**Role of $T_H2$ Cytokines in Regulatory B Cell Biology**

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Immunology)
at the University of Michigan
2018

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**DEDICATION**

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**ACKNOWLEDGMENTS**

I would first like to thank my family. The lessons I have learned throughout my life from you are what make me who I am. Your love and wisdom carries me through the hard times and inspires me in the good times. I am grateful to be a part of such a supportive and inseparable family, even with the great distance between us.

To my mentor Steve, it has been a wild ride. Throughout the years we have grown a lot together and we know it hasn't always been easy. I want to thank you for all your support and enthusiasm in the lab and on paper. Your constant excitement for science inspires me to find that same passion as I prepare for my next steps.

To my lab, thank you for all the comradery and support. Lucien, thank you for making me a better scientist and for all the laughs and tears we shared. Thank you for all your funny Dutch quirks, sayings, and spirit. Thank you for being the most genuine friend. Molly, you have taught me more than you know and I have enjoyed mentoring you through these past two years. Your fun loving spirit is a friendly reminder to relax and enjoy life as it comes, that you for being your sweet innocent self.

To my Immunology program family, these have been the most stressful, exciting, depressing, and inspiring years of my life and we have all gone through these growing pains together. I chose Michigan because it felt like you all actually cared about the each other. I now leave Michigan knowing this to be true throughout the students, faculty, and staff. As I start my next chapter, I know I will take with me the many meaningful friendships I have built with you.

Thank you Beth, you are also why I chose Michigan, you made it feel like home here and have never been anything but supportive and caring. You make this program a family, thank you. Zarinah, we are so lucky to have you and I have enjoyed working with you on all the fun immunology events we have planned. You are the glue that keeps us all from unraveling. Thank you for everything you have done and continue to do for all us.

To my PIBS and friends 2013, thank goddess for you all. First year was rough and if it had not been for intramural sports every semester and post game beers, I would have surely lost my mind. Thank you for all the PIBsgivings, birthday parties, and the best tailgating on that entire golf course.

I also would like to thank my committee and my co-mentor Nick. You have all been nothing but supportive throughout this process. Thank you for your insight, advice, and your time. Nick, thank you for all the long morning harvests that I am sure we are both glad are done. Thank you for always being available to talk about life and science.

Aric, hagu gue niya hu todu i tempu. Hamyo acho' hu. Magof yu' na matto kon hao. Gi sagan ora para ta fanhanao. Ben lana hao mangge, yanggen kon mu. Nihi ta fanhanao para i salon ta chule' gimen-mami. Ta miresi-na. Hu guaiya hao ginen fondo korason mu.

Thank you to my funding sources: Rackham Graduate School Merit Fellowship, American Association of Immunologists Careers in Immunology Fellowship, and NIH-NIAID AI007413-21

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## ABSTRACT

B cells are now appreciated to be far more than antibody secreting cells. Throughout the last two decades B cells have been described to utilize a vast array of mechanisms to influence immune responses. Immune tolerance is achieved by a network of cells composed of regulatory T cells, tolerogenic DC and now regulatory B cells are held as a significant part of this network. However, much of the biology of regulatory B cells has yet to be uncovered. This thesis set out to understand how regulatory B cells may be modulated during $T_H2$ responses in order gain insight into their dysregulation that may contribute to allergic disease.

To understand how regulatory B cells are modulated during allergic responses, we investigated the effects of $T_H2$ cytokines (IL-5 and IL-4) on their regulatory function and growth *in vitro*. Previously, the lab reported that culture with CD40 ligand (CD40L) and interleukin 5 (IL-5) stimulated the secretion of IL-10 from murine B cells. In this thesis we further described this stimulation to induce IL-10 production specifically from B-1a cells which was inhibited in the presence of IL-4. The suppressive capacity of the IL-10 producing B cells was proven *in vivo* in an allergic airway disease model and found to be dependent on their secretion of IL-10. Adoptive transfer of CD40L and IL-5 stimulated B cells reduced airway resistance measured using plethysmography, and cellular infiltration and cytokines in the airways of sensitized mice. We suggest that regulatory B cell growth and function may be limited in the context of allergic inflammation due to the presence of IL-4 and may allow for the continuation of inappropriate

responses to allergens. However, enhancing the number and function of these B cells could lead to reduced disease severity and potentially the induction of tolerance.

Similar *in vitro* cultures were performed with human B cells from the peripheral blood. Inhibitors were used to target signaling pathways downstream of CD40L and IL-5 stimulation to understand the necessary pathways for regulatory B cells growth and function in both mice and human B cells. Bruton's tyrosine kinase, nuclear factor of activated T cells, JAK, and STAT signaling may all play a part in regulating suppressive function and growth of IL-10 producing B cells.

Together this work provides insight into the modulation of regulatory B cells during allergic inflammation. We also identify possible targets to further investigate as therapeutics to enhance or inhibit regulatory B cell activity. Many protective and pathogen roles have been described for regulatory B cells spanning autoimmunity to infectious disease. While the focus of this thesis was in the context of allergic inflammation, modulation of regulatory B cells with the identified targets could be employed throughout human disease

# CHAPTER 1

# Introduction

## 1.1 Regulatory B cells

The immune system is complex and dynamic. Immune regulation is a necessary companion to immune activation, preventing excessive tissue damage from an unchecked immune response. Many cells are involved in maintaining tolerance to non-pathogenic antigens. Structural cells assist in delivery of antigens, antigen presentation, and homing of other regulatory cells. $CD103^{+}$ dendritic cells (DC) and the regulatory T cells they induce are the two most well studied immune cell types that play crucial roles in peripheral tolerance (Berin, Sampson 2013, Chistiakov et al. 2015). However, many other cells including innate lymphoid cells and basophils have been implicated in immune tolerance, growing the immune tolerance network (Chirumbolo et al. 2017, Hepworth et al. 2015). This chapter will present evidence for the role of B cells in maintaining immune tolerance to self and to innocuous exogenous antigens, adding another layer of complexity to the DC and Treg centered model of tolerance. Over the past two decades, B cells, which are well established as antibody producers, have been increasingly recognized as important modulators of the immune response spanning throughout human disease from autoimmunity and allergy to infectious disease and cancer (Mauri, Menon 2015, Dai, Zhong & Xu 2017). Suppressive functions of B cells were first suggested in a delayed hyper-sensitivity model in guinea pigs (Neta, Salvin 1974). This response is transient, the guinea pigs will start to respond 4 days post sensitization and no longer respond 8 days post

sensitization. The authors found that pretreatment of the guinea pigs with cyclophosphamide, to preferentially deplete B cells, prolonged the response past 8 days (Neta, Salvin 1974, Turk, Poulter 1972). After this study, the field went quiet for more than two decades until Wolf et al. found that B cell deficient mice could not recover from experimental autoimmune encephalomyelitis (EAE) compared to self-remitting disease in wild type mice. This reignited the field and soon thereafter the term "regulatory B cell" was coined by Mizoguchi et al. They found that B cells played a suppressive role in TCR$\alpha^{-/-}$ spontaneous chronic colitis model, as B cell deficiency resulted in more severe disease but could be restored by B cell transfer (Mizoguchi et al. 2002). Regulatory B cells are now well appreciated to play a principal role in the immune regulatory network and to utilize multiple mechanisms including secretion of anti-inflammatory cytokines such as interleukin 10 (IL-10) and transforming growth factor beta (TGF-β), and expression of Fas ligand among many others (Ray, Wang & Dittel 2015). This thesis however, is specifically focused on IL-10 production from B cells. While most of the work on regulatory B cells to date has been in the context of autoimmunity, this thesis investigates the role of regulatory B cells in allergic asthma, and the influence that allergic immune responses have on growth and function of regulatory B cells. In this chapter we review the biology of IL-10-producing regulatory B cells and what is known of their functions in allergic disease. For simplicity, regulatory B cells from here on will specifically refer to IL-10 producing regulatory B cells.

### 1.2 Stimulation of IL-10 production from B cells

A variety of stimulations are known to induce IL-10 production and suppressive function of B cells (Fig1.1). Stimulation through toll like receptors (TLRs), the B cell receptor (BCR), or through interactions with other cells types either directly through CD40-CD40 ligand or

indirectly through the cytokines they produce. The following section reviews the stimulations found to induce regulatory B cells.

Toll like receptor ligands

Toll like receptor (TLR) ligands activate an integral arm of innate immunity that is critical for host defense. TLRs recognize pathogen associated molecular patterns that serve to detect early infection and activate innate immune responses. Human B cells have been shown to express TLR1-4, 6, 7, 9, and 10 with expression levels varying between subsets and activation states (Hornung et al. 2002, Mansson et al. 2006, Agrawal, Gupta 2011, Bernasconi, Onai & Lanzavecchia 2003). Murine B cells express TLR1-4, 6, 7, and 9, again with various expression levels in different subsets (Gururajan, Jacob & Pulendran 2007, Genestier et al. 2007, Marshall-Clarke et al. 2007). TLRs also play a significant role in the induction of regulatory B cell function. LPS stimulation *in vitro* induces IL-10 production from multiple B cell subsets (Lampropoulou et al. 2008, Yanaba et al. 2008, Madan et al. 2009). Mice with B cell specific deficiencies in TLR2, TLR4, or MyD88, a signaling molecule downstream of many TLRs, display reduced B cell suppressive abilities. These mice develop unremitting EAE in contrast to self-remitting wild type mice (Lampropoulou et al. 2008). Salmonella typhimurium infection is enhanced by the presence of regulatory B cells in wild type mice while deficiency of TLR2, TLR4, or MyD88 in the B cell compartment leads to resistance (Neves et al. 2010). Additionally, TLR9 stimulation by CpG also increases IL-10 production from neonatal B cells, dampening potentially fatal inflammatory responses to infection during early life (Sun et al. 2005). Interestingly, variations in CpG have been found to dictate cytokine production from B cells. Whereas CpG type A will elicit secretion of proinflammatory IFN-γ, type B promotes IL-10 production (Mauri, Bosma 2012). The seemingly opposing roles of TLRs during the immune

response suggest that certain cells within a subset may be programmed for an anti-inflammatory role and/or the diversity of TLR ligands play a more prominent role in directing immune responses than previously appreciated (Fig 1.1).

CD40 Ligand

CD40 is a membrane associated protein of the tumor necrosis factor (TNF) superfamily. While CD40-CD40 ligand (CD40L) interaction on B cells is important for maturation of antibody secreting cells, it is also one of the most well defined stimuli for IL-10 production from B cells and can come from a variety of cell types (Wykes 2003, Komlosi et al. 2017, Kim et al. 2015). One of the first reports of the involvement of CD40 in acquisition of B cell regulatory capacity was in TCRα mutant (TCR$\alpha^{-/-}$) mice which spontaneously develop chronic colitis. When crossed with Ig$\mu^{-/-}$ B cells deficient mice ($\alpha\mu^{-/-}$), these double knockout mice develop more severe colitis, suggesting a suppressive role for B cells in colitis (Mizoguchi et al. 1997). B cell transfers attenuated disease development and reduced pathogenic T cells during established colitis (Mizoguchi et al. 2000). Disease regulation was dependent on CD40 and CD86 expression on the B cells. Transfer of B cells deficient in CD40 or CD86, or the use of blocking mAb for these proteins eliminated protective effects. In an inflammatory bowel disease model, IL-33 peritoneal injection in mice induced IL-10 producing regulatory B cells (Sattler et al. 2014). *Ex vivo* anti-CD40 stimulation of these B cells, enabled IL-10 dependent suppression of T cell expansion and protected against spontaneous IBD *in vivo*. Similar results were found in the murine model of multiple sclerosis, EAE (Fillatreau et al. 2002). Recovery from EAE was found to be dependent on IL-10 production from B cells. B cells from recovered mice produced IL-10 in response to anti-CD40 and antigen stimulation. Mice with CD40 deficient B cells were unable to recover from EAE. In the collagen induced arthritis (CIA) model, treatment of mice with

agonistic anti-CD40 has been shown to prevent and alleviate arthritis (Mauri, Mars & Londei 2000). Additionally, treatment of splenocytes from arthritic mice with anti-CD40 prevented transfer of arthritis to SCID mice. In a follow up study, antigen and anti-CD40 treatment of arthrogenic splenocytes resulted in IL-10 producing B cells which were responsible for protection (Mauri et al. 2003). Adoptive transfer of anti-CD40 treated B cells to MLR/lpr lupus prone mice improved disease and survival in an IL-10 dependent manner (Blair et al. 2009).

In the context of allergy CD40 ligation has also induced protective regulatory B cells. In models of anaphylaxis, transfer of IL-10-producing $CD5^+$ B-1a cells suppressed mast cell activation (Kim et al. 2015). Protection was dependent on mast cell B cell interaction dependent on CD40-CD40L in which CD40 ligation by CD40L on the mast cell enhanced IL-10 production from B cells.

CD40 activation is also implicated in regulatory B cell function in tumor models. When splenocytes from C57Bl/6 or uMT mice (B cell deficient) were co-cultured with murine tumor lines increased antitumor activity was seen in the absence of B cells. B cells also limited $CD8^+$ T cell and NK cell activation, in a manner dependent on CD40-CD40L interaction (Inoue et al. 2006, Shah et al. 2005).

Taken together, these observations indicate that stimulation through CD40 induces regulatory function of B cells in multiple disease contexts (Fig 1.1). CD40 ligation alone and in addition to other stimuli has consistently shown promise as a potential target for expansion and activation of regulatory B cells. While CD40 ligation is a potent inducer of IL-10 production, CD40 expression is not necessary for IL-10 B cell development (Yoshizaki et al. 2012a).

B cell receptor stimulation

B cell receptor (BCR) signaling influences regulatory B cell function and development (Fig 1.1). This is evidenced in studies of CD19 deficient mice. CD19 is a positive regulator of BCR signaling. In its absence IL-$10^{+}$ B cells are absent while CD19 over-expression increases IL-$10^{+}$ B cell compared to wild type (Yanaba et al. 2008). $CD19^{-/-}$ mice experienced exacerbated T cell mediated inflammation in models of contact hypersensitivity, EAE and IBD (Yanaba et al. 2008, Yanaba et al. 2011, Matsushita et al. 2008). The calcium sensors, STIM1 and STIM2, downstream of BCR signaling are required for BCR induced IL-10 production (Matsumoto et al. 2011). B cells deficient in both sensors fail to produce IL-10 in response to BCR stimulation resulting in more severe EAE disease. Moreover, MD4 mice have fixed BCR specificities for hen egg lysozyme and therefore would never naturally encounter their antigen. The number of regulatory B cells *ex vivo* and TLR9 stimulated IL-10 producing B cells are significantly reduced in these mice (Fillatreau et al. 2002, Yanaba et al. 2009, Miles et al. 2012). In turn, bone marrow chimeras with MD4 B cells are unable to recover from EAE (Fillatreau et al. 2002). Finally, in multiple disease models B cells are induced during the inflammatory response. These B cells must be restimulated with the relevant antigen in addition to other stimuli to produce IL-10(Fillatreau et al. 2002)(Mauri et al. 2003). Furthermore, regulatory B cells from mice immunized with one antigen cannot suppress responses to a different antigen.

Cytokines

While activity of regulatory B cells during inflammation seems contradictory to the purpose of an immune response, it is clear they are present and exert regulatory function. Regulatory B cells are reported to increase in number during the inflammatory phase of multiple autoimmune and allergic diseases (Mizoguchi et al. 2002, Evans et al. 2007, Singh et al. 2008). Their functional role during inflammation is evidenced by the development of exacerbated

disease in their absence (Fillatreau et al. 2002, Singh et al. 2008, Carter et al. 2011, Carter, Rosser & Mauri 2012). Presence of regulatory B cells during inflammation suggests that inflammatory cytokines may play a role in Breg induction and expansion (Fig 1.1). A study of IL-10 regulatory B cells in EAE investigated multiple cytokines and their effects on the expansion of regulatory B cells (Yoshizaki et al. 2012b). They found that IL-21 increased regulatory B cell IL-10 production to similar levels as LPS stimulation, where as IL-4, 6, 10, 12, 23, and 17 had no effect. However, they did find that IFN-γ and TGF-β decreased IL-$10^{+}$ B cells after culture. Transfer of wild type regulatory B cells reduced EAE severity in $CD19^{-/-}$ mice by inhibition of T cell proliferation, but transfer of $IL\text{-}21R^{-/-}$ Bregs had no protective effect (Yoshizaki et al. 2012b). IL-21 from T follicular helper (Tfh) cells in MRL/lpr lupus prone mice has also been shown to promote IL-10 production and regulatory function from B cells. While IL-6 did not increase regulatory B cells in the previous study (Yoshizaki et al. 2012b), another group found that IL-6 and IL-1β production during induction of arthritis promoted differentiation of regulatory B cells and was dependent on the gut microbiota (Rosser et al. 2014). Mice with IL-6R or IL-1βR deficient B cells had reduced numbers of regulatory B cells and exacerbated arthritis. *In vitro* stimulation of naïve B cells with CD40L, IL-1β, and IL-6 induced IL-10 production from multiple reported subsets of regulatory B cells. Additionally, an interesting GM-CSF IL-15 fusion protein termed GIFT-15 was shown to induce regulatory B cells after *ex vivo* treatment of mouse splenocytes (Rafei et al. 2009). Infusion of these B cells induced remission in EAE mice, dependent on B cell IL-10 production.

IL-33 is an innate cytokine associated with inflammatory conditions of the mucosa (Bamias et al. 2012). Intra-peritoneal administration of IL-33 in $IL\text{-}10^{-/-}$ colitis prone mice accelerated development of IBD symptoms. However, WT mice were protected from gut

inflammation following IL-33 administration which induced IL-10 producing B cells in the blood. Transfer of the induced B cells was sufficient to prevent spontaneous IBD induction in the IL-$10^{-/-}$ mice (Sattler et al. 2014). Using the dextran sulfate sodium model of IBD in C57Bl/6 mice, IL-33 was again found to exacerbate disease symptoms although an increase regulatory B cells in the mesenteric lymph nodes was found (Zhu et al. 2017). Whether the increased inflammation or the IL-33 itself was responsible for IL-10 induction in B cells was undetermined.

IL-35 is a heterodimeric cytokine composed of Ebi3, a β chain subunit, and the IL-12p35 α subunit (Devergne, Birkenbach & Kieff 1997). IL-35 is produced by natural regulatory T cells and contributes to their suppressive abilities (Collison et al. 2010). Mice treated with IL-35 had increased IL-10 and IL-35 producing B cells and were protected against development of experimental autoimmune uveitis (EAU) (Wang et al. 2014a). Mice lacking the p35 subunit of IL-35 or lacking the IL-12Rb2 receptor component for IL-35 developed severe EAU. Transfer of B cells induced by IL-35 treatment *in vivo* suppressed established EAU. Moreover, *in vitro* stimulation of human B cells with IL-35 also induced IL-10 production. Stimulation with just the IL-12p35 subunit of IL-35 was sufficient to induce regulatory B cells and T cells to ameliorate EAU and EAE (Choi et al. 2017, Dambuza et al. 2017).

