
# INVESTIGATING THE IMMUNOBIOLOGY OF IgE+ B CELLS AND REGULATORY B CELLS IN ALLERGIC ASTHMA

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# B CELL RESPONSES IN ALLERGIC ASTHMA

# INVESTIGATING THE IMMUNOBIOLOGY OF IgE+ B CELLS AND REGULATORY B CELLS IN ALLERGIC ASTHMA

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**ABSTRACT**

Global prevalence of allergic diseases has been on the rise for the last 30 years. In Canada, this upward trend in allergic diseases has resulted in over 3 million Canadians being affected by allergic asthma. Allergic asthma is triggered by inhalation of environmental allergens resulting in bronchial constriction and inflammation, which leads to clinical symptoms such as wheezing, coughing and difficulty breathing. Asthmatic airway inflammation is initiated by the release of inflammatory mediators (-eg- histamine) released by granulocytic cells (-eg- mast cells and basophils). However, immunoglobulin E (IgE) antibody is also necessary for the initiation of the allergic cascade, and IgE is produced and released exclusively by memory B cells and plasma cells. Allergen crosslinking of IgE:FcεRI complexes on the surface of mast cells and basophils causes degranulation of pro-inflammatory mediators. Acute allergen exposure has also been shown to increase IgE levels in the airways of patients diagnosed with allergic asthma; however, more studies are needed to better understand local airway inflammation. Our group's work, in accordance with the literature, has shown an increase of IgE in the airways of subjects with mild allergic asthma following allergen inhalation challenge. Although regulatory B cells ($B_{regs}$) have been shown to modulate IgE-mediated inflammatory processes in allergic asthma pathogenesis, particularly in mouse models of allergic airway disease, the levels and function of these IgE+ B cells and $B_{regs}$ remain to be elucidated in human models of asthma. The overall objective for this dissertation was to investigate the biology of B cells in allergic asthma pathogenesis, specifically investigating the frequency of IgE+ B cells and $B_{regs}$ in allergic asthma, and the kinetics of these cells after allergen exposure.

First, we characterized IgE+ B cells in the blood and sputum of allergic asthmatics and healthy controls with and without allergies (Chapter 2). We showed that IgE+ B cell levels were higher in sputum, but not blood, of allergic asthmatics compared to controls. We further demonstrated that these findings were consistent across airway IgE+ B cell subsets, which include IgE+ memory B cells and

IgE+ plasma cells. Additionally, IgE+ B cells in sputum positively correlated with sputum eosinophils, total IgE and B cell activating factor (BAFF) measured in sputum fluid phase. These findings highlight the association of airway IgE+ B cells with allergic asthma, and suggest that local IgE+ B cell functions contribute to the pathogenesis of asthma.

Second, we measured the trafficking of IgE+ B cells in periphery (blood, bone marrow and tonsil) and locally (sputum) in allergic asthmatics following whole lung allergen challenge (Chapter 3). IgE+ B cells only increased in the airways of allergic asthmatics following allergen inhalation challenge; there were no allergen-induced changes in IgE+ B cell levels in blood, bone marrow and tonsil. In addition, we showed allergen-induced increases in BAFF and total IgE, but not allergen-specific IgE in sputum fluid phase. Taken together, chapters 2 and 3 show that allergic asthmatics have elevated levels of IgE+ B cells in the airways, that can be further increased after allergen exposure. Therefore, local B cell production of IgE in the lungs may be an important source of IgE for initiation of acute inflammatory responses in allergic airways.

Third, we evaluated the levels of $B_{regs}$ in allergic asthmatics compared to controls, and examined the kinetics, function and distribution (bone marrow, blood and sputum) of $B_{regs}$ following allergen inhalation challenge (Chapter 4). We showed that $B_{regs}$ were 2-fold lower in the blood of allergic asthmatics compared to controls, highlighting a possible dysregulation of this regulatory cell type in allergic asthmatics, which may contribute to disease pathology. Furthermore, after whole lung allergen challenge $B_{regs}$ decreased in the bone marrow with a co-incident increase in the blood and sputum of allergic asthmatics. This pattern reflects potential trafficking of these cells from bone marrow to the airways after exposure to allergic stimuli. Lastly, we stimulated CD19+ B cells purified from blood of allergic asthmatic with IL-4 in vitro. IL-4 is a type 2 cytokine known to isotype-switch B cells to IgE+ B cells, as well as differentiates naïve T cells into Th2 cells, thus propagating the allergic cascade. We found that IL-4 promoted higher proportions of IL-10+ and FoxP3+ $B_{regs}$, which

demonstrates that $B_{regs}$ may have a role in dampening IgE-mediated inflammation in a type 2 environment. However, further functional studies are warranted.

Taken together, the findings of this dissertation highlight the local compartmental changes in IgE+ B cells and $B_{regs}$ following allergen challenge of allergic airways. Better understanding the temporal and compartmental shifts in B cell subpopulations, particularly IgE+ B cells and $B_{regs}$, may aid in future development of therapeutics.

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## LIST OF ABBREVIATIONS AND SYMBOLS

AA - allergic asthmatic

AHR - airway hyperresponsiveness

AIC - allergen inhalation challenge

AIT - allergen immunotherapy

ANA - allergic non-asthmatic

APC - antigen presenting cell

BAFF - B cell activating factor

BAFF-R - B cell activating factor receptor

BAL - bronchoalveolar lavage

BCMA - B cell maturation antigen

BCR - B cell receptor

Blimp-1 - B lymphocyte induced maturation protein 1

BM - bone marrow

$B_{regs}$ - regulatory B cells

C/EBP - CCAAT enhancer binding protein

CD - cluster of differentiation

CD40L - cluster of differentiation 40 ligand

COPD - chronic obstructive pulmonary disease

D - diversity genes

DC - dendritic cell

EAE - experimental autoimmune encephalomyelitis

EAR - early asthmatic response

EBF - early B cell factor

ELISA - enzyme linked immunosorbent assay

FcεRI - high affinity IgE receptor

FcεRII - low affinity IgE receptor

$FEV_1$ - forced expiratory volume in one second

FMO - fluorescence minus one

FoxP3 - forhead box p3

FVC - forced vital capacity

FVD - fixable viability dye

GINA - global initiative for asthma

GrB - granzyme B

HC - healthy control

HDM - house dust mite

HIV - human immunodeficiency virus

HSC - hematopoietic stem cell

ICOS - inducible T cell costimulator

ICS - inhaled corticosteroid

IFN - interferon

Ig - immunoglobulin

IgA - immunoglobulin A

IgD - immunoglobulin D

IgE - immunoglobulin E

IgG - immunoglobulin G

IgM - immunoglobulin M

IL - interleukin

iNKT - invariant natural killer T cell

IV - intravenous

J - joining genes

JAK - janus kinase

LABA - long acting beta agonist

LAR - late asthmatic response

LPS - lipopolysaccharide

LTRA - leukotriene receptor antagonist

MBC - memory B cells

MCh $PC_{20}$ - provocative concentration of methacholine inducing at least a 20% fall in $FEV_1$

MHC II - major histocompatability complex class 2

NFκB - nuclear factor kappa light chain enhancer of B cells

NK - natural killer

NKT - natural killer T cell

OCS - oral corticosteroid

OVA - ovalbumin

PAX5 - paired box 5

PB - peripheral blood

PC - plasma cell

$PC_{20}$ - provocative concentration of methacholine resulting in a minimum of 20% fall in $FEV_1$

PLA - phospholipase

PPC - pre-plasma cell or plasmablast

PRR - pathogen recognition receptor

RAST - radioallergosorbent test

SABA - short acting beta agonist

SAC - segmental allergen challenge

SLE - systemic lupus erythematosus

SOCS3 - suppressor of cytokine signaling 3

STAT - signal transducer and activator of transcription

TACI - transmembrane activator and CAML interactor

TCR - T cell receptor

TGF - transforming growth factor

TLR - toll like receptor

TNF - tumour necrosis factor

Tr1 - T regulatory cell type 1

$T_{regs}$ - regulatory T cells

TSLP - thymic stromal lymphopoeitin

TSP-1 - thrombospondin 1

V - variable genes

α - alpha

δ - delta

ε - epsilon

μ - mu

ϒ - gamma

## DECLARATION OF ACADEMIC ACHIEVEMENT

The research documented in this thesis is presented as a "sandwich doctoral thesis". The four articles presented in Chapters 2-4 are three independent, but thematically related bodies of research that, as of April 2017, have been or are being peer-reviewed prior to publication. Although I was the major contributor for the work presented in this thesis, the work required a collaborative effort from several individuals. As such, my contributions, along with those who assisted with the content of each publication are highlighted and outlined below.

## CHAPTER 2

**TITLE:** Allergic asthmatics with allergy have elevated levels of IgE+ B cells in the airways

**AUTHORS:** John-Paul Oliveria, Brittany M. Salter, Stephanie Phan, Caitlin D. Obminski, Caroline E. Munoz, Steven G. Smith, Tara X. Scime, Richard M. Watson, Roma Sehmi, Gail M. Gauvreau

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**CITATION OF PUBLICATION:** Oliveria JP, Salter BM, Phan S. Obminski CD, Munoz CE, Scime TX, Watson RM, Sehmi R, Gauvreau GM. Allergic asthmatic subjects have elevated levels of IgE+ B cells in the airways. Journal of Allergy and Clinical Immunology. 2017. [Epub ahead of print]. DOI: 10.1016/j.jaci2016.12. 981. PMID: 28213181.

**AUTHOR CONTRIBUTION:** JPO, BMS, SGS, RS and GMG designed the experiments and provided technical input on the project. JPO, CDO, CEM, TXS and RMW coordinated the clinical procedures. JPO, BMS, SP, CDO, CEM and SGS handled the biological specimens. JPO analyzed the data and prepared figures and tables for the manuscript. JPO, SP, RS and GMG completed the writing of the manuscript.

**SCIENTIFIC CONTRIBUTION:** The levels of B cells, IgE+ B cells, IgE+ memory B cells and IgE+ plasma cells are higher in the airways, but not in the blood of allergic asthmatic subjects compared to non-asthmatic controls. Airway IgE+ B cells are positively correlated with airway eosinophils and total IgE levels.

**CHAPTER 3**

**TITLE:** Increased IgE+ B cells in sputum, but not in blood, bone marrow or tonsils, after inhaled allergen challenge in asthmatic subjects

**AUTHORS:** John-Paul Oliveria, Brittany M. Salter, Jonathan MacLean, Shweta Kotwal, Ashley Smith, Jeffrey M. Harris, Heleen Scheerens, Roma Sehmi, Gail M. Gauvreau

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**AUTHOR CONTRIBUTION:** JPO, GMG, RS, JMH and HS designed the experiments. All authors contributed to writing of the manuscript.

**SCIENTIFIC CONTRIBUTION:** The frequency of IgE+ B cells is elevated in the airways after allergen inhalation in allergic asthmatics, and increases in CD19+ B cells correlated with worsened airway hyperresponsiveness and increased eosinophilia. This suggests localized inflammation in allergic airway is driven by B cells.

**CHAPTER 4**

**TITLES:** Changes in the levels of FoxP3+ regulatory B cells in bone marrow, blood and sputum of asthmatic subjects following whole lung allergen challenge

**AUTHORS:** John-Paul Oliveria, Amani I. El-Gammal, Michelle Yee, Caitlin D. Obminski, Tara X. Scime, Rick M. Watson, Karen Howie, Paul M. O'Byrne Roma Sehmi, Gail M. Gauvreau

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**AUTHOR CONTRIBUTION:** JPO, PMO, RS and GMG designed the experiments and provided technical input on the project. JPO, AIEG, CDO, TXS, RMW and KH coordinated the study, consented the subjects, performed the allergen inhalation challenge, obtained clinical measurements and acquired the blood and sputum samples. AIEG obtained the bone marrow aspirate samples. JPO and MY completed the experiments. JPO, MY and CDO completed the data analysis. JPO, PMO, RS and GMG completed the writing of the manuscript.

**SCIENTIFIC CONTRIBUTION:** In subjects with allergic asthma, the level of CD19+FoxP3+ B cells and the level of $B_{reg}$ phenotypes, CD19+CD5+FoxP3+ and CD19+CD24+CD27+ cells are lower in the blood compared to non-allergic controls. After allergen inhalation by subjects with allergic asthma, CD19+FoxP3+ B cells in sputum and blood increase with a corresponding decrease in the bone marrow. Expression of FoxP3 in several phenotypes of $B_{reg}$ cells consistently decrease in bone marrow and increase in blood 24 hours post-allergen challenge. Following allergen exposure in the airways of subjects with allergic asthma, $B_{regs}$ appear to efflux from the bone marrow and accumulate in the lungs with a possible role in dampening local inflammatory responses.

# CHAPTER 1: Introduction

## 1.1 Allergic Asthma

Asthma is a chronic, inflammatory lung disease that affects both children and adults, but although we have developed therapeutics to control and manage the disease, there are still subsets of the population suffering from uncontrollable asthma symptoms[1,2]. Asthma is a disease with a high degree of heterogeneity in regards to both clinical manifestations and signs of the inflammatory profile, especially during asthmatic exacerbations[3]. This high level of variation has a likely role in the spectrum of asthma severity[4,5].

Allergic asthma is a multifactorial disease that involves the interplay between genetic and environmental factors, which manifest into the signs and symptoms individuals face[6]. It is characterized by early and late phase allergic asthmatic responses, mediated by IgE and allergic immune cells[7,8]. Allergic asthma is the most common type of asthma making up a large proportion (50% to 80%) of diagnosed individuals. Allergic asthma is defined as a disease that results from the exposure, sensitization, and reaction to environmental allergens, and involves the presence of both asthma signs (airway eosinophilia) and symptoms (airway hyperresponsiveness), and a positive skin prick test[9]. Allergic asthma is a type 2 cytokine-mediated inflammatory airway disease[10–12], commonly characterized and hallmarked by eosinophilic airway inflammation[13], airway hyperresponsiveness[14–16], and airway remodeling[17,18], which ultimately leads to reversible airway obstruction of the lungs[19–24].

### 1.1.1 Epidemiology

Asthma is a common chronic non-communicable disease of the airways, affecting over 300 million people globally across all age ranges[25]. The Global Burden of Asthma estimates that roughly 14% of the global pediatric population experience asthmatic symptoms[25]. Specifically, Canadian demographics data show that approximately 3 million people are affected by asthma, which is about 10% of the population[26]. Due to their higher prevalence, as a whole, respiratory diseases, which

include: lung cancer, asthma, and chronic obstructive pulmonary disease (COPD), are placing a large burden on the Canadian economy, where approximately $12 billion dollars are required for both direct health care costs (hospitalizations, therapeutics, and healthcare providers) and indirect costs (premature mortality and long term disability) in 2010[27,28]. Unfortunately, of all respiratory illnesses, asthma contributes significantly to these costs[27]. The Conference Board of Canada projects that the cost of asthma management by the Canadian Healthcare system will rise to $4.2 billion by 2030 if there are no efforts to better manage this disease[29]. Taken together, it is apparent that the burden of asthma is rising, both in incidence and economic demand. Thus, the understanding behind the complex pathogenesis of allergic asthma is essential in alleviating economic burden on the healthcare system, and more importantly, improving the quality of life for the individuals suffering from this disease.

### 1.1.2 Diagnosis

Due to improving technologies and better awareness of the disease, the diagnosis of allergic asthma has improved in accuracy and precision over the years[4]. One of the first reliable methods used to diagnose patients with allergic asthma involves a thorough assessment of medical history to evaluate the presence of common asthmatic symptoms, which include: wheezing, coughing and shortness of breath[9,20], especially during peak allergy seasons[30–32]. In addition to past medical history of asthmatic symptoms, physicians also conduct physical examinations to assess airway function, which includes performing spirometry and methacholine challenge tests[9,19]. Spirometry is used to assess lung function, which is measured as a function of volume against time. During the test, the patient is asked to quickly exhale as much air as possible after maximal inhalation[33]. Forced expiratory volume in 1 second ($FEV_1$) and forced vital capacity (FVC) are then determined to calculate the ratio of $FEV_1$/FVC, where a value of $FEV_1$/FVC less than 70% of that predicted for a given gender, race, age and height, would indicate restricted airflow[33,34]. Furthermore, a reversibility test that involves the administration of

inhaled bronchodilators is conducted in order to determine if airway obstruction is reversible, and if lung function improves after administration of the treatment[22,33,35,36].

Additionally, the methacholine challenge test is used to determine the presence of airway hyperresponsiveness by measuring the provocative concentration of methacholine, or histamine that induces at least a 20% fall in $FEV_1$ ($PC_{20}$)[15,19,37,38]. Histamine is able to induce airway hyperresponsiveness by binding to histamine receptors; specifically, binding of histamine to H1 receptors on airway smooth muscle and endothelial cells induces bronchoconstriction and vasodilation leading to airway hyperresponsiveness[39,40]. Alternatively, methacholine, a synthetic derivative of acetylcholine, is delivered into the airways as a wet aerosol. Methacholine induces bronchoconstriction by directly binding to muscarinic receptors on smooth muscle in the airways, thus leading to airway hyperresponsiveness[37,38]. Airway hyperresponsiveness is a hallmark of asthma and is characterized as a state of bronchoconstriction and increased sensitivity of the airways ("twitchy" airways), as a response to an inhaled agonist[15]. The methacholine challenge test is a useful diagnostic tool that can be conducted if spirometry test results fall within normal range. Methacholine is a muscarinic receptor agonist that stimulates bronchoconstriction[19]. During the challenge, individuals inhale increasing (doubling) concentrations of methacholine until a 20% fall in $FEV_1$ is obtained[19,38]. A positive methacholine challenge test is defined as a $PC_{20}$ of less than 16 mg/mL of inhaled methacholine[41].

In order to determine allergic status, skin prick tests are used to induce IgE-mediated reactions against various environmental allergens (pet dander, pollens, dust mite, mold), and a positive result is characterized by a wheal diameter of more than 2 mm compared to a negative saline control[20,42]. In the skin prick test, histamine is used as a positive control to ensure skin reactivity occurs for a reliable test result[42]. Furthermore, blood tests can be performed to assess serum IgE levels through the enzyme-linked immunosorbent assay (ELISA), radioallergosorbent test (RAST) or ImmunoCAP diagnostic testing methods[20].

Based on the Expert Panel Report 3 guidelines, asthma can be classified into 4 different categories: intermittent, persistent-mild, persistent-moderate and persistent-severe[4]. The guidelines used to determine the level of severity focuses on the factors, which include: daytime symptoms, nighttime sleep disturbance, frequency of using short-acting beta-agonists, interference with daily activities, lung function, and exacerbations[4].

### 1.1.3 Gold Standard Treatment

Although allergic asthma is a heterogeneous disease and different individuals have their own fingerprint in how asthma affects them, there are guidelines in place to best manage this disease[12]. According to GINA guidelines, the first-line therapy in the management of asthmatic symptoms is the use of short acting beta agonists (SABA) as needed[43]. If symptoms are unmanaged, this treatment would then progress to using low dose inhaled corticosteroids (ICS), with potential substitution to a leukotriene receptor antagonist (LTRA) if ICS use was ineffective[43]. If low dose ICS or LTRA were ineffective in managing asthma, increasing the doses of both ICS and LTRA would be explored[43]. Finally, if conventional treatments fail in controlling asthma symptoms, specialized treatments like omalizumab (anti-IgE) or mepolizumab (anti-IL-5), in addition to low dose oral corticosteroids (OCS) would be utilized[43].

The classic interventions in managing asthma are showing to be ineffective in a subset of asthmatics due to the heterogeneity behind the pathogenesis of asthma, and the inefficacy of gold standard treatments in managing all asthmatic patients. Furthermore, the use of ineffective treatments in managing asthma is putting a large economic burden on the healthcare system due to increased exacerbations and patient hospitalizations[27]. Thus, there is a large push towards personalized treatment regimens for patients where conventional therapies are ineffective, and for patients known to have severe refractory asthma[43]. Severe refractory asthma has been characterized by persistent airway obstruction with frequent and often severe asthma exacerbations, leading to increased

hospitalizations[25]. These exacerbations continue despite the patients being on high doses of ICS given with or without long acting beta agonist (LABA) or OCS[44,45]. Patients with severe refractory asthma represent roughly 5-10% of the overall asthmatic population; however, contributing disproportionately to health care expenditures, and thus highlighting a need for novel therapeutics for asthma management[46,47].

Overall, although concrete guidelines for treating the asthmatic population do exist and already a wide range of therapeutics available for use, a small subset of the asthmatic population continues to create a large economic burden for the healthcare system, moving toward the need for new treatment options and therapy regimens for more personalize medicine strategies. However, in order to develop novel therapies, a better understanding of asthma pathogenesis is necessary.

### 1.1.4 Pathogenesis

Asthma is understood as a heterogeneous disease with differences in severity, natural history, comorbidities, and responses to treatment[31]. Asthma has been defined as a chronic inflammatory disorder associated with variable airflow obstruction and bronchial hyperresponsiveness[31]. Furthermore, the asthmatic response has been elucidated as a complexly integrated and multifaceted inflammatory response in the conducting airways of the lungs[31]. The allergic asthma phenotype is one of the more dominant forms of asthma in early life[31]. Allergic diseases are hyperactive responses against common environmental allergens. These allergic responses are propagated by the development of memory T and B cell responses to specific environmental antigens, leading to the production and secretion of type 2 inflammatory cytokines and IgE. The production of these inflammatory mediators aid in the recruitment of allergic immune effector cells upon exposure to allergen[48]. The pathogenesis of allergic asthma has been associated with an increase in T lymphocytes, specifically CD4+ T cells, and the release of type 2 cytokines, which include IL-4, IL-5, and IL-13; where IL-4 and IL-13 specifically play pivotal roles in the development of IgE-mediated inflammatory processes[49,50].

Furthermore, these cytokines are associated with complex pathways leading to IgE production, and the proliferation of inflammatory cells including eosinophils, mast cells, and basophils, which contribute to bronchial hyperresponsiveness in asthmatics[49].

Allergen bronchoprovocation tests have been a key tool in providing further information on the intricate pathways associated with allergic asthma pathogenesis[24,51]. The general paradigm for allergic disease development revolves around the ability of the initial allergen exposure to prime the immune system towards a shift to type 2 inflammation mediated by IgE-dependent mechanisms[31]. The presentation of the allergen by antigen-presenting cells (APCs) to naive CD4+ T cells has been elucidated as a primary mechanism in the development of allergy[50]. The airways of allergic asthmatics have been characterized by higher levels of resident lymphocytes, eosinophils, basophils and mast cells, in addition to epithelial desquamation, goblet cell hyperplasia, and the thickening of the basement membrane[50]. Allergen binding on high affinity IgE receptors (FcERI) on the surface of dendritic cells, granulocytic cells; such as mast cells and basophils, leading to the induction of cross-linking of the membrane-bound receptors and initiation of degranulation and release of inflammatory mediators by these immune cells[51]. Specifically, mediators, such as; histamine and leukotrienes, are released by these cells, in addition to the type 2 cytokines, IL-4, IL-5, and IL-13, which ultimate lead to an influx of CD4+ T cells and eosinophils into the airways of the asthmatics[51]. In allergic asthma, airway inflammation is hallmarked by eosinophilia, and changes in the levels of eosinophils can be used to determine disease severity and treatment efficacy[51]. It is apparent that the pathogenesis of allergic asthma has a very broad range of interacting cellular and molecular processes, leading heterogeneous presentations of this disease, and ultimately difficulty in disease management. Thus the development of novel therapeutics is crucial in being able to treat patients who are unresponsive to conventional therapies. Specifically, since allergic asthma pathogenesis is classically thought to be initiated by IgE-

dependent mechanisms, an effective and clinically approved anti-IgE therapy has shown clinical efficacy in the management of allergic asthma[52] (Figure 1.1 and Figure 1.2).

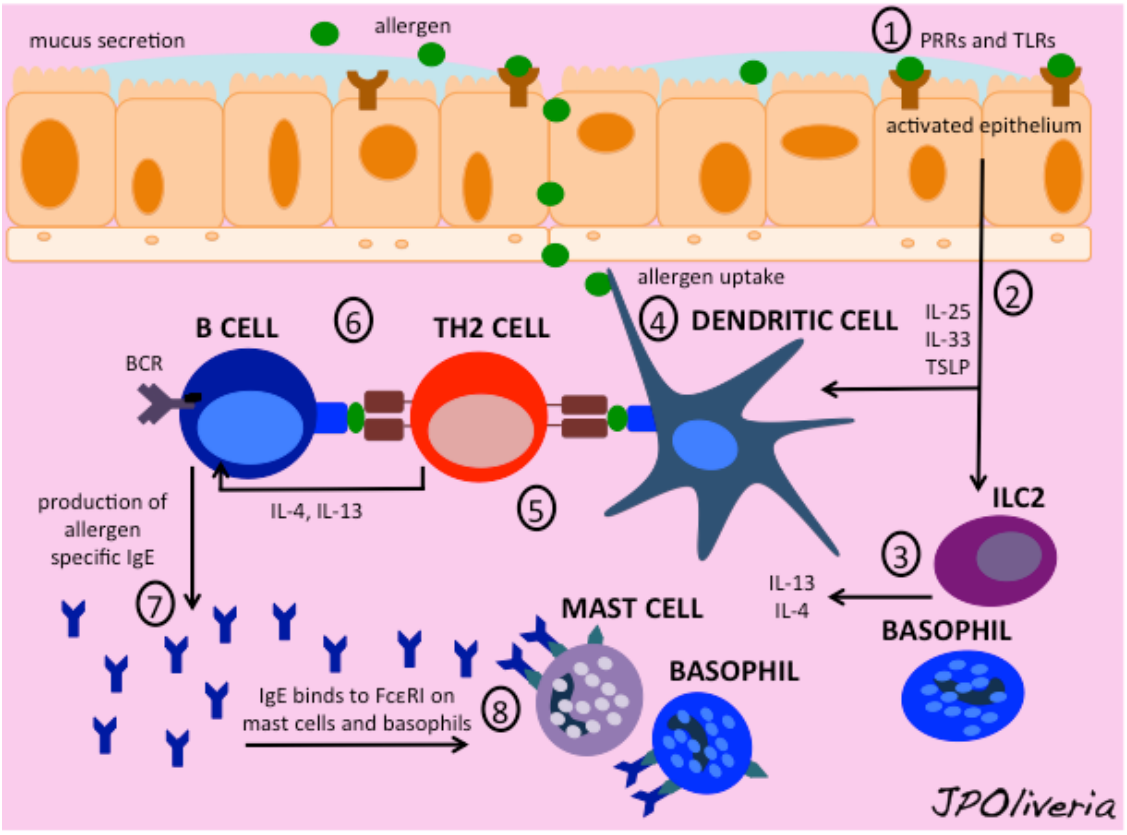


**Figure 1.1: Initiation of the allergic asthma cascade.** (1) Upon inhalation of certain environmental allergens, pathogen recognition receptors (PRRs) and toll like receptors are able bind allergens, which induce the activation of the epithelium and release of (2) alarmin cytokines (IL-25, IL-33, TSLP). (3) Alarmin cytokines prime immune cells (ILC2s, basophils, dendritic cells) and induces the release of type 2 cytokines (IL-4, IL-5 and IL-13) by these aforementioned cells. (4) Dendritic cells (DCs) present allergen peptides via their MHC class II molecules to (5) naïve T cells, thereby activating them, and driving their differentiation and maturation into CD4+ T cells. (6) Activated CD4+ T cells produce IL-4 and IL-13, cytokines crucial for the induction of IgE isotype-switching to occur in B cells. (7) IgE production of B cells increase and the IgE binds to high affinity IgE receptors on the surface of mast cells and basophils. (8) Upon future exposure of allergic asthmatics to allergens, mast cells and basophils are armed to quickly respond and initiate the allergic, IgE-mediated inflammatory cascade.

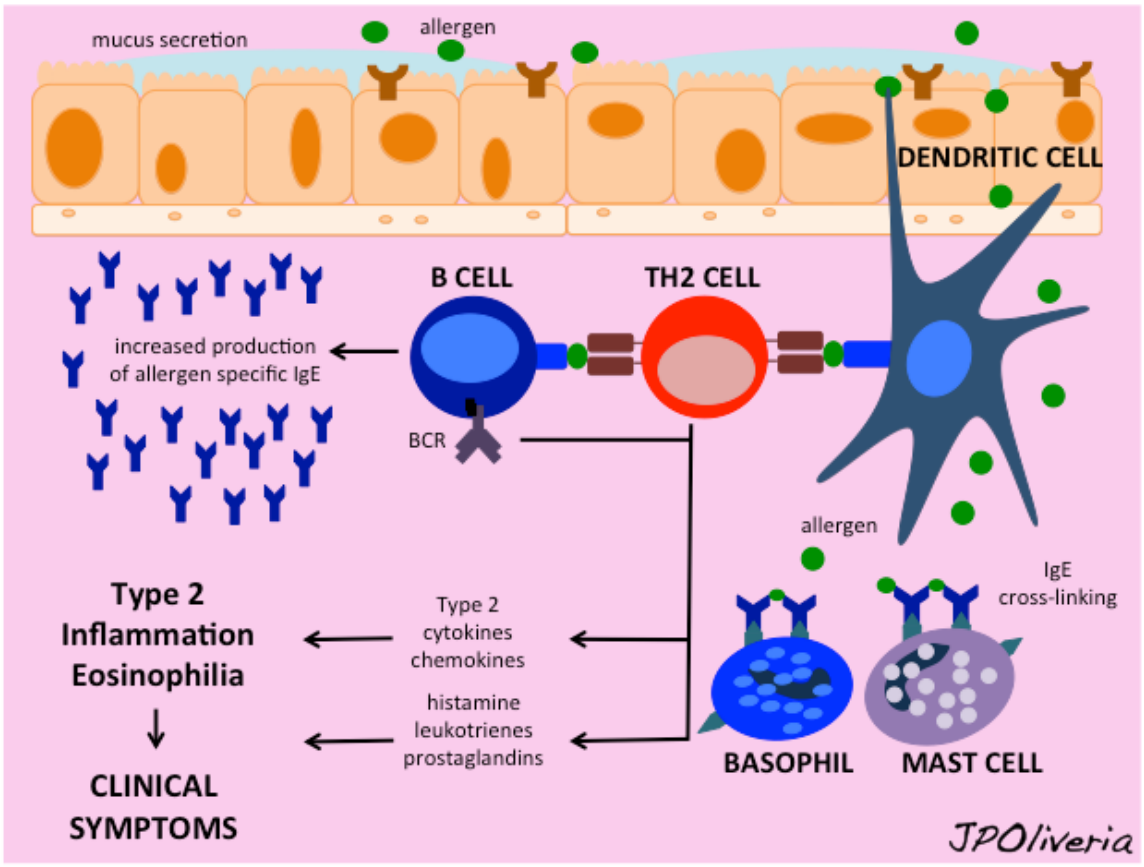


**Figure 1.2: Re-exposure to environmental allergen from an IgE and B cell perspective.** Re-exposure to environmental allergens induce allergen-specific responses whereby memory B cells are able to mature into plasma cells and produce allergen specific IgE. Additionally, allergen-specific IgE bound to their high affinity IgE receptors on the surface of immune cells, are able to complex with allergen, cross-link with neighbouring IgE:IgE receptor complexes, thereby activating basophils and mast cells, inducing their degranulation and release of inflammatory mediators. This initiation of the allergic cascade induces type 2 inflammation characterized by eosinophilia, which leads to clinical symptoms; such as: wheezing, coughing and shortness of breath.

### 1.1.5 Anti-IgE and Anti-IgE+ B Cell Therapies

IgE, an important initiator of allergic disease, is a molecule targeted by therapeutics in order to control the allergic cascade in allergic asthma. There are data showing that anti-IgE therapies are able to deplete serum free IgE by directly binding to IgE and forming complexes, and thereby inhibiting IgE-FcεRI binding on the surface of mast cells and basophils, which is necessary for IgE cross-linking, activation and degranulation of mast cells and basophils[53]. In addition, the number of high affinity IgE receptors (FcεRI) was found to be decreased on basophils and mast cells acutely after treatment, which decreases the chance of IgE cross-linking, and in turn, decreases basophil and mast cell sensitivity to allergens. Furthermore, anti-IgE therapies do not bind to IgE already bound to IgE receptors. Although anti-IgE is effective in diminishing free serum IgE, it does not target IgE-producing B cells, such as IgE+ memory B cells or plasma cells. Milgrom et al treated allergic asthmatics with anti-IgE and found that after 20 weeks of treatment, subjects undergoing treatment had a significantly decreased concentration of unbound or free serum IgE compared to subjects in the placebo group[53]. In addition, subjects undergoing treatment also rated their asthma symptoms more improved compared to subjects in the placebo group. This shows that anti-IgE therapy alleviates clinical symptoms of asthma by reducing free IgE. Furthermore, Wegmann et al also evaluated anti-IgE treatment in mice with allergic airway disease[54]. They found that short-term treatment with anti-IgE decreased the number of eosinophils in the airway, while long-term treatment with anti-IgE decreased the serum concentrations of IgE. However, anti-IgE treatment, short-term or long-term, has yet been shown in human models to decrease plasma cells, which are primary producers of IgE[54]. In addition to omalizumab, ligelizumab is another anti-IgE therapy that has shown efficacy in managing allrgic asthma symptoms[55,56]. Benefits that propel ligelizumab in favour of omalizumab is its higher binding affinity to IgE, which has the potential to translate to more efficacious clearing of IgE in circulation[55–57]. Although, both omalizumab and ligelizumab have shown efficacy and promise in the treatment of asthma, their route of

administration to patients is intravenous (IV), while gold standard asthma therapeutics has been inhalation of $\beta_2$-agonists and corticosteroids. In order to test the efficacy of inhaled anti-IgE, Fahy et al tested the effects of aerosolized anti-IgE treatment after allergen inhalation in allergic asthmatics, and found that the aerosol route was not superior to the intravenous route of administration of anti-IgE therapy[58]. Regardless, anti-IgE therapies do have positive effects in controlling allergic asthma symptoms mainly through reducing serum IgE; however, the source of IgE, IgE-expressing B cells, are not being reduced with this method of therapy.

