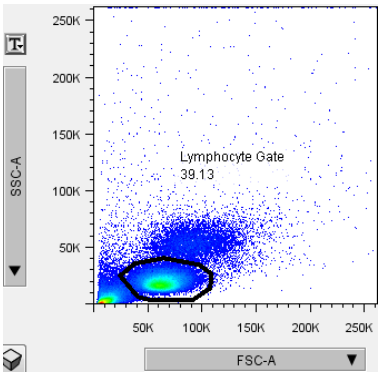


Figure 2 – The allergen-induced early and late asthmatic responses.

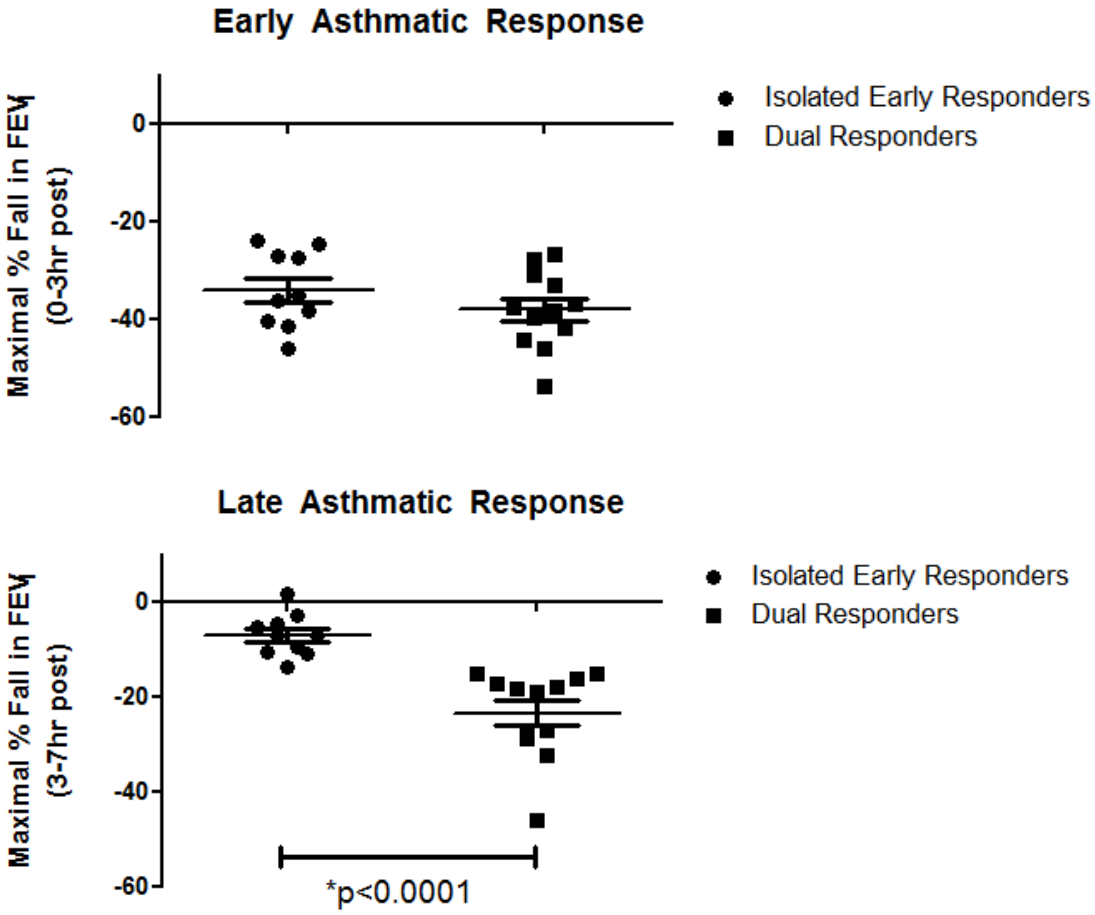
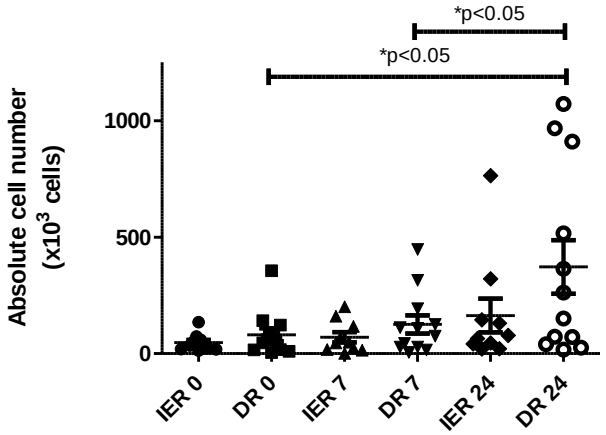


Figure 3 – Changes in CD4+ absolute number and percentage.

a) Sputum absolute number



b) Sputum percentage

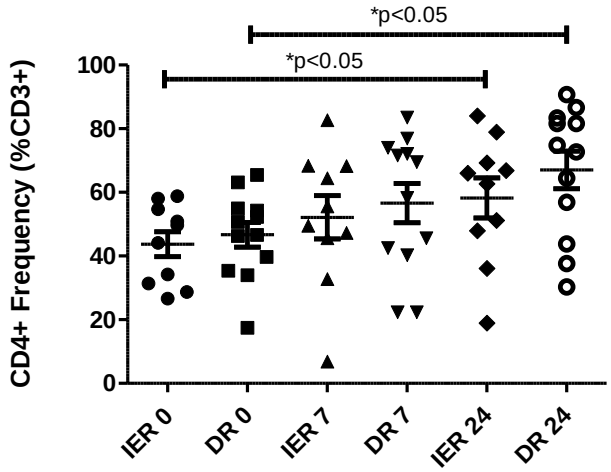
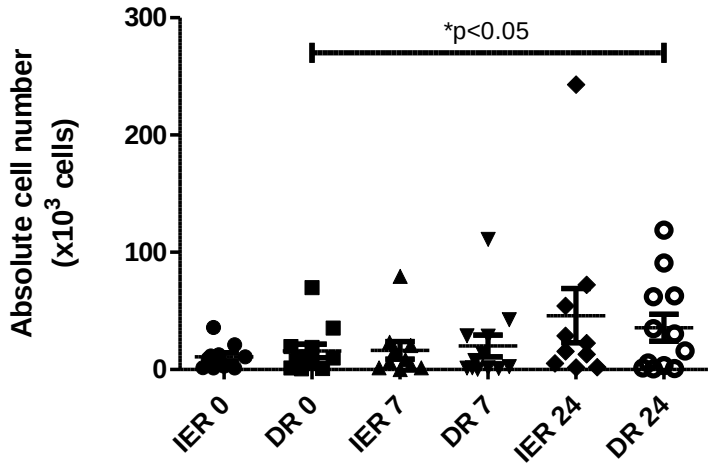