B cell activating factor (BAFF) is a key regulator of B cell development. *In vitro* and *in vivo* stimulation with BAFF alone induced IL-$10^{+}$ B cells. BAFF-induced B cells were able to suppress *in vitro* proliferation and IFN-γ production from activated T cells in an IL-10 dependent manner. Upon adoptive transfer, BAFF induced B cells reduced incidence and delayed onset of CIA. An artherosclerosis study also found that stimulation by BAFF and antigen induced IL-10 B cells that prevented progression of atherosclerosis (Ponnuswamy et al. 2017).

Interestingly, little is known about the effects of $T_H2$ cytokines on regulatory B cell function and growth. Yoshizaki et al. included IL-4 in their cultures to expand total B cells, then re-cultured the cells to investigate IL-10 production (Yoshizaki et al. 2012b). However, IL-4 stimulation alone did not affect IL-10 production from splenic B cells. Our lab found IL-5 to be a potent inducer of IL-10 production and expansion of regulatory B cells (Klinker et al. 2013). The role of regulatory B cells in allergic disease has only recently been investigated. Additional studies exploring aspects of the allergic milieu and their effects on regulatory B cells are expected in the near future.

## 1.3 Phenotypes of IL-10 producing B cells

The field of regulatory B cells has expanded immensely over the past two decades. The increased interest and appreciation of the immunomodulatory roles of B cells has led to the discovery of regulatory B cells in disease models ranging from autoimmunity to infectious disease. B cells from all stages of maturation and differentiation can be induced to secrete IL-10, suggesting that IL-10 production and subsequent regulation by B cells may be an inducible program rather than a separate lineage (Table 1.1). However, there is no unifying transcription factor for IL-10-producing B cells, such as FoxP3 for regulatory T cell, to define such a program. Therefore, when describing IL-10-producing regulatory B cells it is necessary to not only show they can produce IL-10 but also that they have suppressive abilities.

### 1.3.1 Mouse regulatory B cells

<u>B-1a Cells</u>

B-1a cells, which are $CD5^+$, originate from a separate lineage other than B-2 cells. These B cells produce natural IgM as the first line of defense against infection and are the main producers of IL-10 in mice after LPS stimulation (O'Garra et al. 1992). $CD5^+$ B cells from the

peritoneal cavity of mice spontaneously secreted IL-10 and were enhanced by CD40L stimulation (Margry et al. 2014). *In vitro*, B-1a cells could suppress inflammatory cytokine production from activated T cells. They were first found to be protective in TLR mediated inflammation in neonatal mice (Zhang et al. 2007). Inflammation became lethal in the absence of $CD5^+$ B cells, and was dependent on their IL-10 production. Reduction of colitis development in $TCR\alpha^{-/-}$ mice housed in conventional facilities was associated with an increase in B-1a cells. Furthermore, B cell deficient $TCR\alpha^{-/-}$ mice developed severe colitis that was prevented by the transfer of peritoneal B-1a cells from $TCR\alpha^{-/-}$ mice (Shimomura et al. 2008). $CD5^+$ B cells are also protective in models of allergic disease, which will be discussed later. The overlap of B-1a and B10 cell phenotypes suggest that studies of these subsets may be investigating similar cells.

B10 cells

B10 cells, which are $CD5^+CD1d^{hi}$, are able to secrete IL-10 *ex vivo* with PMA, ionomycin, and monensin (PIM) stimulation. They make up 1-3% of splenic B cells and are highly prominent in the peritoneal cavity, even though B cell numbers in general are low in this location. B10 cells can be found in low frequencies in the blood, lymph nodes, Peyer's patches, intestinal tissues, and the central nervous system (Yanaba et al. 2009, Matsushita et al. 2010, Maseda et al. 2013). Adoptive transfers of these B cells have shown therapeutic effects in models of contact hypersensitivity(Yanaba et al. 2008), EAE (Fillatreau et al. 2002, Matsushita et al. 2008, Matsushita et al. 2010), lupus(Haas et al. 2010, Watanabe et al. 2010), IBD(Yanaba et al. 2011, Maseda et al. 2013), and graft versus host disease(Le Huu et al. 2013). B10 cells are hypothesized to come from an undefined B10 progenitor cell that is also preferentially localized within the $CD5^+CD1d^{hi}$ B cell population. B10 progenitor cells will mature into B10 cells and begin secreting IL-10 after stimulations such as LPS or CpG in addition to PIM (Tedder 2015).

In addition to IL-10, regulation by these cells are also dependent on CD40 and MHCII expression as B cells deficient in these co-stimulatory molecules did not confer protection(Yoshizaki et al. 2012a). Since the CD1d marker of B10 cells is not always incorporated in B-1a cell studies, the overlap between these subsets remains unclear.

Transitional-2 Marginal Zone B cells

The immature B cell subset of transitional marginal zone precursor (T2-MZP) B cells, $CD19^{+}CD21^{hi}CD23^{hi}CD24^{hi}IgM^{hi}IgD^{hi}CD1d^{hi}$, is the main producer of IL-10 among splenic B cells in arthritic mice (Evans et al. 2007). Transfer of T2-MZP B cells into arthritic mice reduced disease progression by induction of regulatory T cells and the reduction of $T_H1$ and $T_H17$ cells, all of which was dependent on B cell IL-10 production (Carter et al. 2011, Carter, Rosser & Mauri 2012). T2-MZP cells from lupus prone MRL/lpr mice, on the other hand, lack IL-10 production and suppressive function *ex vivo*. However, this defect can be overcome with anti-CD40 stimulation with improved survival of MRL/lpr mice upon adoptive transfer (Blair et al. 2009). Protection afforded by IL-10 production by T2-MZP cells has also been seen in models of transplantation, Helicobacter-induced gastric inflammation, and allergic airway disease (Moreau et al. 2015, Amu et al. 2010, Sayi et al. 2011).

Marginal Zone B cells

Marginal zone (MZ) B cells of the spleen, $CD19^{+}CD21^{hi}CD23^{-}CD24^{hi}IgM^{hi}IgD^{lo}CD1d^{hi}$, can also secrete IL-10 in a regulatory capacity. MZ B cells increased IL-10 secretion in response to apoptotic cells, and augmented T cell IL-10 production in co-cultures. Treatment of mice with apoptotic cells limited disease symptoms of CIA and protection could be transferred by B cells from treated mice (Miles et al. 2012, Gray et al. 2007). In a model of colitis, transfer of splenic MZ B cells or MZ like B cells from the mesenteric lymph nodes delayed the onset of disease

(Wei et al. 2005). In a *Leishmania donovani* infection model, MZ B cells were found to inhibit $CD8^+$ T cells responses during the early infection stage (Bankoti et al. 2012).

$CD138^+$ B cells

$CD138^+$ plasmablasts in the draining lymph nodes of EAE mice were found to be crucial in suppression of EAE by suppressing dendritic cell functions. They also found that mice with B cells lacking important genes for plasma cell differentiation, Prdm1 and IRF4, developed more severe EAE (Matsumoto et al. 2014). B10 cells were reported to differentiate into $CD138^+$ plasmablasts after *in vitro* or *in vivo* activation however, regulatory capacity of these differentiated B10 cells was not assessed(Maseda et al. 2012). IL-35 was found to induce $CD138^+$ B cells that secreted IL-10 and IL-35. Transfer of these IL-35-stimulated B cells protected from experimental autoimmune uveitis (EAU) development. Lack of IL-35 or IL-35 signaling in mice led to development of more severe EAU. Whether protection in this model was dependent on secretion of IL-10, IL-35, or both from the B cells was not determined (Wang et al. 2014a). However, in another study, mice with B cell specific deficiency in IL-35 were unable to recover from EAE. $CD138^+$ plasma cells were found to be the main B cell subset producing IL-35 and IL-10(Shen et al. 2014). Whether IL-10 and IL-35 play redundant regulatory roles is unclear.

TIM-$1^+$ B cells

TIM-1 was reported to be a marker that could unify multiple IL-10 producing cells, B10, T2-MZP, MZ, and $CD138^+$ (Shen et al. 2014, Ding et al. 2011, Shalapour et al. 2015). Ligation of TIM-1 prolongs allograft survival, which is dependent on B cells, as B cell depletion reduced allograft survival (Ding et al. 2011, Yeung et al. 2015). Expression of TIM-1 on B cells allows recognition of apoptotic cells which has been shown to reduce EAE severity. Mice with a B cell

deficiency in TIM-1 lose protection generated from treatment with apoptotic cells (Xiao et al. 2015). TIM-1 deficient mice also suffer from spontaneous systemic autoimmunity and severe multi-organ inflammation as they age (Xiao et al. 2015, Xiao et al. 2012). Investigation into TIM-$1^+$ B cells warrants further confirmation before TIM-1 can be considered a unifying marker.

### 1.3.2 Human Regulatory B cells

$CD24^{hi}CD38^{hi}$ regulatory B cells

The immature population of B cells, $CD19^+CD24^{hi}CD38^{hi}$, in the peripheral blood of healthy controls produce IL-10 in a regulatory capacity and are impaired in autoimmune patients. CD40 stimulated $CD24^{hi}CD38^{hi}$ B cells from healthy individuals can suppress $T_H1$ and $T_H17$ differentiation and induce regulatory T cells (Flores-Borja et al. 2013). T cell proliferation and proinflammatory cytokine production were also reduced, partially dependent on B cell IL-10 production and independent of TGF-β (Blair et al. 2010). Cell contact was necessary for suppression as blocking antibodies for CD80/CD86 reversed suppression. Suppressive capacity of $CD24^{hi}CD38^{hi}$ B cells was found to be defective in SLE patients. B cells from these patients were refractory to CD40 stimulation but not TLR9 and produced less IL-10 after *in vitro* activation (Flores-Borja et al. 2013, Blair et al. 2010) (Jin, Weiqian & Lihuan 2013). Further, $CD24^{hi}CD38^{hi}$ B cells were also numerically and functionally defective in rheumatoid arthritis (RA) patients (Flores-Borja et al. 2013). B cells from RA patients did not suppress differentiation of $T_H17$ and regulatory T cells however; they maintained suppression of $T_H1$ development. Active RA correlates with a reduction in $CD24^{hi}CD38^{hi}$ B cells compared to inactive disease. Whether the reduction in $CD24^{hi}CD38^{hi}$ regulatory B cells during inflammation is due to their migration from the blood to site of inflammation is unclear.

SLE patients have multiple B cell abnormalities and some have undergone B cell depletion therapy with rituximab in hopes of resetting their B cell populations. Rituximab treated patients have delayed reconstitution of memory $CD27^+$ B cells. This delay correlates with expansion of $CD24^{hi}CD38^{hi}IgD^+$ B cells during the B cell reconstitution phase (Palanichamy et al. 2009, Anolik et al. 2007). Long term remission in SLE patients after rituximab treatment was associated with prolongation of an increased transitional to memory B cell ratio. Differences in regulatory capacity of these cells before and after rituximab treatment were not determined. Similarily, in neuromyelitis optica patients, rituximab treatment increased the ratio between $CD24^{hi}CD38^{hi}$ and memory B cells (Quan et al. 2015). IL-10 production was also enriched in this population. These studies support the idea that during autoimmunity, suppressive effects of regulatory B cells are overcome by expansion of pathogenic B cells.

Beyond autoimmunity, this regulatory B cell subset has also been associated with success in transplantation. Tolerant renal transplant patients, who have stable graft function without immunosuppression, had increased IL-10 producing $CD24^+CD38^+$ B cells compared to patients receiving immunosuppression and healthy controls (Newell et al. 2010). This increase in $CD24^{hi}CD38^{hi}$ B cells was also associated with reduced rejection rates (Shabir et al. 2015). A study in kidney transplantation found that the ratio of IL-10:TNF-α produced may be a better measure of regulatory function than IL-10 alone (Cherukuri et al. 2014). When comparing patients with stable kidney transplants to patients with graft rejection, $CD24^{hi}CD38^{hi}$ B cells produced similar levels of IL-10 however, stable patients had higher IL-10:TNF-α ratios and reduced $T_H1$ cytokine production.

<u>$CD24^{hi}CD27^+$ Regulatory B cells</u>

The B10 equivalent in humans, $CD24^{hi}CD27^{+}$, are potent producers of IL-10 *ex vivo* in response to PIM+LPS (Iwata et al. 2011). The B10 progenitor cells are also enriched in this subset and produce IL-10 48 hours after CD40L and LPS or CpG stimulation. IL-10 producing B cells were found in the peripheral blood, spleen, and tonsils of healthy individuals. Co-culture with $CD24^{hi}CD27^{+}$ but not $CD24^{lo}CD27^{-}$ B cells from healthy individuals reduced TNF-α production from activated T cells and monocytes. This effect on monocyte TNF-α production was blocked by addition of anti-IL-10 blocking antibodies while effects on T cells were IL-10 independent. In this study, $CD24^{hi}CD27^{+}$ B cells were increased in patients with autoimmunity including SLE, but Jin et al found that at the onset of SLE in patients before treatment this subset was significantly reduced compared to healthy individuals (Jin, Weiqian & Lihuan 2013). Additionally, IL-10 production after CpG and PIM stimulation in total $IL\text{-}10^{+}$ B cells and $IL\text{-}10^{+}CD24^{hi}CD27^{+}$ B cells were reduced at SLE onset. After 1 year of treatment, $CD24^{hi}CD27^{+}$ B cells increased to normal levels accompanied by reduced SLE disease activity. Decreases in this B cell subset were also found during disease flares, further supporting an inverse correlation between $CD24^{hi}CD27^{+}$ B cells and SLE disease activity. These studies suggest that in SLE, at the onset of disease $IL\text{-}10^{+}$ B cells are reduced and after disease progression this subset may expand. Decreased frequencies and function of $CD24^{hi}CD27^{+}$ regulatory B cells have also been seen in patients at the onset of Graves' disease (Zha et al. 2012). In recovered patients frequencies normalize however, function is still reduced compared to healthy controls.

Changes in $CD24^{hi}CD27^{+}$ B cells were assessed in RA patients requiring biologic drug treatment (Salomon et al. 2017). Before treatment these $CD24^{hi}CD27^{+}$ B cells were not significantly different in number from healthy controls. However, increased frequencies of these B cells at baseline predicted better remission rates at 6 months of treatment.

In studies of chronic graft versus host disease (cGVHD), healthy individuals were found to express IL-10 in multiple B cell subsets including $CD24^{hi}CD27^{+}$ B cells, $CD27^{hi}CD38^{hi}$ plasmablasts, and $IgM^{+}CD27^{+}$ memory B cells (Khoder et al. 2014, de Masson et al. 2015). $CD24^{hi}CD27^{+}$ and $IgM^{+}CD27^{+}$ B cells suppressed T cell proliferation and IFN-γ production, which was dependent on IL-10 production and cell contact (Khoder et al. 2014). In the study investigating $CD24^{hi}CD27^{+}$ and $CD27^{hi}CD38^{hi}$ B cells, suppressive function was not determined (de Masson et al. 2015). In both studies, $IL\text{-}10^{+}$ B cells frequencies and IL-10 production were significantly reduced in cGVHD patients when compared to patients in remission, patients without cGVHD, and healthy controls (Khoder et al. 2014, de Masson et al. 2015). This reduction in $IL\text{-}10^{+}$ B cells and their function was due to reduced STAT3 and Erk phosphorylation. Thus, regulatory B cells can be found in multiple subsets and are usually reduced during autoimmunity and transplant rejection.

Human B1 cells

Human B1 cells are phenotypically defined as $CD20^{+}CD27^{+}CD43^{+}$ and can be further divided by the expression of CD11b (Griffin, Holodick & Rothstein 2011, Griffin, Rothstein 2011). $CD11b^{-}$ B1 cells secrete more antibodies but do not stimulate T cells efficiently. Conversely, $CD11b^{+}$ B1 cells express high levels of CD86 and strongly stimulate T cells but, secrete less antibody than $CD11b^{-}$ B1 cells. $CD11b^{+}$ B1 cells from human peripheral blood were shown to spontaneously secrete IL-10 and suppress T cell activation similar to murine B1a cells (Griffin, Rothstein 2012). B1 cells are also found in the skin of healthy individuals and a higher percentage of B1 cells secrete IL-10 compared to other $CD43^{-}$B cells (Geherin et al. 2016).

## 1.4 Mechanisms of regulation

Regulatory B cells can interact with multiple cell types to mediate their suppression (Fig 1.1). They can inhibit activation and effector functions of $CD4^+$ and $CD8^+$ T cells as well as antigen presenting cells. In addition to inhibiting proinflammatory cell types, regulatory B cells also induce regulatory T cells. These interactions and the subsequent effects on immune responses are summarized here.

Induction of Regulatory T cells

B cells are crucial for the maintenance of $FoxP3^+$ regulatory T cells (Treg) as Tregs are reduced in B cell deficient μMT mice (Boonpiyathad et al. 2017). Reduced $FoxP3^+$ Tregs in μMT mice enhances anti-tumor immunity rendering the mice resistant to mammary carcinoma development (Tadmor et al. 2011). Additionally, adoptive transfer of B cells can restore $FoxP3^+$ Treg frequencies and responses during oral tolerance and arthritis in μMT mice (Carter et al. 2011, Sun et al. 2008). IL-10 production specifically from B cells was shown to be necessary for $FoxP3^+$ Tregs using chimeric mice with IL-10 deficiency restricted to B cells. These mice are unable to recover from self-remitting EAE or CIA which was associated with decreased $FoxP3^+$ and IL-$10^+$ (Tr1) Tregs. Induction of Tr1 cells *in vitro* and *in vivo* by *Heliobacter felis* stimulated B cells are also protective in controlling excessive $T_H1$ inflammation (Sayi et al. 2011). IL-$10^+$ B cells not only induce and maintain regulatory T cells, they can also enhance their proliferation and migration, which is dependent on B cell expression of CD80/CD86 (Amu et al. 2010, Mann et al. 2007) (Fig 1.2).