Moreover, instead of an anti-IgE therapy, Brightbill et al, evaluated another treatment that uses anti-M1 prime antibodies to specifically deplete IgE-switched B cells. Targeting the M1 prime segment, which is exclusively found on human membrane-bound IgE of IgE-switched B cells and IgE plasmablasts, will potentially deplete the source of IgE, thus reducing circulating IgE[59]. Brightbill et al showed that anti-M1 prime treatment depleted IgE-switched B cells and serum IgE in a humanized mouse model of allergic airway disease[59,60]. Taken together, anti-M1 prime treatment has the potential to be an effective way to deplete IgE, by targeting the source (membrane-bound IgE+ B cells). Furthermore, Gauvreau et al showed that quilizumab treatment (anti-M1 prime) of subjects with allergic rhinitis and mild allergic asthma reduced allergen-specific IgE levels when compared to placebo treatment[61]. Furthermore, the allergic asthmatics were found to have reduced allergen-induced increases in sputum eosinophilia after quilizumab treatment compared to placebo[61]. Thus, targeting the cellular source of IgE was found to be an effective way to decrease IgE production in mild allergic asthmatics. More studies evaluating the role B cells play in allergic pathogenesis is required due to the very limited literature available. Thus by better elucidating the roles B cells play in allergic asthma pathogenesis, better therapeutic targets can be developed (Figure 1.3).

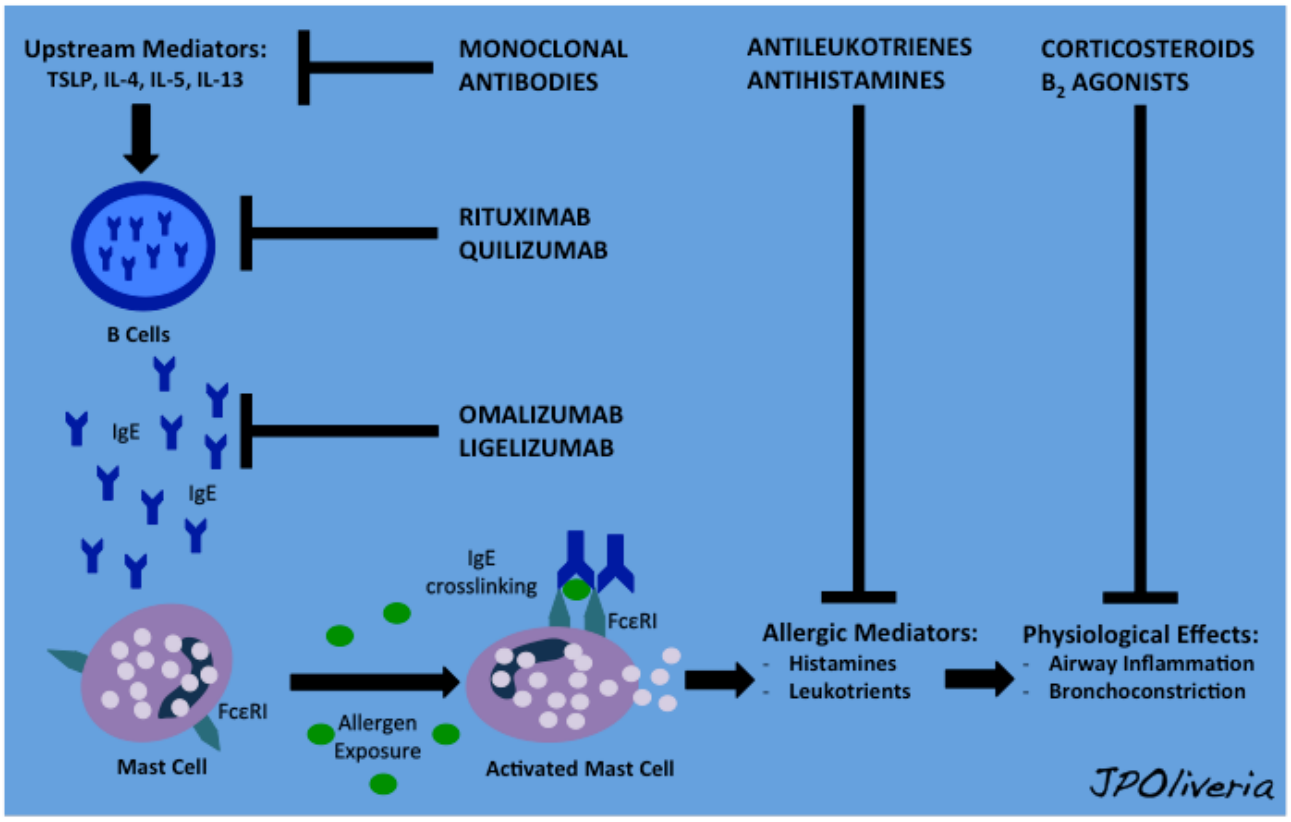


**Figure 1.3: Therapeutic strategies in the management of allergic asthma.** Gold standard therapies in the management of allergic asthma includes the use of: corticosteroids (inhaled and oral), as well as, beta-2 agonists, antihistamines, and antileukotrienes. Monoclonal antibodies have been a large proponent in managing difficult to control asthma, where by omalizumab (anti-IgE) and mepolizumab (anti-IL-5) have been approved for clinical use. Other anti-IgE (ligelizumab) and anti-B cell (rituximab, quilizumab) therapies exist, but are still being studied.

## 1.2 B Cells

B cells have been implicated in many different diseases, including, but not limited to, autoimmune disorders (multiple sclerosis[62,63], systemic lupus erythematosus[64]), cancer (leukemia[65,66]), and allergy (rhinitis[67], asthma[68–72]). Briefly, B cells mature from hematopoietic stem cells. Most of their development and maturation occurs in the bone marrow, where they differentiate and mature into functional effector or regulatory B cells[73–77], and are released into the peripheral compartments of the body (peripheral blood, secondary lymphoid organs, lymph nodes, and local tissues)[78]. Characterization of B cells at different stages of development are currently based on the cell surface markers that they express, which primarily include: CD19, CD20, CD27, CD38, and CD138[73–75,79].

### 1.2.1 Ontogeny and Development

In allergic asthmatics, IL-4 and IL-13, are important for B cell maturation and isotype switching into IgE-expressing cells[73]. Furthermore, other cytokines including thymic stromal lymphopoietin protein (TSLP) and B cell activating factor (BAFF) are also involved in the differentiation, maturation, and proliferation of B cells[80–82]. The main stages of B cell development include: progenitor B cells, immature B cells, naïve B cells and mature B cells, where naïve B cells efflux out of the bone marrow and enter into the periphery to encounter antigens[74,78]. Mature B cells can be further sub-categorized into memory B cells (MBCs), plasmablasts or pre-plasma cells (PPCs) and plasma cells (PCs)[74,78]. Common cell surface markers used to phenotype MBCs are CD19+CD27+, while PPCs are identified by CD19+CD27+CD38+, and PCs by CD138+ (Figure 1.4).

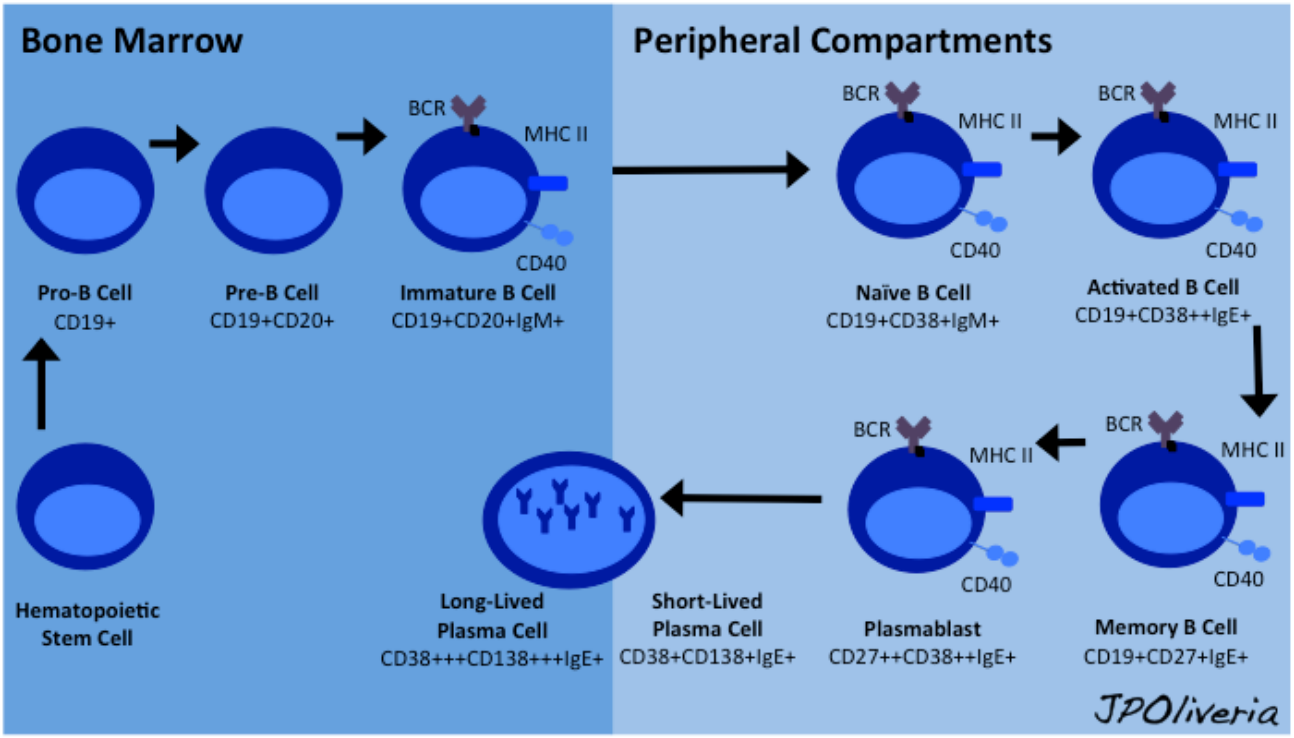


**Figure 1.4: Development of B cells.** All B cell development occurs in the bone marrow from hematopoietic stem cells (HSCs). VDJ recombination occurs and once B cells reach the immature stage, they are able to emigrate out of the bone marrow and into the peripheral compartments (lymphatics, lymph nodes, circulation and tissues). Upon B cell activation from encountering antigens, B cell proliferation and maturation occurs, a small subset of memory B cells form for subsequent exposure to antigens. Short-lived plasma cells are theorized to remain within local tissue compartments, while long-lived plasma cells are known to reside in the bone marrow until subsequent antigen exposure.

This dissertation aims to explore and better understand whether the frequency of these B cell subsets are altered in allergic asthma after allergic stimulation, which can be evaluated by utilizing the allergen bronchoprovocation model in mild allergic asthmatics. There are currently very few published studies that have evaluated the development and kinetics of B cells in a murine model, and currently no studies have evaluated the kinetics of multiple B cell subsets across different compartments in humans after an allergen inhalation challenge.

Since B cells are the sole source of antibodies that are responsible for mediating IgE-dependent inflammation in allergic asthma, it is important to better understand the biology of B cells when studying allergic asthma. As mentioned before, B cells develop from hematopoietic stem cells in the bone marrow, where they undergo a sequence of developmental stages, which involve the rearrangement of immunoglobulin gene segments. Successful completion of each developmental stage ensures the survival and function of B cells[7]. During the first stage of early B cell development, the D (diversity) and J (joining) gene rearrangements occur in the heavy chain of the early pro-B cell. Subsequently, the V (variable) to DJ gene rearrangement occurs[74]. Successful VDJ rearrangement results in the production of a pre-B cell. In the pre-B cell phase, rearrangement of the gene segments encoding the light chains, kappa and lambda, occurs[74]. With the synthesis of the light and heavy chains, the cell develops into an immature B cell expressing an IgM molecule on the cell surface[74].

In addition, there are several transcription factors that play a role in the different stages of B cell development, which help to regulate the expression of genes to facilitate B cell development. PU.1, E2A and early B cell factor (EBF) are known to be critical for the early stages of B cell development[75]. PU.1 is an important transcription factor that is involved in regulating gene transcription during both the early and late stages of B cell development. It promotes the transcription of IL-7Rα and EBF[75]. E2A is required for B cell differentiation, while EBF is regulates the transcription of B-cell specific genes like paired-box protein 5 (PAX5)[75]. PAX5 is a transcription factor that drives B cell lineage

commitment[74]. Furthermore, B lymphocyte-induced maturation protein 1 (Blimp-1) is a transcription factor that regulates terminal B cell differentiation, and is required for the development of plasma cells[74,83].

### 1.2.2 Central Tolerance and Survival

After reaching the immature, pre-B cell stage, B cells undergo positive and negative selection during the process of maturation[83]. Positive and negative selections are important processes that occur throughout B cell development and maturation in the bone marrow and peripheral sites. Positive selection allows for lineage commitment of B cells, B cell survival, differentiation, and maturation, and upon successful VDJ recombination in B cells, positive selection promotes entry to peripheral tissues and recognition of specific foreign antigens[7,84]. Negative selection eliminates B cells bearing receptors that recognize self-antigens through cell death or apoptosis, this process prevents the propagation of autoimmune-related diseases[7]. After positive and negative selection, the surviving B cells that home in the marginal zone of the lymph nodes become marginal zone B cells, while others become follicular B cells in the follicle of the lymph node[83]. Marginal zone B cells ($IgM^{hi}IgD^{lo}CD21^{hi}CD23^{lo}$) are non-circulating B cells that remain in the marginal zone of the lymph nodes and secondary lymphoid organs. Follicular B cells ($IgM^{int}IgD^{hi}CD21^{int}CD23^{hi}$) may recirculate in the bone marrow or lymphatics[7,74,83].

B cell survival is dependent on the expression of the B-cell receptor (BCR) on the cell surface of developing and mature B cells. In addition, B cells require the presence of B-cell activating factor of the TNF-α family (BAFF), which is a signal provided by follicular dendritic cells essential for the differentiation, maturation, and proliferation of B cells[85]. BAFF binds to three different receptors: the BAFF receptor (BAFF-R) or the transmembrane activator, the calcium modulator and cyclophilin ligand (TACI), and the B-cell maturation factor (BCMA)[82]. The signals received through receptor-ligand interactions result in the development of marginal zone B cells and follicular B cells[74].

### 1.2.3 Activation

In order for B cell activation to occur, B cells must encounter non-self antigens to which they can mount an immune response against. B cells encounter antigens directly through their BCRs if the antigenic protein is in a free soluble form or by dendritic cells, a professional APC. Upon encountering antigens, marginal zone B cells and follicular B cells differentiate into memory B cells and plasma cells[74,83]. The memory B cells and plasma cells that form from marginal zone B cells may leave the marginal zone and enter other areas of the lymph node, allowing them room to clonally expand and proliferate. However, the majority of these clonally expanded cells express low-affinity IgM, and are short-lived, with a lifespan of approximately 3 days prior to undergoing apoptosis[74,83]. Activated follicular B cells enter the follicles from the germinal center, where they interact with follicular T cells and follicular dendritic cells. T cells expressing CD40L can interact with CD40 on the surface of B cells, acting as a co-stimulatory factor for B cell activation, while follicular dendritic cells concentrate antigens to the germinal center. Upon activation, B cells can undergo proliferation, affinity maturation, and class switch recombination of immunoglobulin genes, from IgM to IgD, IgG, IgE or IgA[74].

Affinity maturation is a process by which B cells produce antibodies that possess high affinity for specific antigens. This process is a result of somatic hypermutation, where point mutations occur in the V regions of the immunoglobin gene, creating a variety of B cell antibodies with varying affinities for specific antigens[7,74]. Class-switch recombination is a process where the heavy chain, constant region on the surface of B cells is replaced to produce a different Ig isotype, for example switching from IgM to IgE in the context of allergic asthma[7]. The result of these events is the ability of B cells to differentiate into IgE-switched memory B cells or plasmablasts[74]. Plasmablasts are precursors of plasma cells that exit the germinal center prior to proliferation and differentiation into plasma cells[83]. Memory B cells are long-lived B cells that provide a robust response upon re-exposure to the same

antigen. Plasma cells generated from the germinal center are also long-living B cells that secrete large amounts of high-affinity antibodies[83].

### 1.2.4 Memory

Memory B cells and plasma cells are commonly known as the memory subsets of B cells due to their ability to mount antigen-specific responses upon re-exposure to the same antigens. Memory B cell subsets can be analyzed using flow cytometry, and co-expression of specific cell surface markers (CD19, CD20, CD24, CD27, CD38 and CD138)[78,86] are used to differentiate the various B cell subsets. Immature and naïve B cells can be identified by the co-expression of pan-B cell markers, CD19 and CD20, which are known to be expressed on all B cells[78,87]. Transitional B cells, or B cells transitioning from one compartment in the body to another, are characterized by their co-expression of CD24 and CD38[88]. CD27 acts as a surface marker for memory B cells. Both plasmablasts and plasma cells can be identified by their expression of CD38, with plasma cells co-expressing CD138[74,78,86].

Furthermore, a limited number of studies have been done to evaluate the effect of allergen on the kinetics of B cells, which is important for the understanding B cell driven immune responses following antigen exposure. Zhang et al studied the kinetics of memory B cells and plasma cells in mice immunized with plague vaccines[89]. They measured levels of memory B cells and plasma cells in bone marrow and spleen at different time points and found that the percentage of memory B cells and plasma cells increased after immunization, where both cell types in the spleen reached their highest peak 42 days after immunization[89]. Memory B cells gradually declined after the peak, whereas plasma cell levels were sustained for 98 days after immunization[89]. However, the kinetics of B cells after immunization or allergen stimulation has not been examined in humans.

### 1.3 IgE+ B Cells

The five classes of Ig antibodies produced by B cells are IgM, IgD, IgG, IgE and IgA[74]. The isotype expressed by B cells and functions elicited are dependent on the heavy chain, constant region on the BCR[74,90]. When B cells first develop and mature, they express the IgM isotype. The mechanism of class-switch recombination or isotype switching is facilitated by the activation-induced cytidine deaminase (AID) enzyme[90]. Upon the activation of the B cells through antigen exposure and co-stimulation via CD40L, B cells undergo isotype-switching to rearrange the heavy chain genes. During this process, AID deaminates residues along switch regions, which are gene segments upstream of the heavy chain genes. The enzyme activity results in breaks at two switch regions, facilitating subsequent double-stranded breaks to occur[90]. Portions of the strand carrying heavy chain genes are then excised, leaving the gene encoding the heavy chain of interest to be expressed [91]. Mu (μ) encodes the heavy chain for IgM, delta (δ) for IgD, gamma (γ) for IgG, epsilon (ε) for IgE, and alpha (α) for IgA[74,90].

As mentioned previously, IgE plays an important role in the allergic asthma pathogenesis, as it is responsible for binding to FcεRI receptors to initiate the activation and degranulation of mast cells and basophils in the presence of a specific allergen[92]. In a study by van de Pol et al, total serum IgE and allergen-specific IgE were increased in allergic asthmatics after a single allergen inhalation challenge with house dust mite extract[93]. In support, Wilson et al studied local production of IgE in allergic asthmatics, and found that bronchoalveolar lavage (BAL) fluid from allergic asthmatics had increased levels of allergen-specific IgE after allergen challenge compared to saline challenge[94]. Taken together, these studies support the notion that B cells play a critical role in mediating inflammatory responses locally in the airways through the production of IgE antibodies.

#### 1.3.1 IgE

The synthesis of IgE is important in the pathogenesis of allergic asthma[69]. However, IgE serum concentration is one of the lowest among the 5 immunoglobulin subclasses (IgA, IgD, IgE, IgG, IgM),

and has the shortest serum half-life of only up to 48 hours[68,95]. Free serum IgE has two receptors; FcεRI (high affinity) and FcεRII (low affinity). The low affinity IgE receptor (FcεRII/CD23) is expressed on the surface B cells[96], while the high affinity IgE receptor (FcεRI) is expressed on the surface of mast cells and basophils[68,95]. Furthermore, FcεRI, unbound to IgE, has a short half-life, and thus IgE and FcεRI have a short window to bind to one another in order to propagate IgE-mediate inflammatory processes[68,95]Allergens cross-link IgE-FcεRI complexes on the surface of mast cells and basophils. When IgE-FcεRI complexes cross-link, mast cells and basophils become activated, and consequently degranulate their granule contents in the local tissue promoting an inflammatory milieu, which leads to the initiation for local allergic immune cell infiltration and inflammation[95].

As mentioned previously, allergic asthma involves the production of IgE by long-living antibody-secreting B cells, plasma cells, against a specific antigen [78]. The synthesis of IgE in B cells is a highly regulated process that requires stimulation by two important signals, type 2 cytokines IL-4 and IL-13, and co-stimulation with the co-stimulatory ligand (CD40L) of CD40 expressed on the surface of B cells [97,98]. Upon the binding of IL-4 and IL-13 to their respective receptors, IL-4R and IL-13R, the tyrosine kinases, Janus kinase 1 and 3 (JAK1 and JAK3), are activated to phosphorylate tyrosines on the intracellular domain of the receptor[99]. This process results in the recruitment of STAT6 to the phosphorylated residues, where it subsequently becomes phosphorylated by JAKs, causing it to homodimerize and translocate into the nucleus[97,99]. STAT6 is essential for activating the transcription of the germline epsilon (ε) gene[100]. Thus, IL-4 and CD40L co-activate STAT6 to promote germline ε transcription from the Iε promoter to stimulate class-switching of B cells from IgM to IgE [97]. Similarly, IL-13 also contributes to IgE production by binding to the type II IL-4 receptor, consisting of an IL-4α chain and IL-13α1 chain, to activate the transcription of the germline ε gene[98]. Transcription factors that are responsible for activating this process include: nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), PU.1, Pax-5, AP1, C/EBP and E2A[97]. Once the B cells are IgE class-

switched, re-exposure to allergens allows for B cells to differentiate into IgE-producing plasma cells, and the production of IgE sustains local and systemic IgE levels, which bind to low and high affinity IgE receptors[8].

### 1.3.2 IgE+ B Cells Kinetics and Compartmental Distribution

Although there are no studies evaluating IgE+ B cells in humans after allergen stimulation, there are limited studies evaluating B cells in murine models. Nduati et al studied the kinetics of circulating murine B cells (MBCs and PCs) in a parasitic infection model[101]. They found that MBCs were detectable in the blood; however, PCs were only detectable in the blood shortly after parasitic infection (primary and secondary infections)[101]. Thus, the detection of B cells was dependent on the time point of sample collection after a challenge, such as a parasitic infection challenge. This alludes to the notion that a short timeframe is needed to mobilize B cells into the blood after an inflammatory event. Luger et al studied the kinetics of PCs after a mucosal allergen challenge with ovalbumin (OVA) in a murine model[102]. They found that their challenge model induced the production of PCs in the lungs, spleen and bone marrow. Similar to Nduati et al's study, PCs were found in all compartments during a short window after the allergen challenge. However, PCs were found to be long-lived in the spleen and BM. After an OVA-challenge, a small pool of OVA-specific PCs was able to survive in the BM and spleen. In addition, the long-lived OVA-specific PCs were found to secrete OVA-specific IgE, IgA and $IgG_1$[102]. Additionally, Chvatchko et al showed in a murine inhalation challenge model an increase in antigen-driven differentiation of B cells in the lungs of the challenged mice[103]. Specifically, an increase in OVA-specific IgE and IgG1 PCs were measured in the lungs of the challenged mice[103]. Although the kinetics of B cells remain unclear, even in murine models, measuring B cells in multiple compartments (lungs, circulation, lymphatics, bone marrow) at various times points post-allergen challenge may provide crucial information regarding the expansion of these cells in allergic asthma.

Although, IgE+ B cells are very rare cells and are difficult to enumerate, there have been various technical advances in flow cytometry allowing for the measurement of these rare cell populations[104].

### 1.3.3 Allergen-Induced IgE Production

Since it has been established that IgE is important in allergic asthma pathogenesis, the detection of allergen-specific IgE is important to evaluate allergen-specific responses. Wilson et al and Peebles et al quantified allergen-specific IgE production after segmental allergen challenge (SAC) and allergen inhalation challenge (AIC), respectively[94,105]. Both studies found increased allergen-specific IgE in bronchial alveolar lavage (BAL) fluid[94,105]. However, unlike the study conducted by van de Pol et al, Wilson et al found no significant increase in allergen-specific IgE in the serum. Further disputing van de Pol et al study, Eckl-Dorna et al also studied allergen-specific IgE in allergic subjects and found that the majority of allergen-specific IgE in the serum was not produced by IgE-producing cells from the blood[106]. Eckl-Dorna et al further postulated that the increases in allergen-specific IgE were produced locally in the tissues, such as the lung in allergic asthmatics[106]. Although, allergen-specific IgE is increased after lung challenges, the frequencies and kinetics of IgE+ B cells responsible for producing local IgE are still not clear after allergen challenge in humans.

### 1.3.4 Allergen-Induced Type 2 Cytokine Production

Exposure of B cells to type 2 cytokines, specifically to IL-4 and IL-13, initiates IgE isotype-switching of B cells, ultimately leading to the expansion of IgE-producing plasma cells (PCs) and a small sustained subpopulation of IgE memory B cells (MBCs) in the bone marrow[102]. In addition to the production of IgE antibodies, B cells are also able to secrete cytokines, such as IL-4 and IL-13, which aids in the progression of the allergic cascade. One of the main effector functions of mature B cells in the allergic cascade is the ability to produce cytokines, which include IL-4 and IL-13[73,91,107,108]. Thus, B cells may be able to self-propagate IgE production and IgE-switched B cell pools. However, although B cells have been deemed drivers in allergic asthma pathogenesis, the recent discovery of their ability

to produce IL-10 makes B cells potential players in dampening inflammatory processes in allergic asthma[77,109,110].

### 1.4 Regulatory B Cells ($B_{regs}$)

The suppressive functions of B cells were first postulated in the early 1970s, when it was observed that B cell-depleted, splenocytes adoptively transferred into OVA-sensitized guinea pigs had decreased suppressive function in dampening inflammatory responses[111,112]. However, the exact mechanisms that were responsible for these initial observations had never been further explored until recently[113]. In 1996, Wolf et al demonstrated that B cell deficient mice were unable to recover from experimental autoimmune encephalomyelitis (EAE)[114]. Further discoveries also demonstrated B cell dependent immunomodulation in mouse models of inflammatory bowel disease[115], autoimmune diseases[116], and allergy[117,118]. Additionally, studies began to show a role for IL-10 as one of the primary mechanisms of suppression by B cells[119]. Specifically, IL-10 suppression was seen as a primary immunomodulatory pathway in models of colitis[115], EAE[119], and arthritis[120]. However, a universally accepted B cell phenotype is not clear in literature, regardless of disease. Furthermore, the study of regulatory B cell ($B_{reg}$) subsets and their functional mechanisms, specifically in allergic asthma, is still in its infancy and plenty of questions remain unanswered.

#### 1.4.1 Development of $B_{regs}$

Over 40 years ago, the discovery of distinct lymphocyte subpopulations participating in unique aspects of the adaptive immune response brought significant attention to the origins and functions of B and T cells[121]. The B (*b*ursal) lymphocyte subpopulation can be characterized as a population of cells that express a cell surface immunoglobulin receptor specific to antigenic epitopes[121]. The early studies in the 1960s and 1970s demonstrated that the B cell was primarily responsible for the basic functions of antibody production and mounting a humoral immune responses[113]. Several years later, studies observed the continuum of complexities associated with B cell responses, and B cell development, differentiation and maturation. Specifically, the role of B cells functioning as antigen presenting cells was elucidated, with their ability to present peptides to T cells via MHC II molecules on their cell

surface[121]. Additionally, their ability to secrete a diverse array of cytokines to influence immune cell functions, and contribute to inflammatory processes was also demonstrated[121]. Furthermore, the role of these B cells undergoing somatic hypermutation within germinal centers was elucidated, revealing B cell ability to mount a robust secondary immune response with higher affinity to antigens[121]. Identification of the role of B cells in adaptive memory was a crucial advancement in understanding the capacity of these cells, with the discovery of long-lived plasma cells and memory B cells[122,123]. B cells have a notable role in allergy pathogenesis, wherein B cells possess the ability to class switch to IgE isotype in type 2 inflammatory environments[48], with IgE inducing a downstream inflammatory cascade[48]. However, the recent discovery of regulatory B cells, allows for another avenue of B cells research within allergic asthma pathogenesis. Since the study of regulatory B cells is relatively novel in literature, the development and ontogeny of these cells remain to be elusive and future research is required.

### 1.4.2 Functions of $B_{regs}$

The discovery of a tolerogenic role for B cells created a paradigm shift in the understanding of B cell functions, and roles in disease. The cell itself has the ability to take on a regulatory phenotype in response to certain stimulation[113]. Specifically, TLR ligands, such as; CpG and LPS, the CD40:CD40L axis, and BCR agonists have been elucidated as factors contributing to the activated state of these $B_{regs}$[113]. The term "activated" is variable in literature, but often refers to the $B_{regs}$ ability to produce IL-10 either constitutively or in response to stimulation. Regarding their immunomodulatory function, $B_{regs}$ have primarily been studied based on their ability to modulate T cell function, in favor of a regulatory phenotype, both in mice[124] and in humans[125]. Additionally, $B_{regs}$ have also been shown to play an indirect immunomodulatory role in suppressing inflammatory processes. $B_{regs}$ also possess the ability to induce regulatory phenotypes in a variety of other immune cell subsets (-eg- DCs, T cells, and mast cells) and produce anti-inflammatory cytokines (-eg- IL-10, TGF-β, and IL-35), indirectly aiding

in the suppression of Th1 and Th17 differentiation of $CD4^+$ T cells[126]. $B_{regs}$ also play a crucial role in the maintenance of $T_{reg}$ populations[127,128]. When analyzing both the $T_{reg}$ and $B_{reg}$ regulatory functions, they have many distinct similarities and differences. Like thymus-derived $T_{regs}$, $B_{regs}$ are a specific lineage of B cells in which distinct factors control the expression of genes responsible for their suppressive phenotype; however the development and ontogeny of these cells remain unclear[113]. Furthermore, $B_{regs}$ and $T_{regs}$ both demonstrate similar regulatory functions with the ability to secrete anti-inflammatory cytokines to provide direct exhaustion and deletion of effector cells[129–132]; however the mechanisms by which regulatory B cells elicit their function requires further research. Nevertheless, $B_{regs}$ have a unique ability to mount a suppressive humoral response, primarily through the $IgG_4$ isotype, known to functionally be a blocking antibody, which competes with IgE in binding allergens[133]. Overall, understanding the role B cells play in the immune response is growing in the literature, but the persistence of scientific endeavors are crucial to providing transparency into the diverse effects $B_{regs}$ play in the suppression of immune responses.

### 1.4.3 IL-10 Production by $B_{regs}$

IL-10 plays a role as an anti-inflammatory cytokine in its ability to suppress the immune response[134,135]. IL-10 is predominantly produced by clonally expanded Tr1 (regulatory T cells) cells and professional antigen presenting cells, but can also be produced by mast cells, monocytes and NK cells[134,136]. IL-10 is known to primarily suppress naïve T cell activation through establishing a blockade for co-stimulatory signaling through CD2, CD28, and inducible T-cell co-stimulation (ICOS) in a rapid signal transduction cascade[137]. Specifically, IL-10 inhibits CD28 tyrosine phosphorylation, preventing the binding of phosphatidylinositol 3-kinase p85, consequently inhibiting the CD28 signaling pathway, which is important in T cell co-stimulatory pathways[137,138]. IL-10 has also been demonstrated to induce expression of the suppressor of the cytokine signaling 3 (SOCS3) gene that plays a role in the inhibition of IFN-γ-induced tyrosine phosphorylation of STAT1, which hinders the type 1 mediated

response[139]. IL-10 also plays a role in allergy and immune tolerance in its ability to suppress both total and allergen-specific IgE antibodies, while simultaneously skewing the humoral response from the IgE isotype to the non-inflammatory $IgG_4$ and IgA isotypes[140,141,142].

Classically, IL-10 is a regulatory cytokine that is able to suppress inflammation by suppressing the activation of effector cells and decreasing the production of inflammatory cytokines[143]. Regulatory T cells have been studied in allergic asthma for their ability to suppress inflammation via the secretion of IL-10[144,145]. In the early 2000s, studies showed subsets of B cells that were also able to produce IL-10[146], and this novel subset of B cells were loosely termed regulatory B cells. In current literature, the nomenclatures for IL-10 producing B cells are B10 cells, regulatory B cells or $B_{regs}$. One of the main controversies when studying $B_{regs}$ is that their phenotype is still widely variant in literature[147,148]. However, the common attribute that $B_{reg}$ papers have is that the $B_{regs}$ are able to produce IL-10. Furthermore, in Iwata et al's study, $B_{regs}$ were studied in mice and humans using the same phenotype (CD19+CD24+CD27+)[148]. These $B_{regs}$ were competent in producing cytoplasmic IL-10[148]. However, the pathways leading to the manifestation of these B cells is still not known. In addition to the production of IL-10, $B_{regs}$ were also found to produce $IgG_4$ antibodies[110,149]. In Maseda et al's study, $B_{regs}$ capable of producing IL-10 were shown to also produce antibodies, specifically $IgG_4$[150]. Thus, regulatory B cells were shown to produce IL-10 and antigen-specific antibodies[150].

According to James et al and van de Veen et al, IL-10 induces B cells to produce $IgG_4$, which can act as a blocking antibody for IgE-specific antigens. This blockade prevents the downstream allergic cascade mediated by the IgE pathway. James et al showed that grass pollen-specific $IgG_4$ antibodies were able to neutralize grass pollen allergen, which in turn reduced IgE-mediated events[149]. In van de Veen et al, IL-10-producing $B_{regs}$ were able to suppress inflammatory immune responses, induced by bee venom, by producing anti-inflammatory $IgG_4$ antibodies[110]. Based on these studies, $B_{regs}$ produce IL-10 and $IgG_4$, and these anti-inflammatory mediators suppress CD4+ T cells

proliferation, IgE, and the allergic inflammatory cascade[110,149]. Noh et al studied cow's milk allergy and $B_{regs}$ in humans, and found that $B_{regs}$ were able to produce IL-10 when stimulated with allergen. However, allergen stimulation also increased apoptosis in the regulatory B cell subpopulation[151–153]. Overall, $B_{regs}$ occupy many phenotypically defined subpopulations; however, competency in producing IL-10 remains one of the most effective markers for identification of $B_{regs}$.