Figure 4 – Changes in CD4+Foxp3+ Treg absolute number and percentage.

a) Sputum absolute number



b) Sputum percentage

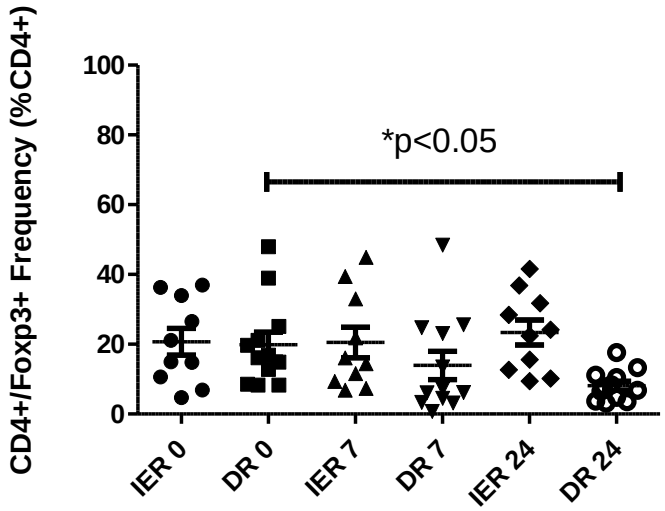


Figure 5 – Ratio of absolute Treg to CD4+ cell number in sputum of IER vs DR.

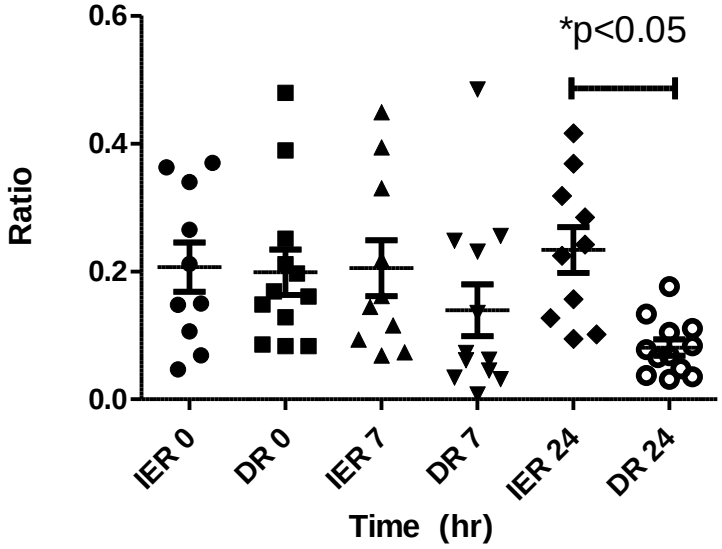
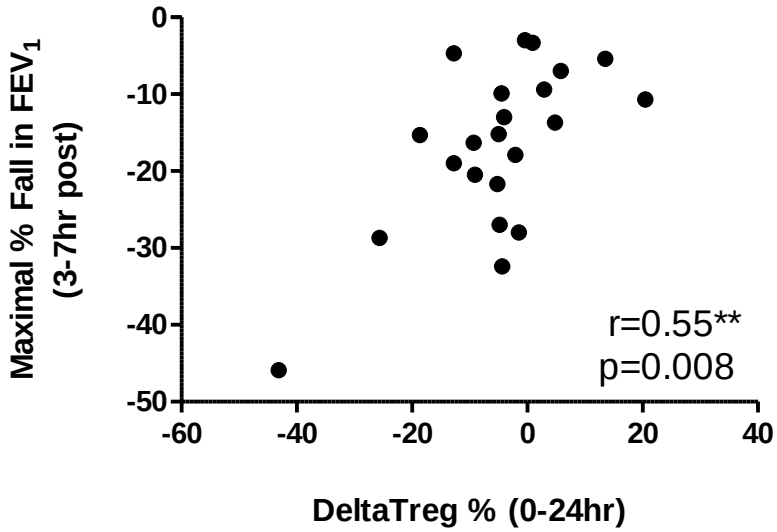


Figure 6 – Correlation of change in sputum CD4+Foxp3+Treg percentage (0-24hr) vs LAR



CHAPTER 4

Title

Treatment with anti-OX40L or anti-TSLP does not alter the frequency of T regulatory cells in allergic asthmatics

Authors

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Statement of Contribution

The clinical studies were developed by Drs. Paul O'Byrne and Gail Gauvreau. I developed the Treg component as an exploratory measurement. Subject recruitment was performed by Rick Watson. I carried out the majority of the experimental work with assistance from Drs. Steven Smith and Benny Dua. I prepared the manuscript in collaboration with Dr. Paul O'Byrne.

Allergy: Brief Communication

Treatment with anti-OX40L or anti-TSLP does not alter the frequency of T regulatory cells in allergic asthmatics

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Capsule Summary: *In vivo* treatment of allergic asthmatic subjects with monoclonal antibodies to OX40L or TSLP does not alter the frequency of circulating Foxp3^{+} regulatory T cells.

Key Words: allergic asthma, flow cytometry, OX40L, T regulatory cell, TSLP

Abstract

OX40-OX40L interactions and TSLP are important in the induction and maintenance of Th2 responses in allergic disease whereas T regulatory cells (Treg) have been shown to suppress pro-inflammatory Th2 responses. Both OX40L and TSLP have been implicated in the negative regulation of Treg. The effect of anti-asthma therapies on Treg is not well known. Our aim was to assess the effects of two monoclonal antibody therapies (anti-OX40L and anti-TSLP) on Treg frequency using a human model of allergic asthma. We hypothesized that the anti-inflammatory effects of these therapies would result in an increase in circulating Treg ($CD4^+CD25^+CD127^{low}Foxp3^+$ cells) frequency. We measured Treg using flow cytometry and our results showed that neither allergen challenge nor monoclonal antibody therapy altered circulating Treg frequency. These data highlight the need for assessment of airway Treg and for a more complete understanding of Treg biology so as to develop pharmacologics/biologics that modulate Treg for asthma therapy.