Suppression of effector T cells

In addition to interactions with regulatory T cells, Bregs also interact with pathogenic T cells to influence immune responses (Fig 1.2). Suppression of EAE by adoptive transfer of

regulatory B cells is dependent on B cell expression of CD40 and IL-21R suggesting that T cell interaction is necessary (Yoshizaki et al. 2012a). In an islet allograft model, transfers of regulatory B cells prolong graft survival and reduce $CD4^+$ T cell proliferation and IFN-γ production (Ding et al. 2011). These T cells are instead skewed towards a $T_H2$ response. Reduced proliferation and proinflammatory cytokine production from $CD4^+$ T cells has also been found in multiple models of allergic disease and autoimmunity both *in vivo* and *in vitro* (Fillatreau et al. 2002, Mauri et al. 2003, Carter, Rosser & Mauri 2012, Margry et al. 2014, Braza et al. 2015).

While less described, B cells can also exert suppressive activity on $CD8^+$ T cells. During infection with *Leishmania donavoni*, B cells suppress the generation of a protective $CD8^+$ T cell response, which is attributed to marginal zone B cells as their depletion led to a decrease in parasite burden (Bankoti et al. 2012). Studies of $CD8^+$ T cell responses in cancer models have found that B cells can enhance tumor susceptibility by limiting $CD8^+$ T cell activity and migration (Qin et al. 1998, Schioppa et al. 2011). Thus, IL-10 production from B cells can limit immune activation by suppression of effector T cell expansion, function, and migration to sites of inflammation.

Suppression of antigen presenting cells

Beyond their affects on T cells, regulatory B cells can also modulate immune responses through their influence on antigen presenting cells (Fig 1.2). Regulatory B cell IL-10 production dampens fatal $T_H1$ responses in neonates after infection. Suppression of proinflammatory cytokine production by dendritic cells mediated this protection (Sun et al. 2005, Zhang et al. 2007). In $CD19^{-/-}$ mice lacking IL-$10^+$ B cells, macrophages are more able to control *Listeria monocytogenes* infection through enhanced phagocytosis (Horikawa et al. 2013). Depletion of

mature B cells or preferential depletion of regulatory B cells by CD22mAb in mice similarly enhances macrophage apoptosis and production of proinflammatory cytokines and nitric oxide. Transfer of B cells reduced clearance and increased bacterial load in an IL-10 dependent manner. *In vitro* activated B cells suppressed nitric oxide, TNF-α, and IFN-γ production from macrophages. Regulatory B cells thus influence cells central to immune activation and maintenance of immune responses, achieving significant immune modulation by this rare B cell population.

Fas Ligand expression

In addition to IL-10 production, our lab is also interested in B cell expression of FasL and the ability of B cells to induce apoptosis in activated T cells through Fas-FasL interaction. FasL expressing B cells have been found in both humans and mice (Hahne et al. 1996, Lundy 2009). FasL expression and cytolytic activity of murine B cells was first reported following PMA/ionomycin or LPS stimulation (Hahne et al. 1996). Transfer of LPS stimulated B cells but not control B cells, inhibited spontaneous type 1 diabetes in NOD mice (Tian et al. 2001). Co-transfer of LPS activated B cells also inhibited transfer of diabetes by diabetogenic splenic T cells. Activation induced FasL expression on B cells was also seen in MRL/lpr lupus prone mice (Bonardelle et al. 2005). MRL/lpr mice have increased $FasL^+$ B cells and expression of FasL increased with cell size and expression of the activation marker CD69. These B cells had potent cytotoxic activity on $Fas^+$ targets, comparable to natural killer cells. These B cells also play a role in tolerance, as transfer of male wild type B cells but not FasL mutant (Gld) B cells, induced tolerance and acceptance of male to female skin grafts (Minagawa et al. 2004). Additionally, $CD5^+$ $FasL^+$ B cells were inversely correlated with disease severity in a collagen induced arthritis model (Lundy, Fox 2009). Schistosome infection also increases FasL expression on B cells,

which can then kill $CD4^+$ T cells *in vitro* (Lundy, Lerman & Boros 2001). Subsequently, infection of B cell deficient mice resulted in decreased splenic T cell apoptosis (Lundy, Boros 2002). $CD5^+$FasL expressing B cells have been described following chronic cockroach antigen exposure in an allergic asthma model. Lung T cell apoptosis was reduced in Xid mice lacking $CD5^+$ B cells (Lundy et al. 2005).

### 1.5 Allergic Asthma

While asthma is now appreciated to be a syndrome with multiple endotypes ranging from $T_H2^{hi}$ to $T_H17$ responses in the absence of $T_H2$ (Holgate et al. 2015), the work presented in this thesis focuses on classical $T_H2$ allergic asthma and is introduced here. Asthma is a chronic inflammatory disease of the airways involving many cells of both the innate and adaptive immune systems (Lambrecht, Hammad 2015). The characteristic features of asthma include bronchial hyper reactivity (BHR), mucus over-production, and eventually, remodeling of the airways. Epithelial cell damage and TLR activation by allergens induce chemokine, cytokine, and danger signals from the epithelial cells to recruit and activate group 2 innate lymphoid cells (ILC2), basophils, and dendritic cells (DC). Immature DCs migrate to the lungs where they encounter the antigen, then migrate to the lymph node to activate the adaptive immune system and promote differentiation of $T_H2$ cells. ILC2 cells contribute to the activation of DCs in the lungs through their production of IL-13 and later act as non-professional antigen presenting cells for T cells in the lungs. Within the lymph node, activated T cells induce IgE class switching of allergen-specific B cells which binds FCεRI on mast cells, mediating degranulation upon antigen cross linking. $T_H2$ differentiated $CD4^+$ T cells leave the lymph node and migrate to the lungs where they act as conductors of the allergic response through their secretion of $T_H2$ cytokines IL-4, IL-5, and IL-13. IL-5 directly mediates the maturation of eosinophils in the bone marrow

while recruitment to the lung is mediated through eotactic chemokines induced by IL-4 (CCL11, CCL24, and CCL26) (Moser et al. 1993, Mochizuki et al. 1998, Mochizuki et al. 1999). Eosinophils are increased in the brochoalvelolar lavage fluid (BAL), induced sputum, and brochial biopsies of asthmatic patients (De Monchy et al. 1985, Humbert et al. 1996, Bentley et al. 1992, Bousquet et al. 1990). Eosinophils directly cause BHR through release of granule proteins, enhance DC activation, and contribute to airway remodeling through production of TGF-β (Song et al. 2009, Coyle et al. 1994, Coyle et al. 1995, Chu et al. 2014). IL-4 is necessary for B cell production of IgG1 and IgE as well as vessel priming for eosinophil extravasation (Thornhill, Haskard 1990, Schleimer et al. 1992, Patel 1998, Lundgren et al. 1989, Isakson et al. 1982). Lastly, IL-13 is important for goblet cell metaplasia, resulting in increased mucus production and clogging of the airways. Additionally IL-13 has also been found to be important in BHR (Wills-Karp 2004, Grunig et al. 1998).

### 1.6 Effects of $T_H2$ cytokines on B cells

Interleukin 4

IL-4 acts synergistically with CD40 ligation to promote B cell survival, proliferation, and differentiation (Wurster et al. 2002, Rush, Hodgkin 2001). It induces IgG1 and IgE class switching in LPS activated murine B cells, while suppressing IgG2b and IgG3 responses (Isakson et al. 1982, Sideras et al. 1985, Coffman et al. 1986). IL-4 induction of IgG1 and IgE class switching is dependent upon activation of STAT6 (Kaplan et al. 1996). STAT6 deficient B cells do not respond to IL-4 and deficient mice do not develop IgG1 or IgE in response to nematode infection (Takeda et al. 1996). Additionally, IL-4 upregulates B cell expression of MHCII, suggesting that IL-4 enhances antigen presentation abilities (Thieu et al. 2007). In humans, IgG4 and IgE are induced by IL-4 (Lundgren et al. 1989).

Recently, autophagy related allele polymorphisms have been associated with asthma in children and adults (Martin et al. 2012). Autophagy was enhanced in B cells from asthma-prone mice and autophagy deficient B cells attenuated disease severity in these mice (Xia et al. 2018). B cell autophagy was found to be dependent on IL-4 *in vivo* and *in vitro*. IL-4 induced B cell autophagy enhanced B cell survival and antigen presentation dependent on JAK3 signaling. These studies explore a novel role for IL-4 in the pathogensis of asthma and regulation of B cells.

Interleukin 5

IL-5 is an important growth factor for B-1a cell development. B-1a cells are increased in mice expressing the IL-5 transgene and are reduced in IL-5R deficient mice (Tominaga et al. 1989, Tominaga et al. 1991, Yoshida et al. 1996). Signaling of IL-5 through Bruton's tyrosine kinase regulates B-1a cell development as evidenced by the absence of B-1a cells in the periphery of mice carrying a mutation in this gene (Xid) (Scher, Berning & Asofsky 1979).

IL-5 affects production of multiple immunoglobulin isotypes. IL-5 stimulation enhances IgA production from $IgA^+$ B cells but, cannot induce IgA secretion by itself (Sonoda et al. 2009, Coffman, Lebman & Shrader 2009). Further, IL-5 does not induce Cα transcripts nor Igα specific switch circular DNA, necessary for class switching (Iwasato et al. 1992). IL-5 however, can synergize with TGF-β to enhance IgA production from switched B cells (Sonoda et al. 2009, Coffman, Lebman & Shrader 2009). A similar situation is seen with IgG1 and IgE production (Purkerson, Isakson 1992). IL-4 is necessary to induce class switching to these isotypes but, IL-5 can enhance production of γ1 and ε chain transcripts. Therefore, IL-5 alone cannot induce class switching but, can enhance production of multiple antibody isotypes.

### 1.7 Regulatory B cells in murine models of allergic disease

Contact hypersensitivity

The first report of B cells with regulatory functions was in a model of contact hypersensitivity in guinea pigs (Neta, Salvin 1974). Protection from delayed skin hypersensitivity could be transferred from previously sensitized guinea pigs. However, B cell depleted splenocytes did not confer protection. More recently, the $CD5^{+}CD1d^{hi}$ B cell subset from the spleen was found to inhibit CHS in an antigen specific and IL-10 dependent manner (Yanaba et al. 2008). LPS stimulated IL-10 production in this subset was found to be controlled by B cell linker protein (BLNK) expression and downstream STAT3 phosphorylation (Jin et al. 2013). $BLNK^{-/-}$ mice developed exacerbated CHS and EAE which could be rescued by adoptive transfer of splenic $CD5^{+}CD1d^{hi}$ wild type B cells.

Anaphylaxis

Helminth infection has been associated with reduced allergic disease in humans (van den Biggelaar et al. 2001). Mice infected with the helminth *Schistosoma mansoni* had increased regulatory B cells and were protected from passive Ab-mediated anaphylaxis (Mangan et al. 2006). Depletion of B cells or blockade of IL-10R diminished protection. *In vitro* stimulation of B cells with *Schistsoma mansoni* increased IL-10 producing B cells that protected mice from lethal anaphylaxis upon adoptive transfer. $IL\text{-}10^{+}CD5^{+}$ B cells were recently found to expand in the spleens, peritoneal cavity, lymph nodes, and blood of animals with IgE and antigen induced passive systemic anaphylaxis (PSA) (Kim et al. 2015). Transfer of $CD5^{+}$ but not $CD5^{-}$ B cells protected $CD19^{-/-}$ mice from PSA. Protection was dependent on CD40-CD40L interaction between the B cells and mast cells, which enhanced IL-10 production from the B cells. IL-10 was necessary to inhibit mast cell activation and confer protection.

Allergic Airway Disease

Helminth infection with *Schistosoma mansoni* has also been found to be protective in mouse models of allergic airway disease (AAD). As previously reported, helminth infection caused a general expansion of B cells which included IL-$10^{+}CD19^{+}CD5^{+}CD1d^{hi}CD21^{hi}CD23^{+}IgD^{+}IgM^{hi}$ B cells (Amu et al. 2010). Transfer of $CD1d^{hi}$ but not $CD1d^{lo}$ B cells prevented and reversed AAD dependent on B cell IL-10 production and induction of $FoxP3^{+}$ regulatory T cells. Importance of $CD1d^{hi}$ B cells from infected mice in ablation of AAD after transfer was further confirmed by van der Vlugt et al (van der Vlugt et al. 2012).

Regulatory B cells in the absence of helminth infection also prove protection in AAD. The importance of the $CD5^{+}$ subset was revealed in a cockroach antigen model of AAD in Xid mice lacking $CD5^{+}$ B cells (Lundy et al. 2005). These mice have increased allergic inflammation compared to wild type. While the role of IL-10 was not assessed, the authors suggest expression of FasL by $CD5^{+}$ B cells was the mechanism. In another study using the house dust mite model, IL-10 expressing lung B cells were reduced which may contribute to the development of AAD (Braza et al. 2015). Transfer of $CD9^{+}$ but not $CD9^{-}$ splenic B cells in a HDM model also ameliorated AAD by reducing effector T cell/ $FoxP3^{+}$ regulatory T cell ratios, in a manner dependent on IL-10. B cells from the Peyer's patches of mice induced antigen specific Tregs, which was partially dependent on IL-10 (Chu, Chiang 2012). While the study did not determine the suppressive abilities of the B cells directly, they found that Tregs induced by regulatory B cells prevented and suppressed AAD.

Chronic antigen exposure can result in resolution of AAD (Singh et al. 2008). Transfer of B cells from the hilar lymph nodes (HLN) of tolerant mice attenuated AAD while HLN B cells

from allergic mice were not protective. Protection was antigen-specific and accumulation of FoxP3$^{+}$ Tregs was observed. HLN B cells from tolerant mice induced FoxP3$^{+}$ Tregs *in vitro*, dependent on B cell TGF-β production. These B cells were later identified as CD5$^{+}$ and shown to co-localize with FoxP3$^{+}$ Tregs at the T-B border of HLNs of tolerant mice (Natarajan et al. 2012). However, respiratory tolerance has been reported in B cell deficient mice suggesting that B cells may be induced during tolerance but are not always necessary for tolerance development (Habener et al. 2017).

### 1.8 Regulatory B cells in Human Allergic disease

Asthma

While multiple studies describe regulatory B cells in autoimmunity, not much work has been done in allergic diseases. The first paper to describe regulatory B cells in allergic asthma investigated CD1d$^{hi}$, CD24$^{hi}$CD38$^{hi}$, and CD24$^{hi}$CD27$^{+}$ B cell subsets (van der Vlugt et al. 2014). Total IL-10$^{+}$ B cells were reduced in asthmatic patients compared to controls only after stimulation with LPS but not anti-IgG/M or CpG. IL-10 production was observed in all three subsets after LPS stimulation, but asthmatic patients had reduced IL-10$^{+}$CD24$^{hi}$CD27$^{+}$ B cells while numbers of cells in the other subsets were similar to controls. Overlap of the subsets did occur with 55-60% of CD1d$^{hi}$ B cells in the CD24$^{hi}$CD27$^{+}$ subset and 18% in the CD24$^{hi}$CD38$^{+}$ subset. LPS primed CD24$^{hi}$CD27$^{+}$ B cells induced IL-10$^{+}$ T cells in co-cultures, which was dependent on B cell IL-10 production. B cells from allergic patients had reduced ability to induce IL-10$^{+}$ T cells. Allergic rhinitis (AR) precedes asthma in 10-40% of cases (Shaaban et al. 2008). Frequencies of CD24$^{hi}$CD27$^{+}$ B cells were decreased in AR patients and further reduced in AR patients with asthma (Kamekura et al. 2015). The ratio of T follicular helper 2 cells to Breg frequencies positively correlated with increased disease parameters in patients with AR and

asthma. While the study suggests a role for $CD24^{hi}CD27^{+}$ Bregs in the progression of AR to co-morbidity with asthma, regulatory functions of these cells and other subsets were not assessed. These studies suggest that regulatory B cells are decreased in frequency and function during allergic responses and that rescue of these populations may lead to attenuation of disease.

Allergen immunotherapy

Allergen immunotherapy (AIT) aims to restore tolerance to allergens by treatment with multiple allergen doses subcutaneously or sublingually over an extended period (Dominguez-Ortega et al. 2017). AIT efficacy has been extensively evaluated in allergic rhinitis and rhinoconjunctivitis. Some patients suffer from concomitant asthma which has allowed for some asthma related outcomes to be assessed. Only recently have trials been designed to investigate efficacy of AIT in asthma, and no mechanistic data has been reported. Overall AIT has been found to reduce disease symptoms and increase quality of life for allergic patients suffering from rhinitis, rhinoconjunctivitis, and asthma (Dominguez-Ortega et al. 2017).

More detailed studies of immune modulation in AIT have been primarily in bee venom and milk allergy patients. *In vitro* stimulation of PBMCs from milk allergic or tolerant patients found that the milk protein casein elicited increased production of IL-10 from $CD5^{+}$ B cells in tolerant patients compared to allergic patients (Noh et al. 2010). In similar *in vitro* conditions, $CD5^{+}$ B cells were also found to produce TGF-β. Frequencies of TGF-β producing $CD5^{+}$ B cells were decreased in allergic patients compared to tolerant patients (Lee et al. 2011). Induction of tolerance by milk and IFN-γ treatment in allergic patients was accompanied by an increase in IL-$10^{+}$ B cells, which were enriched in the $CD5^{+}$ subset (Noh et al. 2012). In milk allergy both IL-10 and TGF-β production from B cells may be the difference between tolerance and allergy. Whether the same B cells express IL-10 and TGF-β was not determined.

Van de Veen et al. investigated B cell phenotypes in bee venom allergic patients and tolerant beekeepers. IL-10 production was enriched in $CD73^{-}CD25^{+}CD71^{+}$ B cells specific for phospholipase $A_2$ (PLA), the major bee venom allergen. These cells suppressed antigen specific proliferation of $CD4^{+}$ T cells *in vitro* and were significantly reduced in allergic patients. This deficiency could be rescued by venom specific immunotherapy (VIT). Production of protective IgG4 is a hallmark of a healthy immune response to allergens. IgG4 expression was confined to $IL\text{-}10^{+}$ B cells in tolerant beekeepers who also displayed low IgE:IgG4 ratios of serum PLA specific antibodies. After VIT, IgE:IgG4 ratios were reduced in allergic patients but, remained higher than beekeepers. Another study confirmed that increases in $IL\text{-}10^{+}$ and $IgG4^{+}$ B cells from patients undergoing VIT paralleled changes seen in beekeepers after multiple bee venom exposures during the season (Boonpiyathad et al. 2017).

These studies support a role for regulatory B cells in the induction of tolerance by allergen specific immunotherapy. Multiple mechanisms of regulation are suggested including production of anti-inflammatory cytokines and allergen specific immunoglobulins. Investigation of these B cells in other allergic diseases shown to benefit from AIT will be informative.