Overall, the IL-10+ $B_{regs}$ are phenotypically diverse and occupies a variety of B cell subpopulations; however, these cells play a significant role in many autoimmune diseases, allergies, cancers, and infectious diseases. Based on current literature, it is clear that $B_{regs}$ are able to mediate inflammatory processes through IL-10, but the function and phenotype of $B_{regs}$, particularly in allergic asthma, remain elusive.

### 1.4.4 Role of $B_{regs}$ in Allergic Disease

The pathogenesis of allergic disease is heterogeneous and complex. Allergic diseases can be established through the sensitization and development of B and T cell responses to a specific allergen, followed by type 2 mediated inflammatory responses[48]. Current therapies are designed with the goal of modifying type 2 inflammatory responses, shifting toward a healthy tolerogenic state[48]. Allergen immunotherapy (AIT) is one of the only potentially curative treatments currently for allergic disease, representing a clinical *in vivo* model for tolerance induction upon the regimented and controlled administration of increasing allergen exposure[48]. The role of $B_{regs}$ in the allergic response can be broken down into two mechanisms. First, there are cell-mediated suppressive responses through the release of anti-inflammatory mediators (-eg- IL-10 and TGF-β). Second, there are suppressive humoral responses, with the B cells ability to isotype switch in order to produce $IgG_4$ blocking antibodies.

One of the very first discoveries for the role of $B_{regs}$ in allergy was observed in the context of the ovalbumin (OVA)-induced skin hypersensitivity reaction in guinea pigs[111]. The evidence for $B_{regs}$ role in human allergic disease was first reported in 1998, a study that evaluated the frequency of IL-10+

B cells in human patients with B venom allergy receiving venom immunotherapy[140]. Upon comparison to IL-10- B cells using transcriptome analysis, the study revealed that IL-10+ cells were characterized as cells with cell surface markers consisting of CD19+CD73-CD25+CD71+[133]. These inducible IL-10+ B cells were termed $B_{regs}$, which parallels with the similarities with Tr1 cells due to their ability to suppress antigen-specific T cell proliferation through an IL-10-mediated response[133]. The Br1 CD19+CD73-CD25+CD71+IL-10+ B cells specific for the major bee venom phospholipase A2 (PLA) demonstrated a 2 to 5-fold increase in patients with bee venom allergy, approximately 4 months post-venom immunotherapy[133]. Thus, there is evidence of the expansion of the $B_{reg}$ population in response to high-dose allergen exposure, which emphasizes the functional role for IL-10-producing B cells in inducing and maintaining a tolerogenic response to specific allergens[133]. Furthermore, $B_{regs}$ demonstrated humoral effects in allergic disease with their ability to increase IgG4 production when compared to serum IgE levels[133]. Specifically, there was a 100-fold decrease in the IgE/$IgG_4$ ratio, with IL-10 playing a crucial role in the B cells ability to differentiate into $IgG_4$-producing plasma cells[133]. Overall, the data demonstrates that $B_{regs}$ have the ability to induce a tolerogenic response to allergens, both through natural suppression of effector T cells and the $IgG_4$ skewed humoral response. The role of $B_{regs}$ not only shows promise in their ability to modulate selective treatments for allergies in the future, but aids the understanding of allergic pathogenesis.

A limited number of studies have provided mechanistic insight into the functions of $B_{regs}$ in allergic airway inflammation[154]. In murine models, CD9+ B cells were identified as a critical source for suppressive mediators in a house dust mite (HDM) model of airway hyperresponsiveness[155]. CD9 functions as an adhesion molecule primarily expressed on marginal zone B cells, B1 cells, and plasma cells[155]. Adoptive transfer of these CD9+ B cells effectively suppressed Th2 and Th17 mediated inflammatory responses, predominantly through IL-10 mechanisms[155]. Additionally, the stimulation with cockroach allergen was shown to up-regulate FasL expression on CD5+ B1 cells, which controlled

the cockroach allergen-induced airway inflammation through the apoptosis of effector CD4+ T cells[156]. Furthermore, in human trials, IL-10+ $B_{regs}$ have been shown to play a pivotal role in the suppression of AHR mediated inflammation. Specifically, CD19+CD1d$^{hi}$CD5+CD21$^{hi}$CD23+IgD+IgM$^{hi}$ $B_{regs}$ help reverse the allergic airway inflammation through IL-10 production, which was also associated with the positive expression of CD1d[118]. Additionally, $B_{regs}$ suppress airway inflammation through the recruitment of $T_{regs}$ to the lungs[127,154].

A few studies have also looked at the implications of regulatory B cells in allergic airway disease using murine models[71,72,157]. Amu et al, Singh et al, and Lundy et al showed that a deficiency in $B_{regs}$ was associated with increased serum IgE, increased type 2 cytokines (IL-4 and IL-5) and increased eosinophilia[71,72,157]. Furthermore, $B_{regs}$ were shown to induce pulmonary infiltration of $T_{regs}$, which further had suppressive effects on murine allergic airway disease[71,72,157]. Most importantly, Amu et al, Singh et al, and Lundy et al conducted adoptive transfer experiments, where regulatory B cells were adoptively transferred into mice with allergic airway disease. Adoptive transfer of $B_{regs}$ into mice with allergic airway disease resulted in the reversal of allergic airway inflammation[71,72,157]. Taken together, $B_{regs}$ have the potential ability to suppress the allergic cascade of allergic airway disease through IL-10 and $IgG_4$ production. To date, the role of $B_{regs}$ in the pathogenesis and pathobiology of allergic asthma is still not clear, as the literature is still very limited.

### 1.4.5 Phenotypes of $B_{regs}$

The scientific literature has elucidated partial understanding of role of the $B_{regs}$ in various disease states; however, the phenotypic characterization of the $B_{regs}$ has not been standardized. Currently, there have been several $B_{reg}$ phenotypes investigated within different diseases utilizing both human and murine models. Nevertheless, researchers have not yet been able to clearly identify how these $B_{reg}$ phenotypes are induced within specific disease contexts. Whether these $B_{regs}$ are specific to a distinct B cell lineage or the immunological microenvironment induces a transient change in the

phenotype and function of the B cell, remain as two suggested hypotheses. Miyagaki et al highlights that IL-10-enriched B cell populations do exist with varying phenotypic characterizations of $B_{regs}$, and the primary goal will be to elucidate a universal phenotypic characterization of these $B_{regs}$, highlighting those with the most suppressive function, enabling researchers to gain a more in-depth understanding of these cells and their role in modulating disease. Within allergy, it would be beneficial to elucidate $B_{reg}$ trafficking (-eg- compartmental localization of these cells during the early and late phase allergic responses) and mechanisms of action within *in vitro* co-culture experiments. However, within the allergy literature, there have been several phenotypes including, CD1d+CD5+ $B_{regs}$, CD5+FoxP3+ $B_{regs}$, CD24+CD38+ $B_{regs,}$ and CD24+CD38+ $B_{regs}$ that have shown some promise in reversing allergic airway inflammation, particularly in murine models.

#### *1.4.5.1 CD1d+CD5+ Bregs*

CD1d+CD5+ $B_{regs}$ are shown to originate from a splenic B cell subset, predominantly producing IL-10 to exert their immunomodulatory effects[116]. From an allergy context, these cells play an integral role in the suppression of the acute exacerbation phase of contact hypersensitivity[158,159]. Additionally, CD19+IL-10+CD1d$^{hi}$CD5+CD21+CD23+ $B_{regs}$ have been shown to play a role in suppressing allergic airway inflammation in a murine model, with $B_{reg}$ function mediated through IL-10 secretion and CD1d expression[118]. Outside the context of allergic disease, these cells are implicated in both human and mouse disease models of autoimmunity (-eg- systemic lupus erythematosus and rheumatoid arthritis. Specifically, it was found that in SLE patients, invariant natural killer T (iNKT) cell numbers and functions were impaired due to defective $B_{reg}$ stimulation associated with altered CD1d expression[160]. Specifically, CD1d is able to activate natural killer T (NKT) cells throught interactions with T cell receptors (TCRs) on the surface membrane of NKT cells[161–163]. Overall, this distinct phenotype is implicated in both allergic and non-allergic diseases, and has unique suppressive mechanisms directly through CD1d expression.

#### *1.4.5.2 CD5+FoxP3+ Bregs*

FoxP3 has been extensively studied as a major transcription factor in $T_{reg}$ development. In 2010, Noh et al identified FoxP3 expression for the first time in the CD19+CD5+ B cell subpopulation[164]. FoxP3-expressing CD5+ B cells were identified in human PBMCs, which consisted of 8.5±3.5% of the CD19+ B cell pool[164]. Additionally, these cells showed a significantly elevated apoptotic frequency compared to other cell populations (63.44%)[164]. Furthermore, these CD5+FoxP3+ $B_{regs}$ have been studied within the context food allergy. One study analyzed patients allergic to cow's milk protein (-eg-casein) (N=12), where the allergic patients demonstrated a decrease in CD5+FoxP3+ $B_{reg}$ fraction upon casein allergen stimulation compared to the milk tolerant group, where this cell population increased[165]. Notably, in both the milk allergy group and milk-tolerant group, the CD4+FoxP3+ $T_{reg}$ population both were increased after allergen stimulation[165]. This suggests that allergen-specific $B_{reg}$ responses may have a unique influence on the immune response to food allergens beyond $T_{reg}$-mediated suppression. With this discovery, further studies on the role CD5+FoxP3+ $B_{regs}$ plays in allergic asthma pathogenesis remains to be elucidated.

#### *1.4.5.3 CD24+CD38+ Bregs*

The CD24+CD38+ $B_{reg}$ subset has been shown to predominantly localize in peripheral tissues, and exist within the transitional B cell stage of development[116,126,166,167]. Upon CD40 stimulation, CD24+CD38+ $B_{regs}$ produced the highest levels of IL-10 among all other $B_{regs}$ (CD24+CD27+ $B_{regs}$, CD1d+ $B_{regs}$) in peripheral blood[116]. Furthermore, these $B_{regs}$ were the only subset known to suppress Th1 differentiation of CD4+ T cells. The mechanism behind the Th1 suppression was speculated to be partially IL-10 dependent, and required cellular contact between CD80-CD86 on B cells and CD28 on T cells[166]. In addition to the direct Th1 suppression, these cells possess the capacity to differentiate CD4+ T cells into $T_{regs}$ or Tr1 cells[166]. Additionally, Blair et al demonstrated a defective CD24+CD38+ $B_{reg}$ subpopulation in patients with systemic lupus erythematosus (SLE)[166]. Specifically, these B cells

showed impaired IL-10 production upon CD40 stimulation[166]. Furthermore, the CD24+CD38+ $B_{reg}$ subpopulation was unable to suppress Th1 responses due to defective STAT3 phosphorylation in SLE patients[166]. It was hypothesized that there may be a genetic pathology behind this disease pathogenesis, or that this dysfunctional $B_{reg}$ subpopulation may be the result of $B_{reg}$ overstimulation due to the chronic inflammatory environment associated with SLE, leading to $B_{reg}$ exhaustion and loss of suppressive function[168]. Additionally, the CD24+CD38+ $B_{reg}$ cell subset has shown to contribute to an immune dysfunction in HIV infection, and hinders viral clearance[169]. Finally, this subset has been implicated in chronic graft versus host disease, aiding in the establishment of graft tolerance by suppressing effector T cells[170,171]. Thus these cells have demonstrated a diverse role in a number of disease states, giving rise to possible biomarkers for therapeutic intervention, and transplantation tolerance in human subjects. Although these cells have been extensively studied in autoimmune diseases, their implication in allergic disease is not as well elucidated in literature.

#### *1.4.5.4 CD24+CD27+ $B_{regs}$*

The CD24+CD27+ $B_{regs}$ subset has been primarily found within the memory B cell subset of B cells[116,126]. Additionally, a majority of the IL-10-producing B10 cells possess the CD24+CD27+ cell phenotype[116]. This subset has primarily been associated with the regulation of TNFα production by circulating monocytes[167]. In the context of allergy, it was shown that allergic asthmatics had impaired IL-10 production from CD24+CD27+ $B_{regs}$ upon LPS stimulation compared to healthy controls[172]. Furthermore, not only was this subset impaired in IL-10 production for asthmatics, but the subset was significantly reduced in number as well[172]. Additionally, the impaired IL-10 production from CD24+CD27+ $B_{regs}$ in response to LPS led to a weaker induction of $T_{regs}$ in response to Der p1, and this impaired response may play a role in propagating allergic asthma pathogenesis[172]. Thus, this promising data warrants further research on the role CD24+CD27+ $B_{regs}$ play in allergic asthma pathogenesis.

#### *1.4.5.5 Other $B_{regs}$ Phenotypes*

Due to the heterogeneity of phenotypic characterization of $B_{regs}$, there are other $B_{reg}$ phonotypes implicated in disease, many of which diverge away from IL-10 mediated suppression. Firstly, an IL-10 rich B cell subset, $CD27^{hi}CD38^{hi}$, is primarily within the plasmablast B cell subpopulation, and may possibly have a role in the anti-inflammatory humoral response[173]. Furthermore, in the context of allergy, a subset of $B_{regs}$ has been shown to display a unique $CD25^{hi}CD71^{hi}CD73^{lo}$ phenotype[133]. These $B_{regs}$, as mentioned previously, play an essential role in allergen tolerance through suppression of antigen specific CD4+ T cell proliferation and production of $IgG_4$ antibodies[133]. Furthermore, there are a number of less common phenotypes beyond the IL-10 enriched $B_{reg}$ subsets[174].

Specifically, granzyme B- (GrB) expressing B cells with a CD19+CD38+CD1d+IgM+CD147+ phenotype have been shown to play a role as tolerant B cells able to regulate T cell responses within the tumor microenvironment[175]. These cells were induced primarily through IL-21 production by T cells, and expressed IL-10 and CD25[175]. Additionally, CD39+CD73+ B cells have shown the ability drive the microenvironment away from an ATP-mediated pro-inflammatory environment, facilitated through adenosine[176]. Finally, a subset of CD35+TSP-1+ $B_{regs}$ have been shown to promote $T_{reg}$ differentiation via TGF-β production and down-regulation of co-stimulatory molecules on dendritic cells[177]. Upon adoptive transfer of CD35+TSP-1+ $B_{regs}$, they demonstrated the ability to suppress allergic inflammation in the intestine[177]. Thus, although there is still no consensus on phenotypic characterization of $B_{regs}$, especially within allergic asthma literature, these cells have been shown to modulate inflammatory processes and require future studies.

**1.5 Summary**

Overall, data shows that B cells have functions with the potential to influence the severity of allergic asthma through various mechanisms: (1) production of inflammatory mediators[68,69,107,178] and (2) regulation[71,72,157,179] (Figure 1.5). A study by Kidney et al showed that B cells are elevated in the airways of allergic asthmatics[180], but not much more is known about the function and kinetics of different subpopulations of B cells in the pathobiology of allergic asthma. Thus, the main purpose of this research is to investigate the biology of B cells in subjects with allergic asthma, and the response of B cell subsets to allergic stimulation of the airways. Further knowledge on the kinetics and functions of IgE+ B cells and $B_{regs}$ after allergic stimulation in subjects with allergic asthma would provide further insight on the role B cells play in allergic asthma pathogenesis (Figure 1.6).

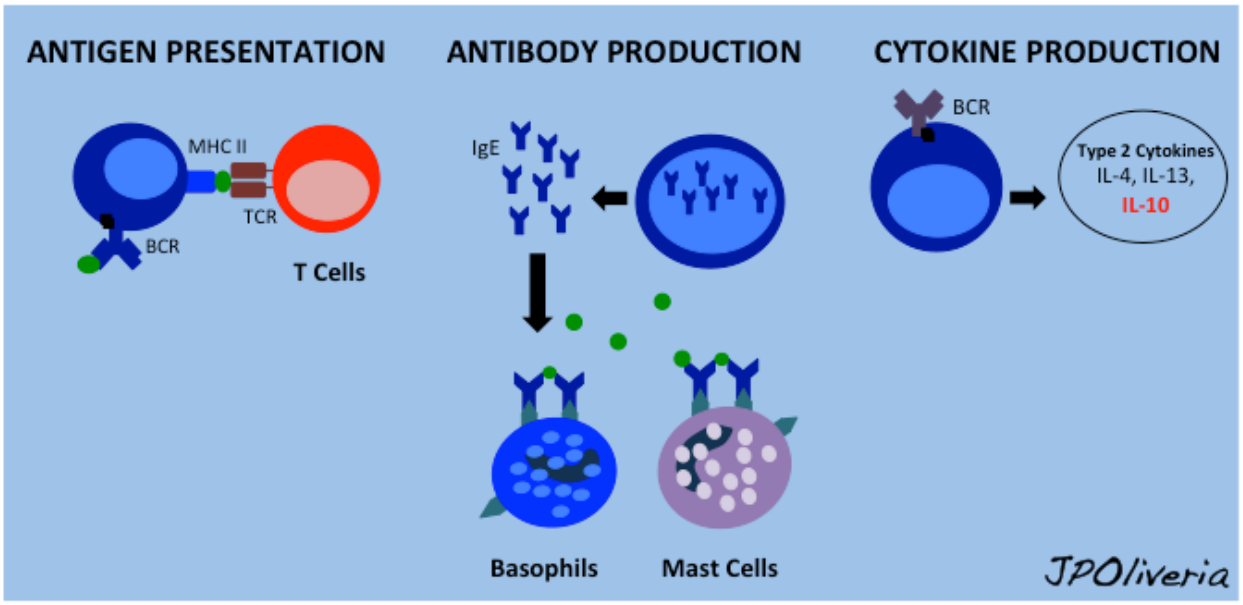


**Figure 1.5: B cell functions in allergic disease.** Canonically, B cells have been shown to have the ability to present antigens to T cells via MHC class II molecules. However, they are more commonly known to be able to produce IgE, a molecule important in initiating the allergic cascade. Lastly, B cells have been shown to have the ability to produce type 2 cytokines, such as: IL-4 and IL-13; however, they have been recently implicated to produce IL-10, a cytokine involved in dampening the allergic cascade.

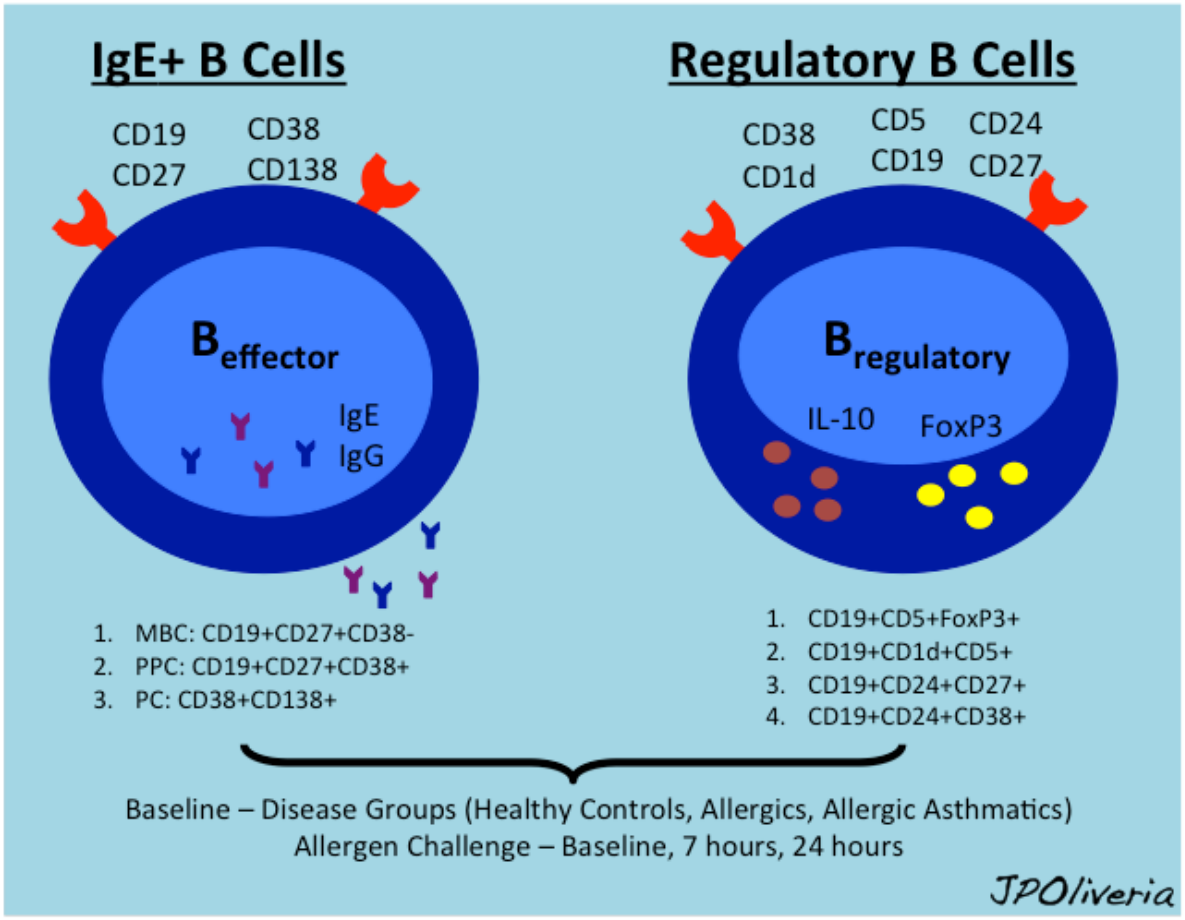


**Figure 1.6: IgE+ B cells and regulatory B cells.** This dissertation aims to evaluate IgE+ B cells and regulatory B cells in allergic asthma pathogenesis. These cells will be evaluated at baseline levels in allergic asthmatics, and non-asthmatics with and without allergies. Additionally, IgE+ B cells and regulatory B cells will be evaluated in allergic asthmatics following allergen inhalation challenge.

## 1.6 Central Hypothesis, Specific Hypotheses and Specific Aims

**CENTRAL HYPOTHESIS**

**The pathogenesis of allergic asthma is regulated by an imbalance in IgE+ B cells and regulatory B cells.**

**SPECIFIC HYPOTHESES AND AIMS**

**Specific Hypothesis 1: IgE+ B cells and regulatory B cells vary in frequency in allergic asthma compared with healthy individuals (Chapter 2 and 4)**

Aim 1a: To investigate the frequency of IgE+ B cells in allergic asthmatics

Aim 1b: To evaluate compartmental differences in the frequency IgE+ B cells (PB and sputum)

Aim 2a: To investigate the frequency of regulatory B cells in allergic asthmatics

Aim 2b: To evaluate compartmental differences in the frequency of regulatory B cells (PB and sputum)

**Specific Hypothesis 2: Allergen inhalation challenge increases IgE+ B cell numbers and function in subjects with allergic asthma - (Chapter 3)**

Aim 1: To determine the kinetics of IgE+ B cells following allergen inhalation challenge

Aim 2: To quantify the magnitude of total and specific IgE in PB serum and sputum supernatant following allergen inhalation challenge

Aim 3: To evaluate compartmental differences in the frequency of IgE+ B cells (PB, BM, tonsil, sputum)

**Specific Hypothesis 3: Allergen inhalation challenge reduces regulatory B cell in subjects with allergic asthma - (Chapter 4)**

Aim 1: To determine the kinetics of regulatory B cells following allergen inhalation challenge

Aim 2: To evaluate compartmental differences in the frequency of regulatory B cells (PB, BM, sputum)

Aim 3a: To evaluate the levels of regulatory B cells following stimulation with IL-4

Aim 3b: To determine the proportions of FoxP3+ and IL-10+ B cells following stimulation with IL-4

## CHAPTER 2: Allergic asthmatics with allergy have elevated levels of IgE+ B cells in the airways



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### CAPSULE SUMMARY

The levels of B cells, IgE+ B cells, IgE+ memory B cells and IgE+ plasma cells are higher in the airways, but not in the blood of allergic asthmatic subjects compared to non-asthmatic controls. Airway IgE+ B cells are positively correlated with airway eosinophils and total IgE levels.

**ABSTRACT**

**Background:** IgE is important for allergen-mediated inflammation. B cells express IgE, including memory B cells (MBC) and plasmablast (PPC) intermediates, which differentiate into IgE-producing plasma cells (PC). However, the comparative levels of IgE+ B cells in blood and sputum of allergic asthmatics and controls remain to be elucidated.

**Objectives:** This study compared the levels of IgE+ B cell subsets in the blood and sputum of allergic asthmatics, and non-asthmtic controls with and without allergies.

**Methods:** Allergic asthmatics (n=18), allergic non-asthmatics (n=19) and healthy controls (n=10) donate blood and sputum samples. Cells from blood and sputum were isolated and stained with fluorescent antibodies to detect IgE+ B cell subsets using flow cytometry.

**Results:** The levels of CD19+ B cells and IgE+ B cell subsets in sputum were higher in allergic asthmatics compared to non-asthmatics with and without allergies; however, there were no difference between groups in the blood. Furthermore, in sputum, IgE+ memory B cells and IgE+ plasma cells were higher in allergic asthmatics compared to controls. Lastly, IgE+ B cells in sputum were positively correlated with sputum eosinophils and total IgE and B cell activating factor (BAFF) measured in sputum fluid phase.

**Conclusions:** Allergic asthmatic subjects have higher levels of IgE+ B cell subsets, BAFF and total IgE in their airways compared to control groups, where IgE+ B cells positively correlate with eosinophils in the airways. Taken together, the data suggests that IgE+ B cells may contribute to local production of IgE, and in turn, maybe lead to the development of eosinophilic airway inflammation in allergic asthma.

*To the editor:*

Allergic asthma is an IgE-mediated disease whereby inhaled allergens cross-link membrane-bound IgE on the surfaces of mast cells and basophils, inducing the activation and release of pro-inflammatory mediators, and driving type-2 inflammation and the manifestation of clinical symptoms[1]. Since B cells solely produce IgE, it is evident that these cells play a crucial role in initiating allergen-induced inflammatory processes.

The level of IgE+ B cells in the airways of asthmatics has not been examined previously. There have been reports of increased allergen-specific IgE in the blood of allergic asthmatics approximately one month after whole-lung allergen challenge[2], and in bronchoalveolar lavage fluid 24-hours after segmental allergen challenge[3], suggesting allergen exposure to the airways induces an acute localized accumulation of allergen-specific IgE, followed by a persistent systemic increase. We sought to quantify the level of IgE+ B cells in the airways and blood of subjects with allergic asthma to delineate their role in local inflammation in the airways.

The three groups of subjects recruited for the study were all 18 to 65 years of age and non-smokers with either allergic asthma (n=18), or allergies without asthma (n=19), or they were healthy controls without allergies or asthma (n=10) (Table E2.1). No subjects required medication for treatment of asthma or allergies and all subjects provided written informed consent. Each subject provided a sample of blood and induced sputum. Sputum cells were extracted from mucus plugs, and surface-stained with antibodies to CD45, CD19, CD27, CD138, or CD4, CD8; while peripheral blood monomuclear cells (PBMCs) were isolated from blood were surface-stained with antibodies to CD45, CD19, CD27, CD138, CD38. Sputum cells and PBMCs were stained intracellularly with antibodies to IgE and IgG. Cells were acquired with a Becton Dickenson LSR II flow cytometer, and cell analyses were completed using FlowJo software. The total IgE level in serum, and total IgE and B cell activating factor (BAFF) in sputum fluid-phase were measured using a commercially available ELISA kit. The

subject criteria, sample processing, cell staining procedures and gating strategies are detailed in the Supplementary Methods and Fig E2.1. All data are presented as mean±SEM.

When expressed as a percentage of total lymphocytes, the frequency of B cells in the sputum of asthmatic subjects was 11.8±2.0%, which was over 2-fold higher than 5.4±0.9% in allergic subjects and 4.9±0.6% in controls (Fig 2.1A, $p<0.05$). The frequency of IgE+ B cells expressed as a percentage of total B cells in sputum was 26.2±3.2% in asthmatic subjects, which was over 3-fold higher than 7.3±1.8% in allergic subjects and 4.5±1.4% in controls (Fig 2.1B, $p<0.05$). Furthermore, the frequency of IgE+ memory B cells and IgE+ plasma cells, expressed as a percentage of total B cells in sputum was also higher in asthmatic subjects compared to the non-asthmatic subjects (Fig 2.1C-D, $p<0.05$). The level of sputum BAFF was elevated in asthmatic subjects compared to non-asthmatic subjects (Table E2.1),

In blood, there was no difference in the frequency of B cells or IgE+ B cell subsets across groups (Fig 2.1E-H, $p>0.05$). When B cells were expressed as absolute number of cells per-milliliter of blood, or per-gram of sputum, we found higher levels of IgE+ B cell subsets in the airways of asthmatic subjects compared to the other groups (Fig E2.2A-D, $p<0.05$), with no differences detected in blood (Fig E2.2E-H, $p>0.05$). In contrast, we did not observe any difference in the level of sputum memory B cells, plasma cells, CD4+T-cells, CD8+T-cells or total lymphocytes between groups, nor was there a difference in blood between groups (Fig E2.3-E2.5, $p>0.05$). The frequency and absolute numbers of IgG+ B cells, evaluated as a control cell population, showed a higher tendency, but no statistical difference in asthmatics compared to the non-asthmatic subjects when measured in the sputum or blood (Fig E2.6, $p>0.05$). Furthermore, a positive correlation between IgE+ and IgG+ B cells were found in sputum (Fig E2.6C, $r=0.36$, $p=0.08$) and blood (Fig E2.6F, $r=0.39$, $p=0.03$).

In sputum, the frequency of IgE+ B cells positively correlated with eosinophils (Fig 2.2A, $r=0.65$, $p=0.0005$), sputum total IgE levels (Fig 2.2B, $r=0.68$, $p=0.0002$), and sputum BAFF levels (Fig

2.2C, r=0.42, p=0.04). The frequency of sputum B cells also positively correlated with sputum eosinophils (Fig 2.2D, r=0.60, p=0.002) and sputum BAFF levels (Fig 2.2F, r=0.47, p=0.02). There was no relationship between blood B cells and IgE+ B cells, and plasma total IgE and blood eosinophils (Fig E2.7, p>0.05).

Kidney et al reported higher a sputum level of B cells in asthmatics (16.5±3.4% of lymphocytes) compared to non-asthmatic smokers (4.0±1.1% of lymphocytes)[4]; however, no further reports on human B cells have been published since. Using a more stringent panel of B cell markers, the current study found similarly elevated levels of B cells in asthmatic subjects (11.8±2.0% of lymphocytes), thereby corroborating previous findings.

Historically, IgE+ B cells have been difficult to detect in blood, however methodological advances including availability of fluorochrome-labeled antibodies and flow cytometers with more lasers has allowed for B cell subsets to be measured. Recent papers have reported allergen-specific IgE+ B cells measured in the blood[5], nasal biopsies and nasal lavage of patients with allergic rhinitis[6,7]. With our methods we were able to consistently detect IgE+ B cells in the blood and sputum of our subjects, which allowed us to show, for the first time, significantly higher levels of IgE+ B cell subsets in the sputum of asthmatics compared to non-asthmatic controls. These findings, along with higher levels of sputum BAFF in our allergic asthmatic subjects and a positive correlation between sputum BAFF, and B cells and IgE+ B cells, support a mechanism whereby local maturation and proliferation of B cells occur in the airways of allergic asthmatic subjects[8], and together with IL-4 and IL-13[9], drives local increases in IgE+ B cell subsets and IgE levels.

We also observed a positive correlation between sputum eosinophils, and B cells and IgE+ B cells, supporting the findings of Kidney et al that described a relationship between sputum eosinophils and B cells[4]. We also found a positive correlation between sputum total IgE levels and IgE+ B cells,

indicating that IgE-secreting B cells in the airways, but not in the blood, are directly related to IgE levels[10].

The results of this study highlight the close relationship between airway IgE+ B cells, local production of IgE, and the development of eosinophilic airway inflammation in allergic asthma. We propose that the findings of this study support the development of therapeutic strategies targeting IgE+ B cells for treatment of asthma.