The majority of naturally occurring T regulatory cells (Treg) develop in the thymus and characteristically express the forkhead box 3 (Foxp3) transcription factor along with CD4, CD25 and CD127 cell surface molecules (1). Treg possess potent immuno-suppressive properties, making them potentially key regulators of inflammatory responses in many diseases, including asthma (2). Evidence suggests that the balance between effector T cells and Treg is important in the initiation, propagation and resolution of T- helper (Th) 2 inflammatory responses in asthma (3). Existing asthma therapies focus predominantly on the inhibition of effector cells. Limited research exists examining the effects of these therapies on Treg and results indicate variable effects on effector T cells versus Treg depending on the therapy administered (4,5).

Research into novel asthma therapies has recently focused on the development of biologics targeted at specific inflammatory molecules and cytokines (6). Two such targets, OX40 ligand (OX40L) and thymic stromal lymphopoietin (TSLP) have been shown to play a role in allergic responses. OX40/OX40L interactions lead to the induction and maintenance of Th2 inflammation by means of TSLP-activated dendritic cells (7-9). The *in vivo* blockade of OX40L has been shown to inhibit atopic inflammation mediated by TSLP (10).

While the role of OX40L and TSLP in the allergic response has been well characterized, little information exists regarding their effects on

Treg. It has been shown that the OX40/OX40L interaction leads to the expansion of effector T cells while inhibiting Treg function (11). Studies examining the effects of TSLP on Treg provided evidence that TSLP inhibited the generation of Treg and also directly impaired Treg function (12,13). This suggests that OX40L and TSLP are not only potent contributors to the allergic inflammatory response, but they may also exert significant negative effects on Treg number and function.

We have previously reported on the effects of anti-OX40L and anti-TSLP monoclonal antibody therapy on allergen-induced airway responses using an inhaled allergen challenge model of human allergic asthma (14,15). In these same studies, we evaluated the effect of anti-OX40L and anti-TSLP monoclonal antibody therapy on the frequency of circulating Treg. We hypothesized that blockade of OX40L or TSLP would result in an increase in PB Treg frequency.

The studies were carried out as two separate randomized, double-blind, placebo-controlled, parallel group clinical trials (ClinicalTrials.gov numbers NCT00983658 and NCT01405963). Studies were approved by the Hamilton Integrated Research Ethics Board and written informed consent was obtained from all study participants. The study design for the anti-OX40L clinical trial has been described by Gauvreau *et al* (14). Briefly, 28 subjects with mild, allergic asthma were recruited and received four doses of treatment (drug or placebo) over three months. Allergen

challenges were performed at baseline and 56 and 113 days after the first treatment dose. Peripheral blood (PB) was drawn at baseline and on days 55, 57, 112 and 114 for assessment of Treg. The study design for the anti-TSLP clinical trial has been described by Gauvreau *et al* (15). Briefly, 31 subjects were recruited and received three doses of treatment (drug or placebo) over two months. Allergen challenges were performed at baseline and 42 and 84 days after the first treatment dose. PB was drawn at baseline and on days 41, 43, 83, and 85 for assessment of Treg.

Venous blood was collected, diluted and layered over Lymphoprep density gradient medium (STEMCELL Technologies Inc., Vancouver, British Columbia, Canada). Peripheral blood mononuclear cells (PBMC) were isolated via density gradient centrifugation. Freshly processed PBMC underwent primary staining for surface markers using the following fluorescently labelled antibodies: CD3-AmCyan (Clone SK7; BD Biosciences), CD4-eFluor®450 (Clone OKT4; eBioscience, San Diego, California, USA), CD25-PE (Clone M-A251; BD Biosciences), CD127-FITC (Clone eBioRDR5; eBioscience), and relevant isotype controls. Secondary staining for intracellular markers was done using Foxp3-APC (Clone 236A/E7; eBioscience) and its relevant isotype control. Data were acquired on a 15 colour LSRII flow cytometer (BD Biosciences, Mississauga, Ontario, Canada). Treg were identified as $CD4^{+}CD25^{+}CD127^{low}Foxp3^{+}$ cells (gating strategy outlined in Fig. 1A).

These surface markers were used as they are the most comprehensive and would detect the majority of human Treg. The addition of $CD127^{low}$ to the standard $CD4^+CD25^+$ markers helps to discriminate Treg from activated T cells that can transiently express Foxp3. The frequency of Treg was expressed as a percentage of the $CD4^+$ population.

Comparisons were made between pre-treatment and post-treatment values. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, California, USA). Data were analyzed using a two-way repeated measures ANOVA. Data were expressed as mean + SEM.

The clinical outcomes of the two trials were different. Treatment with anti-OX40L MAb did not attenuate the early- or late-phase asthmatic responses at days 56 or 113 compared with placebo (14). By contrast, treatment with anti-TSLP MAb reduced the maximum percent fall in FEV_1 during the late response by 34% compared to the placebo group on day 42 ($p=0.09$) and by 45.9% on day 84 ($p=0.02$)(15). Treg frequency ranged between 5%-11% of PB $CD4^+$ cells in these allergic asthmatic subjects. PB $CD4^+CD25^+CD127^{low}Foxp3^+$ Treg frequency did not change after allergen challenge in either study and there was no effect of treatment with either anti-OX40L or anti-TSLP on Treg frequency (Fig. 1B, 1C).
The results of these two studies differed in that treatment with anti-OX40L had no effect on the late asthmatic response or on PB Treg frequency,

whereas treatment with anti-TSLP AMG 157 attenuated allergen-induced early and late asthmatic responses, but also showed no effect on PB Treg frequency. Taken together, these studies show that neither an inflammatory stimulus, such as inhaled allergen, nor a pharmacological intervention, such as anti-TSLP, which demonstrated efficacy on allergen-induced responses, induces changes in circulating Treg frequency. This suggests that PB is not an appropriate compartment to study changes in Treg frequency in asthma. As reported by Kinoshita *et al.* (16), measuring Treg in a more relevant compartment (the airway) may have yielded different results. It is likely that in airways disease, Treg in the relevant compartment (ie. the lung) may be more sensitive to change.