## 1.9 Conclusion

IL-10 production from regulatory B cells has been proven to be beneficial in multiple diseases spanning human health. Studies in humans have shown inverse associations between disease severity and frequency and function of regulatory B cells. Rescuing regulatory B cell frequencies by B cell depletion with rituximab or, allergen immunotherapy correlates with a decrease in disease severity. While current human studies can only make associations between therapies and regulatory B cells, studies in mice have provided great insight into the protection that regulatory B cells afford. In models of autoimmunity, allergy, transplantation, and cancer,

production of IL-10 from regulatory B cells has been proven to play a significant role in modulating the immune response. Protection conferred can be mediated through direct interactions with multiple cellular targets. While the field of regulatory B cells has expanded greatly in the last few decades, further investigation into their phenotypes and origin are necessary before utilization in the treatment of human disease can be considered. The thesis looks to contribute to the understanding of what signals can contribute to regulatory B cell expansion and function to provide insight for the development of targeted drug therapies.

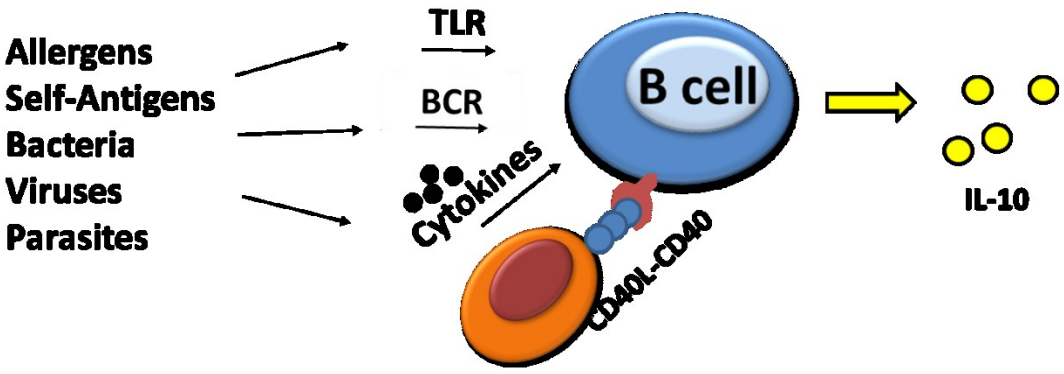



**Figure 1. 1 Stimulations of IL-10 producing regulatory B cells**

Following exposure to allergens, self-antigens, and various microbes, B cells can be stimulated through their toll like receptors, B cell receptors, and by interactions with other cells through the cytokines they produce and CD40-CD40 ligand interaction to induce IL-10 secretion.

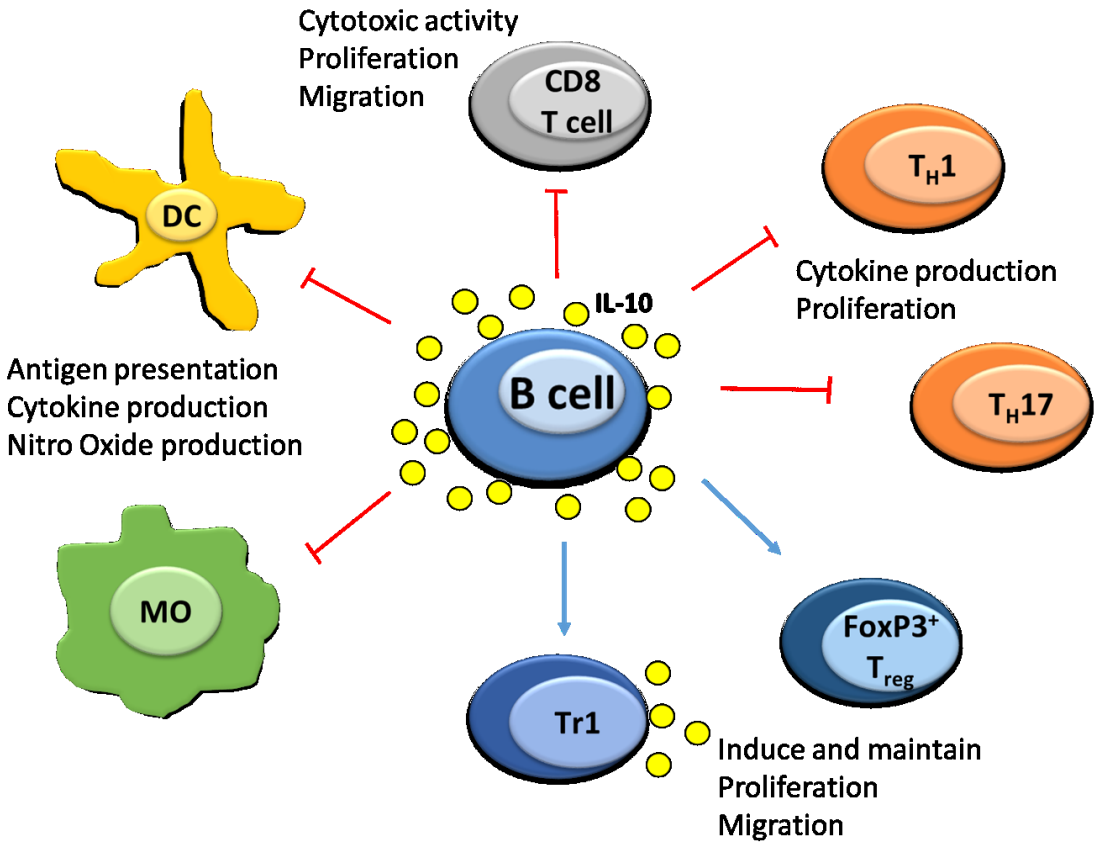



**Figure 1. 2 Mechanisms of suppression**

IL-10 producing regulatory B cells can act on multiple cells types to mediate their suppression. Regulatory B cells can inhibit activation and effector functions of $CD4^+$ and $CD8^+$ T cells, reducing cytokine production, proliferation, cytotoxicity and migration. Regulatory B cells can also inhibit effector functions of antigen presenting cells by downregulation of CD80/CD86/MHCII and reducing cytokine and nitric oxide production. In addition, regulatory B cells also induce and maintain regulatory T cells, $FoxP3^+$ and IL-10 producing Tr1, which can further aid in suppression of immune responses.

| Regulatory B cell subset | Mouse | Human | Ref |
|---|---|---|---|
| **B-1a** | **$CD5^+$** | **$CD11b^+CD20^+CD27^+CD43^+$** | (Kim et al. 2015, Griffin, Rothstein 2012) |
| **B10** | **$CD19^{hi}CD5^+CD1d^{hi}$** | **$CD24^{hi}CD27^+$** | (Tedder 2015) |
| **T2-MZP** | **$CD19^+CD21^{hi}CD23^{hi}CD1d^{hi}IgM^{hi}$** | **--** | (Evans et al. 2007) |
| **MZ** | **$CD19^+CD21^{hi}CD23^-CD1d^{hi}IgM^{hi}$** | **--** | (Gray et al. 2007) |
| **Immature** | **--** | **$CD19^+CD24^{hi}CD38^{hi}$** | (Blair et al. 2010) |
| **Plasmablast** | **$CD138^+CD44^{hi}$** | **$CD19^+CD38^{hi}CD27^{int}$** | (Matsumoto et al. 2014) |
| **Plasma Cell** | **$CD138^{hi}CD1d^{hi}IgM^+$** | **--** | (Shen et al. 2014) |

**Table 1. 1 Phenotypes of regulatory B cells**

Phenotypes of mouse and human regulatory B cells are summarized in the table above. (T2-MZP, transitional 2 marginal zone precursor cells) (MZ, marginal zone B cells)

# CHAPTER 2

## Differential influence on regulatory B cells by $T_H2$ cytokines affects protection in allergic airway disease.

This work was performed in collaboration with Luciën E.P.M. van der Vlugt, Molly M. Shea, Jennifer Yang, Nicholas W. Lukacs, and Steven K. Lundy. S.H.T, E.P.M.V, S.K.L, and N.W.L designed and performed the experiments. M.M.S and J.Y assisted in performing experiments and data analysis. S.H.T wrote the manuscript and S.H.T, E.P.M.V, M.M.S, J.Y, S.K.L, and N.W.L assisted in editing.

At the time of defense, this paper was submitted to the *Journal of Immunology* and awaiting reviewer comments.

### 2.1 Abstract

The role of regulatory B cells (Breg) in modulating immune responses and maintaining tolerance are well established. However, how cytokines present during immune responses affect the growth and function of these cells is not as well defined. Previously, our lab reported that IL-5 and mCD40L-expressing fibroblast (mCD40L-Fb) stimulation induced IL-10 production from B cells. The current study investigated the phenotype and functional relevance of IL-10 producing B cells from this culture. We found that IL-5/mCD40L-Fb stimulation induced IL-10

production exclusively from $CD5^+$ splenic B cells of naïve mice. After stimulation, the resulting IL-$10^+$ cells displayed markers of multiple reported Breg phenotypes. Interestingly, when investigating effects of IL-4, a critical $T_H2$ cytokine, on IL-5/mCD40L-Fb induced IL-10 production we found that IL-4 inhibited IL-10 production in a STAT6 dependent manner. Upon adoptive transfer, $CD5^+$ B cells previously stimulated with IL-5/mCD40L-Fb were able to reduce development of OVA induced allergic airway disease (AAD) in mice. Using B cells from IL-10 mutant mice differentiated by IL-5/mCD40L, we found that protection from AAD development was dependent on the IL-10 production from the transferred B cells. Regulatory B cells have been shown to play crucial roles in the immune tolerance network and understanding stimuli that modulate their growth and function may be key in development of future treatments for disease of immune dysregulation.

## 2.2 Introduction

Allergic airway disease (AAD), also known as allergic asthma, is a chronic inflammatory disease of the lungs induced by inhalation of otherwise innocuous antigens such as pollen, pet dander, and house dust mite (Lambrecht, Hammad 2015). These antigens trigger an inappropriate T helper 2 ($T_H2$) cell response resulting in an influx and accumulation of lymphocytes and granulocytes into the lungs. IL-4 from $T_H2$ cells induces class-switching of antigen specific B cells to IgE, which binds receptors on mast cells that activate degranulation upon antigen binding. Inflammatory components from mast cells and eosinophils induce bronchial hyper-reactivity of the smooth muscles causing airway narrowing. Eosinophilopoiesis and efflux from the bone marrow is driven by the type 2 cytokine IL-5, and results in hyperplasia and metaplasia of mucous producing goblet cells, airway remodeling, and obstruction of the

airways. While treatments to control symptoms are available, there is currently no cure for AAD and patients are often on life-long medications.

IL-4 and IL-5 are products of $T_H2$ cells that drive protective humoral immunity against extracellular microorganisms, but also mediate pathogenesis in allergic diseases. While IL-13 is also a $T_H2$ cytokine, B cells do not express the IL-13 receptor and therefore IL-13 will not be discussed further (Kuhn, Rajewsky & Muller 1991, Andrews et al. 2001). In mice, IL-4 and CD40 ligand stimulation from $T_H2$ cells is well-documented to induce antibody class-switching, proliferation, and differentiation of follicular B cells into antibody-secreting memory and plasma cells that produce IgG1 and IgE antibody isotypes. IL-5 supports IL-4 induced class-switching to IgG1 and IgE but is not sufficient by itself (Purkerson, Isakson 1992). IL-5 also acts on terminally differentiated IgA-producing B cells in the mucosal lymphoid tissues to increase IgA secretion (Sonoda et al. 2009, Harriman et al. 1988).

Beyond follicular B cells, studies in IL-5Rα deficient mice demonstrate IL-5 as a critical growth and/or survival factor for $CD5^+$ B-1a cell development (Yoshida et al. 1996, Moon et al. 2004, Erickson, Foy & Waldschmidt 2001). B-1a cells are also the only B cells to constitutively express the IL-5Rα chain of the IL-5 receptor heterodimer, IL-5Rα and common B chain. B-1a cells are the major source of natural IgM contributing 80-90% of resting serum levels, and are also estimated to make 50% of resting serum IgA (Ishida et al. 1992, Kroese, Ammerlaan & Kantor 1993, Sidman et al. 1986). These cells reside in the pleural and peritoneal cavities with a minority present in the spleen, and arise from progenitors, distinct from B-2 cells, that are present in the fetal liver and omentum (Solvason, Lehuen & Kearney 1991). The antibodies expressed by these cells are poly-reactive and self-reactive, allowing for broad protection from pathogens and a potential housekeeping role in apoptotic cell removal (Pennell et al. 1989b,

Hardy et al. 1989, Shaw et al. 2003). In addition, B-1a cells have also been found to be as potent antigen presenting cells (APC) as dendritic cells (DC), and are able to induce naïve T cell proliferation and activation directly *ex vivo* without additional stimulation (Mohan et al. 1998, Sato et al. 2004, Murakami et al. 1995). Their self-reactivity, and their APC abilities have led to speculation of their involvement in autoimmune diseases, and there is evidence in autoimmune mouse models to support this (Mohan et al. 1998, Sato et al. 2004). Conversely, their reported IL-10 production and immune suppressive abilities suggest self-reactivity and APC functions position them as dominant regulators of the immune system (Klinker, Lundy 2012, Rothstein et al. 2013). $CD5^+$ B cells have had reported regulatory functions for over two decades (O'Garra et al. 1992). Multiple groups have reported regulatory function of $CD5^+$ B cells in several models of allergic disease and autoimmunity (Kim et al. 2015, Yoshizaki et al. 2012a, Margry et al. 2014, Shimomura et al. 2008, Amu et al. 2010, Lundy, Fox 2009, Lundy et al. 2005, Natarajan et al. 2012).

Bregs are now considered important modulators of the immune response displaying suppressive capacity in multiple mouse models of autoimmunity and allergy. They employ an array of mechanisms to alter the immune response including surface expression of Fas ligand (FasL), PD-L2, and the secretion of anti-inflammatory cytokines TGF-β and IL-10 (Klinker et al. 2013, O'Garra et al. 1992, Hahne et al. 1996, Tian et al. 2001, Lundy, Boros 2002, Zhong et al. 2007b). Secretion of IL-10 is the most extensively studied regulatory mechanism used by Bregs in both mice and humans. Almost every subset of B cells has in part been shown to produce IL-10 after a variety of different stimuli. These include stimulation through CD40 ligand, B cell receptor, multiple Toll like receptors, IL-35, IL-21, and IL-4 (Mauri, Menon 2015). Previously, we reported that stimulation with IL-5 and mCD40L-Fb for 5 days, resulted in growth of B cells

with regulatory functions (Klinker et al. 2013), with increases in both FasL expression and IL-10 production.The breadth of B cells able to produce IL-10 lends evidence to the belief there is no unifying phenotype that defines IL-10 producing Bregs. Therefore, demonstration of functional capacity defines regulatory B cells.

In the present study, the phenotypic and functional responses of mouse B cells to stimulation through CD40 in the presence of $T_H2$ cytokines IL-4 and IL-5 were compared. In combination with mCD40L-Fb stimulation, IL-4 and IL-5 had independent and cooperative growth promoting effects on mouse B cells. Stimulation with IL-5/mCD40L-Fb induced the production of IL-10 exclusively from $CD5^+$ B cells while the presence of IL-4 blocked IL-10 production through a STAT6 dependent signaling pathway. The functional capacity of these IL-10 producers was assessed in OVA induced AAD model in BALB/c mice. $CD5^+$ B cells from the IL-5/mCD40L-Fb cultures attenuated the development of AAD *in vivo* in an IL-10 dependent manner. Our findings provide insight for the development of more effective treatments for severe allergies and other $T_H2$-mediated diseases through the use of Bregs.

## 2.3 Materials and Methods

### 2.3.1 Mice

All experiments, unless otherwise specified, were performed using 8-12 week old female BALB/cJ (Strain #000651) mice obtained from Jackson Labs (Bar Harbor, ME). Female DBA/1 mice were compared with female C57BL/6J (Strain #000664) mice and female BALB/cJ mice purchased from Jackson Labs. STAT6 knockout (Strain # 002828) and IL-10 mutant (Strain # 004333) breeding pairs were purchased from Jackson labs on the BALB/cJ background. All mice

were housed in specific pathogen-free facilities. Animal protocols and husbandry practices were approved by the Institutional Animal Care and Use Committee of the University of Michigan.

### 2.3.2 Mouse B Cell Culture on mCD40L-Fibroblasts

NIH3T3 fibroblasts stably transduced with the gene for mouse CD40 ligand (mCD40L-Fb) were generously provided by Dr. David Fox and Dr. Kevin McDonagh, and a detailed description of their generation has been previously published (Morita et al. 2005). The non-transduced NIH3T3 fibroblast line was purchased from ATCC (Rockville, MD). Fibroblast lines were maintained in DMEM/10% calf serum and grown to 80-90% confluence before removal from culture flasks with 0.25% Trypsin solution. Prior to coculture with B cells, fibroblast lines were exposed to 30 Gy of gamma radiation to inhibit their proliferation. Purified mouse B cells were obtained from single cell suspensions derived from the spleens of naive 8-12 week old mice by anti-mouse CD19 MACS magnetic bead positive selection (Miltenyi Biotec, Auburn, CA) using the manufacturer protocol. Purified B cells and mCD40L-Fb fibroblasts were washed and resuspended in B cell culture medium containing 1×DMEM (Hyclone, SH30243.01), 10% heat-inactivated FCS, 5% Penicillin-Streptomycin, and 0.5% Insulin/Transferrin/Selenium (Gibco, 51500-056). B cells and fibroblasts were plated at a 4:1 ratio in flat-bottom plates. Recombinant murine cytokines IL-4 (R&D Systems) and IL-5 (Peprotech) were added to the cultures at 50ng/mL. Primary cultures of naïve splenic B cells were stimulated for 5 days and secondary cultures were stimulated with fresh mCD40L-Fb and cytokines for 3 days. Lipopolysaccharide (LPS) from *E. coli* strain O111:B4 was purchased from Chemicon/Millipore and used at a concentration of 10 μg/mL. Control cultures were performed for all assays that are presented using non-transduced NIH3T3 fibroblasts, none of which yielded viable B cells after five days.

### 2.3.3 Flow Cytometry

A list of fluorochrome-conjugated monoclonal antibodies specific for mouse cell markers, their clone and dilutions used are listed in Supplemental Table 2.1 7AAD (Sigma Aldrich) was used to label and exclude dead cells and non-specific binding to Fc receptors was blocked using anti-CD16/CD32 (Fc Block, BD Biosciences). Cells were stained with the indicated antibodies in a suspension of PBS containing 0.5% bovine serum albumin and 0.1% sodium azide for 30 minutes at 4°C. Staining for apoptosis was done in Annexin V staining buffer (140mM sodium chloride and 2.5mM Calcium Chloride dehydrate) for 30 minutes at 4°C Samples were analyzed with a BD$^{TM}$ LSRII flow cytometer and the data were analyzed using FlowJoX10 software (Tree Star Inc.).