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**TABLES AND FIGURES**

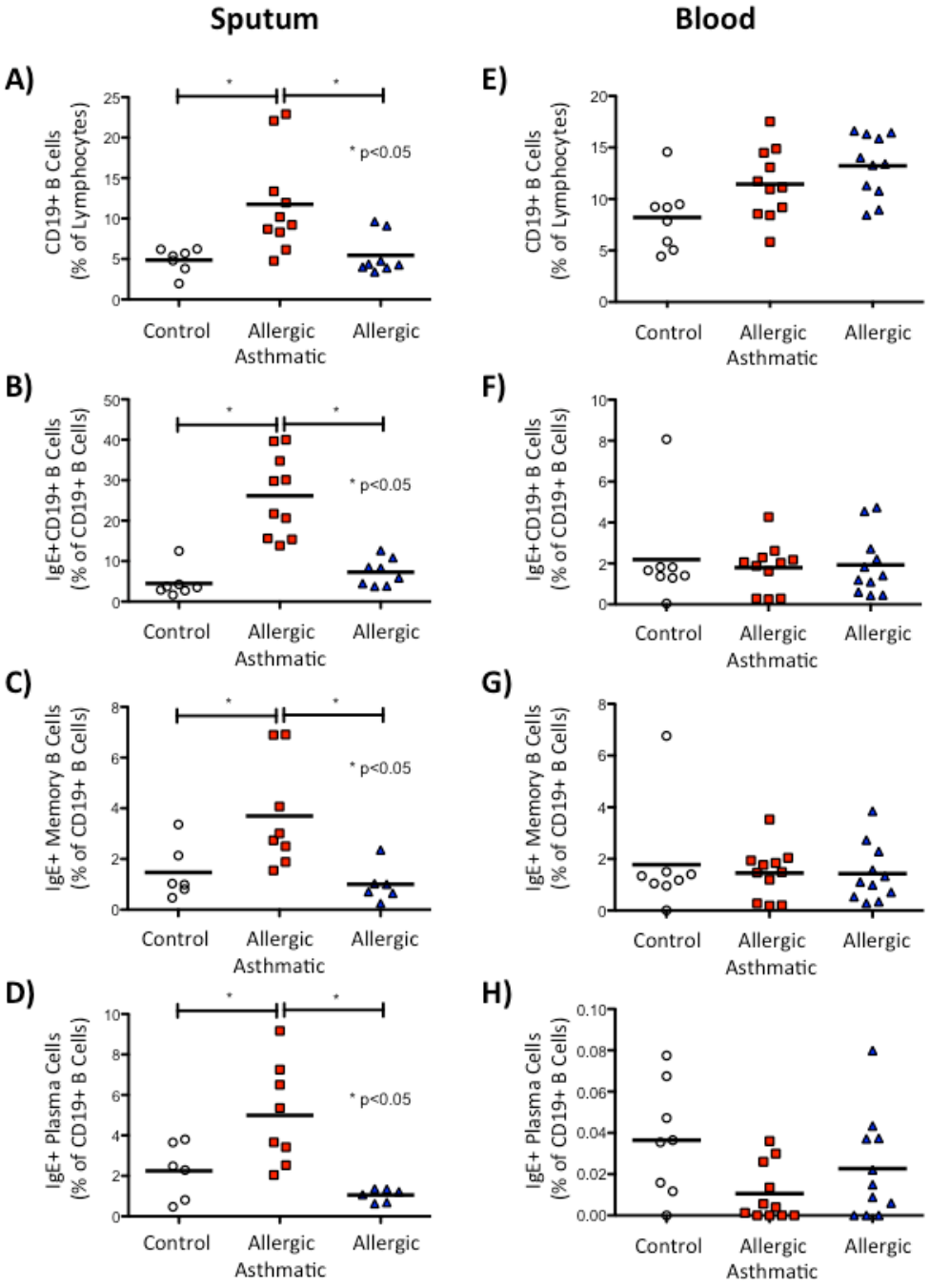



**Figure 2.1: The levels of B cells, IgE+ B cells, and IgE+ B cell subsets (memory B cells and plasma cells) in the sputum and blood of subjects with allergic asthma and non-asthmatic controls.** Data were analyzed using a 1-way ANOVA with Tukey post-hoc tests and are presented as mean±SEM. In sputum analysis of B cells and IgE+ B cells: control n=7, allergic asthmatic n=10, allergic n=8, while sputum analysis of IgE+ B cell subsets: control n=6, allergic asthmatic n=8, allergic=6. In blood analysis of all cells: control n=11, allergic asthmatic n=11, allergic n=8. Significant findings are denoted by *, where $p<0.05$.

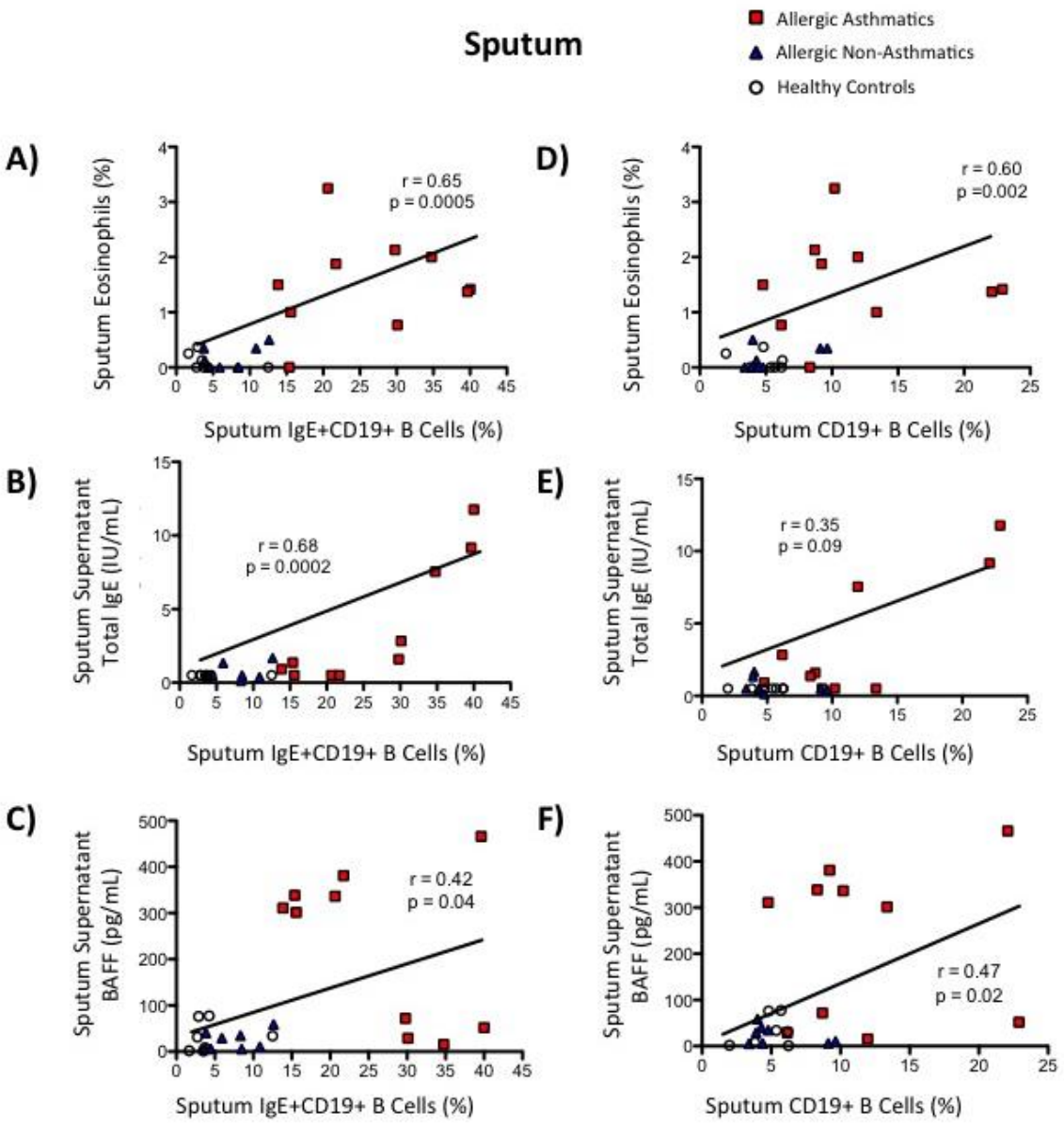



**Figure 2.2: The relationship between B cells and IgE+ B cells, and eosinophils and total IgE and B cell activating factor (BAFF) in sputum of subjects with allergic asthma and non-asthmatic controls.** Data were correlated with Spearman rank r-values and linear regression lines of best fit. In sputum: control n=7, allergic asthmatic n=10, allergic n=8. Exact p-values were denoted, where $p<0.05$ was considered a significant correlation.

## SUPPLEMENTARY METHODS

### Subject Criteria and Study Design

Allergic asthmatics (n=18), allergic non-asthmatics (n=19) and healthy controls (n=10) all between 18 and 65 years of age were recruited to participate in the study. No subjects required medication for treatment of asthma or allergies. All allergic asthmatic subjects were atopic with mild asthma; the percent predicted forced expiratory volume in one second ($FEV_1$) was ≥70% of predicted, and the methacholine provocative concentration causing at least a 20% fall in $FEV_1$ ($PC_{20}$) ≤16 mg/ml[1]. Subjects were excluded if they had asthmatic exacerbations or respiratory tract infections within four weeks of the study start date, or underlying diseases other than asthma. Participating subjects underwent a screening procedure to determine allergic status by skin prick test, and lung function by spirometry. All subjects provided written informed consent. Subjects donated peripheral blood and induced sputum.

### Clinical Tests

Spirometry was performed following ATS guidelines[2]. Skin prick test was performed using common aeroallergens and histamine and saline as positive and negative controls, respectively. A wheal diameter of >2mm was considered to be positive[3–5]. A methacholine challenge test using tidal breathing from a Wright Nebuilizer was completed to determine the provocative concentration that resulted in a 20% fall in $FEV_1$ ($PC_{20}$)[6].

### Sample Collection and Cell Processing

Sputum was induced by inhalation of 3, 4 and 5% saline and mucous plugs were selected and processed as previously described[7,8]. Briefly, sputum was treated with Dulbecco's phosphate buffered saline (DPBS) and dithiothreitol (DTT) and filtered through a nylon mesh, the cells were pelleted by centrifugation. Two cytospins were prepared and stained with Diff Quik for cell differentials and the

remaining cells were stored at 4°C flow cytometry staining. The fluid phase was frozen for measurements of total IgE.

Peripheral blood was collected into vacutainers containing heparin. A total cell count was performed using a hemocytometer and smears were prepared and stained with Diff Quik for cell differentials. A portion of the blood was centrifuged at 1000 g for 10 minutes to collect plasma for measurement of total IgE levels. The remaining blood was diluted with 10% fetal bovine serum (FBS) Roswell Park Memorial Institute (RPMI) buffer, layered on AccuPrep density gradient,and then centrifuged at 800 g for 20 minutes without a break. The peripheral blood mononuclear cell (PBMC) layer was collected and stored at 4°C for flow cytometry staining.

**Cell Staining and Gating Strategy for B Cells**

Cells were stained with fluorochrome-conjugated antibodies for flow cytometry analysis of B cells and B cell subsets. Cells were initially washed twice with FACS buffer. Antibodies to surface markers were incubated with the cells for 30 minutes, and the cells were washed, fixed and permeabilized. Antibodies to intracellular markers were then incubated with the cells for 30 minutes. Appropriate isotypes were used as negative controls. The cells were acquired using a Beckton Dickinson (BD) LSRII flow cytometer (BD Bioscience, ON, CAN) and the files were analyzed with the FlowJo software (TreeStar, OR, USA).

Sputum cells were surface-stained with antibodies to CD45 (APC-H7), CD19 (PE-Cy7), CD27 (V450), CD138 (FITC), CD4 (APC-H7), CD8 (FITC) and intracellularly stained with IgE (PE) and IgG (APC). From the forward scatter (FSC) and side scatter (SSC) plot, a gate was placed around all the cells, excluding debris. SSC and CD45 were then plotted and a gate was placed around the lymphocyte population ($SSC^{low}CD45+$). CD45 and CD19 were then plotted and a gate was placed around the CD19+ population. CD19+ B cells were used to plot CD19 with IgE and IgG to isolate IgE+ and IgG+ B cells in the airways. Furthermore, CD19+ B cells were used to plot CD19 with CD27 and

CD138 to isolate memory B cells (MBCs) and plasma cells (PCs), respectively. IgE+ MBCs and PCs were also enumerated in the airways.

PBMCs were surface-stained with CD45 (APC-H7), CD19 (PE-Cy7), CD27 (V450), CD138 (FITC), CD38 (PE) and intracellularly with IgE (APC) and IgG (APC). The following was the gating strategy used to enumerate memory B cells (MBCs) (CD27+CD38-), plasmablasts (PCs) (CD27+CD38+) and plasma cells (PCs) (CD38+CD138+) from peripheral blood. From the forward scatter (FSC) and side scatter (SSC) plot, a gate was placed around the lymphocyte population. SSC and CD19 were then plotted and a gate was placed around the CD19+ population. SSC and CD27 were plotted and a gate was placed around the CD27+ population. CD38 and CD27 were then plotted and this was used to obtain each of the required phenotypes, MBCs and PPCs. MBCs and PPCs were then used to plot CD19 with IgE and IgG isolate IgE+ and IgG+ MBCs and PPCs in circulation. Furthermore, CD19+ B cells were used to plot CD38 and CD138 and this was used to obtain PCs. PCs were then used to plot CD19 with IgE and IgG to isolate IgE+ and IgG+ PCs in blood.

For a representative gating strategy in sputum, refer to Fig E2.1.

**Total IgE and BAFF Measurements**

Total IgE (Abcam, ON, CAN) was measured in plasma and sputum fluid and BAFF (R&D Systems, MN, USA) was measured in sputum fluid phase using commercially available sandwich, enzyme linked immunosorbent assay (ELISA) kits.

**Statistical Analyses**

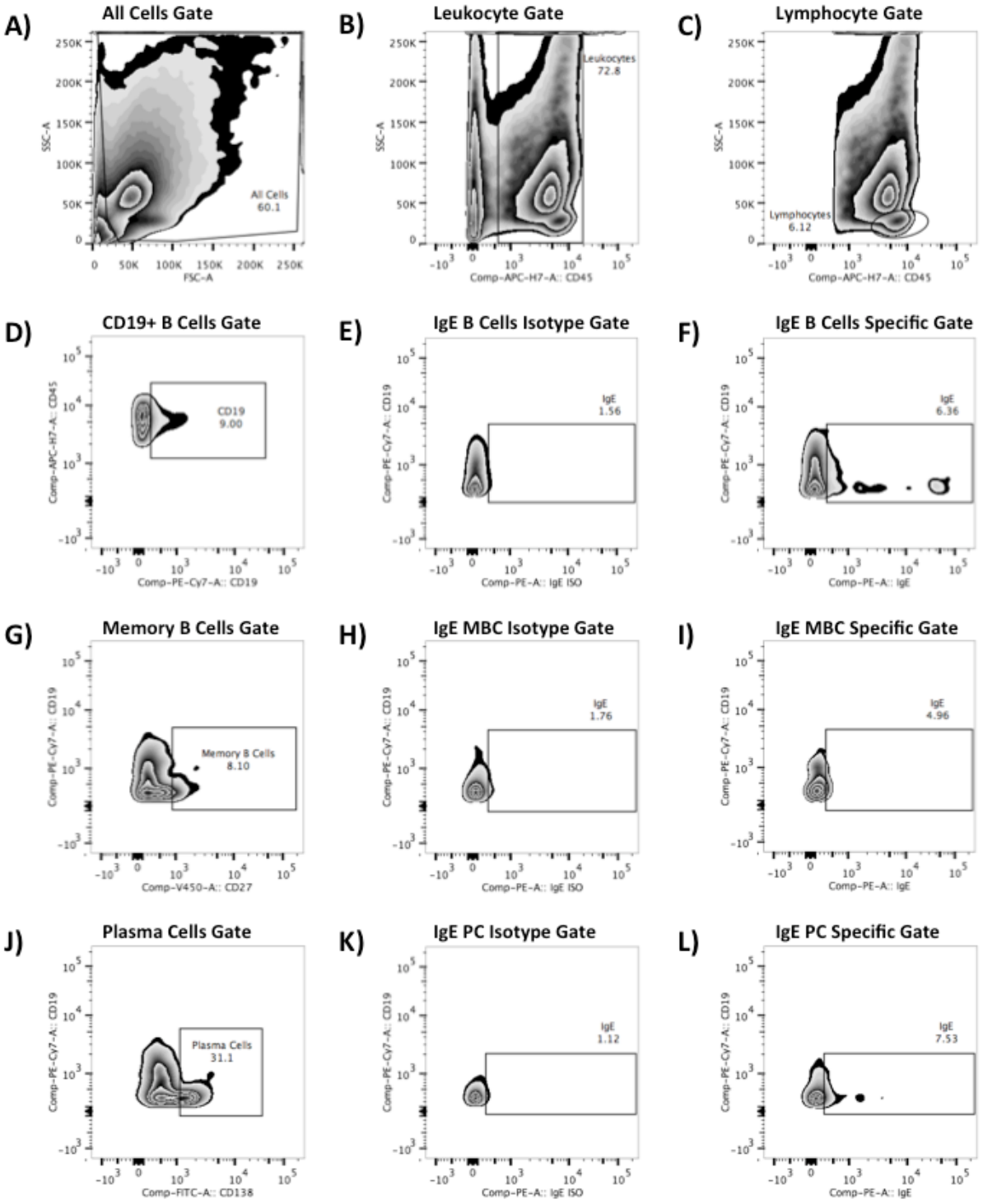
Data were presented as the mean±SEM with the exception of methacholine $PC_{20}$ presented as geometric mean (95% confidence interval). Airway physiology, sputum and blood cells (frequencies and magnitudes), and total IgE and BAFF levels were analyzed using 1-way ANOVA with Tukey post-hoc tests. In cases where total IgE in sputum fluid phase was not detected, we assigned the lower limit of detection (0.5 IU/mL). Spearman rank tests were used to evaluate the relationship between CD19+ B

cells and IgE+ B cells, and eosinophils, total IgE, and BAFF in the airways. Significance was accepted at $p<0.05$.

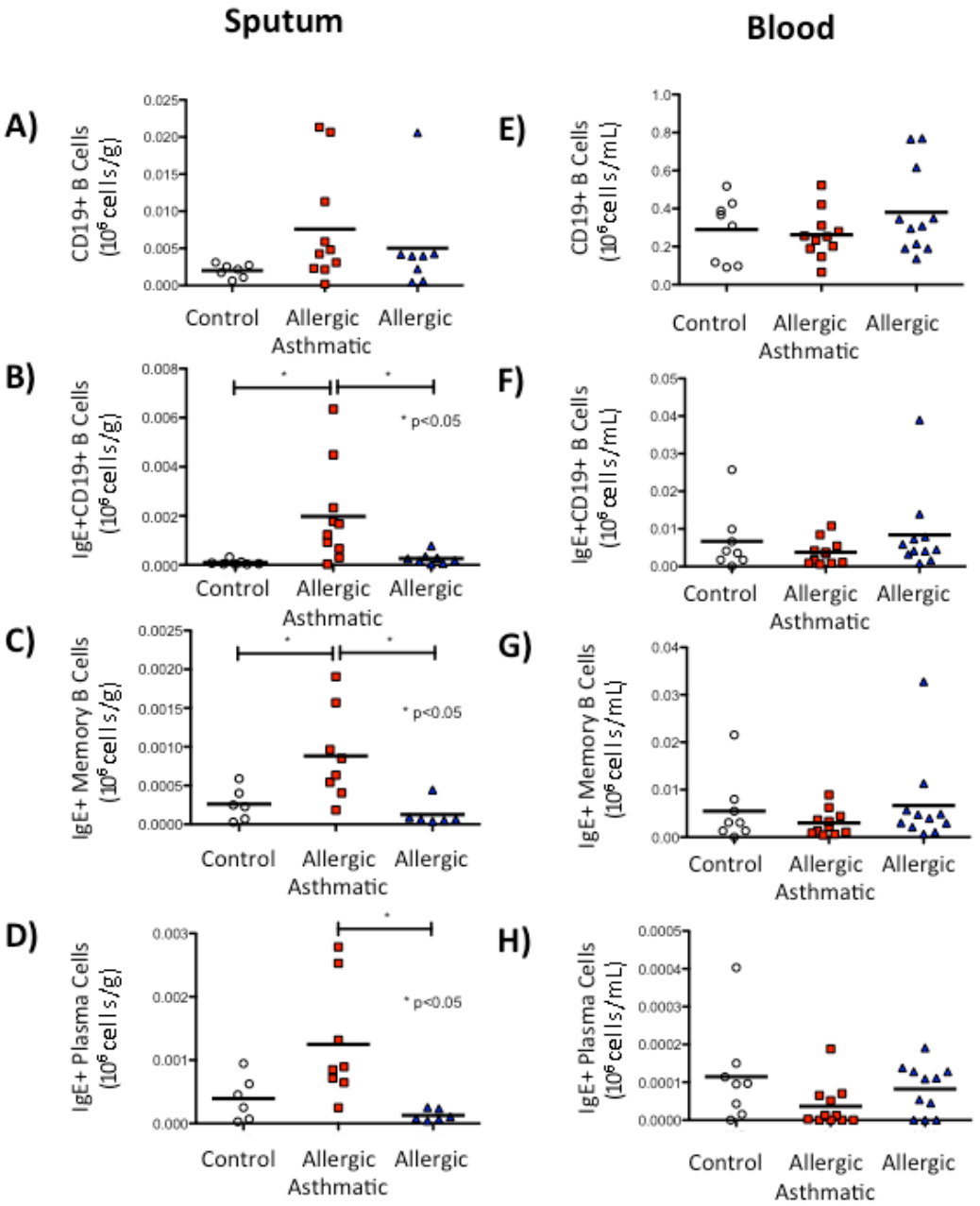
## SUPPLEMENTARY TABLES AND FIGURES

**Supplementary Table E2.1: Subject demographics.** Data are shown as mean±SEM, except methacholine $PC_{20}$, which is shown as geometric mean (95% CI). Significant findings are denoted by * where $p<0.05$ compared to healthy control subjects and † where $p<0.05$ compared to allergic non-asthmatic subjects.

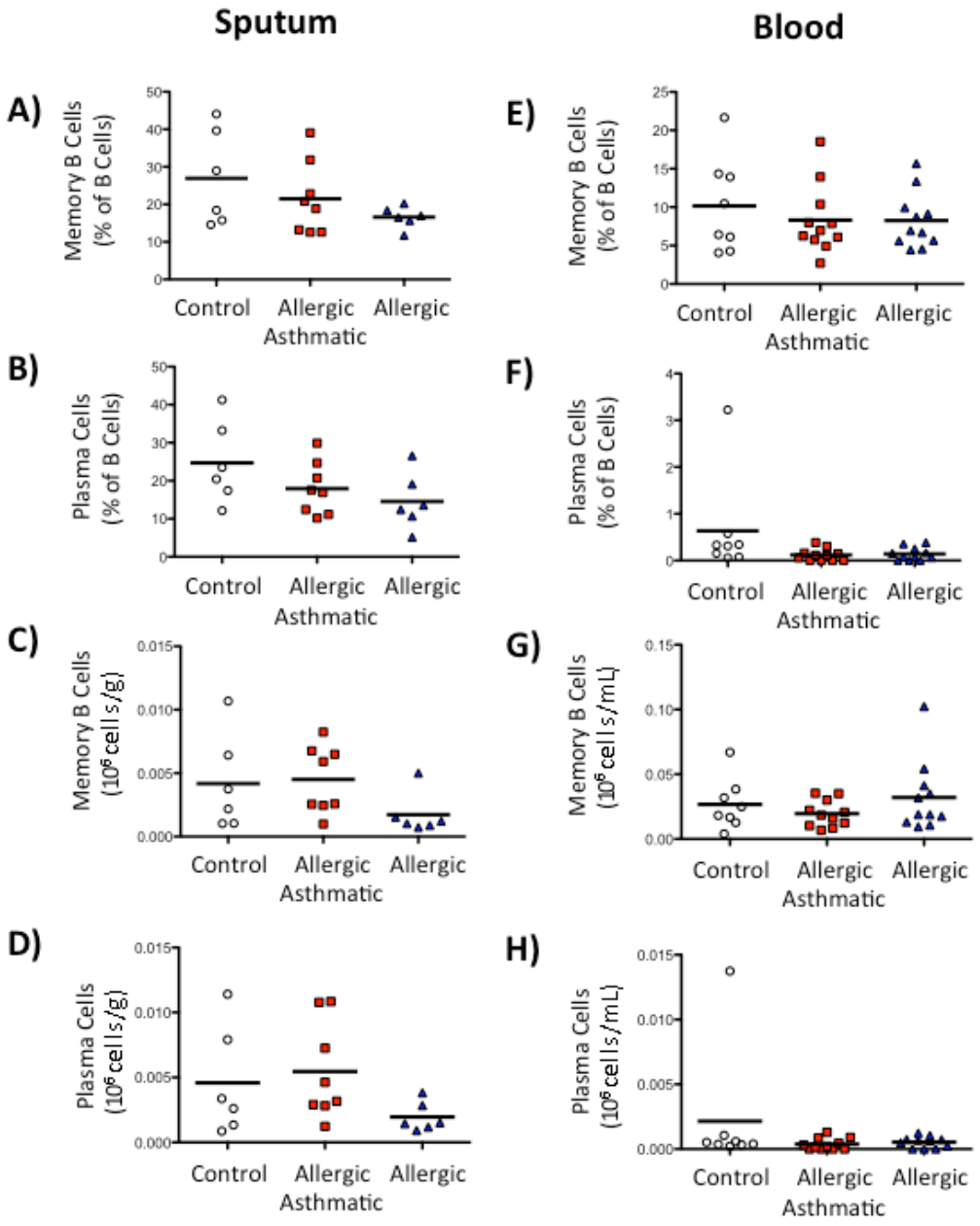
| | Controls (n=10) | Allergic Asthmatics ( n=18) | Allergic (n=19) |
|---|---|---|---|
| **Sex (M/F)** | 4/6 | 8/10 | 10/9 |
| **Age (years)** | 37.1±3.8 | 31.8±3.3 | 26.2±2.4 |
| **$FEV_1$ (% of predicted)** | 94.9±2.6 | 91.6±1.6 | 98.2±2.8 |
| **Methacholine $PC_{20}$ (mg/mL)** | >16 | 3.7 (2.2, 6.4) *† | >16 |
| **Sputum Eosinophils (%)** | 0.08±0.04 | 1.8±0.3*† | 0.6±0.1 |
| **Sputum Total IgE (IU/mL)** | 0.5±0.0 | 3.7±1.3* | 0.8±0.2 |
| **Sputum BAFF (pg/mL)** | 32.8±12.3 | 230.3±53.4*† | 24.0±7.0 |
| **Blood Eosinophils (%)** | 1.8±0.4 | 4.8±0.5* | 3.2±0.4 |
| **Plasma Total IgE (IU/mL)** | 19.8±3.4 | 288.7±34.3*† | 107.6±29.9 |
| **Skin test positive to:** | | | |
| Cat Dander | 0 | 15 | 12 |
| Horse Dander | 0 | 12 | 5 |
| House Dust Mite | 0 | 17 | 16 |
| Trees | 0 | 14 | 6 |
| Grass | 0 | 11 | 6 |
| Ragweed | 0 | 14 | 8 |



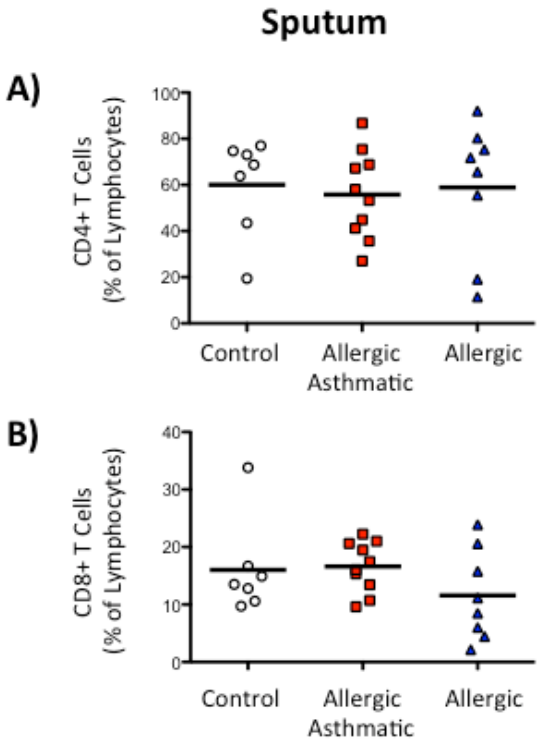

**Supplementary Figure E2.1: Representative gating strategy for IgE+ B cell subsets with isotype controls in sputum.**



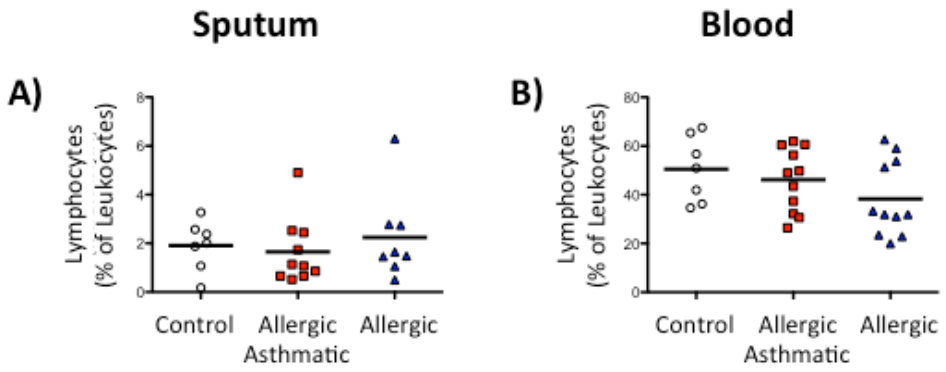

**Supplementary Figure E2.2: The number of B cells, IgE+ B cells and IgE+ B cell subsets (memory B cells and plasma cells) in the sputum and blood of subjects with allergic asthma and non-asthmatic controls.** Data were analyzed using a 1-way ANOVA with Tukey post-hoc tests and are presented as mean±SEM. In sputum analysis of B cells and IgE+ B cell numbers: control n=7, allergic asthmatic n=10, allergic n=8, while sputum analysis of IgE+ B cell subset numbers: control n=6, allergic asthmatic n=8, allergic=6. In blood analysis of all cell numbers: control n=11, allergic asthmatic n=11, allergic n=8. Significant findings are denoted by *, where $p<0.05$.



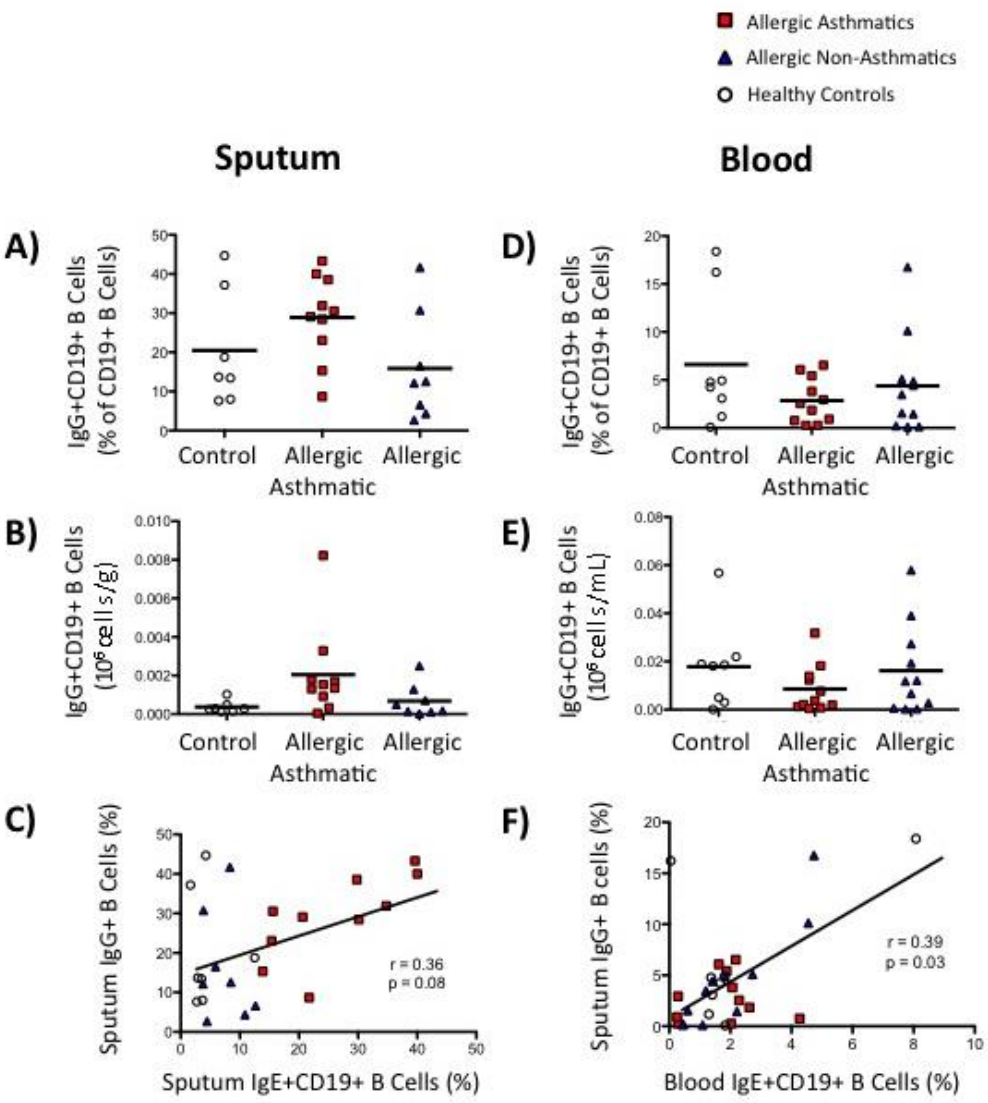

**Supplementary Figure E2.3: The frequency and number of memory B cells and plasma cells in the sputum and blood of subjects with allergic asthma and non-asthmatic controls.** Data were analyzed using a 1-way ANOVA with Tukey post-hoc tests and are presented as mean±SEM. In sputum: control n=6, allergic asthmatic n=8, allergic n=6. In blood: control n=11, allergic asthmatic n=11, allergic n=8. Significant findings are denoted by *, where $p<0.05$.