The control of the development, survival, and function of Treg is crucial for the effective regulation of immune responses. Treg have the ability to suppress the activation, proliferation and effector function of other lymphocytes in physiological and pathological immune responses. However, it remains uncertain what ultimately determines the efficacy of Treg, the absolute number of each cell subtype, their frequency compared with other cell subtypes, the location of the cells, their functional capacity or some combination thereof. Thus, a complete understanding of the biological functions of Treg *in vivo* should facilitate the development of pharmacologic and biologic agents that can be used to modulate Treg number/function in a therapeutic setting (17). Additionally, Treg as stand-

alone or adjunct cell-based therapy may provide an alternative avenue in the treatment of airway diseases such as asthma (18).

Author contributions

The studies were designed by GM Gauvreau and PM O'Byrne. The experimental work was performed by AJ Baatjes, SG Smith, and B Dua. Analyses were performed by AJ Baatjes. The paper was written by AJ Baatjes and PM O'Byrne. All authors reviewed and approved the manuscript before submission.

Conflicts of interest

The authors declare they have no conflicts of interest.

Funding

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Table I – Subject characteristics summarized from two separate clinical trials (14,15). Data expressed as mean (SD) or mean ± SEM. Methacholine PC_{20} (the provocative concentration causing a 20% fall in FEV_1) expressed as the geometric mean with 95% CI.

	Anti-OX40L MAb Clinical Trial		**Anti-TSLP MAb Clinical Trial**	
	Placebo (n=14)	**Drug (n=14)**	**Placebo (n=15)**	**Drug (n=16)**
Age, years	33.9 (12.0)	33.4 (13.3)	31.5 ± 2.9	30.8 ± 2.7
Sex (M/F)	7/7	8/6	4/11	6/10
FEV_1, % predicted	84.9 (14.7)	91.7 (11.4)	97.6 ± 3.9	95.4 ± 3.3
Methacholine PC_{20}, mg/ml	0.79 (0.05-13.5)	1.62 (0.3-11.6)	1.87 (0.97-3.61)	1.31 (0.48-3.64)

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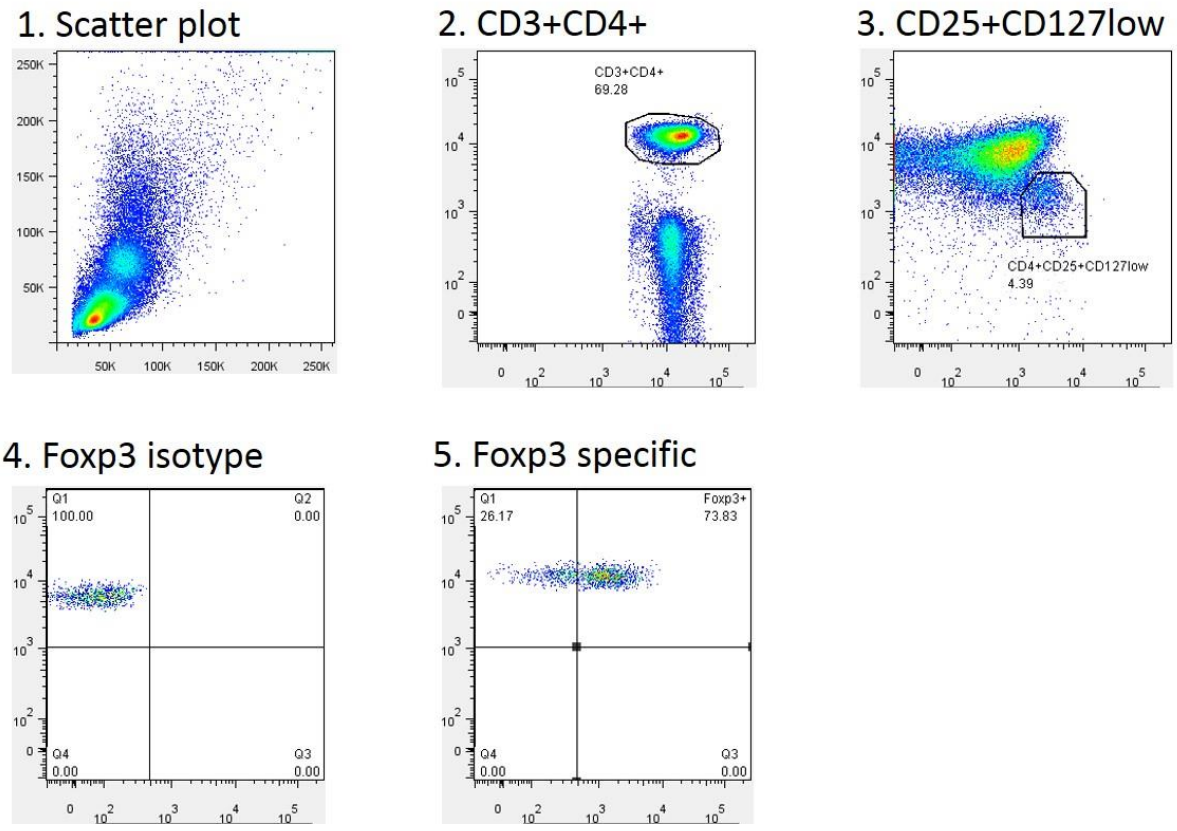
Figure 1 A. Flow cytometric Treg gating strategy using a representative peripheral blood sample. 1. PBMC forward scatter (FSC) vs side scatter (SSC); 2. T cell $CD3^{+}CD4^{+}$ gate; 3. CD25 vs CD127; 4. CD4 vs isotype control; 5. CD4 vs Foxp3. **B.** No changes in PB Treg frequency after anti-OX40L MAb treatment. Data expressed as mean ± SEM. **C.** No changes in PB Treg frequency after anti-TSLP MAb treatment. Data expressed as mean ± SEM.