IL-10 intracellular staining on B cells was done after 5 days of culture with IL-5 or IL-4 and mCD40L-Fb. B cells were further stimulated at 6 million/mL with 50ng/mL phorbol myristate acetate (PMA) (Sigma Aldrich) and 500ng/mL ionomycin (Sigma Aldrich) (PI) for 4 hours at 37°C. During the last hour of PI stimulation Golgi Plug (BD) containing Brefeldin A was added at 10 µg /mL. After stimulation the cells were stained using zombie violet (Biolegend) fixable viability dye according to manufacturer's protocol. The cells were stained with surface markers as previously described then fixed and permeabilized (eBioscience Foxp3 Fixation/Permeabilization Kit) for IL-10 intracellular staining following the manufacturer's protocol.

To sort CD5$^{+}$ splenic murine B cells, CD19$^{+}$ B cells were first isolated using anti-mouse CD19 MACS magnetic bead positive selection (Miltenyi Biotec) using manufacturer's protocol. The cells were stained with CD5-PE (Biolegend) then sorted using BD FACS Aria II.

### 2.3.4 ELISA

IL-10, IL-6, IL-5 and IL-4 proteins were assessed in the supernatant by sandwich ELISA kits purchased from BD Biosciences using the manufacturer protocols.

### 2.3.5 Real-time Quantitative PCR-Supplementary

Purified mouse B cells from naïve DBA/1 spleens were cultured in triplicate for five days with irradiated mCD40L-Fb and the indicated cytokines and/or LPS. Cells were collected from the cultures without further stimulation and RNA was purified using a QIAcube machine and RNeasy Mini QIAcube kit (QIAGEN, Valencia, CA). Complimentary DNA was reverse transcribed using Oligo dT and MMLV-RT following standard protocols. Mouse IL-6, IL-10, and GAPDH gene expression were determined by Taqman$^{TM}$ quantitative polymerase chain reaction using manufacturer's inventoried primers and a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). Cytokine gene expression was normalized to GAPDH within each sample and expressed as parts per million of GAPDH message. Control samples not shown in the figure consisted of equal numbers of B cells grown on non-transduced NIH3T3 cells with the same cytokines and LPS conditions, as well as NIH3T3 and mCD40L fibroblasts cultures without B cells, none of which led to measurable amounts of IL-6 or IL-10 mRNA expression after five days of culture.

### 2.3.6 Allergic Airway Disease Model

To induce allergic airway disease, BALB/cJ mice were sensitized with 20 µg OVA protein in 2mg of Alum on day 0 and 7. On day 19, $CD5^+$ or $CD5^-$ B cells were sorted from IL-5/mCD40L-Fb cultures of splenic BALB/c or IL-10 mutant B cells. Two million of the indicated B cell subset or PBS was tail vein injected into sensitized mice. Twelve hours after B cell transfer, mice were challenged with 10 µg OVA protein in 50 µL by forced aspiration daily for 3

days (day 20-22). Mice were anesthetized for each challenge with a 4% xylazine, 10% Ketamine, and 86% PBS solution. On day 23, the mice were evaluated for airway hyperresponsiveness, and then sacrificed prior to further analysis of lung physiology and immune responses.

### 2.3.7 Airway Hyperresponsiveness (AHR) by Plethysmography

The mouse to be tested was anesthetized with sodium pentobarbital and subsequently intubated intratracheally with an 18-gauge metal tube. The mouse is attached to a Harvard pump ventilator (tidal volume, 0.4 ml; frequency, 120 breaths/minute; positive end-expiratory pressure, 2.5 to 3.0 cm $H_2O$; Harvard Apparatus, Holliston, MA) and the plethysmograph is sealed to determine baseline resistance. The Buxco software calculates resistance by dividing the change in pressure (Ptp) by the change in flow (F) (Ptp/F; units = cm $H_2O$/ml/second). The mouse is then challenged with 0.2 mg of methacholine, previously determined as the optimal dose, by tail vein injection. Peak resistance is recorded and the difference between the peak and baseline resistance was reported as a measure of AHR.

### 2.3.8 Bronchoalveolar lavage fluid and lung collection

A solution of of 3mM EDTA in PBS (3 x 1mL/mouse) was used to lavage the bronchoalveolar space for extraction of cells and cytokines. Lungs were then perfused with 10 mL 1x PBS before collection of the tissue that was then minced and digested with 0.7 mg/mL of Collagenase IV in RPMI for 50 min at 37°C. Cells were extracted from the lung tissue by mechanical force then used for flow cytometry.

### 2.3.9 Statistical Analysis

Data presented are from representative experiments of at least three experiments performed for each assay. Cultures were done in triplicate wells in every experiment. Asterisks signify: *$P$<0.05, **$P$<0.01, ***$P$<0.001, ****$P$<0.0001, and n.s.= not significant in all graphs.

## 2.4 Results

### 2.4.1 $CD5^+$ B cells are induced by IL-5 to produce IL-10

We have previously shown that culture of purified mouse splenic B cells from DBA/1 mice with mCD40L-Fb in the presence of IL-5 supported the growth of $FasL^+$ B cells (Klinker et al. 2013). In addition to having enhanced killer function against antigen-specific T cells, these B cells also produced IL-10. To confirm IL-5 stimulated production of IL-10 from B cells was not a strain specific phenomenon, we cultured B cells from DBA/1, C57BL/6, and BALB/c mice. Splenic B cells and mCD40L-Fb were cultured with or without IL-5 for five days. IL-5/mCD40L-Fb induced IL-10 production from B cells of all three strains, however, B cells from DBA/1 mice produced slightly more IL-10 than those from BALB/c mice, and B cells from C57BL/6 mice made significantly less IL-10 than B cells from DBA/1 and BALB/c mice (Fig 2.1A). To further explore the relevance of this finding in the context of allergic airway disease, B cells from BALB/c strain background were used for all subsequent experiments unless otherwise indicated.

$CD5^+$ B cells are one of the subsets extensively reported to produce IL-10 after a variety of stimulations (Tedder 2015). To determine if $CD5^+$ B cells were the IL-10 producers, we sorted $CD5^+$ and $CD5^-$ splenic B cells from naïve BALB/c mice by fluorescence activated cell sorting and cultured them with IL-5/mCD40L-Fb. Only $CD5^+$ B cells but not $CD5^-$ B cells produced

detectable levels of IL-10 in culture supernatants (Fig 2.1B). Similar results were found using DBA mice (data not shown). Intracellular staining for IL-10 was assessed after IL-5/mCD40L-Fb culture to further investigate phenotypic similarities to other reported Bregs. IL-$10^+$ B cells were highly enriched for expression of CD5, CD9, IgM, CD43, and rarely expressed CD23 (Fig 2.1C, Fig 2.2). IL-$10^+$ B cells were also enriched in $CD1d^{hi}$ B cells, a marker of a $CD5^+$IL-$10^+$ subset termed B10 cells. However, $CD1d^{hi}$ B cells made up merely 6% of IL-$10^+$ B cells. While there was phenotypic overlap with multiple reported Breg subsets including marginal zone B cells, B10, and B-1a cells, the cells induced in these cultures do not fit into any one subset. In conclusion, $CD5^+$ B cells from naïve mice are induced to produce IL-10 after IL-5/mCD40L stimulation and are phenotypically unique from other reported IL-$10^+$ Bregs.

### 2.4.2 IL-4 inhibits IL-10 production from $CD5^+$ B cells via the STAT6 signaling pathway

IL-5 is a major product of $T_H2$ cells activated during allergic reactions, and it was not clear why the stimulation of Breg functions by IL-5 is not sufficient to reduce development of AAD endogenously. In addition to IL-5, IL-4 is also present at high levels during $T_H2$ responses, and therefore, we investigated the effects of IL-4 on IL-5-induced IL-10 production by B cells. Sorted $CD5^+$ splenic B cells were cultured with mCD40L-Fb alone (no cytokine indicated as Nock), or with IL-5, IL-4, or both cytokines. Higher cell numbers were observed when $CD5^+$ B cells were stimulated with IL-5/mCD40L-Fb, but not IL-4/mCD40L-Fb, compared to mCD40L-Fb alone (Fig 2.3A), suggesting that IL-5 was utilized as a growth factor while IL-4 was not. IL-4 stimulation blocked IL-10 production, irrespective of the presence of IL-5 (Fig 2.3B), and instead induced IL-6 production (Fig 2.4A). Further, IL-4 resulted in suppression of IL-10 mRNA similar to protein levels while IL-6 mRNA did not increase (Fig 2.4B) The release of IL-

10 did not occur until day 4 of B cell stimulation with IL-5/mCD40L-Fb while IL-6 was released as early as 24 hours after stimulation (Fig 2.4C).

Based on our findings, we questioned whether IL-4 blockade of the generation of IL-10$^+$ B cells would also affect existing IL-10$^+$ B cells. To address this question we sorted splenic CD5$^+$ B cells and cultured them first with IL-5 or IL-4 and mCD40L-Fb which will be referred to as the "primary culture". After primary culture, B cells were re-cultured in a "secondary culture" of mCD40L-Fb alone or in the presence of IL-5, IL-4, or both cytokines. B cells from IL-4 primary culture had enhanced survival in the secondary culture with mCD40L-Fb alone and did not require additional cytokines as growth/survival factors (Fig 2.3C). B cells from the IL-5 primary culture did not expand with mCD40L-Fb stimulation, but were responsive to both IL-5 and IL-4 as growth/survival factors (Fig 2.3C). CD40-induced IL-10 production from IL-5/mCD40L-Fb primary cultured CD5$^+$ B cells was not affected by IL-4 in secondary culture. However, IL-5 enhancement of mCD40L induced IL-10 production was significantly reduced (Fig 2.3D). Additionally, CD5$^+$ B cells from the IL-4 primary culture did not produce IL-10 in the presence of IL-5 in the secondary culture (Fig 2.3D), suggesting that the effects of IL-4 are stable and long-term. LPS stimulation is another reported stimulus of IL-10$^+$ Bregs (Tedder 2015). To determine whether IL-4 inhibition was specific to IL-5-stimulated culture, we tested the effects of IL-4 on LPS/mCD40L-Fb induced IL-10 production. Splenic B cells were cultured in the same primary culture conditions used previously with or without LPS. LPS/mCD40L-Fb induced IL-10 production similarly to IL-5/mCD40L-Fb stimulation which was significantly reduced with the addition of IL-4 (Fig 2.4A). Overall, in primary culture, IL-4 inhibited mCD40L-induced production of IL-10. After primary culture with IL-5/mCD40L-Fb, IL-4 did not inhibit IL-10 production in the secondary culture but reduced IL-5 enhancement of IL-10

production. The inhibitory effect of IL-4 on IL-10 production was not unique to this culture system as LPS stimulated IL-10 production was also reduced.

To further explore the mechanism of IL-4 inhibition of IL-10 production, splenic B cells from STAT6$^{-/-}$ mice were used to determine whether the effects of IL-4 were dependent on activation of this major signaling pathway. The frequency and number of CD5$^{+}$ B cells from naïve STAT6$^{-/-}$ mice were similar to BALB/c, therefore differences in starting numbers of CD5$^{+}$ B cells were not a factor (Fig 2.5A & B). In contrast to effects on BALB/c B cells, IL-4 failed to inhibit IL-10 production induced by IL-5/mCD40L-Fb stimulation in STAT6$^{-/-}$ B cells (Fig 2.5C). IL-6 production in response to IL-4 stimulation was also severely affected by loss of STAT6 signaling (data not shown). In conclusion, the data suggests that in the primary culture, IL-4 blocks the initiation of IL-10 production in a STAT6 dependent manner.

### 2.4.3 IL-5/mCD40L-Fb stimulated CD5$^{+}$ B cells reduced allergic airway disease

In addition to the effects of IL-5/mCD40L-Fb stimulation on *in vitro* IL-10 production, an OVA induced AAD model was used to determine the suppressive abilities of the IL-5/mCD40L-Fb stimulated CD5$^{+}$ B cells following adoptive transfer. CD5$^{+}$ and CD5$^{-}$ B cells sorted from IL-5/mCD40L-Fb cultures were intravenously injected into OVA-sensitized mice, followed by three daily challenges by forced aspiration. Using plethysmography, a decrease in airway resistance was observed only in CD5$^{+}$ B cell recipient mice compared to allergic controls (Fig 2.7A). CD5$^{+}$ B cell transfer significantly reduced the amount of IL-4 detected in the bronchoalveolar lavage (BAL) fluid (Fig 2.7B) while IL-5 levels were unchanged and IL-10 was undetectable (data not shown). CD5$^{+}$ B cell recipients also had significant reduction in total cells and eosinophils in the BAL fluid (Fig 2.7C & D). T cells and B cells were not significantly reduced (Fig 2.8A & B). In contrast, transfer of CD5$^{-}$ B cells from IL-5/mCD40L-Fb culture

resulted in similar airway resistance, cytokine levels and cell numbers in the BAL as allergic controls, conferring no protection. Induction of $FoxP3^+$ regulatory T cells (Tregs) in the lungs and mediastinal lymph nodes was assessed, however, no differences were found between the treatment groups (Fig 2.7E & F). To summarize, transfer of $CD5^+$ but not $CD5^-$ B cells from the IL-5/mCD40L-Fb cultures reduced the development of airway hyper-responsiveness of the lungs, and cellular infiltration and IL-4 production in the BAL fluid of mice in this model of acute AAD.

### 2.4.4 Protective effects of $CD5^+$ B cells are IL-10 dependent

Lastly, we investigated the importance of IL-10 production from $CD5^+$ B cells for protection against AAD development. B cells from IL-10 mutant ($IL\text{-}10^{mut}$) mice were cultured with IL-5/mCD40L-Fb and $CD5^+$ B cells were subsequently sorted and adoptively transferred into OVA sensitized mice. Protection of lung function and reduced airway resistance were lost compared to WT $CD5^+$ B cell recipients (Fig 2.9A). Furthermore, the reductions in IL-4 levels and total cell and eosinophils numbers in the BAL was abolished in the absence of IL-10 secretion by the transferred B cells (Fig 2.9B-D). These data showed that protection conferred by $CD5^+$ B cells from the IL-5/mCD40L-Fb cultures was dependent on their IL-10 secretion.

## 2.5 Discussion

Regulatory B cells are essential modulators of the immune system. A fraction of B cells within most subsets have reported regulatory capacity in response to a variety of stimulations (Mauri, Menon 2015). Previously, we reported that IL-5/mCD40L-Fb costimulation induced B cell IL-10 production (Klinker et al. 2013). CD40L stimulation is known to induce IL-10 production from B cells however, that the addition of IL-5 enhanced this effect of mCD40L-Fb is

a novel finding. The current study identified the phenotype of the IL-10 producers and defined the effects of individual and combined stimulation by $T_H2$ cytokines IL-5 and IL-4 on mCD40L-Fb induced IL-10 production from B cells. $CD5^+$ B cells stimulated with IL-5/mCD40L-Fb *in vitro* displayed a potent suppressive capacity *in vivo* that was dependent on IL-10 as assessed in a murine AAD model.

IL-5 enhanced mCD40L-Fb induced IL-10 production exclusively from splenic $CD5^+$ B cells of naïve mice. Confinement of IL-10 producers to the $CD5^+$ B cell compartment suggests they are B-1a lymphocytes. Strain differences in amounts of IL-10 produced were observed. Others also have reported reduced $CD5^+$ B-1a lymphocyte frequencies and IL-10 producing capabilities in C57Bl/6 mice compared to BALB/c mice (Margry et al. 2014, Velupillai et al. 1997). Multiple studies have shown that murine and human B-1a cells produce IL-10 in a regulatory capacity (Kim et al. 2015, Margry et al. 2014, Griffin, Rothstein 2012, Natarajan et al. 2012). Their BCRs recognize microbial pathogens, self-antigens, and potentially allergens (Shaw et al. 2003, Pennell et al. 1989a, Gu, Forster & Rajewsky 1990). and they are potent APCs (Mohan et al. 1998, Sato et al. 2004, Zhong et al. 2007a). The combination of their BCR specificity, regulatory capacity, and APC function positions B-1a cells to be an effective immune regulatory subset in allergy and autoimmunity.

After IL-5/mCD40L-Fb stimulation, CD9 was expressed on the majority of $IL\text{-}10^+$ B cells. CD9 is a tetraspanin-family transmembrane protein found on B-1a, marginal zone, and transitional 2 marginal zone precursor B cells but not on follicular B cells (Braza et al. 2015). All B cell subsets found to express CD9 can produce IL-10 in a regulatory capacity (Mauri, Menon 2015) making it a more all-encompassing marker for $IL\text{-}10^+$ Bregs (Sun et al. 2015). Moreover, adoptive transfers of $CD9^+$ B cells from naïve mice were protective in a murine model of AAD

in an IL-10 dependent manner and have recently been implicated in the immunosuppressive activity of IL-10$^{+}$ Bregs (Braza et al. 2015, Sun et al. 2015). A human IL-10$^{+}$ Breg population, CD27$^{-}$CD24$^{hi}$CD38$^{hi}$, is also reported to express CD9. Our data supports CD9 expression as a marker of activated Bregs as 95% of IL-10$^{+}$ B cells after IL-5/mCD40L-Fb stimulation expressed CD9 on their cell surface. However, stimulation of sorted CD9$^{+}$CD5$^{-}$ B cells from naïve mice with IL-5/mCD40L-Fb did not result in IL-10 production, suggesting that CD9 expression may identify B cells that acquired IL-10 production rather than a population that is sensitive to IL-5/mCD40L stimulation (data not shown).