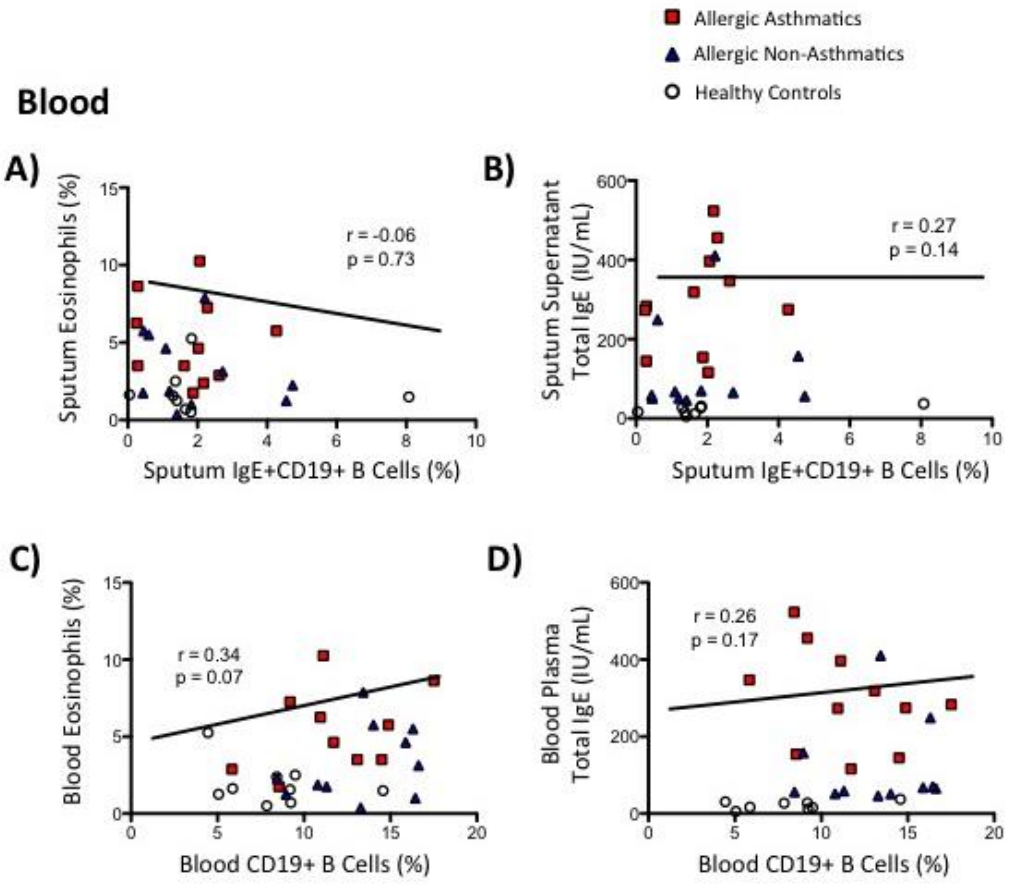

**Supplementary Figure E2.4: The frequency of CD4+ T cells and CD8+ T cells in the sputum of subjects with allergic asthma and non-asthmatic controls.** Data were analyzed using a 1-way ANOVA with Tukey post-hoc tests and are presented as mean±SEM. In sputum: control n=7, allergic asthmatic n=10, allergic n=8. Significant findings are denoted by *, where $p<0.05$.




**Supplementary Figure E2.5: The level of lymphocytes in blood and in the sputum of subjects with allergic asthma and non-asthmatic controls.** Data were analyzed using a 1-way ANOVA with Tukey post-hoc tests and are presented as mean±SEM. In sputum: control n=7, allergic asthmatic n=10, allergic n=8. In blood: control n=11, allergic asthmatic n=11, allergic n=8. Significant findings are denoted by *, where $p<0.05$.




**Supplementary Figure E2.6: The frequency and numbers of IgG+ B cells and correlation with IgE+ B cells in the sputum and blood of subjects with allergic asthma and non-asthmatic controls.** Data were analyzed using a 1-way ANOVA with Tukey post-hoc tests and are presented as mean±SEM. Correlation data are presented as correlations with Spearman rank r-values and linear regression lines of best fit. In sputum: control n=7, allergic asthmatic n=10, allergic n=8. In blood: control n=11, allergic asthmatic n=11, allergic n=8. Significant findings are denoted by *, where $p<0.05$.




**Supplementary Figure E2.7: The relationship between B cells and IgE+ B cells, and eosinophils and total IgE in blood of subjects with allergic asthma and non-asthmatic controls.** Data are presented as correlations with Spearman rank r-values and linear regression lines of best fit. In blood: control n=11, allergic asthmatic n=11, allergic n=8. Exact p-values were denoted, where $p<0.05$ was considered a significant correlation.

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## CHAPTER 3: Increased IgE+ B cells in sputum, but not in blood, bone marrow or tonsils, after inhaled allergen challenge in asthmatic subjects



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### CAPSULE SUMMARY

Exposure to allergen and the release of type 2 cytokines by immune cells are known to induce IgE class-switching of B cells in murine models of asthma. However, the B cell response to inhaled allergen in allergic asthmatic individuals is poorly understood. The frequency of IgE+ B cells is elevated in the airways after allergen inhalation in allergic asthmatics, and increases in CD19+ B cells correlated with worsened airway hyperresponsiveness and increased eosinophilia. This suggests localized inflammation in allergic airway is driven by B cells.

**ABSTRACT**

**Background:** Allergen exposure and resultant type 2 cytokines are known to induce IgE class-switching of B cells in murine models of asthma, but the B cell response to inhaled allergen in allergic asthmatic individuals is poorly understood.

**Objectives:** The current study measured IgE+ B cells in airways, blood, bone marrow and tonsils following an inhaled allergen challenge.

**Methods:** Nineteen subjects with mild allergic asthma were challenged with inhaled allergen extract and diluent control in random order. Samples of sputum, blood, bone marrow and tonsil were collected before and until 28 days after inhalation challenges for measurement of IgE+ and IgG+ B cell subsets using flow cytometry.

**Results:** Inhaled allergen induced a mean±SEM fall in $FEV_1$ of 31.2±2.0% and 13.1±1.9% during the early and late asthmatic responses, respectively. Allergen inhalation increased the frequency of IgE+ B cells (expressed as a percentage of total B cells) in the airways from 11.3±2.0% at baseline, to 38.6±5.8% and 19.6±3.8% at 7 and 24 hours post-allergen, respectively, in association with increased levels of airway total IgE and BAFF. There was no change in the frequency of IgE+ memory B cells, plasmablasts or plasma cells measured in peripheral blood, bone marrow or tonsils.

**Conclusions:** Allergen inhalation in mild allergic asthmatics subjects increased IgE+ B cells and total IgE in the airways without corresponding changes in the circulation, hematopoeitic or lymphatic compartments. These data suggest that allergen-induced increases in IgE occur through the expansion of local IgE+ B cells.

*To the editor:*

The allergic cascade is critically dependent on allergen-specific IgE. Although exposure to allergen is shown to directly induce IgE class switching in the airway of asthmatics[1,2], it is unknown whether elevated IgE levels[2,3] are due to heightened activity of IgE+ B cells or expansion of the IgE+ B cell population. We evaluated the kinetics of IgE+ B cell subsets in the airways, circulation, hematopoeitic and lymphatic compartments after inhaled allergen challenge (clinicaltrials.gov NCT01420003).

We recruited nineteen, non-smoking, stable (no exacerbations or asthma medications) allergic asthmatic subjects with $FEV_1 \geq 70\%$, and methacholine $PC_{20} \leq 16$ mg/ml[4]. Baseline methacholine $PC_{20}$ was measured and we collected sputum in one subset (n=7), and blood and bone marrow in another (n=12). Subjects inhaled allergen until the $FEV_1$ dropped $\geq 20\%$ from pre-challenge, and $FEV_1$ was measured for 7 hours. Blood and sputum samples were again collected 7 hours, 24 hours, 7 days, 14 days and 28 days post-challenge. Bone marrow was collected 7 days and 28 days post-challenge. These methods have been previously described[5,6]. A tonsil biopsy (n=10 subjects who had tonsils) was collected 28 days post-challenge, mechanically disrupted, incubated in 1% collagenase for 2 hours and filtered through a 100-micrometer filter. Subjects underwent inhaled diluent (control) challenge with all measurements repeated.

Sputum cells were surface-stained with antibodies to CD45, CD4, CD8, CD19 to enumerate B and T cells. Blood and tonsil cells were surface-stained with CD45, CD19, CD38, CD27, CD138 to enumerate memory B cells (CD45+CD19+CD27+CD38-) and plasmablasts (CD45+CD19+CD27+CD38). Bone marrow cells were surface-stained with CD45, CD3, CD19, CD38, CD138 to enumerate plasma cells (CD3-CD45+CD38+CD138+) and plasmablasts (CD3-CD45+CD38+CD138-). All cells were stained intracellularly for IgE (immunoglobulin of interest) and IgG (control). Cells were acquired using a BD LSRII flow cytometer and analyzed with FlowJo

software (TreeStar, OR, USA). CD19+B cells, CD4+ and CD8+T cells were expressed as a percentage of total lymphocytes, while B cell subsets were expressed as a percentage of CD19+B cells. Total IgE and B cell activating factor were measured in sputum, and allergen-specific IgE was measured from serum and sputum.

Allergen inhalation induced early and late asthmatic responses and methacholine $PC_{20}$ decreased 24 hours after allergen challenge (data not shown). Sputum eosinophils (data not shown) and CD4+T cells (Fig 3.1A) increased post-allergen confirming the development of an allergic response in the airways.

The frequency of sputum CD19+B cells increased at 7 hours post-allergen ($p<0.05$) but the frequency of sputum lymphocytes ($SSC^{low}CD45+$) (data not shown) and CD8+T cells (Fig 3.1A) was unchanged. The IgE+ B cell frequency and number/gram of sputum (Fig E3.2) increased at 7 hours post-allergen ($p<0.05$) with no change in IgG+ B cells in this small sample size (Fig 3.1B). In sputum, the levels of B cell activating factor increased at 7 and 24 hours post-allergen (both $p<0.05$, Fig 3.2A). Total IgE increased at 7 hours post-allergen ($p<0.05$); however, the 138±22% increase in allergen challenge-specific IgE in sputum at 7 hours post-allergen was not different from diluent control (Fig 3.2A).

Baseline frequencies of IgE+ plasma cells and plasmablasts from bone marrow, and IgE+ memory B cells from blood and tonsils were measured to be 0.8±0.1%, 1.0±0.1%, 0.3±0.1% and 0.7±0.3% as a percentage of CD19+ B cells, respectively. There were no changes in these cell frequencies after allergen challenge compared to diluent (Fig E3.2). Furthermore, in peripheral blood and tonsil tissue the frequency of plasmablasts was too low to quantitate accurately.

Sputum CD19+B cells negatively correlated with methacholine $PC_{20}$ ($r=-0.65$, $p=0.01$) and positively correlated with sputum eosinophils ($r=0.53$, $p=0.049$) and CD8+T cells and $r=0.61$, $p=0.02$). Sputum IgE+ B cells positively correlated with sputum CD4+ T cells ($r=0.63$, $p=0.02$).

Short-lived IgE+ B cells transiently increase in the lungs, spleen and bone marrow of mice 3 days after OVA-inhalation challenge, and long-lived IgE+ B cells increase in the bone marrow 30 days after challenge[7]. We found an increased frequency of IgE+ B cells in the sputum after allergen inhalation but did not observe changes in tonsils, bone marrow or peripheral blood. These different findings could be due to the length of exposure to antigen, whereby ongoing exposure in allergic asthmatic subjects may induce higher underlying B cell responses making it more difficult to see changes post-challenge. Furthermore studies from various murine models suggest there are inherent differences in anatomic and dynamic molecular aspects of IgE class-switching that render comparisons between mouse and human to be complex[8].

The co-incident acute increases in IgE+ B cells and total IgE in sputum after allergen inhalation challenge suggests that local signals in the airway microenvironment may lead to a transient local expansion or influx of IgE+ B cell subsets. Inhaled allergen had no acute effect in circulation, lymphatic or hematopoietic compartments, likely because class-switch recombination typically requires approximately 7 to 14 days to occur[9]. The proliferation and expansion of CD19+B cells and IgE+ B cells in the sputum at 7 hours post-allergen also corresponded with increased sputum B cell activating factor levels, a mediator implicated in B cell proliferation and differentiation[10,11] and produced by dendritic cells and airway epithelium[12].

A large proportion of IgE+ B cells, but not IgG+ B cells, are able to differentiate into plasma cells[13]. Our data demonstrates an acute expansion of IgE+ B cells in the sputum corresponding to higher levels of total IgE and B cell activating factor. That allergen inhalation did not increase allergen-specific IgE in the sputum suggests that the increase in total IgE may be a result of polyclonal switching at 7-hours post-allergen challenge. The acute increase in local levels of total IgE and IgE+ B cells, taken together with the delayed increase in allergen-specific IgE in the serum at 14-days post-challenge may be explained by a prolonged time course of class-switch recombination and

differentiation to allergen-specific plasma cells[9]. Together, these results support a mechanism whereby eosinophilic airway inflammation and airway hyperresponsiveness are elevated post-allergen through local expansion of IgE+ B cells.

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**TABLES AND FIGURES**

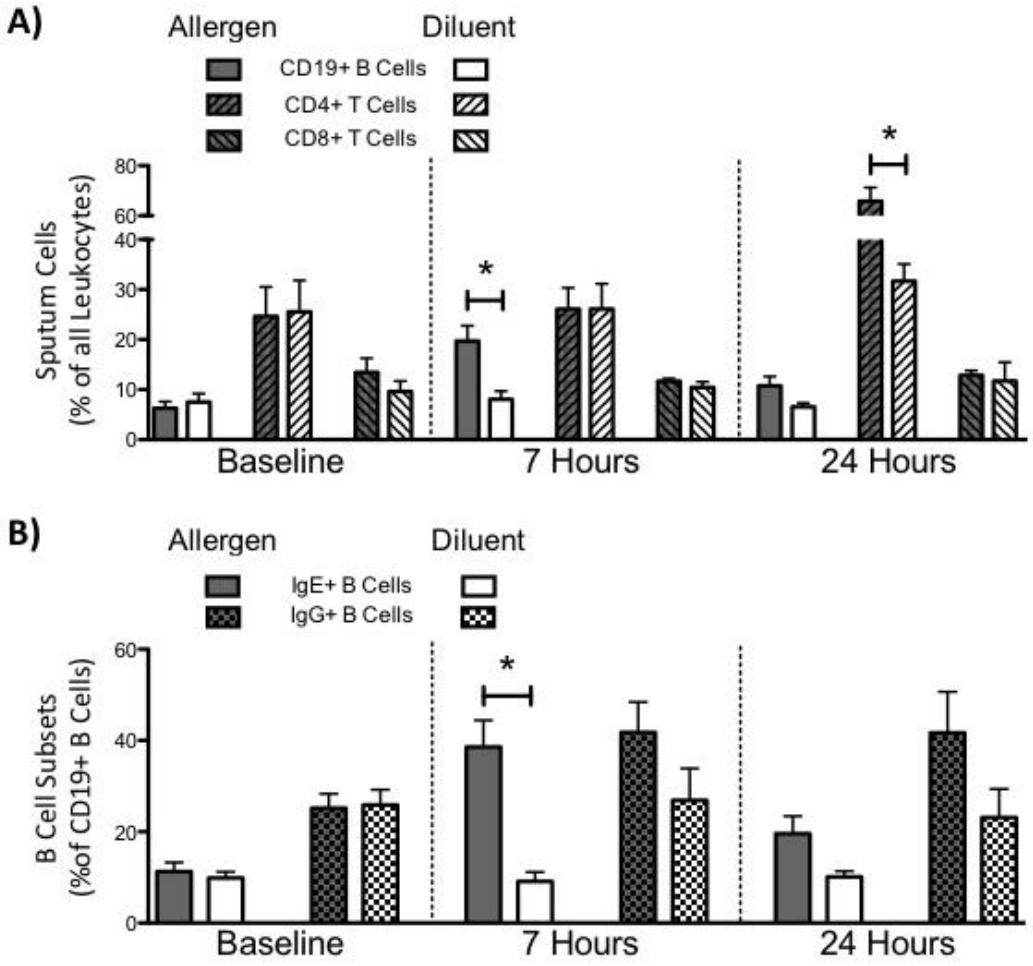



**Figure 3.1: The effects of inhaled allergen on (A) sputum lymphocyte subsets expressed as a percentage of sputum leukocytes, and (B) sputum B-cell subsets expressed as a percentage of sputum B cells, measured at baseline and again at 7 and 24 hours after allergen and diluent challenges (n = 7 subjects).** Data are presented as means±SEM. Significant findings are denoted by an asterisk, where $p<0.05$ compared with diluent.

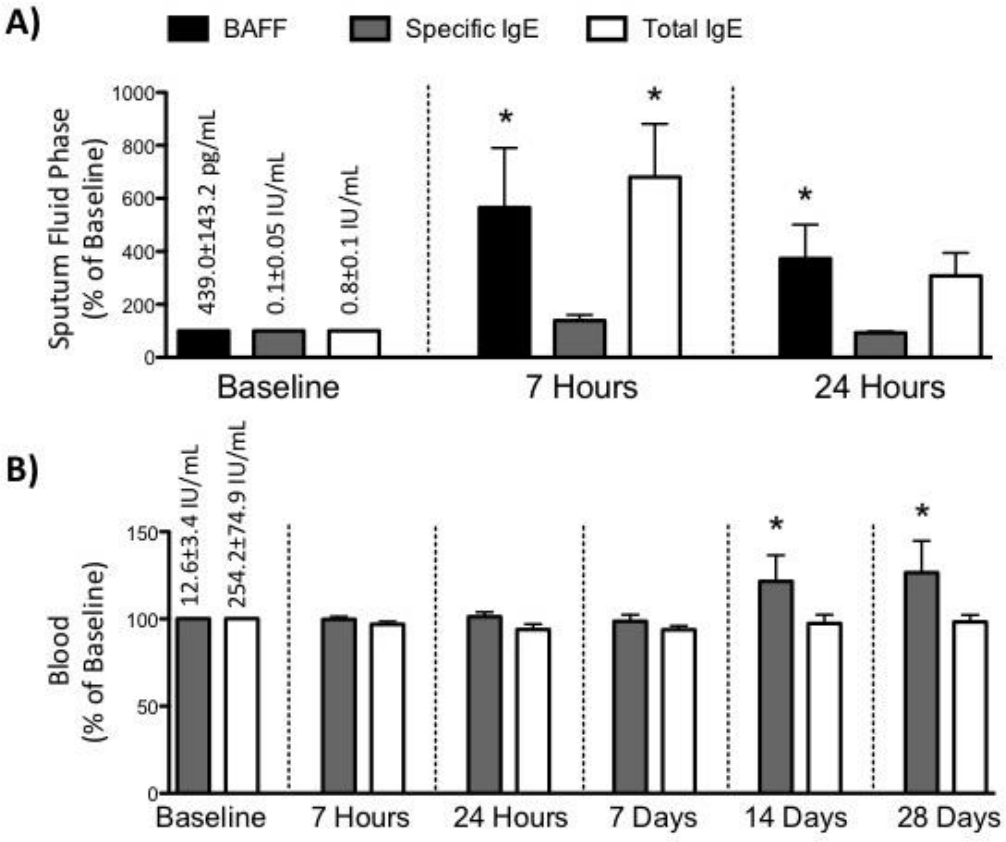



**Figure 3.2: The effect of inhaled allergen on (A) B cell activating factor (BAFF), specific IgE and total IgE levels in sputum fluid phase measured at baseline and again at 7 and 24 hours after challenge (n = 7 subjects), and (B) specific IgE and total IgE levels in blood measured at baseline and until 28 days after challenge.** Data are presented as means±SEM. Significant findings are denoted by an asterisk, where $p<0.05$ compared with pre-challenge baseline.

## SUPPLEMENTARY METHODS

### Clinical Tests

***Spirometry:*** Spirometry was performed following ATS guidelines[1].

***Skin Prick Test:*** Skin prick test were performed with common aeroallergen extracts, to confirm atopic status and enable selection of a suitable extract for allergen inhalation challenge [2–4].

***Methacholine Challenge Test:*** A methacholine test was completed to determine airway responsiveness [5] and together with skin prick test was used to determine the allergen dose for inhalation, as previously described[6] (Table E3.1).

***Allergen and Diluent Inhalation Challenge:*** The allergen challenge was conducted as previously described[7]. Briefly, the allergen challenge was conducted with increasing concentrations of allergen inhaled by tidal breathing for 2 minutes each, and $FEV_1$ was measured 10 minutes post-inhalation. Allergen inhalation was stopped when the $FEV_1$ fell by at least 20%, then $FEV_1$ was measured regularly until 7 hours post-challenge. The early asthmatic response was the lowest fall in $FEV_1$ between 0 and 2 hours, while the late asthmatic response was the lowest fall in $FEV_1$ between 3 and 7 hours. The diluent control challenge consisted of 3 doses of the diluting solution, 0.9% saline.

### Sample Collection and Cell Processing

***Sputum Induction:*** Sputum was induced by inhalation of 3, 4 and 5% saline and mucous plugs were selected and processed as previously described[8,9]. Briefly, sputum was treated with dithiothreitol (DTT) and filtered. The fluid phase was saved for measurements of total and allergen-specific IgE and BAFF. Two cytospins were prepared and stained for cell differentials, and the remaining cells were stained for flow cytometry.

***Peripheral Blood Venipuncture:*** Peripheral blood was collected into heparin tubes and enriched for B cells using a RosetteSep® density gradient (StemCell Technologies, BC, CAN) and the mononuclear

cells were stained for flow cytometry. Blood was also collected in a serum-separating tube to measure total and allergen-specific IgE.

***Tonsil Biopsy:*** Tonsil tissue was collected from ten subjects who had tonsils using a through-cut forceps technique following local administration of lidocaine. The biopsy was mechanically disrupted, incubated in 1% collagenase for 2 hours to further breakdown tissue collagen then filtered through a 100-micrometer filter. Cells were stained for flow cytometry.

***Bone Marrow Aspirates:*** Bone marrow was aspirated from the iliac crest following local administration of lidocaine. The sample was collected into a bone marrow aspiration needle containing 1 milliliter of heparin and diluted with autoMACS® buffer, then layered on AccuPrep® density gradient. The plasma cells were isolated from the mononuclear cell layer by incubating with anti-CD138+ microbeads (Miltenyi Biotech) and magnetic-activated cell sorter (MACS) magnet. The isolated cells were stained for flow cytometry.

**Cell Staining and Gating Strategy for B Cells**

Briefly, cells were stained with fluorescent antibodies compatible for flow cytometry analysis of B cell subsets. Cells were washed twice with FACS buffer. Antibodies to surface markers were incubated with the cells for 30 minutes, and then cells were washed, fixed and permeabilized before antibodies to intracellular markers were incubated with the cells for 30 minutes. Appropriate isotypes were used as negative controls. The cells were acquired using a Beckton Dickinson (BD) LSRII flow cytometer (BD Bioscience, ON, CAN) and the files were analyzed with the FlowJo software (TreeStar, OR, USA).

Sputum cells were surface-stained with antibodies to CD45 (V450), CD4 (APC-H7), CD8 (FITC), CD19 (PE-Cy7) and intracellularly with IgE (PE) and IgG (APC). From the forward scatter (FSC) and side scatter (SSC) plot, a gate was placed around all the cells, excluding debris. SSC and CD45 were then plotted and a gate was placed around the lymphocyte population ($SSC^{low}CD45+$).

CD45 and CD4 were then plotted and a gate was placed around the CD4+ population. CD45 and CD8 were then plotted and a gate was placed around the CD8+ population. CD45 and CD19 were then plotted and a gate was placed around the CD19+ population. CD19+ B cells were then used to plot CD19 with IgE and IgG to enumerate IgE+ and IgG+ B cells in the airways. A sample gating strategy can be found in Fig E3.1.

Peripheral blood and tonsil cells were surface-stained with CD45 (AmCyan), CD19 (APC-Cy7), CD38 (V450), CD27 (PE-Cy7), CD138 (PerCP-Cy5.5), and intracellularly with IgE (PE) and IgG (FITC). The following was the gating strategy used to enumerate memory B cells (MBCs) (CD45+CD19+CD27+CD38-) and plasmablasts (PCs) (CD45+CD19+CD27+CD38) from peripheral blood and tonsil. From the forward scatter (FSC) and side scatter (SSC) plot, a gate was placed around the total population. SSC and CD45 were then plotted and a gate was place around the CD45+ population. SSC and CD19 were then plotted and a gate was placed around the CD19+ population. SSC and CD27 were plotted and a gate was placed around the CD27+ population. CD38 and CD27 were then plotted and this was used to obtain each of the required phenotypes, MBCs and PPCs. MBCs and PPCs were then used to plot CD38 with IgE and IgG enumerate IgE+ and IgG+ MBCs and PPCs in circulation and lymphatics.

Bone marrow cells were surface-stained with CD45 (PerCP-Cy5.5), CD3 (PE-Cy7), CD19 (APC-Cy7), CD38 (FITC), CD138 (PE), and intracellularly with IgE (APC) and IgG (APC). The following was the gating strategy used to enumerate plasma cells (PCs) (CD3-CD45+CD38+CD138+) and plasmablasts (PPCs) (CD3-CD45+CD38+CD138-) from bone marrow. From the forward scatter (FSC) and side scatter (SSC) plot, a gate was placed around the lymphocyte population. CD3 and CD45 were then plotted and a gate was placed around the CD3- population to obtain the B cells. CD138 and CD38 were then plotted and this was used to obtain each of the required phenotypes, PCs

and PPCs. PCs and PPCs were then used to plot SSC with IgE and IgG to enumerate IgE+ and IgG+ PCs and PPCs in the hematopoeitic compartment.

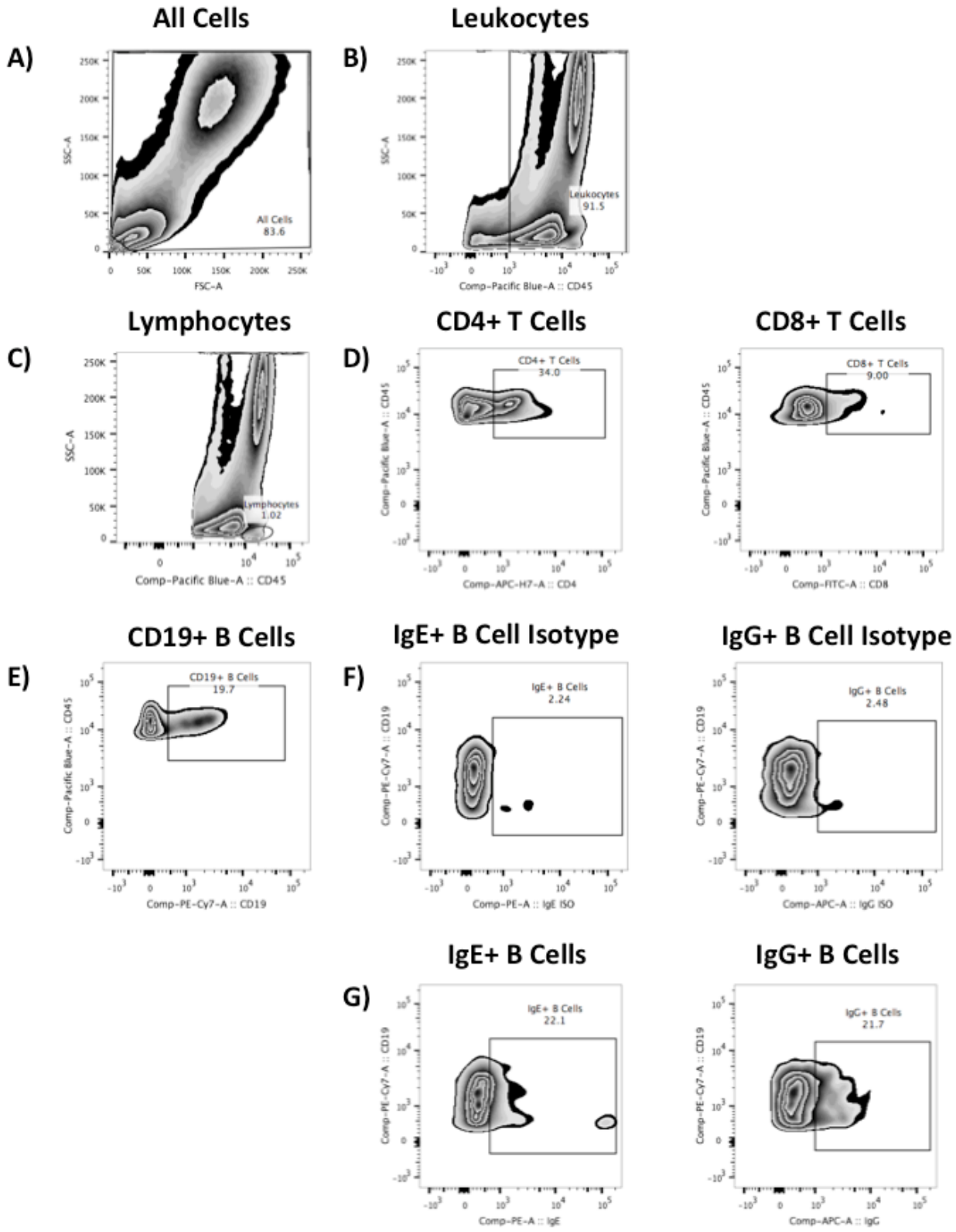
**Total and Specific IgE and BAFF Measurements**

Total IgE from serum and sputum fluid phase was measured using a commercially available sandwich, enzyme linked immunosorbent assay (ELISA) kit (abcam, ON, CAN) with a lower limit of detection (LLOD) of 0.5 IU/mL. BAFF from sputum fluid phase was measured using a commercially available ELISA kit (R&D Systems, MN, USA) with a LLOD of 2.68 pg/mL. Allergen-specific IgE from serum and sputum fluid phase was measured using a Phadia 250 platform by ImmunoCAP® with LLOD of 0.1 IU/mL. Values were multiplied by the sputum processing dilution factor.

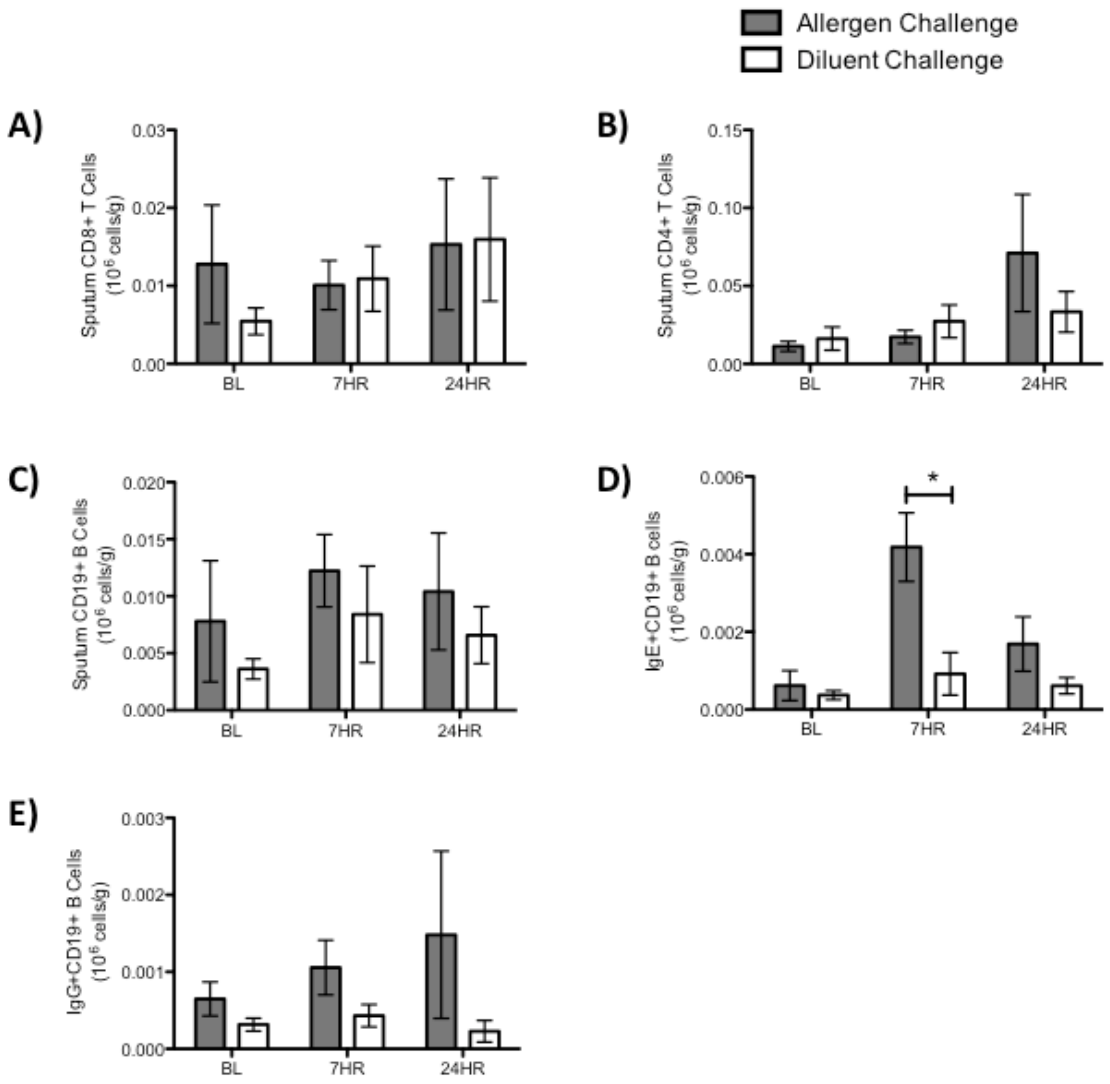
## SUPPLEMENTARY TABLES AND FIGURES

**Supplementary Table E3.1: Allergic asthmatics response to inhalation challenge.** All data are shown as mean±SEM except for MCh $PC_{20}$ shown as geometric mean (95% CI). $FEV_1$ - forced expiratory volume in 1 second, MCh $PC_{20}$ - provocative concentration of methacholine inducing at least a 20% fall in $FEV_1$, EAR – maximum % change in $FEV_1$ from 0-2h post-challenge, LAR – maximum % change in $FEV_1$ from 3-7h post-challenge, HDM – house dust mite, ND - no data, * $p<0.05$ versus diluent.

| **Baseline Demographics** | | | **Allergen Challenge** | | | | | | **Diluent Challenge** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sex** | **Age (years)** | **Predicted $FEV_1$ (%)** | **Allergen Extract Inhaled** | **Dilution of Inhaled Extract** | **MCh $PC_{20}$ Pre-Allergen (mg/mL)** | **EAR (%)** | **LAR (%)** | **MCh $PC_{20}$ 24h Post-Allergen (mg/mL)** | **MCh $PC_{20}$ Pre-Diluent (mg/mL)** | **EAR (%)** | **LAR (%)** | **MCh $PC_{20}$ 24h Post-Diluent (mg/mL)** |
| F | 22 | 92.3 | Cat | 1:4 | 9.4 | -23.7 | -16.0 | 5.5 | ND | ND | ND | ND |
| M | 26 | 73.2 | Cat | 1:32 | 1.1 | -31.4 | -14.2 | 0.9 | 1.5 | -2.5 | 3.0 | 0.8 |
| F | 23 | 102.9 | Cat | 1:2 | 7.3 | -33.4 | -26.0 | 4.0 | 9.4 | -2.2 | -3.0 | 20.8 |
| M | 22 | 99.4 | Grass | 1:32 | 24.4 | -30.1 | -21.3 | 8.6 | 27.4 | -0.8 | -9.2 | 21.6 |
| M | 35 | 95.9 | HDM *Der P* | 1:16 | 6.3 | -27.9 | -16.2 | 3.2 | 1.7 | -4.1 | -0.3 | 1.6 |
| F | 20 | 100.0 | Cat | 1:16 | 3.1 | -22.6 | -14.0 | 4.7 | ND | ND | ND | ND |
| M | 61 | 92.4 | Cat | 1:64 | 10.2 | -36.2 | -17.8 | 1.4 | 12.2 | -6.4 | -9.5 | 3.8 |
| F | 51 | 100.0 | Cat | 1:64 | 5.9 | -27.8 | -7.2 | 5.2 | ND | ND | ND | ND |
| F | 53 | 97.3 | HDM *Der P* | 1:32 | 42.2 | -45.8 | -21.7 | 15.1 | 12.6 | -6.2 | -6.5 | 14.8 |
| M | 20 | 86.9 | HDM *Der P* | 1:16 | 8.5 | -25.2 | -0.5 | 4.5 | 7.0 | -4.8 | 1.0 | 8.1 |
| M | 19 | 76.2 | Cat | 1:256 | 0.3 | -25.8 | -30.6 | 4.5 | 0.2 | 5.3 | 0.3 | 0.1 |
| F | 24 | 95.3 | Cat | 1:16 | 10.1 | -24.4 | -3.5 | 10.1 | 1.8 | -6.6 | -3.5 | 2.8 |
| F | 24 | 106.8 | Ragweed | 1:2 | 8.5 | -29.6 | -7.2 | 1.6 | 1.8 | -4.6 | -4.9 | 1.1 |
| M | 53 | 98.3 | HDM *Der P* | 1:64 | 6.8 | -41.4 | -20.8 | 4.2 | 4.6 | -7.0 | -6.8 | 7.3 |
| M | 33 | 85.1 | Alternaria | 1:64 | 0.6 | -25.6 | 1.3 | 0.5 | 0.6 | 1.0 | -1.0 | 0.6 |
| M | 27 | 102.2 | Ragweed | 1:64 | 5.9 | -25.1 | -3.9 | 2.8 | 2.0 | -2.2 | 0.0 | 2.5 |
| F | 23 | 93.6 | Horse | 1:8 | 3.0 | -25.6 | -8.4 | 1.5 | 3.3 | -6.1 | -7.1 | 4.0 |
| M | 35 | 102.4 | Ragweed | 1:32 | 7.7 | -53.1 | -13.4 | 3.4 | 6.7 | -1.8 | 0.5 | 19.5 |
| F | 22 | 101.3 | Horse | 1:1024 | 0.1 | -32.4 | -24.0 | 4.5 | 0.2 | -1.2 | -1.9 | 0.2 |
| | **31±3** | **94.8±2.1** | | | **4.4 (2.2, 8.8)** | **-30.9±1.9** | **-14.0±2.1** | **3.4 (2.2, 5.1)** | **2.8 (1.4, 5.9)** | **-3.1±1.0** | **-3.1±0.8** | **2.7 (1.1, 6.7)** |




**Supplementary Figure E3.1: Representative gating strategy for IgE+ B cells with isotype controls in sputum.**




**Supplementary Figure E3.2: The effects of inhaled allergen on the number of CD8+ T cells, CD4+ T cells, CD19+ B cells, IgE+ B cells and IgG+ B cells per gram of sputum at baseline (BL) and 7 hours and 24 hours post-challenge.** Data are represented as mean±SEM. Significant findings are denoted by *, where $p<0.05$ compared to diluent.