A



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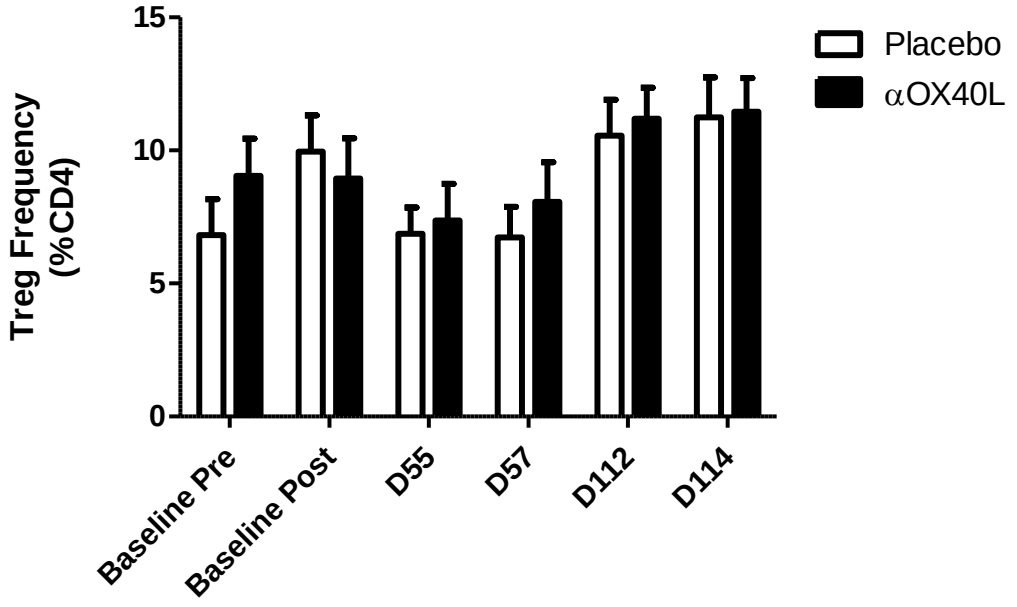
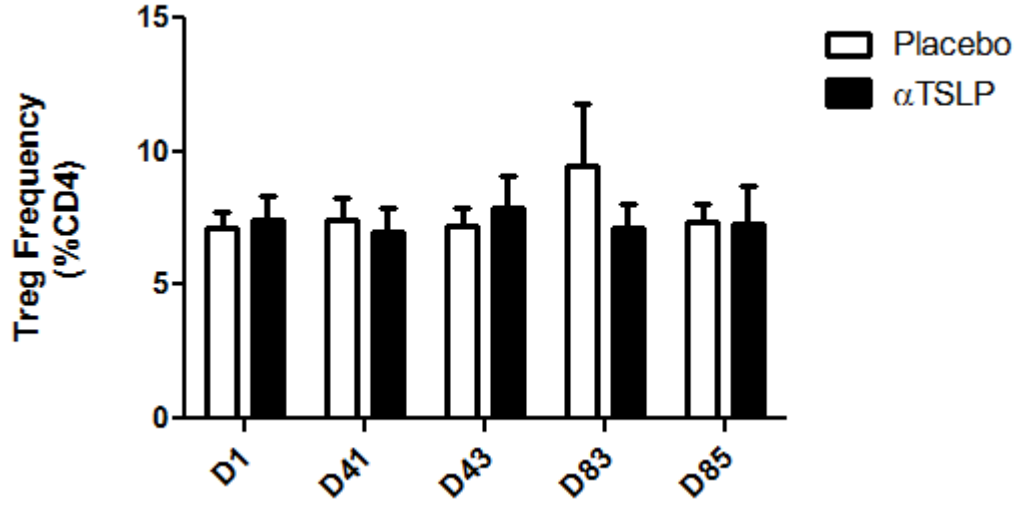

Treg Frequency (%CD4)
15
10
5
0
Placebo
αOX40L
Baseline Pre
Baseline Post
D55
D57
D112
D114

C


Treg Frequency (%CD4)
15
10
5
0
Placebo
αTSLP
D1
D41
D43
D83
D85

Chapter 5 – Discussion

T regulatory cells (Treg) play an essential role in the regulation of immune responses in health and disease, orchestrating the balance between immunity and tolerance. Treg are a heterogeneous population, divided into a number of different subsets displaying unique functional and homeostatic properties that act to suppress the activation, proliferation and effector function of a number of different cell types. The balance between Treg and effector cells determines the outcome of an immune response; thus, the induction, recruitment, maintenance, and function of Treg is pivotal for effective control of these responses (Campbell & Koch 2011). Impaired Treg homeostasis has been implicated in the development of several autoimmune and inflammatory diseases including arthritis and cancer (Mougiakakos et al. 2010; Valencia & Lipsky 2007; Zhang et al. 2014b; Gol-Ara et al. 2012), and more relevant to this thesis, allergy and asthma (Lloyd & Hawrylowicz 2009; Robinson 2009; Larché 2007). A more complete understanding of Treg biology will allow for the development of pharmacological and/or cell-based therapies aimed at the manipulation of Treg for the treatment of various diseases. As such, the overall aim of this thesis was to more thoroughly elucidate the role of Treg in human allergic asthma.

The first objective in this thesis was to examine the biology of three common Treg subsets. In Chapter 2, we performed an observational study comparing three different peripheral blood Treg phenotypes and showed both quantitative and functional heterogeneity in a healthy, non-asthmatic population. We selected our phenotypes based on early work in the field of Treg biology that established both murine and human Treg as being $CD4^+CD25^+$ (Sakaguchi et al. 1995; Baecher-Allan et al. 2001). Subsequent to this, Foxp3 was found to be a nuclear transcription factor important in the development and function of Treg (Fontenot et al. 2003) and was utilized as a specific marker for human Treg (Roncador et al. 2005). The $CD4^+CD25^+Foxp3^+$ Treg phenotype (Treg1) was thought to include $CD25^+$ effector T cells that transiently express Foxp3. As such, we made use of CD127 as a marker to exclude these cells (Seddiki et al. 2006) and provide a more pure $CD4^+CD25^+CD127^{low}Foxp3^+$ Treg population (Treg2). Lastly, we sought to compare Treg1 and Treg2 with the $CD4^+CD25^{high}Foxp3^+$ Treg phenotype (Treg3) as these cells were determined to have the greatest Treg suppressive capacity (Baecher-Allan et al. 2001; d'Hennezel et al. 2011). In comparisons made between all three Treg phenotypes, we demonstrated that the frequency of circulating $CD4^+CD25^{high}Foxp3^+$ Treg (Treg3) was significantly lower than $CD4^+CD25^+Foxp3^+$ Treg (Treg1) and $CD4^+CD25^+CD127^{low}Foxp3^+$ Treg (Treg2). Subsequent functional analysis showed that Treg3 and Treg2

possessed the greatest suppressive capacity, significantly higher than that of Treg1.