$T_H2$ cytokines IL-5 and IL-4 differentially affected CD5$^{+}$ B cell growth and IL-10 production. CD5$^{+}$ B cells constitutively express the IL-5Rα and use IL-5 as a growth or survival factor (Moon et al. 2004). In primary culture, IL-5 stimulated growth of CD5$^{+}$ B cells while IL-4 did not. IL-5-induced proliferation of CD5$^{+}$ B cells likely accounts for increased IL-10 production induced by mCD40L-Fb, as a higher percentage of IL-10$^{+}$ B cells are present in IL-5/mCD40L-Fb compared to mCD40L-Fb cultures (data not shown). IL-4 inhibition of IL-10 is not unique to our culture system as LPS stimulation, another inducer of IL-10$^{+}$ Bregs, was also inhibited in the presence of IL-4. Further, IL-4 was shown to block LPS-stimulated IL-10 production from bone marrow derived DCs (Yao et al. 2005). We speculate that IL-4 renders the IL-10 gene locus inaccessible as IL-4 induced STAT6 signaling is reported to compromise histone acetylation of the IL-10 promoter in LPS stimulated DCs (Yao et al. 2005). STAT6 may also compete with other transcription factors and/or cofactors essential for IL-10 gene expression. Additionally, IL-4 suppression of the common beta chain, which is essential for IL-5 signaling, may explain the inhibition of IL-5 enhancement of IL-10 in the secondary culture (Weber, Isakson & Purkerson 1996). While IL-13 is also a $T_H2$ cytokine murine B cells do not

express the IL-13 receptor (Kuhn, Rajewsky & Muller 1991, Andrews et al. 2001) and cultures including IL-13 had no effect on IL-5/mCD40L-Fb induced IL-10 (data not shown). Ongoing and future studies are focused on understanding the molecular mechanisms of IL-4 inhibition of IL-10 production in our culture system.

Transfer of $CD5^+$ B cells reduced development of OVA induced AAD, reducing airway resistance and total cellular infiltration and eosinophils numbers in the BAL. IL-4 in the BAL was also significantly reduced while IL-5 was unchanged and IL-10 was undetectable (data not shown). Bregs can inhibit cytokine production from activated T cells, dampening immune responses. Reduction in BAL IL-4 suggests effects on T cell cytokine production as a possible mechanism of protection. B cells are central in maintaining the $FoxP3^+$ Treg compartment and induction of $FoxP3^+$ Tregs and IL-10 producing T regulatory cells (Tr1) are also mechanisms utilized by IL-$10^+$ Bregs to regulate immune responses (Carter et al. 2011, Carter, Rosser & Mauri 2012, Tadmor et al. 2011, Sun et al. 2008, Chu, Chiang 2012, Olkhanud et al. 2011). However, no differences were found in percentages or numbers of $FoxP3^+$ Tregs in the lungs or MLN. $FoxP3^+$ Tregs were not increased however, induction of Tr1 cells, which are $FoxP3^-$, could be involved but was not assessed. The full mechanism of protection by $CD5^+$ B cells remains unclear but, protection was IL-10 dependent as transfer of IL-10 mutant $CD5^+$ B cells reversed protective trends of wild-type IL-5/mCD40L-Fb stimulated $CD5^+$ B cells in the AAD model. Further investigations will be focused on improving *in vitro* culture methods and stimulating *in vivo* activation of IL-10 production by endogenous lung $CD5^+$ B cells as a potential treatment for allergic airway disease.

Our results suggest that regulatory $CD5^+$ B cells may be preferentially active in environments where IL-5 and CD40L are present but where IL-4 production is minimal. A

potential cellular source of IL-5 without IL-4 are ILC2 cells (Neill et al. 2010). In the early stages of an allergic response, ILC2s are the initial IL-5 producers and produce minimal IL-4 however, their CD40L expression is unknown. Human ILC3s are known to express CD40L and interact with B cells, enhancing B cell survival, proliferation, and induction of IL-10 producing PD-L1$^{+}$ Bregs (Komlosi et al. 2017). ILC3s and Bregs were found in close contact within regulatory niches of the palatine tonsils in healthy controls. ILC3s frequencies were reduced in tonsil tissue and both ILC3s and Bregs were reduced in peripheral blood of allergic patients (Komlosi et al. 2017). Interactions between ILCs and Bregs may be an important mechanism in the maintenance of immune tolerance.

Most studies suggest IL-4 and IL-5 are produced simultaneously by $T_H2$ cells, however, $T_H$ cells that primarily produce IL-5 have been described and chronic $T_H2$ responses could potentially provide conditions for IL-5-mediated induction of IL-10$^{+}$ Bregs. Murine T cells that had undergone multiple rounds of $T_H2$ polarization *in vitro* expressed CCR8, were responsive to CCL8, and produced abundant IL-5 relative to IL-4 (Islam et al. 2011). Production of IL-5 and not IL-4 was confirmed *in vivo* in a chronic atopic dermatitis model. Intracellular staining of human peripheral T cells for IL-5 and IL-4 paralleled murine results as CCR8$^{+}$ CD4$^{+}$ T cells produced abundant IL-5 and minimal IL-4 (Islam et al. 2011). T cells producing IL-5 dominantly over IL-4 have also been observed during murine and human IL-33-induced $T_H2$ polarization *in vitro* (Kurowska-Stolarska et al. 2008) and murine schistosome infection *in vivo*, another chronic $T_H2$ response (Xie et al. 2013). Bregs are induced during murine chronic helminth infections which may contribute to the transition of the response from $T_H2$ to a regulatory and fibrotic response (Lundy, Lukacs 2013). Adoptive transfer of helminth induced CD5$^{+}$ Bregs prevent and reverse AAD (Amu et al. 2010). Although Bregs can be induced from worm secreted proteins,

this may not be the only mechanism of induction during helminth infection. $CD5^+$ B cells have also been shown to increase in number during chronic house dust mite induced AAD in mice. Moreover, absence of $CD5^+$ B cells in Xid mice resulted in more severe allergic symptoms (Lundy et al. 2005). Chronic OVA stimulation in mice can also result in resolution of pulmonary AAD response (Singh et al. 2008, Yiamouyiannis et al. 1999, Schramm et al. 2004). $CD5^+$ regulatory B cells were induced after 42 days of OVA challenge in hilar lymph nodes (HLN) of tolerant mice. Adoptive transfer of $CD5^+$ HLN B cells into OVA sensitized mice prevented development of AAD in a TGF-β dependent manner (Singh et al. 2008, Natarajan et al. 2012). Similar studies have led to use of chronic antigen stimulation as a strategy for treating allergic diseases. Specific allergen immunotherapy (SIT) is the only current disease modifying treatment for allergic diseases that has curative potential (van de Veen 2017). Multiple high doses of disease causing allergen are received subcutaneously or sublingually to induce permanent tolerance. Studies in mice and humans both suggest induction of tolerance through SIT involves the expansion and function of regulatory B cells (van de Veen 2017, van de Veen et al. 2013). Chronic stimulation induces B cells with multiple regulatory abilities including the production of IL-10. Our results suggest emergence of IL-5 dominant $T_H2$ cells after chronic stimulation may contribute to induction and expansion of Bregs and tolerance seen during helminth infections and SIT. Further investigation is necessary to understand temporal and spatial activation of Bregs.

Future studies are underway to determine the IL-10 dependent regulatory mechanisms of IL-5/mCD40L-Fb induced B cells and the inhibitory effects of IL-4. Understanding the regulation of the suppressive functions of B cells can lead to new and possibly more specific therapeutics and guide current treatment strategies for severe allergic airway disease.

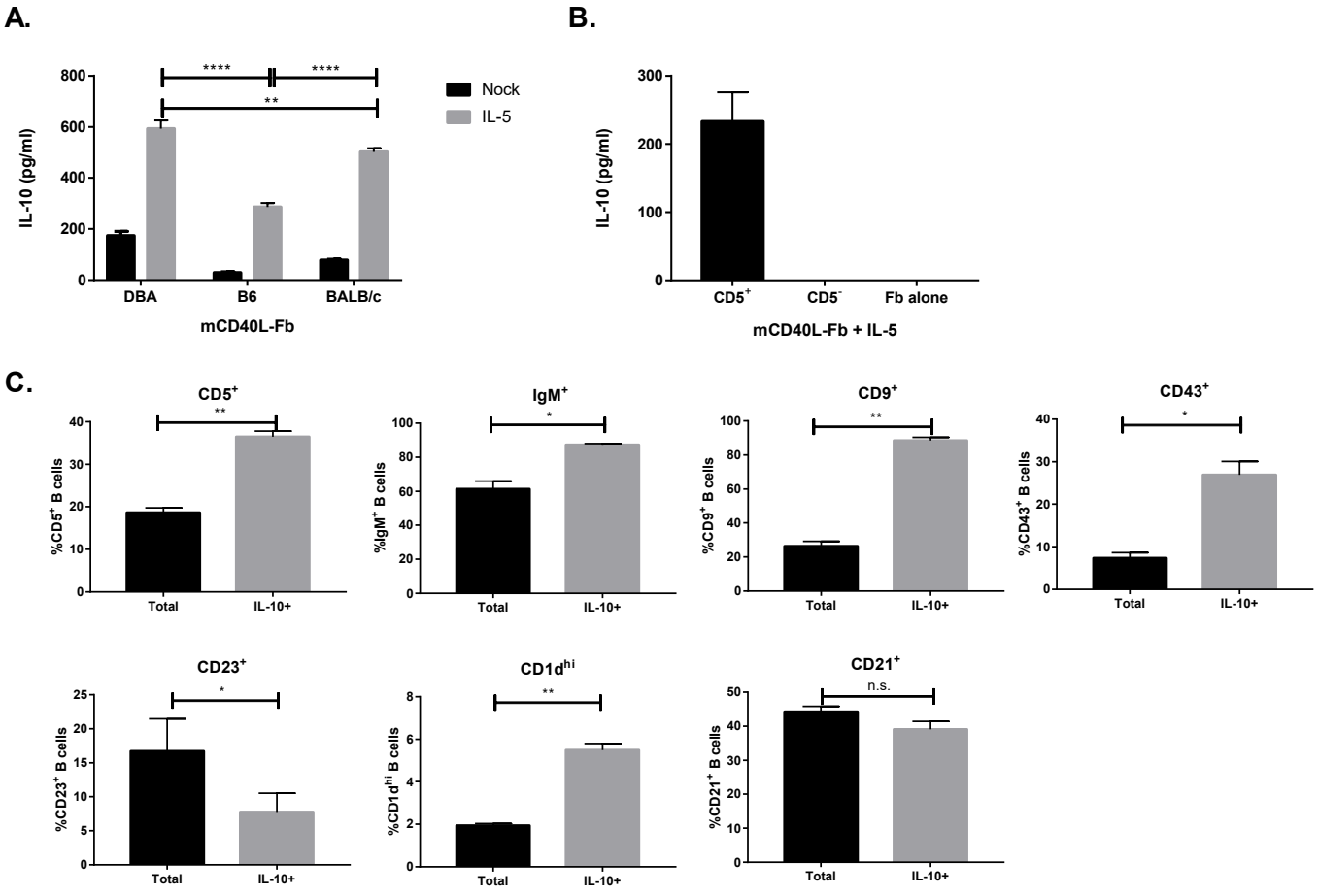
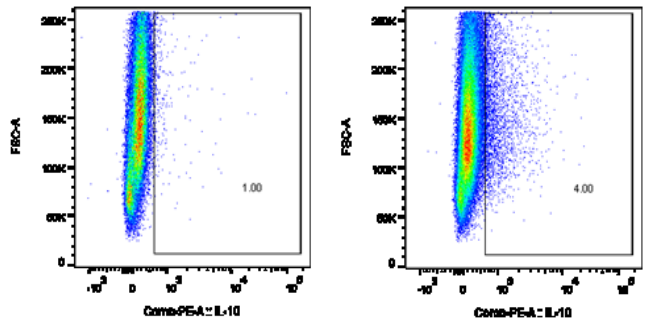



**Figure 2. 1 IL-5/mCD40L-Fb stimulation induces IL-10 production from CD5+ B cells**

**(A and C)** Total or **(B)** sorted $CD5^+$ or $CD5^-$ B cells were cultured for 5 days with IL-5/mCD40L-Fb. **(A and B)** IL-10 in culture supernatant was assessed by ELISA. **(C)** IL-10 intracellular staining was performed on cells from the culture after PMA/ionomycin/Brefeldin A restimulation. Flow cytometry data on B cells markers and their expression in the total B cell population or IL-10+ B cells is summarized in bar graphs. Data are from a single representative experiment of 3 independent experiments (mean ± SEM); at least 3 mice/experiment. **(A and B)** Tukey's multiple comparison test or **(C)** two-tailed paired t test were used for statistics. P values are indicated by: * P>0.05, **P>0.01, ***P>0.001, ****P>0.0001, n.s. not significant. Nock (No cytokine)

**A.**




IL-4/mCD40L-Fb IL-5/mCD40L-Fb

**B.**

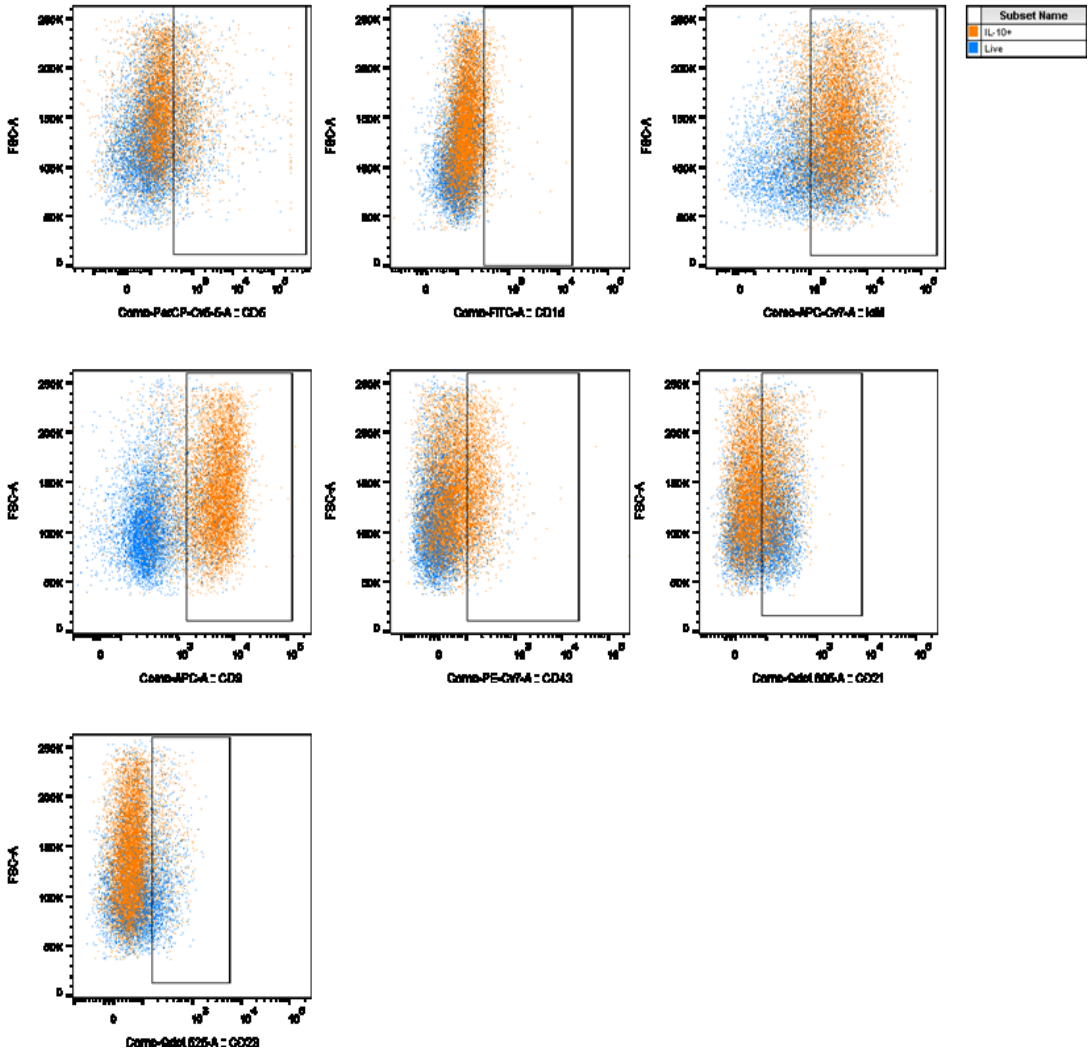


**Figure 2. 2 Representative flow plots of IL-10 intracellular staining**

IL-$10^+$ gate was set according to fluorescence minus one. **(A)** IL-10 intracellular staining was performed on splenic B cells from IL-5 or IL-4/mCD40L-Fb cultures after PMA/ionomycin/Brefeldin A restimulation. **(B)** IL-$10^+$ B cells and total B cells are plotted comparing single markers against forward scatter area. IL-$10^+$ B cells (orange) are overlaid on total B cells (blue).