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## CHAPTER 4: Changes in regulatory B cell levels in bone marrow, blood and sputum of asthmatics following inhaled allergen challenge

This chapter has been submitted to the Journal of Allergy and Clinical Immunology and is currently under peer review.

### CAPSULE SUMMARY

Following allergen exposure in the airways of subjects with allergic asthma, $B_{regs}$ appear to efflux from the bone marrow and accumulate in the lungs with a possible role in dampening local inflammatory responses.

**ABSTRACT**

**Background:** Regulatory B cells ($B_{regs}$) are believed to modulate IgE-mediated inflammatory responses in allergic asthma through IL-10-dependent mechanisms. However, the kinetics and function of $B_{regs}$ after allergic stimulation of asthmatic airways has not been examined.

**Objectives:** This study compared the level of $B_{regs}$ in allergic asthmatics compared to non-asthmatics with and without allergies, and examined the kinetics, function and distribution of $B_{regs}$ in allergic asthmatic subjects following inhaled allergen challenge.

**Methods:** Blood was collected from allergic asthmatic subjects and controls, and $B_{regs}$ were enumerated by flow cytometry. Subjects with allergic asthma also underwent inhaled allergen and diluent challenges; samples of sputum, blood and bone marrow samples were collected before, 7 and 24-hours post challenge for enumeration of various phenotypes denoting $B_{regs}$ by flow cytometry. Purified blood B cells were stimulated with of IL-4, in vitro, and expression of FoxP3 and IL-10 was assessed in CD19+ B cells, and CD5+FoxP3+ and CD24+CD27+ $B_{regs}$.

**Results:** $B_{regs}$ were 2-fold lower in the blood of asthmatics compared to controls ($p<0.05$). Inhaled allergen challenge reduced $B_{regs}$ in bone marrow 24-hours post-challenge, and increased $B_{regs}$ in blood and sputum at 7 and 24-hours post-challenge (all $p<0.05$). Incubation with IL-4-promoted circulating $B_{regs}$ to competently express IL-10.

**Conclusions:** Lower circulating levels of $B_{regs}$ in allergic asthmatics may play a role in disease pathology. Allergen inhalation induced increases in $B_{regs}$ in the airways of asthmatics likely via mobilization from bone marrow. In airways, IL-4 stimulated IL-10 production by $B_{regs}$ may dampen the allergic immune response.

*To the Editor:*

Aeroallergens inhaled into allergic asthmatic airways initiates type 2 inflammation, including production of the cytokines IL-4 and IL-13 known to promote maturation of IgE-producing B cells[1,2]. A novel subset of B cells, termed regultory B cells ($B_{regs}$) have been proposed to suppress IgE-mediated allergic inflammation via secretion of IL-10[3–6]; whereby a deficiency in $B_{regs}$ has been associated with increased serum IgE, increased type 2 cytokines, and increased eosinophilia in murine models of allergic airway disease[3,7]. This study (i) compared phenotypes of $B_{regs}$ across subjects with allergic asthma, and non-asthmatics with or without allergies, (ii) determined the kinetics and relative compartmental distribution (hematopoietic, systemic and local airway compartments) of $B_{regs}$ in allergic asthmatics following allergen inhalation challenge, and (iii) investigated the functional capacity $B_{regs}$ to express IL-10 *in vitro*.

We recruited eleven allergic asthmatics, seven allergic non-asthmatics, and seven healthy controls (Table E4.1-E4.2), all non-smokers and between 18 and 65 years of age. All subjects provided informed consent and underwent screening to determine allergic status by skin-prick-test, and lung function by spirometry. No subjects required medication for treatment asthma or allergies. Asthmatics had a forced expiratory volume in one second ($FEV_1$) ≥70% of predicted, methacholine provocative concentration causing at least a 20% fall in $FEV_1$ ($PC_{20}$) ≤16 mg/ml[8], and were excluded if they had exacerbations or respiratory tract infections within four weeks, or underlying diseases other than asthma. All eligible subjects donated a blood sample for baseline measurements of $B_{regs}$. Ten additional subjects with allergic asthma provided samples of sputum, blood and bone marrow on day 1, followed by randomization to allergen or diluent inhalation challenge on day 2. Spirometry was measured up to 7-hours post-challenge when sputum and blood samples were collected. On day 3 (24-hours post-challenge), methacholine challenge, sputum, blood and bone marrow collection were performed. This

triad was then repeated with the other inhaled agent (allergen or diluent) after a 4-week recovery period.

Sputum cells were surface-stained with antibodies to CD45, CD19, and intracellularly with FoxP3 to enumerate $B_{regs}$ . Peripheral blood and bone marrow mononuclear cells were surface-stained with antibodies to CD19, CD1d, CD5, CD24, CD27, CD38, and intracellularly with IgE, IL-10, and FoxP3 to enumerate four different $B_{regs}$ phenotypes (CD5+FoxP3+, CD1d+CD5+FoxP3+, CD24+CD27+FoxP3+, CD24+CD38+FoxP3+). Appropriate isotypes were used as negative controls. Cells were acquired using a Beckton Dickinson LSRFortessa flow cytometer (BD Bioscience, San Jose, California, United States), and the files were analyzed with the FlowJo software (TreeStar, Ashland, Oregon, United States). The subject criteria, sample processing, cell-staining procedures, and gating strategies are detailed in the Supplementary Methods and Fig E4.1. All data are presented as the mean ± SEM.

In blood, the frequency of CD19+ B cells expressed as a percentage of all lymphocytes was similar between groups (Fig 4.1A). However, the frequency of CD19+FoxP3+ B cells expressed as a percentage of total CD19+ B cells was approximately 2-fold lower in asthmatic subjects (6.1±0.4%) compared to allergic subjects (11.6±0.9%) and controls (12.8±1.3%) ($P<0.05$, Fig 4.1B). The frequency of CD5+ $B_{regs}$ and CD24+CD27+ $B_{regs}$ were also lower in asthmatic subjects compared to controls ($P<0.05$, Fig 4.1C&E). When co-expression of FoxP3 was examined, the frequency of CD5+FoxP3+ $B_{regs}$ and CD24+CD38+FoxP3+ $B_{regs}$ were both lower in asthmatic subjects compared to controls ($P<0.05$, Fig 4.1C&F). In contrast, the frequency of CD1d+CD5+ and CD1d+CD5+FoxP3+ $B_{regs}$ was not different across groups (Fig 4.1D).

After allergen challenge, the frequency of CD19+ B cells expressed as a percentage of lymphocytes did not change in blood or bone marrow compartments, but increased in sputum at 7-hours post-allergen challenge, compared diluent (Fig E4.2). The frequency of CD19+FoxP3+ B cells

expressed as a percentage of CD19+ B cells, decreased in bone marrow at 24-hours post-allergen challenge, but increased in blood at 7-hours and sputum at 24-hours post-allergen challenge, compared to diluent ($P<0.05$, Fig 4.2A).

In bone marrow, there were no allergen-induced changes in any of the $B_{reg}$ phenotypes compared to diluent challenge. In blood, there were significantly lower levels of CD5+ $B_{regs}$ and CD1d+CD5+ $B_{regs}$ at 7-hours post-allergen challenge, while there were significantly higher levels of CD24+CD38+ $B_{regs}$ at 24-hours post-allergen challenge, compared to diluent ($P<0.05$, Table E4.3). Furthermore, compared to diluent, allergen consistently decreased the percentage of FoxP3 positive $B_{reg}$ phenotypes in the bone marrow ($P<0.05$, Fig 4.2B, Fig E4.3A, Table E4.4-E4.6) and increased the percentage of FoxP3 positive $B_{reg}$ phenotypes in blood ($P<0.05$, Fig2B, FigE3B, Table E4.4-E4.6) at 24-hours post-allergen challenge.

CD19+ B cells purified from blood of allergic asthmatics and incubated with 5 ng/ml and 25 ng/mL of IL-4 for 72 hours significantly increased expression of FoxP3 and co-expression of FoxP3 and IL-10 ($P<0.05$, Fig 4.2C). In addition, IL-4 induced significantly higher proportions of CD5+FoxP3+IL-10+ and CD24+CD27+IL-10+ $B_{regs}$ compared to control ($P<0.05$, Fig 4.2C, Fig E4.4). Murine models suggests that $B_{regs}$ have a role in dampening allergic immune responses through IL-10 dependent mechanisms[4–6,9], and further studies are required to confirm this in human models.

In summary, we evaluated phenotypes of $B_{regs}$ implicated in allergic disease[4,9,10] and found lower circulating levels of $B_{regs}$ in allergic asthmatics compared to controls, suggesting allergic immune processes. We observed a consistent decrease in the frequency of FoxP3-expressing $B_{regs}$ in bone marrow and increase in blood at 24-hours post-allergen. Although we were not able to measure $B_{regs}$ in the airways due to limited sample availability, we observed increased frequency of CD19+FoxP3+ B cells in sputum. The observed compartmental differences in the levels of CD19+FoxP3+ B cells after allergen challenge suggests the mobilization of $B_{regs}$ from bone marrow trafficking through the blood,

and homing into the lungs. IL-4 stimulation of B cells induced higher proportions of IL-10-expressing $B_{regs}$ indicating that type 2 cytokine exposure may direct certain B cell subsets towards a regulatory role to dampen the allergic immune response. Taken together, induction of airway inflammation might be partially due to lower levels of $B_{regs}$, while the persistence of inflammation in allergic asthma may be due to impaired local regulation by $B_{regs}$. In conclusion, our data support a role of $B_{regs}$ to dampen the allergic immune response, however, a consensus of how to phenotypically define $B_{regs}$ would be helpful in delving further into the functional capacity of these cells.

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**TABLES AND FIGURES**

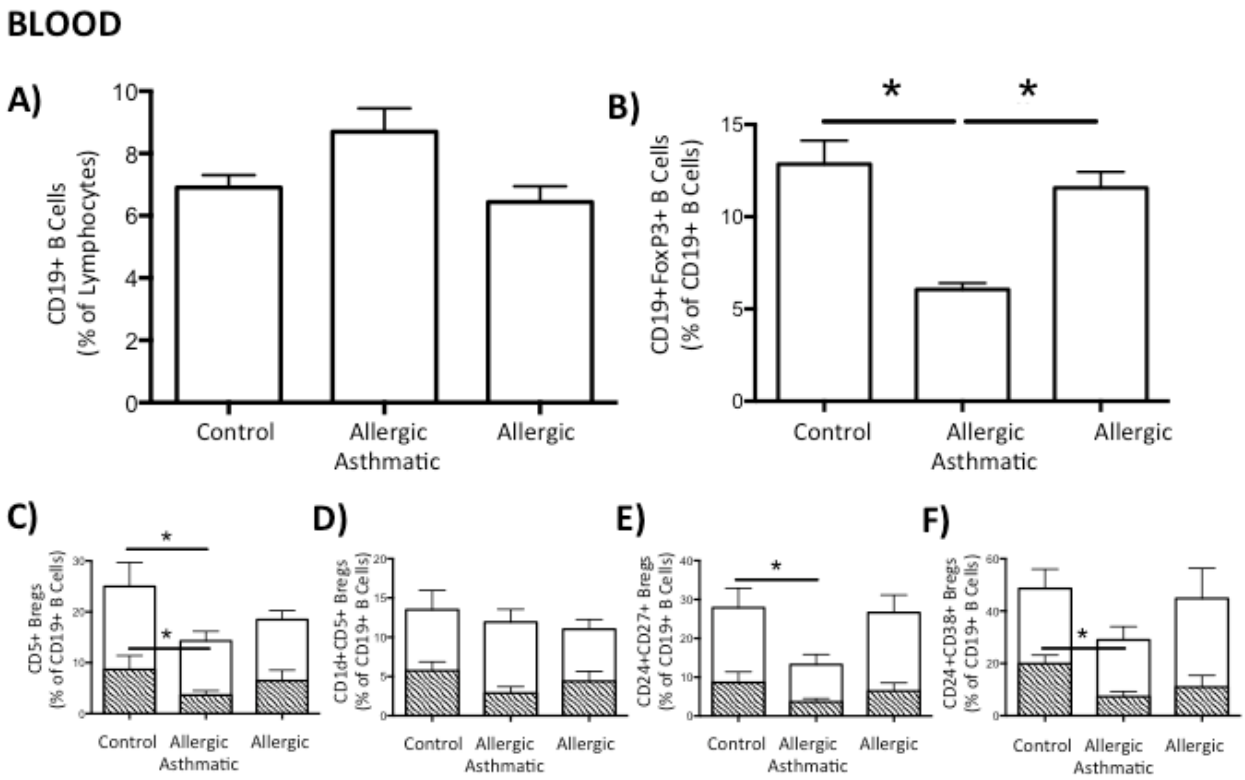



**Figure 4.1: The blood levels of (A) CD19+ B cells, (B) CD19+FoxP3+ B cells, and (C-F) regulatory B cell phenotypes (open bars) and proportions of regulatory B cells expressing FoxP3 (hatched bars) in subjects with allergic asthmatic (n=11), and allergic non-asthmatic (n=7) and healthy controls (n=7).**

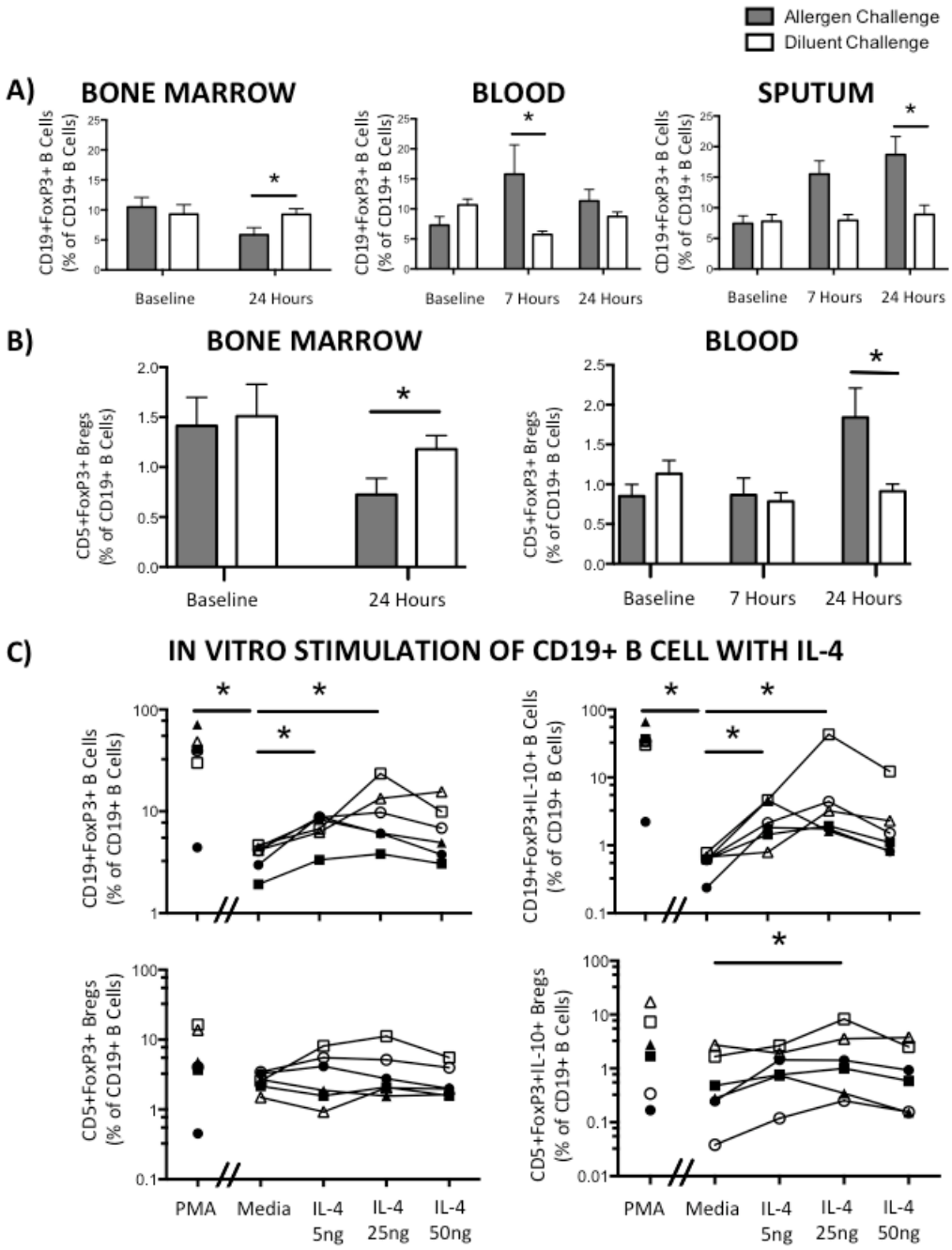



**Figure 4.2: (A) The frequency of CD19+FoxP3+ B cells expressed as a percentage of CD19+ B cells in bone marrow (n=10), blood (n=10), and sputum (n=8) at baseline, 7 hours and 24 hours after inhalation challenge. (B) The frequency of CD5+FoxP3+ $B_{regs}$ expressed as a percentage of CD19+ B cells in bone marrow (n=10) at baseline and 24 hours after inhalation challenge and blood (n=10) at baseline, 7 hours and 24 hours after inhalation challenge. (C) IL-4-induced FoxP3-expression of CD19+ B cells and CD5+ $B_{regs}$, and intracellular IL-10 in CD19+ B cells purified from blood of allergic asthmatic subjects (n=6).**

## SUPPLEMENTARY METHODS

### Subject Demographics and Study Design

***Cross-Sectional Cohorts:*** Allergic asthmatics (n=11), allergic non-asthmatics (n=7) and healthy controls (n=7) (Table EI) all between 18 and 65 years of age provided written informed consent to participate in the study and underwent a screening visit to determine allergic status by skin prick test, and lung function by spirometry. No subjects required medication for treatment of asthma or allergies. Subjects with asthma had a forced expiratory volume in one second ($FEV_1$) ≥70% of predicted, methacholine provocative concentration causing at least a 20% fall in $FEV_1$ ($PC_{20}$) ≤16 mg/ml[1], and were excluded if they had asthmatic exacerbations or respiratory tract infections within four weeks, or underlying diseases other than asthma. All eligible subjects donated a blood sample.

***Inhalation Challenge Cohort:*** Ten additional subjects with allergic asthma provided samples of sputum, blood and bone marrow on day 1, followed by randomization to allergen or diluent inhalation challenge on day 2. Spirometry was measured up to 7-hours post-challenge when sputum and blood samples were collected. On day 3 (24-hours post-challenge), methacholine challenge, sputum, blood and bone marrow collection were performed. This triad was then repeated with the other inhaled agent (allergen or diluent) after a 4-week recovery period.

### Clinical Tests

***Spirometry, Skin Prick Test, Methacholine Challenge:*** Spirometry was performed following ATS guidelines[2]. Skin prick tests were performed with common aeroallergen extracts and histamine and saline were used as positive and negative controls, respectively, to confirm atopic status and determine a suitable extract for inhalation[3–5]. A methacholine challenge test was completed using the tidal breathing method from a Wright Nebulizer to determine the provocative concentration that resulted in a 20% fall in $FEV_1$ ($PC_{20}$)[6].

***Allergen and Diluent Challenges****:* The methacholine $PC_{20}$ and skin prick test were used to determine the allergen dose for inhalation, as previously described[7]. Increasing allergen concentrations were inhaled during tidal breathing for 2 minutes, and $FEV_1$ was measured 10 minutes after each dose. Allergen inhalation was stopped when the $FEV_1$ fell by at least 20%, then $FEV_1$ was measured regularly until 7 hours post-challenge. The diluent control challenge consisted of three 2-minute inhalations of the diluting solution (0.9% saline).

**Sample Collection and Cell Processing**

***Sputum Cells:*** Sputum was induced by inhalation of 3, 4 and 5% saline and mucous plugs were selected and processed as previously described[8,9]. Briefly, sputum was treated with Dulbecco's phosphate buffered saline (DPBS) and dithiothreitol (DTT) and filtered through a nylon mesh, the cells were pelleted by centrifugation[10]. Two cytospins were prepared and stained with Diff Quik for cell differentials and the remaining cells were stored at 4°C flow cytometry staining.

***Peripheral Blood and Bone Marrow Mononuclear Cells:*** Peripheral blood was collected into vacutainers containing heparin. A total cell count was performed using a hemocytometer. Bone marrow was sampled from the iliac crest following local administration of lidocaine, and collected into a bone marrow aspiration needle containing 1 milliliter of heparin. The blood and bone marrow samples were diluted with two times the volume of 10% fetal bovine serum (FBS) in Roswell Park Memorial Institute (RPMI) buffer, layered on AccuPrep density gradient, and then centrifuged at 800g for 20 minutes. The peripheral blood mononuclear cell (PBMC) and bone marrow mononuclear cell (BMMC) layers were collected and stored at 4°C for flow cytometry staining.

**B Cell Isolation and Culture**

A commercially available kit was used to isolate CD19+ B cells from whole blood (STEMCELL Technologies Inc., BC, CAN). Briefly, whole blood was mixed gently and incubated for 5 minutes with magnetic beads and B cell isolation cocktail in a 50mL conical tube. The solution was

washed with PBS and placed in a magnet to incubate for 5 minutes. The clear fraction suspension above a red whole blood cell layer was pipetted into a new 50mL conical tube. This isolation process was repeated 3 times to isolate the B cells, which were suspended at a concentration of $1x10^6$ B cells per millilitre of sterile RPMI media supplemented with 10% FBS, 2.5% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.5% Pennicillin/Streptomycin, and 1% sodium pyruvate.

Isolated B cells were plated in a round-bottom 96-well plate at a starting density of $2x10^5$ cells per millilitre. The B cells were cultured in the presence or absence of IL-4 at three concentrations (5 ng/mL, 25 ng/mL and 50 ng/mL) (R&D Systems, MN, USA) or media alone (negative control) and phorbol 12-myristate 13-acetate (PMA) (positive control) (Invitrogen, CA, USA). B cells were incubated in a humidified 37 degrees Celsius, 5% carbon dioxide incubator for 72 hours. Six hours before the B cells were taken out of culture, a protein transport inhibitor cocktail consisting of brefeldin A and monensin (Invitrogen, CA, USA) was added into the culture suspension to block cytokine secretion. Flow cytometry was used to examine the expression of FoxP3+, CD5+FoxP3+ and CD24+CD27+ as well as expression of intracellular IL-10.

**Cell Staining and Gating Strategy for B Cells**

Briefly, cells were stained with fluorescent antibodies compatible for flow cytometry analysis of B cells and $B_{regs}$ subsets. Cells were washed twice with FACS buffer. Antibodies to surface markers were incubated with the cells for 30 minutes, and then cells were washed, fixed and permeabilized before antibodies to intracellular markers were incubated with the cells for 30 minutes. Appropriate isotypes were used as negative controls. The cells were acquired using a Beckton Dickinson (BD) LSRFortessa flow cytometer (BD Bioscience, ON, CAN) and the files were analyzed with the FlowJo software (TreeStar, OR, USA).

Sputum cells were surface-stained with antibodies to CD45 (V450), CD19 (PE-Cy7) and intracellularly with FoxP3 (Alex Fluor 488). From the forward scatter (FSC) and side scatter (SSC)

plot, a gate was placed around all the cells, excluding debris. SSC and CD45 were then plotted and a gate was placed around the lymphocyte population ($SSC^{low}CD45+$). CD45 and CD19 were then plotted and a gate was placed around the CD19+ population. CD19+ B cells were then used to plot CD19 with FoxP3 to isolate CD19+FoxP3+ B cells in the airways.

Peripheral blood and bone marrow mononuclear cells were surface-stained with antibodies to CD19 (PE-Cy7), CD1d (APC), CD5 (Alexa Fluor 700), CD24 (PE-CF594), CD27 (V450), CD38 (PE) and intracellularly with IgE (APC), IL-10 (PE) and FoxP3 (Alexa Fluor 488). The following gating strategy was used to enumerate $B_{regs}$ from peripheral blood and bone marrow. From the forward scatter (FSC) and side scatter (SSC) plot, a gate was placed around all the cells, excluding debris. SSC and CD19 were then plotted and a gate was placed around the CD19+ population. From the CD19+ B cell population, further gates were created to enumerate (1) CD1d+CD5+, (2) CD5+FoxP3+, (3) CD24+CD27+, and (4) CD24+CD38+ $B_{reg}$ subsets (Fig E4.1).

**Statistical Analyses**

Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., CA, USA). Data are presented as the mean±SEM with the exception of methacholine $PC_{20}$ presented as geometric mean (95% confidence interval). Cross-sectional cohort data and different culture conditions were analyzed with 1-way ANOVAs with Tukey post-hoc tests. Inhalation challenge data were analyzed with 2-way ANOVAs with Bonferroni post-hoc tests. Significance was accepted at $p<0.05$.