Different Treg phenotypes therefore give rise to variability in Treg frequency that is dependent on the phenotype studied. We subsequently examined this in a comparison of healthy, non-asthmatic subjects and those with asthma, where we showed that asthmatics exhibited a significantly lower frequency of peripheral Treg as compared to healthy controls. These data are corroborated with evidence from other diseases, including HIV, cancer, and arthritis (Del Pozo-Balado et al. 2010; Zhuo et al. 2015; Sempere-Ortells et al. 2009). Hence, heterogeneic Treg phenotypes can be found in both healthy and diseased populations, where they exhibit differential frequencies. This is of significance as the determination of Treg phenotype, as well as relative number, in disease will be crucial in assessing Treg effectiveness if they are to be used as a cell-based therapy.

With the discovery of markers that serve to further define Treg sub-populations (Schmetterer et al. 2012), the naming conventions used to define Treg phenotypes are have become increasingly complex. Investigators need to take into consideration several concepts when defining their Treg phenotypes: 1) the location the Treg are derived from, 2) the suppressive capacity of the Treg, and 3) the Treg cytokine profile. For example, the term “natural” Treg was used to define those cells

derived from the thymus – however, this term implies that other Treg are therefore “unnatural”. Treg derived from the thymus could be denoted as tTreg and those peripherally-derived as pTreg. Secondly, the hallmark of Treg is that they are suppressive and, as such, the assessment of Treg suppressive capacity, in studies examining the role of Treg in disease, is essential. Lastly, Treg could be further defined based on the cytokine profile of excreted cytokines such as IL-10 or transforming growth factor (TGF)-β. With the increased interest in utilizing Treg as a therapeutic option for patients, the field is in need of simplification and a consensus on Treg nomenclature so as to ensure accurate and relevant conclusions are drawn (Abbas et al. 2013).

We also emphasized the importance of both Treg number and Treg function in the potential of Treg to be used therapeutically. This concept is shown in the suppression assay we used. The CFSE-based suppression assay is a widely accepted means for evaluating Treg functional capacity (Venken et al. 2007). The assay measures the proliferative capacity of CFSE-labelled responder T cells in the presence of Treg at varying ratios. We observed the greatest Treg suppressive capacity with the highest Treg:Tresp ratio (1:1) and with the Treg phenotype exhibiting the highest frequency of $Foxp3^{+}$ cells. These data highlight the importance of Treg

phenotype, Treg number, and Treg function when evaluating Treg to be used as a potential therapeutic target.

The caveat to the findings in this study is that these three phenotypes are not distinct subsets unto themselves. Treg2 and Treg3 are both derived from Treg1. Treg3 is also a subset of Treg2. So while not separate and unique from one another, they do possess different suppressive capacities. We reasoned that the differences were due to either the expression of Foxp3 and/or the presence of contaminating effector cells. Treg2 and Treg3 displayed the highest frequency of cells expressing Foxp3. The more $Foxp3^+$ cells within a population of Treg, the more suppressive the population. Some non-regulatory, $CD4^+$ cells with non-suppressive activity can transiently express Foxp3 upon activation (Wang et al. 2007). As such, the presence of these cells artificially inflates the frequency of Treg cells while also potentially decreasing the overall functional capacity of the Treg population studied.

There is also evidence to suggest that the level of Treg Foxp3 expression is associated with functional capacity. It has been demonstrated that Foxp3 is an important quantitative regulator of Treg function in that the functional capacity of Treg is dependent on the amount of Foxp3 expressed (Allan et al. 2008). When the $Foxp3^+$ population is further divided based on the level of Foxp3 expressed, it gives rise to functionally heterogeneous sub-populations (Miyara, Yoshioka, et al.

2009). The most effective control of immune responses in disease may rely on the manipulation of Treg that differentially express Foxp3 – the $Foxp3^+$ sub-populations. As such, the determination of the level of Foxp3 expression is likely an important consideration when evaluating Treg for therapy.

The results from the study in Chapter 2 demonstrated that differences in Treg frequency may be dependent on both the phenotype as well as the compartment from where the cells were obtained. The compartmental differences in assessing BAL versus PB Treg highlight the need to study the relevant compartment. It is important to sample secretions from the affected organ, in this instance the BAL from the lung may better reflect the current state of the disease than peripheral blood. Smyth *et al.* (Smyth et al. 2010) assessed the frequency of airway (BAL) Treg using three different phenotypes. In support of our data, they were unable to detect any differences in Treg frequency, regardless of the phenotype studied, when comparing mild asthmatic subjects to healthy controls. However, an increase in airway Treg frequency was observed when comparing moderate/severe asthmatics to healthy controls. This would suggest that airway Treg levels may be an indicator of disease severity but this has yet to be established in allergic asthma.

One of the limitations of this study was the inability to make use of the Foxp3 marker in the suppression assay. Due to the fact that Foxp3 is a nuclear protein, it has limited value for use in the isolation of Treg. In addition, the functional assessment of Treg has also been hampered by the lack of suitable cell surface markers that can distinguish activated effector from regulatory T cells as well as being representative of the $Foxp3^+$ population. We, and others (Yu et al. 2012; Banham 2006), demonstrated that Treg2 ($CD4^+CD25^+CD127^{low}$ phenotype) may be a suitable surrogate to the $CD4^+CD25^+Foxp3^+$ phenotype. The Treg2 population not only comprehensively encompasses most of the $Foxp3^+$ cells within the $CD4^+$ population, with the majority of cells expressing Foxp3, but also exhibits a high suppressive capacity. In fact, it has been shown that CD127 expression is inversely correlated with both Foxp3 expression and suppressive capacity (Liu et al. 2006). As such we were able to isolate viable Treg that were representative of $Foxp3^+$ Treg.