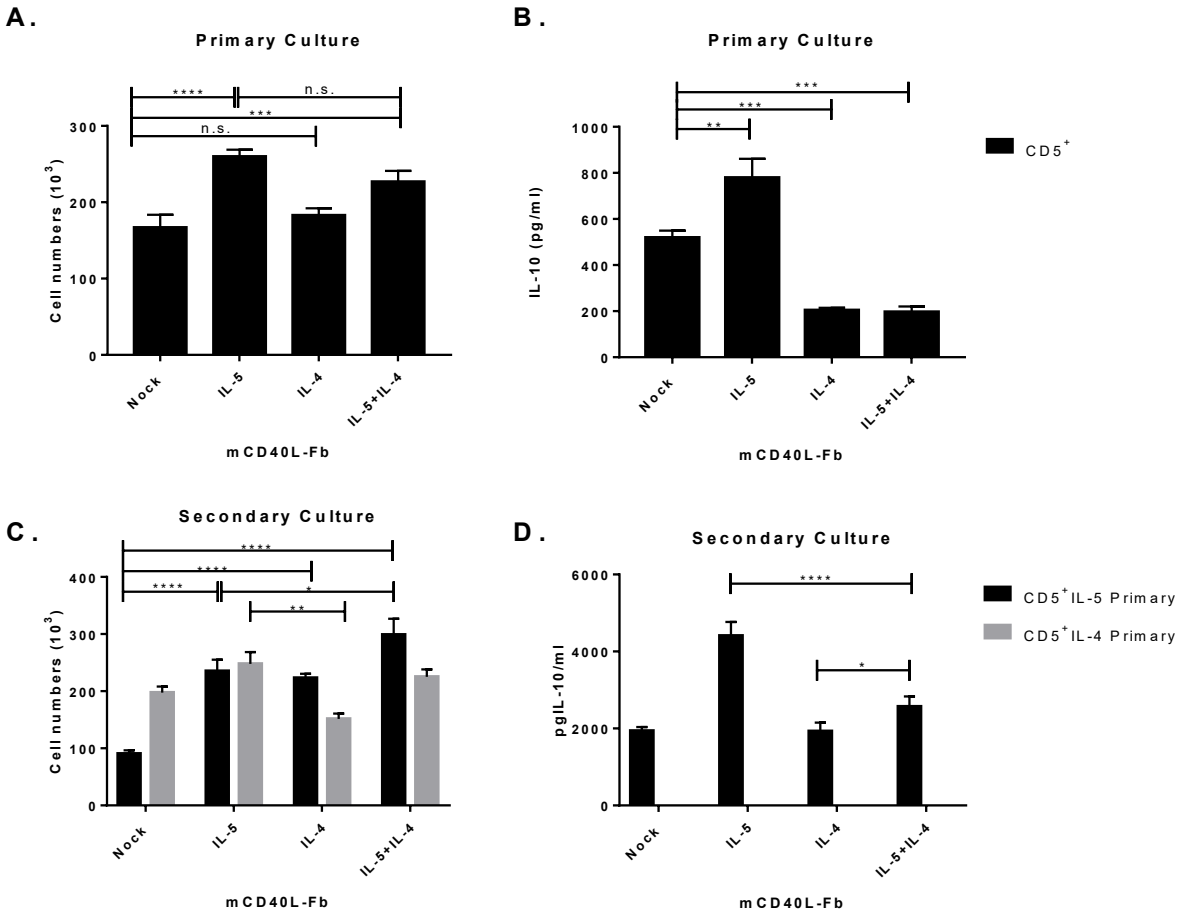



**Figure 2. 3 IL-4 inhibits IL-10 production from CD5$^+$ B cells**

**(A-D)** Sorted CD5$^+$ B cells were cultured with mCD40L-Fb and the indicated cytokines in primary or secondary culture. **(A and C)** Cells were counted after culture and **(B and D)** supernatants were collected for IL-10 ELISA. Data are from a single representative experiment of 3 independent experiments (mean $\pm$ SEM). **(A-D)** Tukey's multiple comparison test was used for statistics. Nock (No cytokine)

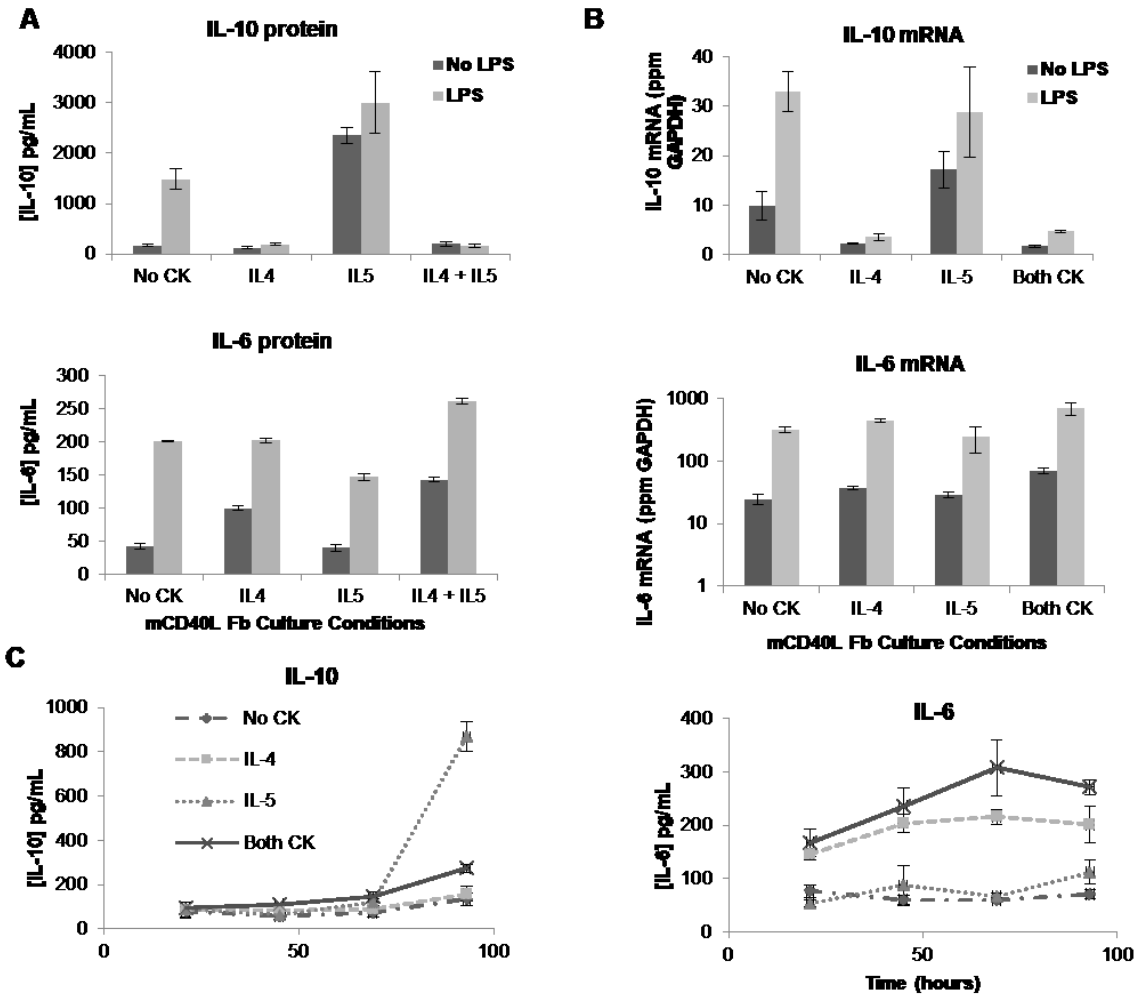



**Figure 2. 4 IL-4 opposes both IL-5 and LPS stimulated IL-10 production from B cell/mCD40L Fb cultures**

B cells (4 million/mL) from naïve DBA/1 mice were cultured in triplicate with mCD40L-Fb, cytokines, and/or 1 mg/mL *E. coli* LPS. **(A)** Supernatants were collected on day 5 and analyzed for the presence of IL-6 and IL-10 by ELISA. Data are mean cytokine concentration ± standard deviation of triplicate samples. **(B)** After five days of culture, cells were collected and mRNA was purified without further stimulation. Taqman™ quantitative PCR was performed on reverse transcribed cDNA samples and the expression of IL-6 and IL-10 genes were compared within each sample to a GAPDH standard. Data are expressed as the mean expression in parts per million of GAPDH message ± standard error for triplicate samples. *P* values in both A and B, which were significant ($P<0.05$) in each case when error bars did not overlap, are omitted for clarity. **(C)** Supernatants were harvested at 24 hour intervals for four days and IL-6 and IL-10 concentrations were measured by ELISA. Data are mean cytokine concentration ± standard deviation of triplicate samples. Nock (No cytokine)

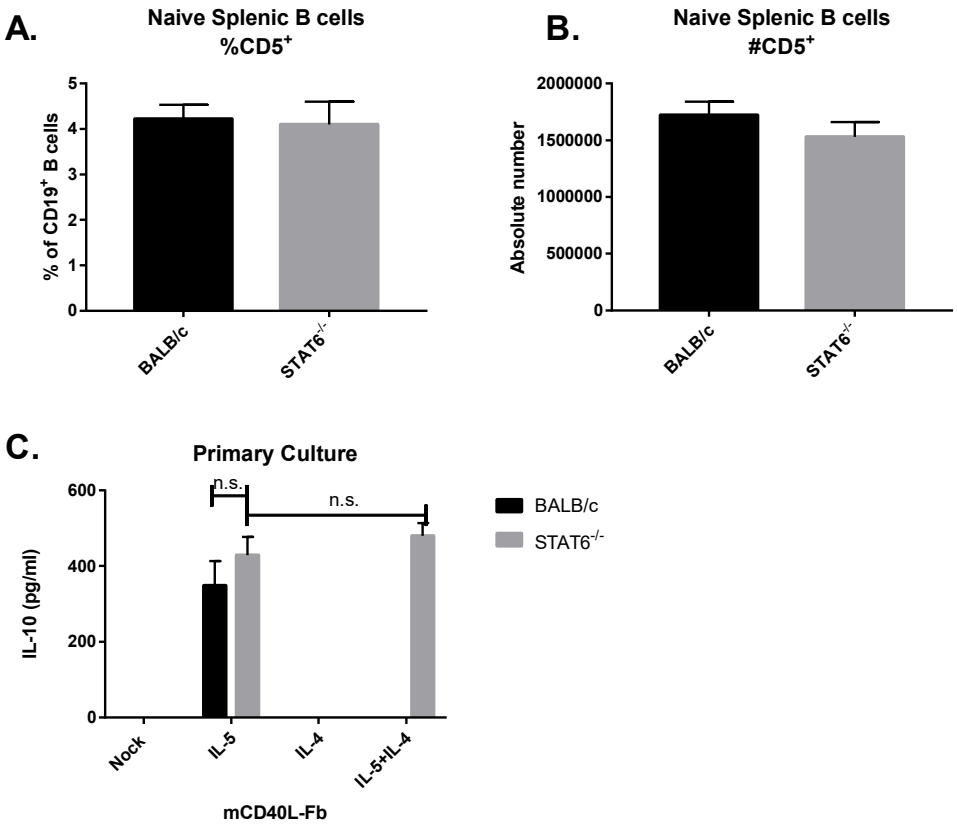



**Figure 2. 5 IL-4 inhibition of IL-10 is mediated through STAT6**

**(A and B)** Percentage and absolute numbers of $CD5^+$ B cells from spleens of naive BALB/c and STAT6-/- mice were assessed by flow cytometry. Data are 10 mice from 3 independent experiments (mean $\pm$ SEM) using Mann Whitney for statistical analysis. **(C)** B cells from these mice were stimulated with mCD40L-Fb and the indicated cytokines for 5 days in primary culture. Culture supernatants were collected for IL-10 ELISA. Data are from one representative experiment of 3 independent experiments (mean $\pm$ SEM); at least 3 mice of each strain in each experiment. Statistics done using Sidak's multiple comparison test. Nock (No cytokine)

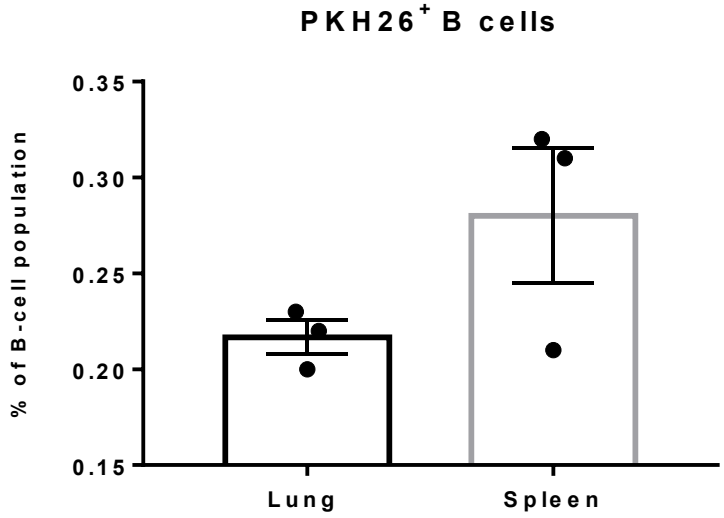



**Figure 2. 6 Homing of transferred B cells**

B cells were labeled with PKH26 membrane dye before transferred into sensitized mice. Upon completion of the allergic model, the lungs, spleen, and draining mediastinal lymph nodes were collected. Flow cytometry was used locate $PKH26^{+}$ transferred B cells in these location. Data are a single representative experiment of 2 independent experiments (mean $\pm$ SEM).

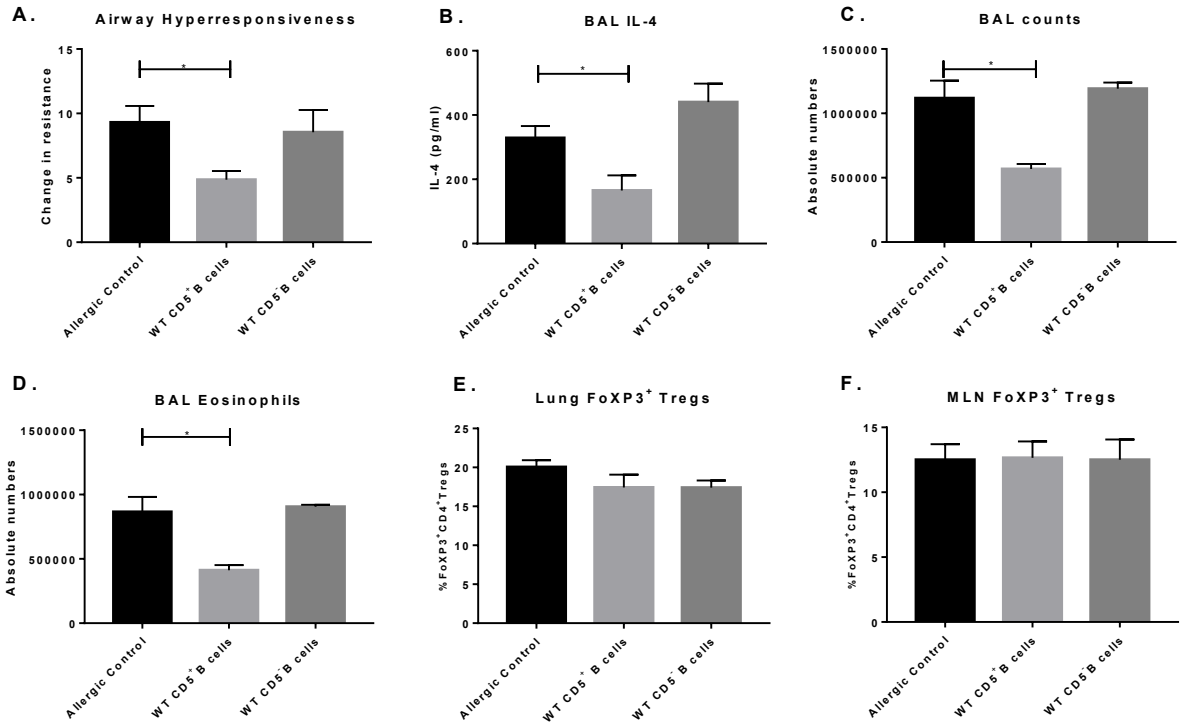



**Figure 2. 7 IL-5/mCD40L-Fb stimulated CD5$^+$ B cells prevent allergic airway disease**

OVA sensitied mice received 2x106 B cells/mouse then, were treated daily with 10µg OVA for 3 days. **(A)** Airway hyperresponsiveness was assessed by plethysmography. **(B)** BAL fluid was collected and assessed by ELISA for IL-4. **(C and D)** Cells in the BAL fluid were counted and assessed by flow cytometry for eosinophils ($CD45^+CD4^-CD19^-Ly6G^-SiglecF^+MHCII^-$). **(E and F)** Regulatory T cells in the lung and mediastinal lymph nodes ($CD45^+CD3^+CD4^+CD25^+FoxP3^+$) were determined by intracellular flow cytometry. Data are a single representative experiment of 3 independent experiments (mean $\pm$ SEM); 3-5 mice/group. Statistics are from Mann Whitney test comparing treatment groups to allergic control.

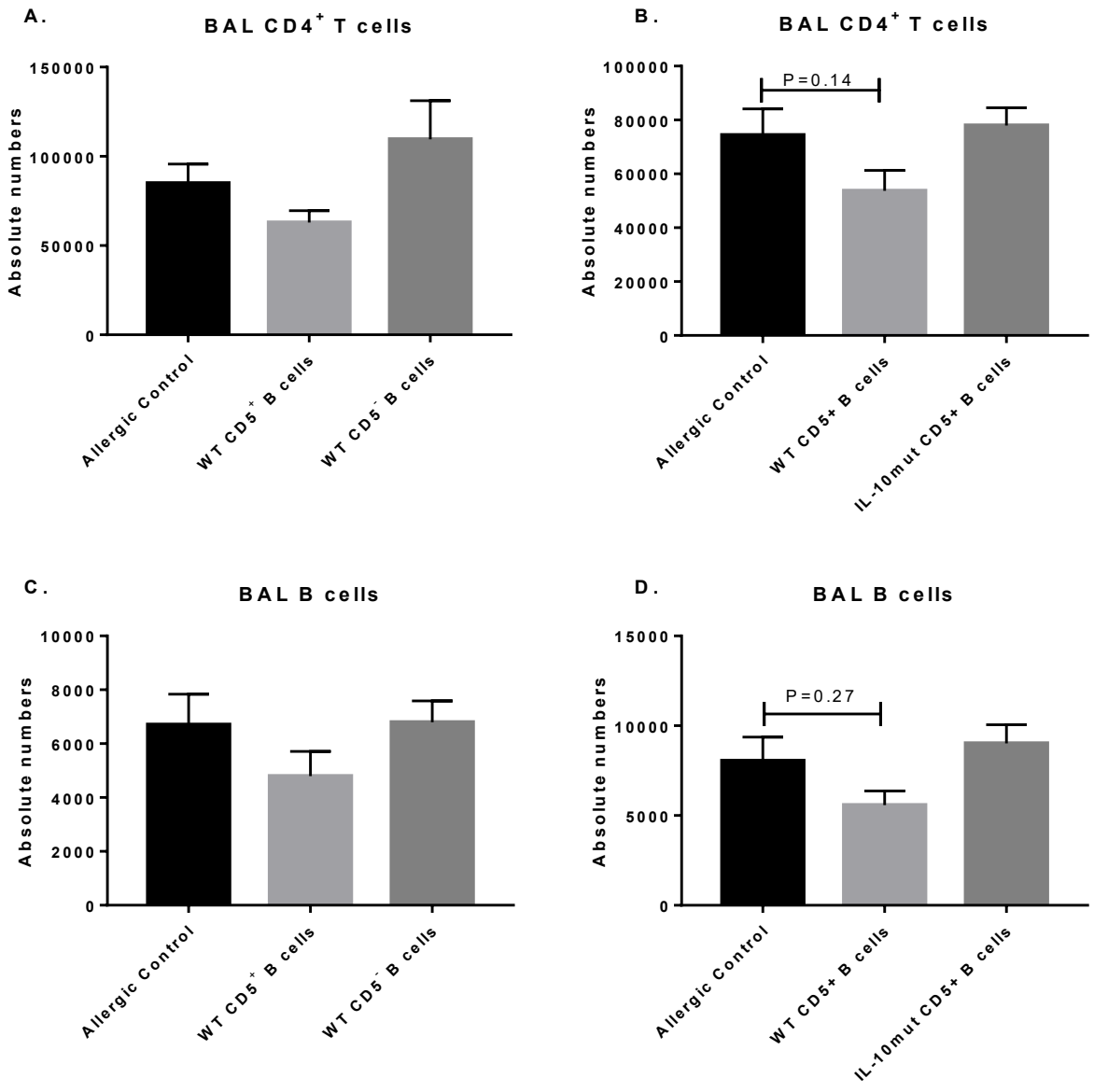



**Figure 2. 8 Effects on T and B cell infiltration into the BAL fluid during AAD**

**(A-D)** Cells in the BAL fluid were counted and assessed by flow cytometry for $CD4^+$ T cells ($CD45^+CD4^+CD19^-$) and B cells ($CD45^+CD4^-CD19^+$). Absolute numbers are given. Data are combined from 3 independent experiments (mean $\pm$ SEM); at least 20 mice/group. Statistics are from Mann Whitney test comparing treatment groups to allergic control.