**SUPPLEMENTARY TABLES AND FIGURES**

**Supplementary Table E4.1: Subject demographics.** All data are shown as mean±SEM except for methacholine $PC_{20}$ (mg/mL) shown as geometric mean (95% CI). $FEV_1$ - forced expiratory volume in 1 second, $PC_{20}$ - provocative concentration of methacholine inducing at least a 20% fall in $FEV_1$, * $p<0.05$ versus controls, # $p<0.05$ versus allergic subjects.

| | Controls (n=7) | Allergic Asthmatics (n=11) | Allergics (n=7) |
|---|---|---|---|
| **Gender (M/F)** | 3/4 | 6/5 | 1/6 |
| **Age (years)** | 38±5 | 30±4 | 23±3 |
| **$FEV_1$ (% of predicted)** | 95.4±1.5 | 94.0±3.3 | 96.7±3.4 |
| **Methacholine $PC_{20}$ (mg/mL)** | >16 | 4.2 (2.1, 8.4) | >16 |
| **Sputum Eosinophils (%)** | 0.06±0.04 | 1.9±0.4*# | 0.6±0.2 |
| **Blood Eosinophils (%)** | 1.3±0.3 | 3.9±1.1* | 1.8±0.4 |

**Supplementary Table E4.2: Allergic asthmatics response to inhalation challenge.** All data are shown as mean±SEM except for MCh $PC_{20}$ shown as geometric mean (95% CI). $FEV_1$ - forced expiratory volume in 1 second, MCh $PC_{20}$ - provocative concentration of methacholine inducing at least a 20% fall in $FEV_1$, EAR – maximum % change in $FEV_1$ from 0-2h post-challenge, LAR – maximum % change in $FEV_1$ from 3-7h post-challenge, HDM – house dust mite, ND - no data, * $p<0.05$ versus diluent.

| Sex | Age (years) | Predicted $FEV_1$ (%) | MCh $PC_{20}$ Pre-Allergen (mg/mL) | MCh $PC_{20}$ Pre-Diluent (mg/mL) | MCh $PC_{20}$ Post-Allergen (mg/mL) | MCh $PC_{20}$ Post-Diluent (mg/mL) | Allergen Extract Inhaled | Dilution of Inhaled Extract | EAR Allergen (%) | EAR Diluent (%) | LAR Allergen (%) | LAR Diluent (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M | 37 | 85.1 | 7.7 | 20.8 | 4.1 | ND | Grass | 1:512 | -34.8 | 1.0 | -12.7 | 4.0 |
| F | 22 | 79.4 | 1.6 | 5.0 | 1.0 | 2.8 | Ragweed | 1:8 | -50.0 | -0.8 | -31.3 | -2.0 |
| M | 24 | 81.0 | 4.0 | 10.0 | 0.88 | 4.8 | HDM | 1:128 | -34.1 | -5.1 | -19.5 | -5.1 |
| M | 23 | 88.9 | 0.3 | 0.6 | ND | ND | Cat | 1:512 | -22.0 | -2.3 | -22.3 | -7.3 |
| F | 23 | 94.5 | 7.5 | 5.7 | 0.7 | ND | Horse | 1:128 | -47.3 | -0.6 | -26.5 | -1.9 |
| M | 66 | 95.4 | 3.4 | 12.3 | ND | ND | Cat | 1:32 | -48.5 | -1.8 | -38.8 | 0.0 |
| M | 53 | 97.6 | 5.4 | 10.3 | 7.4 | 7.3 | HDM | 1:64 | -36.2 | -3.8 | -19.5 | -1.8 |
| M | 28 | 112.4 | 10.5 | 9.6 | 0.1 | ND | HDM | 1:64 | -49.4 | -7.2 | -17.7 | -4.9 |
| F | 22 | 94.3 | 4.7 | 5.3 | 0.2 | ND | Tree | 1:32 | -35.4 | -2.7 | -50.7 | -2.7 |
| M | 51 | 98.4 | 0.3 | 0.4 | 0.8 | 1.7 | Cat | 1:64 | -33.8 | -4.5 | -22.5 | -3.1 |
| | **35±5** | **92.7±3.1** | **2.8 (1.1, 7.0)** | **5.0 (2.0, 12.6)** | **0.8 (0.3, 2.8) *** | **4.2 (1.3, 9.9)** | | | **-39.2±2.9 *** | **-2.8±0.8** | **-26.2±3.6 *** | **-2.5±1.0** |

**Supplementary Table E4.3: Regulatory B Cell frequencies in healthy control, allergic asthmatic and allergic subjects.** All data are expressed as % of CD19+ B cells and shown as mean±SEM, except CD19+ B cells, which are expressed as a % of lymphocytes. ND - no data, * $p<0.05$ versus controls, # $p<0.05$ versus allergic subjects.

| BLOOD | Controls | Allergic Asthmatics | Allergics |
|---|---|---|---|
| CD19+ | 6.9±0.4 | 8.7±0.7 | 6.4±0.5 |
| CD19+FoxP3+ | 12.8±1.3 | 6.1±0.4*# | 11.6±0.9 |
| **Regulatory B Cell Phenotypes** | | | |
| CD5+ | 25.0±4.7 | 14.3±1.9* | 18.5±1.8 |
| CD1d+CD5+ | 13.5±2.5 | 11.9±1.6 | 11.0±1.2 |
| CD24+CD27+ | 27.9±5.0 | 13.3±2.6* | 26.6±4.5 |
| CD24+CD38+ | 48.6±7.4 | 29.0±5.1 | 44.8±11.5 |
| **FoxP3+ Regulatory B Cell Phenotypes** | | | |
| CD5+FoxP3+ | 8.7±2.7 | 3.6±0.9* | 6.4±2.1 |
| CD1d+CD5+ FoxP3+ | 5.7±1.1 | 2.9±0.8 | 4.4±1.3 |
| CD24+CD27+ FoxP3+ | 8.7±2.7 | 3.6±0.9 | 6.4±2.1 |
| CD24+CD38+ FoxP3+ | 19.9±3.2 | 7.3±2.0* | 11.0±4.4 |

**Supplementary Table E4.4: B cell and regulatory B cell frequencies after allergen inhalation challenge.** All data are expressed as % of CD19+ B cells and shown as mean±SEM, except CD19+ B cells, which are expressed as a % of lymphocytes. ND - no data, * $p<0.05$ versus diluent.

| | **Baseline** | | **7 Hours Post** | | **24 Hours Post** | |
|---|---|---|---|---|---|---|
| | Allergen | Diluent | Allergen | Diluent | Allergen | Diluent |
| **BONE MARROW** | | | | | | |
| CD19+ | 15.8±1.5 | 15.3±1.4 | ND | ND | 16.8±2.1 | 15.8±1.1 |
| CD19+FoxP3+ | 10.5±1.6 | 9.3±1.6 | ND | ND | 5.8±1.2* | 9.2±1.0 |
| **BLOOD** | | | | | | |
| CD19+ | 14.4±1.6 | 13.3±1.3 | 18.0±1.9 | 15.3±1.4 | 14.3±1.4 | 13.8±1.4 |
| CD19+FoxP3+ | 7.3±1.4 | 10.7±1.0 | 15.8±4.9* | 5.7±0.6 | 11.3±1.9 | 9.7±0.7 |
| **SPUTUM** | | | | | | |
| CD19+ | 8.6±1.3 | 8.0±1.6 | 16.7±1.6* | 8.5±0.9 | 13.5±2.1 | 9.3±1.8 |
| CD19+FoxP3+ | 7.4±1.2 | 7.8±1.1 | 15.5±8.0 | 7.9±0.9 | 118.7±3.0* | 8.9±1.5 |

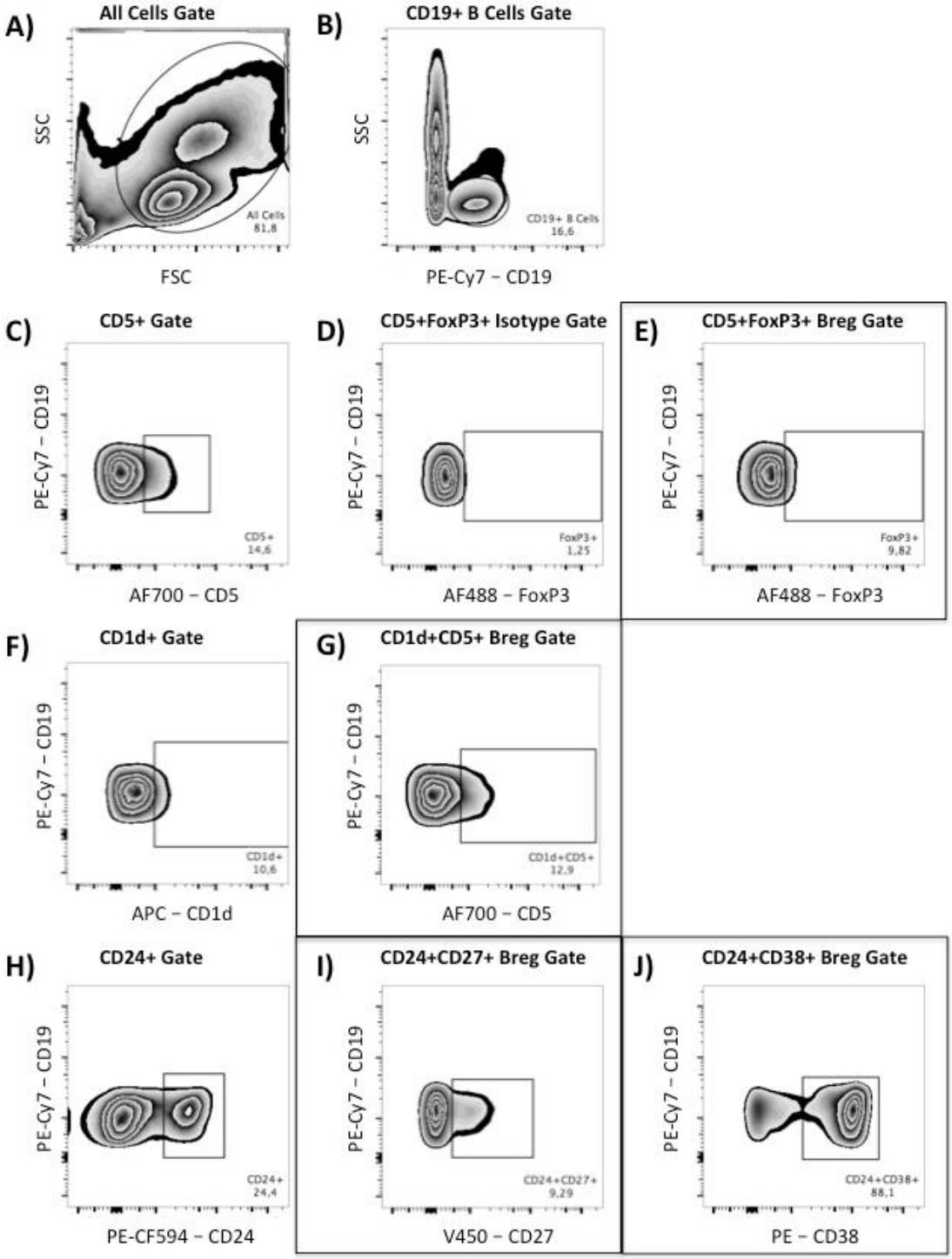
**Supplementary Table E4.5: Frequency of regulatory B cells in blood and bone marrow after allergen challenge.** All data are expressed as % of CD19+ B cells and shown as mean±SEM. ND - no data, * $p<0.05$ versus diluent.

| | **Baseline** | | **7 Hours Post** | | **24 Hours Post** | |
|---|---|---|---|---|---|---|
| | Allergen | Diluent | Allergen | Diluent | Allergen | Diluent |
| **BONE MARROW** | | | | | | |
| CD5+ | 12.1±1.2 | 14.0±1.5 | ND | ND | 11.8±1.3 | 13.2±0.7 |
| CD1d+CD5+ | 4.6±0.3 | 5.6±0.9 | ND | ND | 5.3±0.6 | 6.0±0.6 |
| CD24+CD27+ | 4.5±0.2 | 5.2±0.4 | ND | ND | 6.5±0.9 | 5.4±0.3 |
| CD24+CD38+ | 38.0±2.4 | 34.5±3.4 | ND | ND | 25.4±3.1 | 30.0±4.0 |
| **BLOOD** | | | | | | |
| CD5+ | 11.5±1.3 | 10.6±0.9 | 7.2±1.4* | 12.3±1.2 | 12.7±1.2 | 11.6±0.5 |
| CD1d+CD5+ | 3.2±0.2 | 3.6±0.3 | 2.1±0.4* | 4.3±0.6 | 4.4±0.4 | 3.9±0.4 |
| CD24+CD27+ | 5.2±0.9 | 5.9±0.8 | 7.2±1.1 | 5.4±0.8 | 8.4±1.5 | 5.4±0.6 |
| CD24+CD38+ | 7.0±1.1 | 7.9±1.3 | 9.8±1.5 | 8.7±1.6 | 14.1±2.2* | 7.3±1.0 |

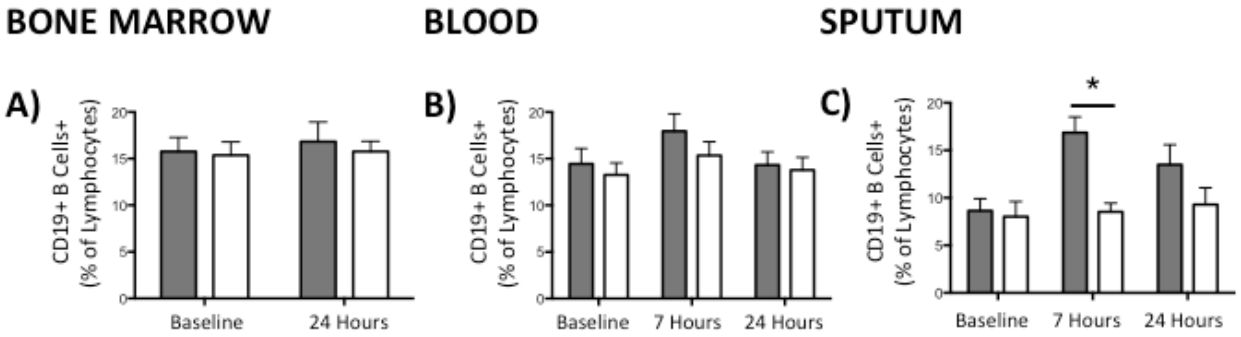
**Supplementary Table E4.6: The effects of inhaled allergen quantified with flow cytometry assessments of FoxP3+ regulatory B cell frequencies.** All data are expressed as % of CD19+ B cells and shown as mean±SEM. ND - no data, * $p<0.05$ versus diluent.

| | **Baseline** | | **7 Hours Post** | | **24 Hours Post** | |
|---|---|---|---|---|---|---|
| | Allergen | Diluent | Allergen | Diluent | Allergen | Diluent |
| **BLOOD** | | | | | | |
| CD5+FoxP3+ | 0.8±0.1 | 1.1±0.2 | 0.9±0.2 | 0.8±0.1 | 1.8±0.4* | 1.0±0.1 |
| CD1d+CD5+ FoxP3+ | 0.32±0.05 | 0.42±0.06 | 0.25±0.06 | 0.31±0.04 | 0.66±0.19 | 0.31±0.05 |
| CD24+CD27+ FoxP3+ | 0.8±0.2 | 0.8±0.2 | 1.3±0.4 | 0.5±0.1 | 1.2±0.4* | 0.5±0.1 |
| CD24+CD38+ FoxP3+ | 0.8±0.2 | 0.8±0.2 | 1.1±0.3 | 0.8±0.2 | 2.2±0.7* | 0.7±0.1 |
| **BONE MARROW** | | | | | | |
| CD5+ FoxP3+ | 1.4±0.3 | 1.5±0.3 | ND | ND | 0.7±0.2* | 1.2±0.1 |
| CD1d+CD5+ FoxP3+ | 0.6±0.1 | 0.7±0.2 | ND | ND | 0.3±0.1* | 0.6±0.1 |
| CD24+CD27+ FoxP3+ | 0.8±0.2 | 0.7±0.2 | ND | ND | 0.4±0.1* | 0.8±0.2 |
| CD24+CD38+ FoxP3+ | 4.5±0.5 | 3.3±0.4 | ND | ND | 1.6±0.3 | 2.8±0.4 |

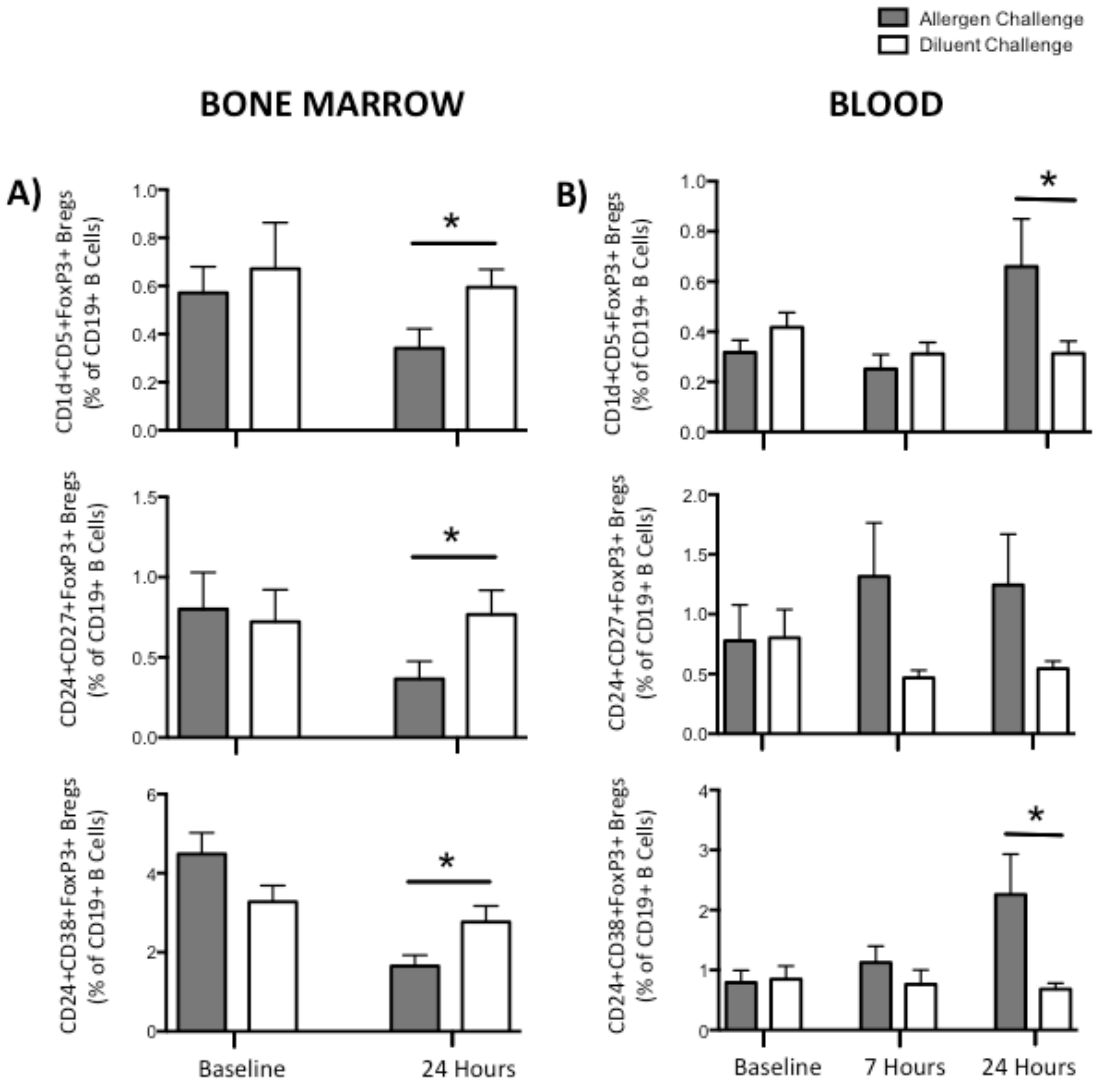
**SUPPLEMENTARY FIGURES**



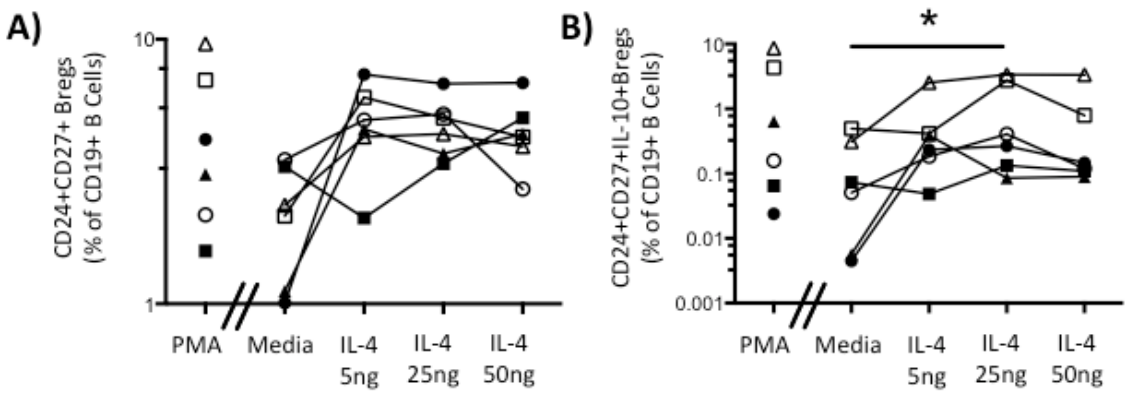

**Supplementary Figure E4.1: Representative gating strategy for regulatory B cell phenotypes with isotype controls.**




**Supplementary Figure E4.2: The frequency of CD19+ B cells expressed as a percentage lymphocytes in (A) bone marrow (n=10), (B) blood (n=10), and (C) sputum (n=8) at baseline, 7 hours and 24 hours after inhalation challenge**.




**Supplementary Figure E4.3: The frequency of FoxP3+ $B_{regs}$ phenotypes expressed as a percentage of CD19+ B cells in (A) bone marrow (n=10) at baseline and 24 hours after inhalation challenge and (B) blood (n=10) at baseline, 7 hours and 24 hours after inhalation challenge.**




**Supplementary Figure E4.4: IL-4-induced FoxP3-expression of CD24+CD27+ $B_{regs}$ (A) and intracellular IL-10 (B) in CD19+ B cells purified from blood of allergic asthmatic subjects (n=6).**

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# CHAPTER 5: Discussion

## 5.1 IgE+ B Cells - Important Effector Cells in Allergic Asthma

IgE produced by B cells is critical for initiation of the allergic cascade, ultimately leading to disease pathogenesis in allergic disorders[181]. Comparing B cell populations between allergic asthmatics and healthy controls with and without allergies, we report significantly higher levels in the proportion and absolute number of IgE+ B cells in sputum, but not blood of allergic asthmatics compared to healthy controls. Furthermore, the proportion and absolute number of IgE+ memory B cells and IgE+ plasma cells, were both significantly higher in sputum, but not blood of allergic asthmatics compared to healthy controls with and without allergies. These higher levels of IgE+ B cell subsets also corresponded with higher levels of total IgE and B cell activating factor (BAFF) in the fluid phase of induced sputum of allergic asthmatics compared to healthy controls with and without allergies. These observed differences in IgE+ B cells subsets and B cell mediators were consistently measured in the airways, but absent in the peripheral compartment, as measured in blood (Figure 5.1) (Chapter 2).

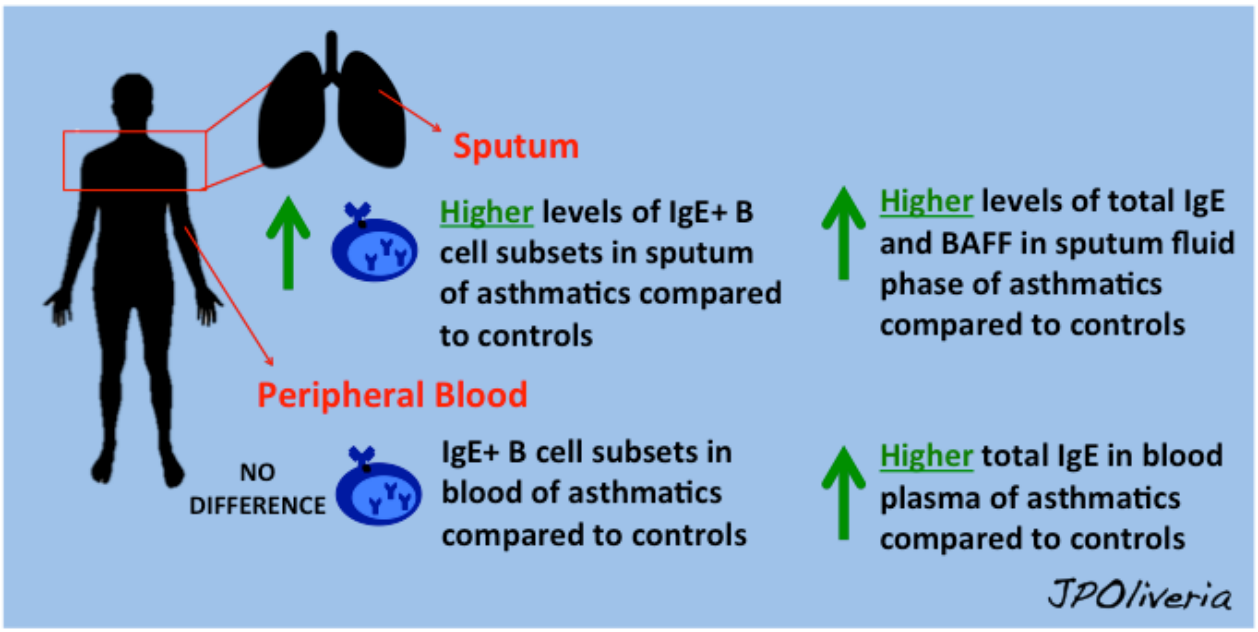


**Figure 5.1: Allergic asthmatics with allergy have elevated levels of IgE+ B cells in the airways.**

In an allergen challenge model, we report a significant increase in the proportion and absolute number of IgE+ B cells in sputum 7-hours post-allergen inhalation, corresponding with the increase in total IgE and BAFF measured in the fluid phase of induced sputum. This observed response was transient, and not detected outside the asthmatic lung (bone marrow, blood, tonsil). Although circulating IgE+ B cells are extremely rare in humans and the levels were low in this small sample size, the levels of IgE+ B cells detected in the lungs were striking and significantly elevated post-allergen challenge in mild allergic asthmatic subjects (Figure 5.2) (Chapter 3).

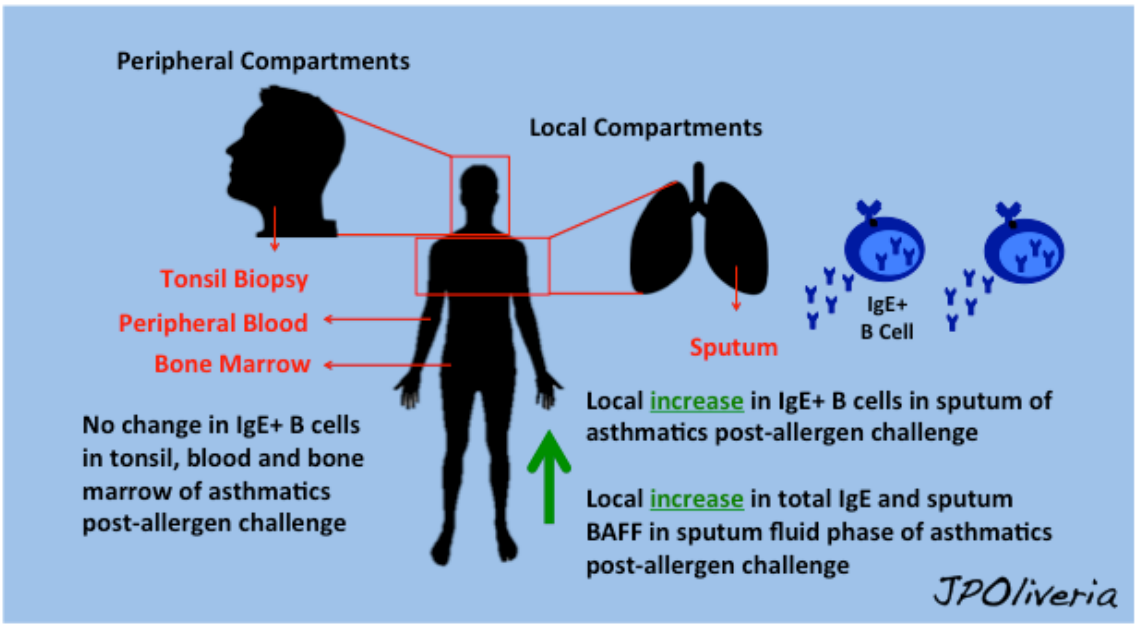


**Figure 5.2: Increased IgE+ B cells in sputum, but not blood, bone marrow or tonsils after inhaled allergen challenge in subjects with asthma.**

Taken together, IgE+ B cell subsets and B cells mediator differences are detectable mainly in the lungs of allergic asthmatic subjects, suggesting the importance of local airway B cells in allergic asthma pathogenesis.

In a study from 1996, Kidney et al reported a higher sputum level of B cells in asthmatics (16.5±3.4% of lymphocytes) compared to non-asthmatic smokers (4.0±1.1% of lymphocytes)[182]; however, no further reports on human B cells, particularly in the lungs of allergic asthmatic subjects, have been published since. Using a more stringent panel of B cell markers, we found similarly elevated levels of B cells in asthmatic subjects (11.8±2.0% of lymphocytes), thereby corroborating previous findings. The higher levels of B cells in the sputum of allergic asthmatics paint the picture of local inflammation in this IgE-mediated inflammatory disease of the airways.

Historically, IgE+ B cells have been difficult to detect in blood; however, methodological advances including availability of fluorochrome-labeled antibodies and flow cytometers with more lasers has allowed for B cell subsets including memory B cells and plasma cells to be measured. Recent papers have reported allergen-specific IgE+ B cells measured in the blood[104], nasal biopsies and nasal lavage of patients with allergic rhinitis[183,184]. With our methods we were able to consistently detect IgE+ B cells in the blood and sputum of our subjects, which allowed us to show, for the first time, significantly higher levels of IgE+ B cell subsets in the sputum of asthmatics compared to non-asthmatic controls. These findings, along with higher levels of sputum BAFF in our allergic asthmatic subjects and a positive correlation between sputum BAFF, and B cells and IgE+ B cells, support a mechanism whereby local maturation and proliferation of B cells occur in the airways of allergic asthmatic subjects[185], and together with IL-4 and IL-13[186], have the potential ability to drive local increases in IgE+ B cell subsets and IgE levels.

We also observed a positive correlation between sputum eosinophils, and B cells and IgE+ B cells, supporting the findings of Kidney et al that described a relationship between sputum eosinophils

and B cells[182]. We also found a positive correlation between sputum total IgE levels and IgE+ B cells, indicating that IgE-secreting B cells in the airways, but not in the blood, are directly related to IgE levels.

The results of this study highlight the close relationship between airway IgE+ B cells, local production of IgE, and the development of eosinophilic airway inflammation in allergic asthma. We propose that the findings of this study support the development of therapeutic strategies targeting IgE+ B cells for treatment of asthma.

Measurements of the kinetics of IgE+ B cells after allergen challenge have been explored in murine models of allergic airway disease using ovalbumin (OVA) sensitization and challenge[102]. Luger *et al* showed that short-lived IgE+ B cells were transiently increased in the lungs, spleen and bone marrow of mice 3 days after OVA-inhalation challenge. Furthermore, long-lived IgE+ B cells were significantly increased in the bone marrow 30 days after OVA-inhalation challenge. Although we also found an increased frequency of IgE+ B cells in the airways after allergen inhalation challenge in allergic asthmatic subjects, we did not observe changes in tonsils, bone marrow or peripheral blood. One of the reasons why the murine studies do not reflect our findings in allergen-challenged subjects could be due to differences in the length of exposure to antigen; ongoing annual or perennial exposure to inhaled allergens in allergic asthmatic subjects may induce higher underlying B cell responses making it more difficult to see changes post-allergen. Furthermore, studies from various murine models suggest that there may be inherent differences in anatomic and dynamic molecular aspects of IgE class-switching that render comparisons between mouse and human data to be complex and difficult to interpret[187].

The allergen inhalation challenge well-characterized allergic asthmatic responses in the airways, including bronchoconstriction, airway hyperresponsiveness and eosinophilic airway inflammation. Consistent with previous studies we found increases in sputum eosinophils and CD4+T

cells[188–191]. We also measured increased levels of IgE+ B cells in the sputum post-allergen challenge, likely contributing to increased local IgE production in the airways. Total and allergen-specific IgE have been previously reported in sputum samples from allergic asthmatic subjects[192–194]. We demonstrated elevated total IgE levels in sputum collected 7 hours after allergen challenge compared to baseline, and this was co-incident with increased levels IgE+ B cells.

In peripheral blood, total IgE remained unaltered after allergen challenge, however the percent change in allergen-specific IgE was significantly increased at 14 days and 28 days from baseline. Increased allergen-specific IgE in serum approximately one month after the whole lung allergen challenge had been reported previously[61], and we believe this is due to activation of long-lived allergen-specific IgE+ B cells. We observed no change in IgE+ B cells or serum total IgE in the peripheral compartments up to 28 days after allergen inhalation challenge.

Overall, the acute increases we observed in airway IgE+ B cell levels, and total IgE and BAFF in sputum supernatant after allergen inhalation challenge suggests that generation of local signals in the airway microenvironment leads to a transient local expansion of IgE+ B cell subsets. This local effect of inhaled allergen on IgE+ B cells or memory B cell subsets was not observed in circulation, lymphatic or hematopoietic compartments for up to 28 days post-allergen challenge. Since class-switch recombination typically requires approximately 7 to 14 days to occur[195], the acute increases in IgE+ B cells in the airways may be driven by resident IgE+ B cells proliferating and clonally expanding following allergen challenge. The proliferation and expansion of CD19+ B cells and IgE+ B cells in the airways at 7 hours post-allergen challenge, also corresponded with increased sputum BAFF levels, a mediator implicated in B cell proliferation and differentiation[82,185] which is produced locally by airway epithelium[196]. Higher levels of BAFF have been reported in sputum of asthmatic children[81] and in bronchial alveolar lavage fluid after segmental allergen challenge[197]. We and others[81,197] have shown

that airway BAFF and eosinophil levels positively correlate in asthmatic subjects, suggesting that BAFF may be indirectly driving eosinophilic inflammation in the asthmatic airways.

We have reported an increased frequency of CD19+ B cells and IgE+ B cells in the airways at 7 hours post-allergen challenge. Ramadani et al[198] showed that a large proportion of IgE+ B cells, but not IgG+ B cells, are able to differentiate into plasma cells. Although the mechanism is unclear as to how IgE+ B cells differentiate into plasma cells, our data shows that an acute expansion of IgE+ B cells in the airways corresponds to higher levels of total IgE in the airways, alluding to an increase in IgE-secreting plasma cells. However, allergen inhalation did not result in a similar increase in allergen-specific IgE in the airways, which suggests that the increase in total IgE is a likely result of polyclonal switching at 7-hours post-allergen challenge. Additionally, the assay used to measure allergen-specific IgE is currently not validated to measure sputum fluid phase samples, which may have impacted the sensitivity and accuracy of the allergen-specific IgE measurements in sputum. Lastly, this acute increase in local levels of total IgE and IgE+ B cells, taken together with the delayed increase in allergen-specific IgE in the serum at 14-days post-allergen challenge may be explained by a prolonged time course of class-switch recombination and differentiation to allergen-specific plasma cells known to take 2 weeks[195,198].

There is an emerging relationship between IgE and eosinophils in the scientific literature. A weak, but positive correlation has been described between serum IgE and blood eosinophils[199], while other reports have shown that therapeutic regulation of circulating IgE leads to a reduction in airway eosinophils[61,200]. In the current study, we observed a positive correlation between allergen-induced levels of sputum eosinophils and CD19+ B cells, and a negative correlation between methacholine $PC_{20}$ and CD19+ B cells. Taken together, these results suggest that activation of the IgE cascade by inhaled allergen, leads to eosinophilic inflammation and increased airway hyperresponsiveness.

To conclude, we demonstrated that IgE+ B cells and total IgE increased acutely in the airways after inhaled allergen challenge without parallel increases in peripheral blood, tonsils or bone marrow, measured over a period of 28 days. The allergen-induced level of airway CD19+ B cells significantly correlated with methacholine $PC_{20}$ and airway eosinophils. These results highlight the role of local IgE+ B cells in the development of allergen-induced airway responses.

**5.2 Regulatory B Cells - New Cell on the Block Modulating Allergic Inflammation**

Canonically, IgE produced by B cells is critical for initiation of the allergic cascade, ultimately leading to disease pathogenesis in allergic disorders[181]. However, with the recent discovery of regulatory B cells ($B_{regs}$) and their potential role in allergic disease, it is pertinent to gain a better understanding of their kinetics and function after allergic stimulation[72,157]. Based on initial findings, we report lower levels of $B_{regs}$ in allergic asthmatics compared to healthy controls. We investigated $B_{regs}$ further, and for the first time, utilizing the allergen inhalation challenge model, we report a shift in $B_{regs}$ populations in bone marrow, blood and sputum of mild allergic asthmatics. Specifically, we observed a decrease in FoxP3+ $B_{regs}$ in bone marrow 24 hours post-allergen inhalation, with a coincident increase in FoxP3+ $B_{regs}$ in blood at 7 hours and sputum at 24 hours post-allergen inhalation compared to post-diluent inhalation. Furthermore, in bone marrow and blood, we examined specific subpopulations of FoxP3+ $B_{regs}$ and found that at 24 hours post-allergen inhalation compared to post-diluent inhalation, FoxP3+ $B_{regs}$ subpopulations were decreased in the bone marrow, but increased in the blood. Due to the rarity of these cells and limited cell yield from the sputum samples obtained, we were unable to measure FoxP3+ $B_{regs}$ subpopulations in cells from the airways (Figure 5.3) (Chapter 4).