A second limitation was that we assessed the frequency of the total $Foxp3^+$ Treg population without distinguishing nTreg versus iTreg. Both of these populations express Foxp3 except nTreg are derived from the thymus and iTreg are generated in the periphery. The field has progressed where markers such as Helios and Neuropilin-1 have been proposed as a means of differentiating the two (Thornton et al. 2010; Yadav et al. 2013). Using a different approach to isolating Treg, Huang *et*

al. examined the relative contribution of nTreg versus iTreg to the induction of tolerance upon exposure to an environmental allergen (Huang et al. 2013). It was shown that while both iTreg and nTreg significant suppress allergen-induced Th2 asthmatic responses, iTreg are more potent. Also, only iTreg were able to completely normalize airway hyper-responsiveness. The results of this murine study is of particular interest to our extrapolations of the role of Treg in allergic asthma. While we may be over-estimating the frequency of nTreg, we argue that the assessment of total Foxp3 Treg is more important than differentiating where the Foxp3 Treg were derived from.

Finally, we were unable to perform functional assays comparing Treg from asthmatics and healthy control subjects due to limitations on the allowable volume of blood to be drawn from each subject and cell recovery from BAL. We acknowledge that the determination of Treg functional capacity, in concert with Treg frequency, would have been most beneficial to the study. In future studies we would look to the possibility of *ex vivo* expansion of PB or BAL-derived Treg to allow for the assessment of functional capacity.

Our second objective in this thesis was to assess Treg frequency in a population of mild asthmatic subjects after allergen challenge. We demonstrated a decrease in the frequency of airway, but not peripheral,

Treg after allergen challenge in dual responder asthmatics only. In addition, we showed that the ratio of Treg to total $CD4^+$ cells was lower in dual responder asthmatics compared to isolated early responders with the change in Treg frequency correlating to the magnitude of the LAR.

Regulatory cells play an important role in regulating immune responses in the periphery. The respiratory tract is constantly exposed to inhaled allergens resulting in aberrant, Th2-skewed inflammatory immune responses to the allergen in susceptible atopic individuals. This gives rise to the characteristic features of allergic asthma, inflammation and AHR.

While a number of cells play a role in the allergic asthmatic response, it is becoming increasingly apparent that the effectiveness of Treg in regulating immune responses relies upon the balance between Treg and effector T cells. Utilizing an established human model of allergic asthma, we demonstrated that while airway Treg numbers do increase after allergen challenge, this increase is insufficient to suppress the late asthmatic response. Our results suggested that a robust allergen-induced Th2 response overwhelms the regulatory response of the Treg. In support of our data, measurement of Treg frequency after *in vitro* allergen challenge yielded an insufficient Treg response in the presence of a pronounced Th2 response (Wang et al. 2009). In assessments of BAL after bronchial allergen challenge, it was found that allergen provocation results in an increase in $Foxp3^+$ Treg that is also associated with a

concomitant increase in Th2 cytokines. These pro-inflammatory cytokines likely negated the Treg suppressive effects and hence the inability to inhibit the allergen-induced Th2 responses (Thunberg et al. 2010). This Treg insufficiency lies not only with numerical but in functional deficiencies as well. It has been shown that inflammatory cytokines have the ability to down-regulate Foxp3 expression and abrogate Treg suppressive function (Lin et al. 2008). Compromised Treg function in conjunction with an imbalance in the ratio of Treg to effector cells may be responsible, in part, for the inability to attenuate the Th2 inflammatory response in allergic asthma.

These data lend credence to the hypothesis that an enhancement in Treg (number and/or function) may lead to the amelioration of the inflammatory T cell infiltrate, reduced inflammation and abrogation of the late asthmatic response. Of particular importance is the fact that the regulatory to effector T cell balance in the control of immune responses in both health and disease may be the most convincing argument in the consideration of Treg as a therapeutic target.

In our assessment of the Treg response after inhaled allergens, we made comparisons of subjects who develop an isolated early asthmatic response (IER), with little or no associated acute inflammatory response, with subjects who develop a dual asthmatic response (DR), with a marked

eosinophilic and basophilic airway response. It has been well established that the trafficking of a T cell infiltrate into the airway is responsible for the asthmatic inflammatory response (Afshar et al. 2008). Our data support the fact that T cell trafficking into the airway occurs in DR asthmatics, but not IER, as evidenced by an increase in sputum CD4 T cell number, both absolute and relative, after allergen challenge. Interestingly, absolute Treg numbers also increased post-challenge in DR; however, IER were lacking of change in either their CD4 or Treg numbers.

We hypothesized that an attenuated Treg response may be associated with enhanced inflammation and, subsequently, the development of the late asthmatic response in dual responders. Thus, IER would have a marked increase in Treg numbers post-allergen, offering a suppressive effect and protecting against the development of the late asthmatic response, while there would be a less robust increase in Treg post-allergen challenge in DR thereby contributing to the development of the late asthmatic response. Surprisingly, there was no increase in airway Treg in IER after allergen challenge. By contrast, there was a significant decrease in airway Treg in DR and a significantly lower ratio of $CD4^+$ to Treg 24h after allergen challenge. These results allow us to propose that an imbalance in airway Treg/effector cell numbers is associated with allergen-induced airway inflammation and the development of a late

response, while maintaining this balance may limit the magnitude of the late response by suppressing inflammation.

We are cognizant of the fact that these conclusions are based on our observation of changes in Treg number only whereas if we had measured Treg function, insight into a more causal role of Treg may have been elucidated. It has been proposed that Treg play a role in the development and control of allergic disease (Venuprasad et al. 2010; Umetsu 2003; Palomares et al. 2014). There are number of animal studies that aim to establish a causal role for Treg in allergic disease but the most convincing evidence may be human patients with IPEX syndrome. This rare disease is caused by mutations in the Foxp3 gene resulting in Treg deficiency and dysfunction and causes, in addition to many autoimmune disorders, allergy (d'Hennezel et al. 2012). Targeting Treg for therapy therefore may not only aid existing therapies in controlling Th2 inflammation and curb symptoms but may also be curative, changing the natural course of allergic disease.

Our results from Chapter 3 also highlight compartmental differences when assessing Treg. Using the clinical model of allergen-induced airway responses, we were able to detect changes in sputum, but not peripheral blood, Treg. As noted earlier, this finding reinforces the importance of studying the relevant compartment, the lung, when trying to assess Treg in asthma. Sputum is used as a reliable measure of inflammation in asthma

and has been compared to BAL and blood (Pizzichini et al. 1998). The evaluation of Treg in the lung can be performed by sampling sputum, BAL or bronchial biopsy tissue. Each is representative of a different compartment of inflammation. Sputum is advantageous in that it provides a higher cell density of recovery, and may be a better medium (more pronounced signal) for the detection of some cytokines. In addition, obtaining induced sputum is a non-invasive procedure as compared to the more invasive procedure needed for the procurement of BAL or bronchial biopsies. As a consequence of this, sputum can be sampled multiple times within a short time period.

There were several limitations to this study. First, we were unable to assess the functional capacity of the Treg measured due to limitations on the allowable volume of blood to be drawn from each subject, as well as the small number of cells recovered after sputum processing. As such, we could only conclude that a Treg insufficiency existed as a consequence of decreased Treg frequency after allergen challenge. However, it would have been beneficial to assess whether Treg function was also compromised, as it has been reported that there is an impairment of Treg function in asthmatics with poorly controlled asthma , as well as those whose disease went into remission (Hartl et al. 2007; Boulet et al.). This is significant in that Treg function is not fully restored after disease

remission, thereby increasing the likelihood of its recurrence. This compromised Treg function could be attributable to a pro-inflammatory environment, but the exact mechanism still needs to be elucidated in order to develop therapeutic strategies that restore and promote Treg function.

Secondly, we measured 'natural' Treg where determination of the relative balance of allergen-specific Treg to Teff may have been more relevant in the context of an allergen-challenge model. Work by Akdis *et al*. (Akdis et al. 2004), elegantly shows that the control of the Th2 immune response to allergens in humans is mediated by IL-10-secreting regulatory cells. Interestingly, both healthy and allergic individuals were shown to possess allergen-specific regulatory and effector T cells. However, it is the relative balance of these two – the ratio of allergen-specific regulatory to effector Tcells – that may determine a healthy immune response from an allergic response.

The third objective in the thesis examined the effect of novel anti-asthma therapies on peripheral Treg. We showed, using a human model of allergic asthma, that neither treatment with anti-OX40L nor anti-TSLP altered circulating Treg frequency. Both of these studies made use of monoclonal antibodies specific for TSLP and OX40L, and as such any potential effects on Treg would have been indirect. The anti-TSLP study demonstrated attenuation of all of the allergen-induced airway responses, as well as the baseline markers of airway inflammation in these mild,

stable asthmatic subjects. By contrast, treatment with anti-OX40L had no effect on any of the baseline inflammatory parameters, or the allergen-induced responses (Gauvreau et al. 2014; Gauvreau et al. 2014). Neither study demonstrated any effect on peripheral blood Treg. These results again emphasize that peripheral blood may not be the compartment of choice to study Treg in allergic asthma.

While these novel asthma therapies had no effect on Treg frequency, established therapies, namely glucocorticoids, have been shown to have significant beneficial effects on Treg number and function. In patients receiving glucocorticoid therapy, higher levels of PB $CD4^+CD25^+$ Treg and Foxp3 mRNA were reported as compared to un-treated asthmatics (Karagiannidis et al. 2004). Thus, glucocorticoids not only exhibit immuno-suppressive and anti-inflammatory effects, but also promote Treg development. Also, asthmatic children have a higher frequency of circulating Treg, compared to non-asthmatic children, that was attributed to inhaled corticosteroid use (Singh et al. 2013). With respect to function, it was shown that fluticasone propionate augments Treg suppression of Teff in an IL-10 dependent manner (Dao Nguyen & Robinson 2004). The efficacy of corticosteroids in asthma may depend in part, on the suppressive function of Treg. With current anti-asthma therapies focussed on the inhibition of the effector Th2 response, it may be relevant in the future to focus on manipulating Treg as a stand-alone or

adjunct therapy with the aim of providing safer, more effective, specific, and longer lasting treatment.

As with the other studies, the limitations to this study included the inability to assess airway Treg due to the low number of recovered cells in sputum. In addition, we were unable to evaluate Treg functional capacity due to restrictions on the allowable volume of blood to be drawn from study participants.

Taken together, the findings of this thesis enhance our understanding of Treg biology and their role in allergic asthma. The observations do not establish a causal role for Treg in the development of allergic asthma. However, the findings do support the hypothesis that a Treg imbalance is associated with the development of allergen-induced responses.

5.1 Future Direction

Regulatory immune mechanisms exist to control allergic inflammatory responses; however, it remains to be elucidated whether these can be exploited to control allergen-induced airway disease. With respect to Treg, there are two major mechanisms by which this could occur, either through the delivery of *ex vivo* expanded Treg as a cell based therapy, or via pharmacologic/biologic manipulation of Treg *in vivo* so as to enhance Treg number and/or function (Miyara, Wing, et al. 2009; De

Serres et al. 2011; Singer et al. 2014). There are some pertinent issues utilizing Treg as a cell-based therapy that need to be addressed (Xystrakis et al. 2007). First, due to the low numbers of Treg, expansion methodologies need to be used. While the expansion of Treg is feasible, the concern lies with the potential of concomitant expansion of effector cells with pathogenic effects. Secondly, non-allergen specific Treg have a wide range of specificities so bystander suppression may result in tumour growth or chronic infection. Thirdly, there is the issue of whether or not Treg homing to the sites of inflammation will occur. Lastly, tissue Treg half-life needs clarification as they need to be around long enough to resolve inflammation, but not remain indefinitely.

In order to best utilize Treg as therapy in disease, it is imperative that we understand how these subsets function in a coordinated manner so as to ensure appropriate immune responses are mounted when needed without the development of inflammatory disease. Relevant to our field of study, important issues moving forward include identifying the significance of different Treg subsets in the control of the allergic asthmatic response, identifying factors that control the differentiation of Treg subsets in the airways, and determining how cytokines and other factors in the immune microenvironment of the airways influence Treg during the initiation, progression and resolution of the allergic asthmatic response.

5.2 Summary

Treg are a diverse T cell sub-population, composed of several phenotypically and functionally distinct subsets, which function to control the development and resolution of immune responses. We hypothesized that an imbalance in Treg versus Teff contributes to the development of allergen-induced asthmatic responses. In support of this, our findings not only highlight the insufficiency of the Treg response in allergic asthma, but also emphasize the importance of Treg phenotype with respect to number and function, and the potential contribution of Treg to the efficacy of asthma therapy. As such, we support further investigation into Treg as a viable therapeutic target for the treatment of allergic asthma.

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