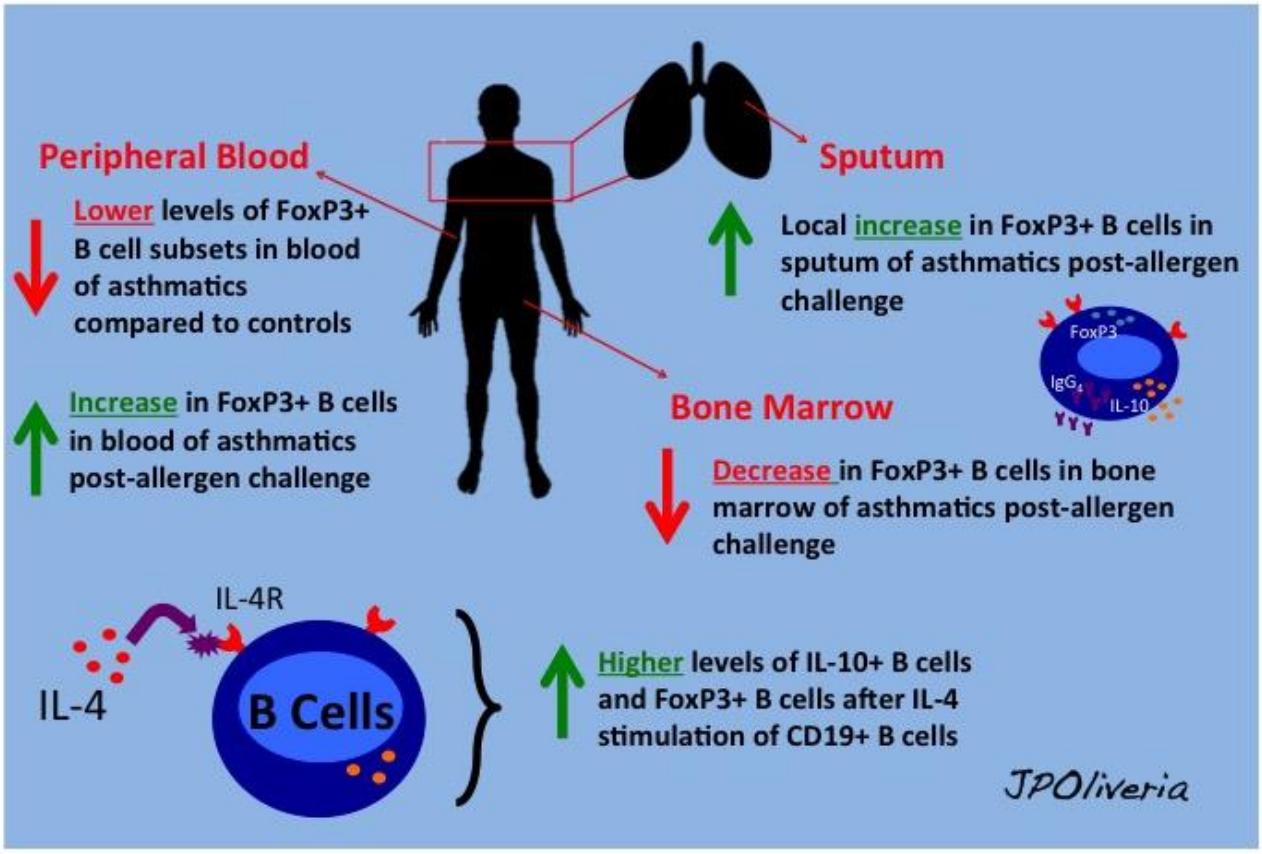


**Figure 5.3: Changes in regulatory B cell levels in bone marrow, blood and sputum of asthmatics following inhaled allergen challenge.**

In 2010, a paper by Amu *et al* highlighted that $B_{regs}$ were shown to prevent and reverse allergic airway inflammation in a murine model of allergic airway disease[72]. However, this model of allergic airway disease was induced using a parasitic helminth infection model. In other studies by Singh *et al* and Natarajan *et al*, an OVA challenge model for mice was used to induce allergic airway disease[157,201]. In these studies, adoptive transfer of B cells from the hilar lymph node resulted in decreased methacholine-induced hyperresponsivness and decreased tissue inflammation, including decreased eosinophilia after OVA challenge of the mice. Furthermore, it is important to highlight that these murine studies showed a potential cell interaction mechanism whereby adoptive transfer of regulatory B cells ($B_{regs}$) also resulted in increased levels of FoxP3+ regulatory T cells ($T_{regs}$) in the lungs of the mice after challenge[72,157,201]. Thus it is important to determine the role $B_{regs}$ play in inducing $T_{regs}$ after allergen inhalation challenge in humans. Of note, Natarajan *et al* also highlighted that the regulatory role of B cells was only observed after adoptive transfer of CD5+ B cells (B1 B cells from the fetal liver), but not CD5- B cells (B2 conventional B cells) from the hilar lymph nodes[201]. Based on these findings, we are able to shed light on specific subpopulations of B cells, specifically CD5+ $B_{regs}$, that may have a more regulatory role than others.

To further this finding, a model of cow's milk allergy evaluated the role of CD5+FoxP3+ $B_{regs}$ in cow's milk allergic subjects. It was important to delve into the role of FoxP3 in relation to $B_{regs}$ due to the importance FoxP3 plays in the development and function of $T_{regs}$. Noh *et al* and Lee *et al* showed that CD5+FoxP3+ $B_{regs}$ were able to regulate cow's milk allergic responses through the production of IL-10[153,202–204]. However, in addition to CD5 and FoxP3, these $B_{regs}$ were also shown to express CD1d. In short, the phenotypic characterization of $B_{regs}$ remains inconsistent in literature. In 2013, the first paper characterizing multiple phenotypes of $B_{regs}$ in allergic asthmatic human subjects was published by van der Vlugt *et al*. Taken together, the major phenotypes of $B_{regs}$ that have been implicated in allergic subjects include: CD5+FoxP3+, CD1d+CD5+, CD24+CD27+ and CD24+CD38+, and these

phenotypes were selected for the current study. In the cross-sectional study conducted by van der Vlugt *et al*, comparing the levels of $B_{regs}$ from allergic asthmatics to healthy controls, it was shown that the levels of CD24+CD27+ $B_{regs}$ were lower in allergic asthmatics (~20% of CD19+ B cells) compared to healthy controls (~30% of CD19+ B cells). In the current study, we were able to replicate van der Vlugt's findings, where we showed allergic that the levels of CD24+CD27+ $B_{regs}$ were significantly lower in asthmatics (~15% of CD19+ B cells) compared to healthy controls (~30% of CD19+ B cells). However, we also demonstrated that the levels of CD5+FoxP3+ $B_{regs}$ were significantly lower in allergic asthmatics (~5% of CD19+ B cells) compared to healthy controls (~10% of CD19+ B cells). Interestingly, the total level of the FoxP3+ $B_{reg}$ population was also significantly lower in allergic asthmatics (~7.5% of CD19+ B cells) compared to healthy controls (~15% of CD19+ B cells). It appears that regardless of phenotype, the proportion of $B_{regs}$ in allergic asthmatics is approximately 50% lower than healthy controls. Furthermore, in a study by Kamekura *et al*, the levels of CD24+CD27+ $B_{regs}$ were lower in allergic asthmatic (~15% of CD19+ B cells) and allergic rhinitic (~25% of CD19+ B cells) subjects compared to healthy controls (~35% of CD19+ B cells). Taken together with the data of van der Vlugt *et al* and Kamekura *et al*, our findings support lower levels of $B_{regs}$ in subjects with allergic asthma. However, there have been no other studies evaluating the changes in frequency of $B_{regs}$ in humans after an allergen inhalation challenge.

In the present study, the allergen inhalation challenge was used to evaluate the levels of different phenotypes of regulatory B cells after allergic stimulation, in addition to determining temporal changes in $B_{reg}$ populations in the bone marrow, blood and lungs of mild asthmatic individuals. We showed that there was a decrease in FoxP3+ $B_{regs}$ in bone marrow 24 hours post-allergen inhalation, with a coincident increase in FoxP3+ $B_{regs}$ in blood at 7 hours and sputum at 24 hours post-allergen inhalation compared to post-diluent inhalation. These findings indicate the potential mobilization of $B_{regs}$ and their precursors in the bone marrow, trafficking through the blood, and ultimately maturation

and expansion in the diseased organ, the lungs. This is the first study to report this apparent efflux of $B_{regs}$ in the bone marrow and subsequent increase in the lungs. As previously mentioned, $T_{regs}$ have been shown to co-localize with $B_{regs}$ in the lungs[72,157] after adoptive transfer of $B_{regs}$ in murine model; however, in a study conducted by Kinoshita et al, it was shown that there is a significant decrease of $T_{regs}$ in sputum at 24 hours post-allergen challenge compared to post-diluent challenge[205]. Taken together, the persistence of chronic inflammation in allergic asthma cannot be ameliorated because there may not be a synergistic anti-inflammatory effect induced by $B_{regs}$ and $T_{regs}$. Thus further studies evaluating the interaction and function of $B_{regs}$ with other effector and regulatory cells are crucial in better understanding the pathogenesis of allergic asthma.

Furthermore, in bone marrow and blood, we were able to track specific subpopulations of FoxP3+ $B_{regs}$ and found that at 24 hours post-allergen inhalation compared to post-diluent inhalation, FoxP3+ $B_{regs}$ subpopulations were decreased in the bone marrow, but increased in the blood. Due to the rarity of these cells and limited cell yield in sputum samples obtained, we were unable to measure FoxP3+ $B_{regs}$ subpopulations in the airways. It has been shown previously that $T_{regs}$ are decreased in the lungs of asthmatic subjects[205], and since FoxP3 is a major marker for the development and function of $T_{regs}$[206], it is important to evaluate FoxP3 in $B_{regs}$. Furthermore, lower levels of FoxP3+ $B_{regs}$ in allergic asthmatics is supported by overall lower FoxP3 expression levels on mRNA[207] and protein[208] levels when comparing allergic asthmatics and healthy controls.

Additionally, there were two phenotypes of $B_{regs}$ that appeared to not only demonstrate interesting results, but consistent results from previously published literature, and these included: CD5+FoxP3+[147,153,204] and CD24+CD27+[77,109] $B_{regs}$. Specifically, we observed significantly lower levels of CD5+FoxP3+ and CD24+CD27+ $B_{regs}$ in allergic asthmatics compared to healthy controls. Also, we observed that 24 hours after allergen inhalation challenge, CD5+FoxP3+ $B_{regs}$ were decreased

in the bone marrow and increased in the blood. Thus we explored the functional capacity of CD5+FoxP3+ and CD24+CD27+ $B_{regs}$ and their ability to express IL-10 in an *in vitro* culture model.

Overall, the acute decreases in bone marrow, and increases we observed in blood and sputum FoxP3+ $B_{reg}$ levels after allergen inhalation challenge suggests that generation of local signals in the airway microenvironment leads to a local expansion or efflux of FoxP3+ $B_{regs}$. This local effect of inhaled allergen on FoxP3+ $B_{regs}$ was supported by observed increases in circulation, and decreases in the hematopoietic compartment post-challenge. Furthermore, the frequency of FoxP3+ $B_{regs}$ were observed to be lower in allergic asthmatics compared to healthy controls. Since the role of $B_{regs}$ is to dampen allergic inflammatory responses[72,110,157], the acute increases in $B_{regs}$ in the airways may be driven by homeostatic responses in offsetting IgE-mediated inflammation and eosinophilia following allergen challenge. The expansion of FoxP3+ $B_{regs}$ in the airways at 24 hours post-challenge may be a regulatory responses counteracting the expansion of IgE+ B cells in the airways at 7 hours post-challenge[209].

### 5.3 Clinical and Therapeutic Implications - IgE, IgE+ B Cells and Regulatory B Cells as Targets in Allergic Asthma

Although B cells are important in the pathogenesis of allergic asthma, there are very few human studies that have evaluated B cells in the airways. Previous reports have primarily focused on the levels of IgE in the lungs (sputum fluid phase and bronchalveolar lavage fluid)[94,194]. There has been a need to understand the function and levels of IgE, specifically allergen-specific IgE due to efficacious monoclonal antibody therapies that reduce IgE levels. Currently, FDA and Health Canada approved, omalizumab is monoclonal antibody to IgE that binds free IgE[210–212], and it has shown efficacy in improving asthma symptoms and reducing exacerbations[188,213]. Since the omalizumab dose is titrated based on IgE levels, increased circulating IgE after allergen exposure could theoretically render omalizumab treatment less effective, which is why it is important to understand the kinetics of B cells and their functions after allergic stimulation. Several other therapeutics targeting IL-4 and/or IL-13 have also been tested and variable effects on IgE reduction were reported[187]. Some of these novel therapies fail like due to a lack of understanding regarding the role of B cells in allergic asthma pathogenesis; for example, we do not understand the mechanism by which $B_{regs}$ affect the production of IgE by IgE+ B cells, which is an avenue for future research. Specifically, targeting IgE+ B cells in the airways may be a positive step forward in therapeutic development; however, when Fahy et al attempted the aerosolize route of delivery for anti-IgE, the therapy failed to attenuate allergen-induced responses (EAR and LAR)[58]. Taken together with increases in IgE+ B cells specifically in the airways after allergen inhalation challenge in asthmatics[209,214], utilizing the aerosol route in targeting IgE+ B cells may be a novel avenue for therapeutic development. Quilizumab with intravenous administration was shown to be effective in treating mild asthmatics[61], but not moderate to severe asthmatics[215]; although the integrated network of inflammatory pathways is more complex in more severe asthmatics,

specifically targeting IgE+ B cells in severe, eosinophilic asthmatics may be an interesting avenue for future research and therapeutic development.

In recent years, there have been several key publications allowing us to better understand the role B cells play in the propagation of allergic asthma; particularly, localized inflammation in the lungs. The kinetics of IgE+ B cells after allergen challenge have been explored in murine models of allergic airway disease using ovalbumin (OVA) sensitization and challenge[102]. Luger *et al* showed that short-lived IgE+ B cells were transiently increased in the lungs, spleen and bone marrow of mice 3 days after OVA-inhalation challenge. To compliment this study, we recently showed that an increased frequency of IgE+ B cells in the sputum, but not blood, bone marrow and tonsils, after allergen inhalation challenge in allergic asthmatic subjects[209]. Furthermore, we showed that there are higher baseline levels of IgE+ B cells, and IgE+ memory B cells and plasma cells in the sputum of allergic asthmatics compared to healthy controls[214]. Taken together, there is growing significance in studying the diseased organ to better understand the cellular interplay of immune cells in the pathogenesis of allergic asthma.

Several therapies have been developed to reduce IgE levels. Omalizumab is monoclonal antibody to IgE that binds free IgE in serum and on the surface of B cells[210–212], and clinically it has shown efficacy in improving asthma symptoms and reducing exacerbations[188,213]. In mechanistic studies using the allergen challenge model, treatment with omalizumab inhibited early and late asthmatic responses, decreased eosinophil levels in sputum[213,216]. Other systemic approaches for reducing circulating levels of IgE have been evaluated. Ligelizumab is an IgE antibody with higher affinity for IgE than omalizumab with greater suppression of IgE-receptor complex formation[56], thereby providing the theoretical potential for enhanced protection compared to omalizumab[217]. Other approaches aimed to reduce the synthesis of IgE by regulating B cell function have been tested in subjects with allergic asthma. Blockade of OX40L was found to decrease total and allergen-specific IgE in the circulation compared to placebo[200], and these decreases were sustained over 250 days after

treatment. Additionally, anti-OX40L treatment reduced eosinophil levels in the sputum. Several different agents targeting IL-4 and/or IL-13 have also been tested and shown variable effects on IgE reduction[187]. Targeted depletion of IgE+ B cells has been tested using an antibody targeting M1-prime, a specific epitope on membrane-bound IgE on B cells. Treatment with anti-M1-prime antibody (quilizumab) reduced serum total IgE and airway eosinophils, ablated the allergen-induced increase in serum total and specific IgE, accompanied by blunted bronchoconstriction response to inhaled allergen[61]. In a larger study in uncontrolled asthmatics quilizumab reduced serum total and allergen-specific IgE approximately 1 year of treatment, but it did not reduce asthma exacerbation and symptoms[215]. The ineffectiveness of this treatment may have been due, in part, to long-lived IgE+ B memory cells and IgE plasma cells that do not express M1-prime, and thus would have remained fully functional, which was supported by the observation that serum IgE was still detectable[215]. Moutsoglou and Dreskin showed that depletion of immature B cells in a murine model did not ablate allergen-specific IgE levels, which they postulated to be due to long-lived plasma cells[218]. Based on these studies, the importance of reducing IgE is clearly evident, however, there is a lack of understanding about the kinetics of IgE+ B cells and IgE synthesis after exposure to an allergic stimulus. However, there is growing literature specifically related to local changes of immune cells and their mediators within the airways in allergic asthma.

Since allergic pathogenesis is driven by effector cells, particularly through IgE dependent mechanisms, the production of IgE by IgE+ B cells is crucial in sustaining chronic lung inflammation[209,214]. However, $B_{regs}$ appear to sustain inflammatory homeostasis through the release of anti-inflammatory mediators, including IL-10[110,152,153]. Specifically, within the allergic context, $B_{regs}$ have been implicated in dampening the allergic immune response through their production of IL-10[109,110]. In cow's milk allergic patients, in vitro stimulation with cow's milk allergen, casein, B cells were induced to produce IL-10[153,202]. Thus, within an allergic context, B cells are able to produce

necessary responses to propagate the allergic response, but there are also regulatory mechanisms that are induced to help control the allergic response[202]. In the present study, we showed that after stimulation of isolated B cells with IL-4, there were higher levels of FoxP3+IL-10+, CD5+FoxP3+IL-10+ and CD24+CD27+IL-10+ $B_{regs}$ compared to media alone stimulation. This data shows that upon B cell exposure to type 2 inflammatory environments, B cells become competent in increasing expression of IL-10, which is the currently known mechanism for which $B_{regs}$ elicit their regulatory role. Additionally, van de Veen *et al* has shown that $B_{regs}$ not only are able to elicit an immunoregulatory role through IL-10, but also through the production of IgG4, an immunoglobulin subtype able to outcompete IgE in binding allergen[110]. However, van de Veen *et al* only showed this phenomenon in bee venom allergic individuals, and they characterized $B_{regs}$ to be CD73-CD25+CD71+. It would definitely be of interest to show allergen-specific $B_{regs}$ responses with relevant environmental allergens to allergic asthma. Furthermore, a consensus in the literature in how to phenotypically define $B_{regs}$ would be helpful in delving further into their functional capacity. Lastly, a better understanding on the ontogeny of $B_{regs}$ is important to understand the dichotomy between effector and regulatory B cells.

**5.4 Limitations and Future Directions**

Although the study of B cells within the context of allergic asthma has rekindled interest in the research community in the last several years, there are several limitations when studying these cells, particularly in the lungs. B cells (1-7% of leukocytes in blood) are relatively rare lymphocyte populations[182,214]. Additionally, studying memory subset populations of B cells is more difficult due to low numbers of memory B cells (0.2-1.7% of leukocytes in blood) and plasma cells (0.2-2% of leukocytes in bone marrow)[104,214]. Since these parent populations of B cells are already rare, further studying smaller subsets of these cells, such as IgE+ B cells and $B_{regs}$, adds to the complexity and difficulty in designing experiments. However, the proposed flow cytometric studies outlined in Chapters 2, 3 and 4 utilized appropriate controls to ensure rigor of the data presented. Specifically, the use of FcR block was necessary and utilized to minimize non-specific binding of fluorescent-labeled antibodies on target cells and false positive results. The use of isotype controls was needed to ensure minimized non-specific binding allowing for clear visualization of positive gates for target cell populations during analysis. Furthermore, fluorescent minus ones (FMOs) were utilized in cases of dim populations in order to correctly identify dim-positive cell populations (-eg- FoxP3+ $B_{regs}$ in Chapter 3) (data not shown). One limitation of the experiments in this dissertation was the lack of live and dead cell gating. In a small quality control experiment, CD19+ B cell proportions remained unchanged between analysis of cells with and without the fixable viability dye (FVD) (data not shown). However, more extensive studies evaluating rare subpopulations of cells (-eg- IgE+ B cells and FoxP3+ $B_{regs}$) with and without FVD need to be evaluated due to the nature of dying cells being more "sticky" and making it more difficult to delineate single cells or singlets. There is a progressive movement in flow cytometric analysis of cells to utilize FMOs and FVDs to ensure correct gating of true positive cell populations of target cells.

A clear strength of this dissertation is the use of well-characterized subject groups (-eg- healthy non-allergic, non-asthmatics, allergic non-asthmatics, allergic-asthmatics) evaluated using diagnostic techniques and past medical history (well described in the methods section of Chapter 2, 3 and 4). Furthermore, in the evaluation of asthmatics before and after allergen inhalation challenge, diluent inhalation challenge was used as an appropriate control. Although it would have been interesting to perform allergen inhalation challenge in healthy, non-allergic, non-asthmatic subjects, there would have been a risk of sensitizing the subjects to allergens and these experiments likely would not have passed the research ethics board. The diluent inhalation challenge remains to be the widely utilized control for allergen inhalation challenges.

Furthermore, there is a growing interest in studying the levels and functions of B cells in the diseased organ, in this case the lungs; however, we are currently limited on the amount of sample we can utilize for experimentation. Sputum-derived cells are a good surrogate for gaining a snapshot view of inflammatory processes occurring in the lungs; additionally, sputum samples provide a non-invasive means of sampling cells in the lung[191,219–222]. Other sample types that would be worth exploring include cells from bronchial brushings or bronchial alveolar lavage fluid; however, getting these samples require more invasive techniques that are not as well tolerated by subjects, and cannot be serially collected to examine kinetics[223,224]. Lastly, the ability to obtain a bronchial biopsy would be an excellent way to not only quantify cells in the lungs, but a great way to also determine localization of cells with other cells and specific lung structures, such as the epithelium and basement membrane[224–226].

Another big limitation when studying IgE+ B cells and $B_{regs}$ is the technological limitation in being able to quantify these cells. With advancement in flow cytometry techniques and an abundance of newly available fluorochromes, it has been possible to adequately study each of these cell subsets (IgE+ cells and $B_{regs}$) in separate flow cytometry experiments. However, the ability to study IgE+ B

cells and $B_{regs}$ in one experiment would be ideal since it would give us the ability to accurately compare the ratio of these cell types; better examining the balance in cellular quantities between inflammation and regulation within the context of allergic asthma pathogenesis. Specifically, utilizing mass cytometry or cytometry by time of flight mass spectrometry (CyTOF), and specifically quantifying and characterizing IgE+ B cells and $B_{regs}$ would be beneficial in better understanding the proportions of these cell subpopulations[227].

Mass cytometry is a novel, versatile technology used for analysis of cells on a single cell level[228–235]. Unlike flow cytometry, mass cytometry allows for simultaneous measurement of more of biomarkers, intracellular and extracellular markers with greater precision[229,231]. In flow cytometry, cells of interest are labeled with fluorochrome-conjugated antibodies, and cell interactions with a laser leads to fluorescent emission of light at variable wavelengths, allowing for the detection and quantification of up to 20 cellular markers[231]. However, mass cytometry involves the labeling of cells with metal-tagged antibodies for the detection and quantification of up to 50 cellular markers, which is almost double compared to flow cytometry[231]. The ability to simultaneously explore more cellular parameters allows for better understanding of complex cellular systems and signalling pathways that contribute to our current understanding of B cell biology. Quantifying cells based on mass and charge allows us to circumvent fluorochrome light detection overlap and autoflouresence, which are large limitations of flow cytometry[231]. Lastly, mass cytometry allows for cellular barcoding, which improves the efficiency and sensitivity of single-cell analysis; allowing researchers to better understand shifts and functional potential of specific cellular subsets and sub-phenotypes[228,231,232]. Capitalizing on the utility of cellular barcoding to increase the efficiency in the acquisition of cells from multiple patients and/or cells from different compartments, such as: spleen, blood and bone marrow, significantly increases the throughput of precious biology samples[228,232]. Specifically, metal-labelled cell barcoding (MCB), explored by Bodenmiller et al, uses a binary combination of seven different lanthanide ions to get 128 different

combinations of lanthanide elements, which increases the combinations of lanthanide ions that can be used for barcoding[228,232]. Thus, a barcode channel would be a combination of metals conjugated to a functionalized antibody specific to a cellular marker. This high-content, high-throughput screening with MCB can be useful for clinical trials investigating novel therapeutics, pre-clinical testing of drugs, and *in vivo* and *in vitro* mechanistic investigation of human disease. Taken together, since there is currently no consensus on surface phenotype of $B_{regs}$ in the literature, the use of mass cytometry to better understand the distribution and quantities of different $B_{regs}$ phenotypes may aid in better understanding the roles these cells play in allergic asthma pathogenesis. Additionally, since we do not know the distribution of $B_{regs}$ in the body after allergic stimulation, we can take advantage of cellular barcoding and mass cytometry to simultaneously analyze multiple compartments (bone marrow, blood, airways) after allergen inhalation challenge. Lastly, mass cytometry can be further used to better understand the single cell proteomic profile of cells by intracellular staining of different cytokines being produced by the cells (-eg- type 2 cytokines; IL-4 and IL-13).

Taken together, the future of B cell research, particularly better understanding the IgE+ B cells and $B_{regs}$ would be to pursue single cell analyses of these rare B cell subpopulations. Utilizing mass cytometry analysis of B cells would allow for the development of a large panel of marker, which could include: all surface receptors associated with regulatory B cells, immunoglobulins, inflammatory and regulatory mediators, type 1, 2 and 17 cytokines, and transcription factors important for B cell development. This shotgun approach for understanding B cells on a single cell level would generate a lot of data, ultimately leading to hypothesis-driven questions and better experimental designs when studying the function of IgE+ B cells and $B_{regs}$. Furthermore, better understanding of B cell genomics, transcriptomics and proteomics, would be beneficial in understanding, which genes, transcription factors and proteins to further study as potential therapeutic targets (-eg- nanostring technology). Better understanding of upregulated and downregulated genes would allow for the development of better

murine knock-out models to understand the development and function of B cells. Additionally, regarding the regulatory B cell population, there is currently no known transcription factor responsible for the development of these cells, thus implementing 'omics' type techniques specifically on B cells, would help shed some light on these currently gaps in literature. While regulatory B cells are known to regulate inflammatory processes through their production of IL-10, proteomic analyses would give us some insight into the importance of other regulatory mediators such as $IgG_4$ and IL-35 (Figure 5.4).

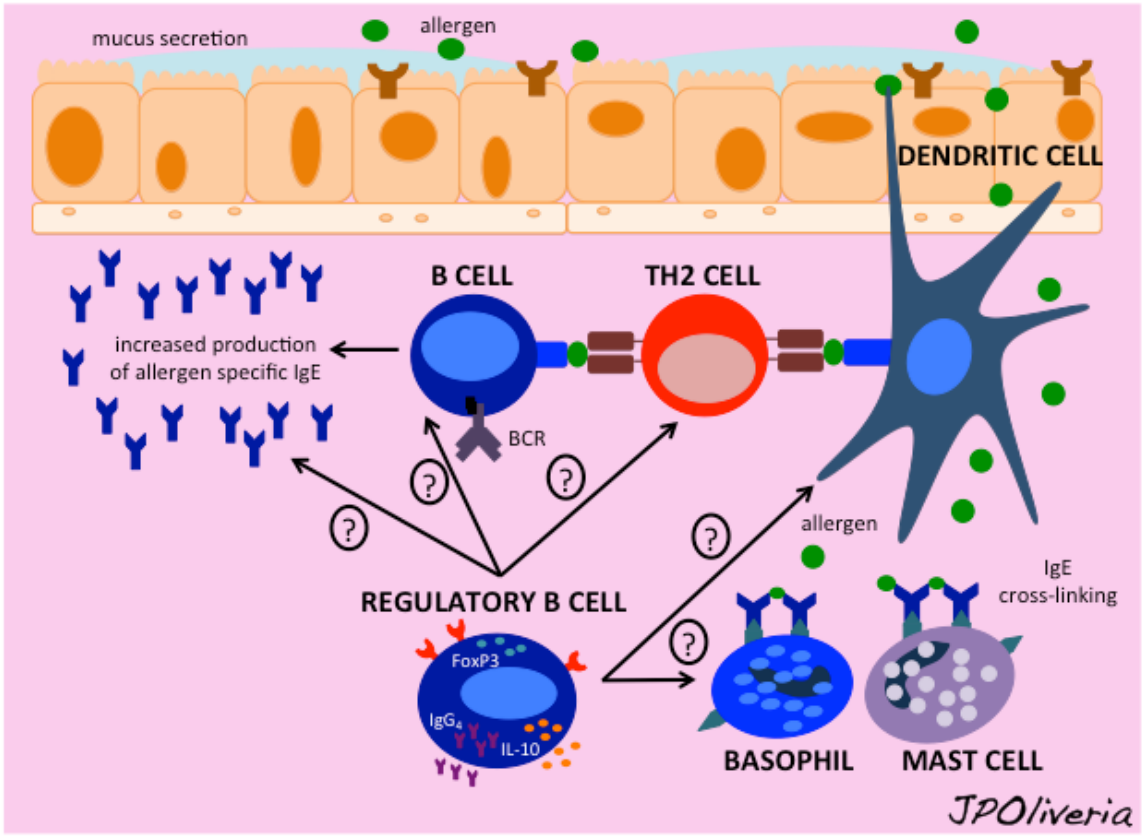


**Figure 5.4: The unknown roles regulatory B cells play in the pathogenesis of allergic asthma.** Future studies can evaluate the potential roles regulatory B cells play in: IgE production by B cells, modulating IgE+ memory B cell responses, Th2 cell responses, dendritic cell responses, and basophil and mast cell responses. Currently, the functional roles regulatory B cells play in allergic asthma pathogenesis is limited.

**5.5 Summary**

To conclude, we demonstrated that IgE+ B cells increased acutely in the airways after inhaled allergen challenge without parallel increases in peripheral blood, tonsils or bone marrow, measured over a period of 28 days. Furthermore, these observations were highlighted by acute decreases in bone marrow, and increases in blood and sputum of FoxP3+ $B_{regs}$ after allergen inhalation challenge. Taken together, these findings demonstrate that there are allergen-induced changes affecting levels of IgE+ B cells and $B_{regs}$, that are closely related to other local inflammatory processes occurring in the airways. However, there is still a large gap in our understanding regarding these cells and their roles in allergic asthma pathogenesis, mainly driven by our limited ability to study rare cell populations. Future studies are warranted to determine the role these cells play in the allergic cascade, particularly regulatory B cells. Overall, the use of 'omics' technologies and single cell analyses of IgE+ B cells and $B_{regs}$ looks to be promising for the future of this field.

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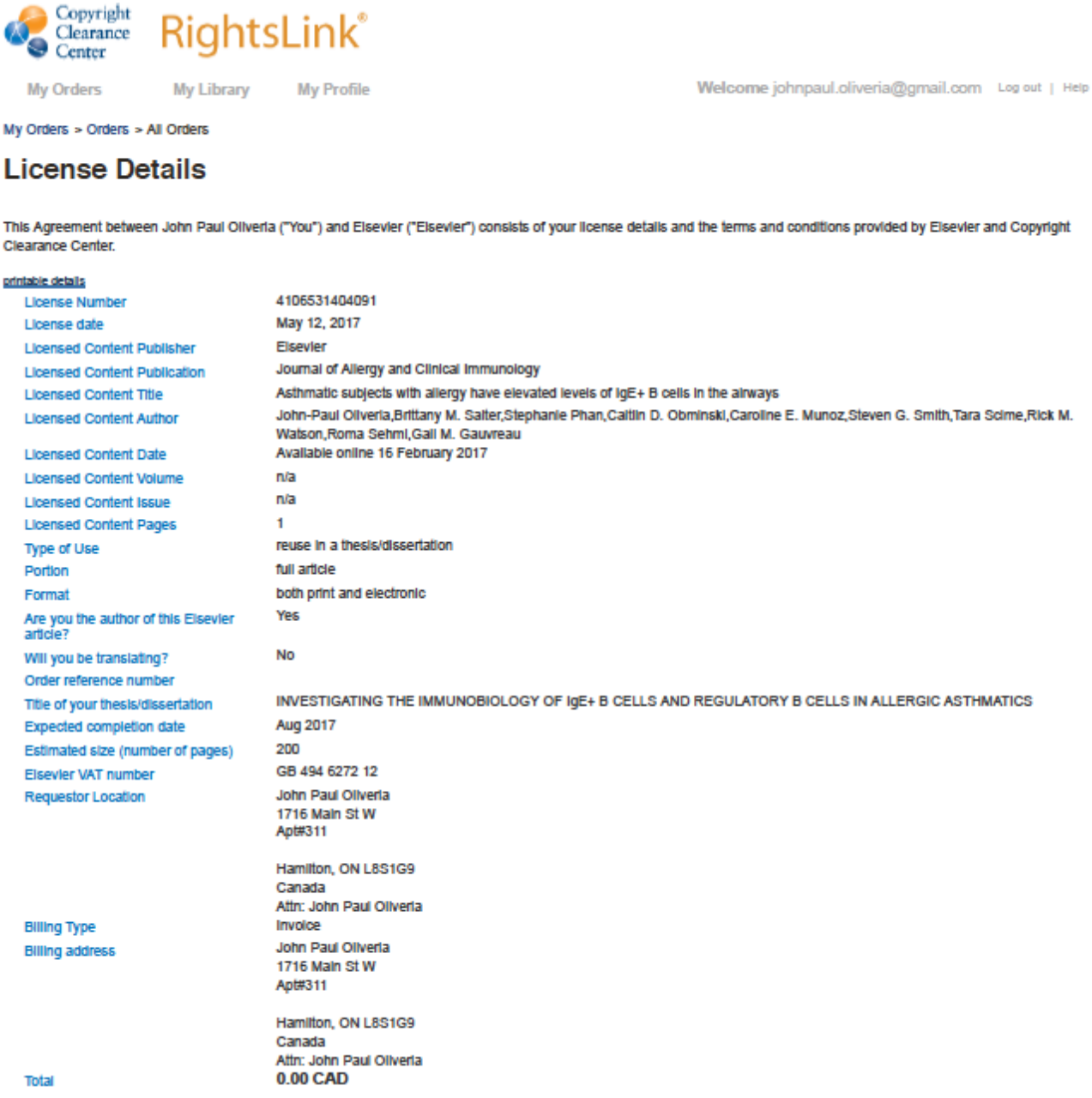

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Chapter 4 of this PhD dissertation has been submitted to the Journal of Allergy and Clinical Immunology and is currently under peer review.