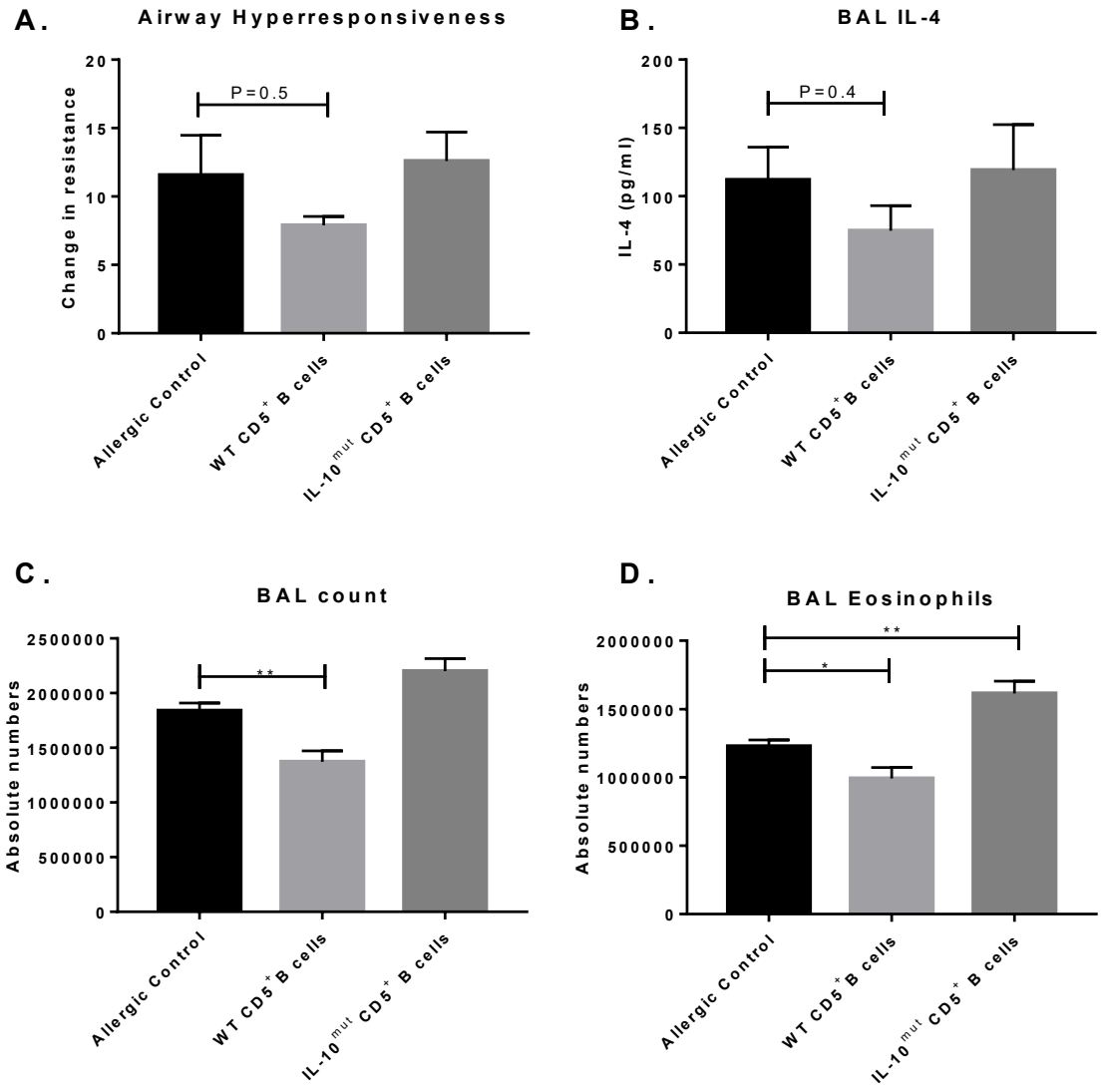



**Figure 2. 9 CD5$^+$ B cell AAD protection is IL-10 dependent**

OVA sensitied mice received 2x10$^6$ B cells/mouse then, were treated daily with 10µg OVA for 3 days. **(A)** Airway hyperresponsiveness was assessed by plethysmography. **(B)** BAL fluid was collected and assessed by ELISA for IL-4. **(C and D)** Cells in the BAL fluid were counted and assessed by flow cytometry for eosinophils (CD45$^+$CD4$^-$CD19$^-$Ly6G$^-$SiglecF$^+$MHCII$^-$). Data is one experiment representative of 3 independent experiments (mean $\pm$ SEM); at least 6 mice/group. Statistics done using Mann Whitney test comparing treatment groups to allergic control.

| IL-10 Intercellular Staining of B cells | | | | |
|---|---|---|---|---|
| Marker | Clone | Color | Dilution | Supplier |
| CD1d | 1B1 | FITC | 1:200 | BD Pharmingen |
| IL-10 | JES5-16E3 | PE | 1:100 | Biolegend |
| CD11b | M1/70 | PEDazzle | 1:800 | Biolegend |
| CD5 | 53-7.3 | PE-Cy5 | 1:1600 | BD Pharmingen |
| CD43 | S7 | PE-Cy7 | 1:1600 | BD Pharmingen |
| CD9 | MZ3 | APC | 1:100 | eBioscience |
| IgM | II/41 | APC-eFluor780 | 1:100 | eBioscience |
| Zombie Violet | | PacificBlue | 1:400 | Biolegend |
| CD23 | B3B4 | BV510 | 1:200 | Biolegend |
| CD21 | 7G6 | BV605 | 1:1600 | BD horizon |
| CD16/32 | 2.4G2 | ------ | 1:100 | BD Pharmingen |
| | | | | |
| Regulatory T cell panel | | | | |
| Marker | Clone | Color | Dilution | Supplier |
| CD73 | TY/23 | AlexaFluor 488 | 1:100 | BD Pharmingen |
| FoxP3 | NRRF-30 | PE | 1:150 | eBioscience |
| CD127 | A7R34 | PeDazzle | 1:200 | Biolegend |
| CD25 | PC61 | PECy7 | 1:200 | Biolegend |
| CD39 | Duha59 | Alexa647 | 1:100 | Biolegend |
| CD45 | 30-F11 | AlexaFluor700 | 1:400 | Biolegend |
| CD3 | 17A2 | APC-eFluor780 | 1:200 | BD Pharmingen |
| CD4 | GK1.5 | BV510 | 1:400 | Biolegend |
| CD16/32 | 2.4G2 | ------ | 1:100 | BD Pharmingen |
| | | | | |
| Cell types in BALF/lung tissue for AAD | | | | |
| Marker | Clone | Color | Dilution | Supplier |
| CD11b | M1/70 | FITC | 1:200 | BD Pharmingen |
| CD11c | HL3 | PE | 1:400 | BD Pharmingen |
| Ly6G | 1A8 | PE-Dazzle594 | 1:800 | Biolegend |
| 7AAD | | PerCP | 1:50 | BD Bioscience |
| CD4 | GK1.5 | PE-Cy7 | 1:400 | Biolegend |
| MHC II (I-A/I-E) | 53-7.3 | APC | 1:1600 | eBioscience |
| CD45 | 30-F11 | AlexaFluor700 | 1:400 | Biolegend |
| F4/80 | BM8 | APCeFluor780 | 1:100 | eBioscience |
| CD19 | 6D5 | PacificBlue | 1:400 | Biolegend |
| Siglec F | E50-2440 | BV510 | 1:100 | BD Bioscience |
| CD335/NKp46 | 29A1.4 | BV605 | 1:200 | Biolegend |
| CD16/32 | 2.4G2 | ------ | 1:100 | BD Pharmingen |

**Table 2. 1 Flow cytometry panels**

The panels of antibodies for IL-10 intracellular staining of B cells, regulatory T cells, and cell populations in the BAL fluid and lungs of sensitized mice are listed. The conjugated fluorochrome, clone, and dilution used for each marker is given.

# CHAPTER 3

## Crucial signaling pathways in regulatory B cells

This work was performed in collaboration with Luciën E.P.M. van der Vlugt, Molly M. Shea, Jennifer Yang, and Steven K. Lundy. S.H.T and S.K.L designed and performed the experiments. E.P.M.V, M.M.S, J.Y assisted in performing experiments and data analysis. S.H.T wrote the manuscript and S.K.L assisted in editing.

Data in this chapter are preliminary and have not been published at the time of submission of this dissertation.

### 3.1 Abstract

Regulatory B cells are critical players in immune regulation, protecting against autoimmune and allergic diseases. Regulatory B cells possess several mechanisms for this purpose, including production of the anti-inflammatory cytokine interleukin 10 (IL-10) and the expression of the death inducing molecule Fas ligand (FasL). Despite the importance of these cells, what governs their activation and regulatory abilities is not fully understood. Our lab has developed *in vitro* cultures using CD40L and IL-5 stimulation to grow mouse regulatory B cells which produce IL-10 and display FasL associated killing abilities. Additionally, CD40L stimulation of human B cells also induces IL-10 production. To elucidate the signaling pathways involved in regulatory B cell activation and function, murine and human cultures were treated with inhibitors to specific signaling proteins downstream of CD40L and IL-5. Effects of

inhibitors on IL-10 production and cell growth of murine B cells were evaluated. Through these inhibitor studies we have identified certain pathways imperative to regulatory B cell growth and function. Interestingly, regulatory B cells may also use unique signaling pathways to respond to CD40L and IL-5 stimulation.

## 3.2 Introduction

In the previous chapter we described a unique stimulation, CD40L and IL-5, which induced IL-10 production from murine B cells that were protective in an allergic airway disease model. Their IL-10 production was limited in the presence of IL-4. In this study we investigated whether stimulation of human peripheral blood B cells would similarly affect IL-10 production. Additionally, we investigated the crucial signaling pathways downstream of CD40L and IL-5 necessary for growth and IL-10 production from murine and human B cells through the use of inhibitors against signaling proteins. Through these studies we hope to identify targets to enhance regulatory B cell function and growth.

CD40 stimulation is an important costimulatory signal for B cells. During a T dependent response CD40-CD40L interaction promotes germinal center formation, Ig class switching, somatic hypermutation, and differentiation of long lived plasma cells and memory B cells (Banchereau et al. 1994, Barrett, Shu & Clark 1991, Clark, Ledbetter 2012, Jabara et al. 1990, Foy, Durie & Noelle 1994, Foy et al. 1994, Foy et al. 1993). Additionally, CD40 stimulation is known to induce human and murine IL-10 producing regulatory B cells however, the signaling pathways that lead to IL-10 production have not been defined. CD40-CD40L interaction has been shown to utilize many signaling pathways (Fig 3.1). Engagement of CD40-CD40L recruits multiple tumor necrosis factor receptor associated factors (TRAFs) including TRAF1-3 and TRAF5-6 which can activate both canonical and non-canonical nuclear factor kB (NFkB)

signaling, mitogen activated protein kinases (MAPK), phosphoinositide 3-kinase (PI3K), and the phopholipase Cγ (PLCγ) pathway (Bishop et al. 2007). In addition to TRAF signaling, Janus family kinase 3 (JAK3) can bind to the CD40 receptor and phosphorylate signal transducer and activator of transcription 5 (STAT5) (Saemann et al. 2003, Saemann et al. 2002). While less described, CD40 stimulation can also activate Bruton's tyrosine kinase (Btk), which is known to be involved in proliferation, survival, and development of B cells.

IL-5 is known for its role in B-1a cell growth and development and eosinophilopoiesis (Yoshida et al. 1996). The IL-5 receptor is comprised of the IL-5 receptor alpha (IL-5Rα) chain and the common beta (βc) chain. IL-5Rα specifically binds IL-5 with low affinity and forms a high affinity receptor upon dimerization with βc (Takaki et al. 1990, Murata et al. 1992, Takaki et al. 1991, Takaki et al. 1993, Tavernier et al. 1991). The βc is not specific for IL-5 and is shared with the receptors for IL-3 and granulocyte-macrophage colony stimulating factor (GM-CSF) (Miyajima et al. 1992). JAK1 and JAK2 are constitutively associated with βc and IL-5Rα respectively and phosphorylated upon IL-5 stimulation (Ogata et al. 1998). IL-5 can activate STAT5 mediated by JAK2 and to a lesser extent JAK1 (Fig3.1) (Ogata et al. 1998, Kouro et al. 1996). Additionally, STAT1 has also been shown to be activated by IL-5 through JAK2 in eosinophils (van der Bruggen et al. 1995, Pazdrak, Stafford & Alam 1995).

In the current study, we found that the stimulation required to induce IL-10 production from human peripheral blood B cells differed from murine B cells. CD40L stimulation was sufficient for IL-10 production but, IL-5 stimulation had no effect. Lastly, we elucidated crucial signaling pathways downstream of CD40L and IL-5 stimulation that may be necessary for IL-10 production and growth of regulatory B cells.

## 3.3 Methods

### 3.3.1 Mice

All experiments, unless otherwise specified, were performed using 8-12 week old BALB/cJ (Strain #000651) mice obtained from Jackson Labs (Bar Harbor, ME). All mice were housed in specific pathogen-free facilities. Animal protocols and husbandry practices were approved by the Institutional Animal Care and Use Committee of the University of Michigan.

### 3.3.2 Mouse B Cell Culture on mCD40L-Fibroblasts

NIH3T3 fibroblasts stably transduced with the gene for mouse CD40 ligand (mCD40L-Fb) were generously provided by Dr. David Fox and Dr. Kevin McDonagh, and a detailed description of their generation has been previously published (Morita et al. 2005). Fibroblast lines were maintained in DMEM/10% calf serum and grown to 80-90% confluence before removal from culture flasks with 0.25% Trypsin solution. Prior to coculture with B cells, fibroblast lines were exposed to 30 Gy of gamma radiation to inhibit their proliferation. Purified mouse B cells were obtained from spleens of naive mice by anti-mouse CD19 MACS magnetic bead positive selection (Miltenyi Biotec, Auburn, CA) using the manufacturer's protocol. Purified B cells and mCD40L-Fb fibroblasts were washed and resuspended in B cell culture medium containing 1×DMEM (Hyclone, SH30243.01), 10% heat-inactivated FCS, 5% Penicillin-Streptomycin, and 0.5% Insulin/Transferrin/Selenium (Gibco, 51500-056). B cells and fibroblasts were plated at a 4:1 ratio in flat-bottom plates. Recombinant murine cytokine IL-5 (Peprotech) was added to the cultures at 50ng/mL. Control cultures were performed for all assays that are presented using non-transduced NIH3T3 fibroblasts, none of which yielded viable B cells after five days.

### 3.3.3 Human B Cell Isolation and Culture

Peripheral blood mononuclear cells (PBMC) were obtained from de-identified donors to the American Red Cross-Detroit Chapter who had passed health screening for plasma donation. Plasmapheresis filters were washed in reverse with 30cc serum-free 1×DMEM to collect cells, which were then layered on 20cc Ficoll/Histopaque and centrifuged at 1200×g for 30 minutes at room temperature. Buffy coats were collected, and PBMC were then washed 2 times in magnetic bead separation buffer (1×PBS/2mM EDTA/0.5% bovine serum albumin/0.01% sodium azide) and counted prior to positive selection with anti- human CD19 MACS bead (Miltenyi Biotec) separation using the manufacturer's protocol. Purified human B cells were resuspended in B cell culture medium (same as mouse media) and plated at a 4:1 ratio with irradiated (30 Gy) human CD40L-transduced NIH3T3 fibroblasts (hCD40L-Fb, a kind gift from Dr. Lee Nadler) Recombinant human IL-4 (Peprotech) was added at a final concentration of 50 ng/mL in all assays.

### 3.3.4 ELISA

IL-10 and IL-6 proteins were assessed in the supernatant by sandwich ELISA kits purchased from BD Biosciences using the manufacturer's protocols.

Intracellular Fas Ligand was measured in cell lysates by sandwich ELISA kits purchased from R&D Systems, using the manufacturer's protocol. Cells from culture were counted and lysed at the same concentrations using RIPA Buffer and proteinase inhibitor cocktail (1:100). Cell debris was spun out and cell free supernatant was used for ELISA.

### 3.3.5 Inhibitors

Inhibitors listed in Table 1 were added at the beginning of each culture at the indicated published concentrations. DMSO was the vehicle control.

## 3.4 Results

### 3.4.1 IL-4 inhibits CD40L induced production of IL-10 from human B cells.

Human B cells are known to secrete IL-10 in response to CD40 ligation. We have shown this stimulation also induces IL-10 production from murine $CD5^+$ B1a cells and addition of IL-4 inhibits IL-10 production. We next wanted to determine if IL-4 inhibited IL-10 production from human CD40L stimulated B cells. Human peripheral blood B cells were cultured with CD40 ligand expressing fibroblasts (HuCD40L-Fb) in the presence or absence of IL-4. Supernatants were collected from these cultures every day for six days to assess the kinetics of cytokine production. Levels of IL-10 and IL-6 were measured by ELISA at each of the time points. CD40L stimulation gradually increased IL-10 production every day over the 6 day culture (Fig 3.3A). When IL-4 was added to the culture, CD40L induced IL-10 production was inhibited (Fig 3.3A). CD40L and IL-4 stimulated B cells produced significantly higher levels of IL-6 by 24 hours compared to CD40L stimulation alone (Fig 3.3A). Inhibition of CD40L stimulated IL-10 production in the presence of IL-4 was consistently found between individuals (Fig 3.3B) as was IL-4 stimulated IL-6 production (Fig 3.3B). While in the murine studies the addition of IL-5 enhanced IL-10 production and cell numbers, IL-5 had no effect on CD40L induced IL-10 production from human B cells (data not shown) (Fig3.2). Thus, similar to results found with murine B cells, CD40L stimulation induced IL-10 secretion from human peripheral blood B cells and was blocked in the presence of IL-4.

### 3.4.2 Btk and NFAT may be important signaling pathways for human regulatory B cell growth and function

To determine important signaling pathways downstream of CD40L stimulation, inhibitors of multiple signaling molecules were included in the 5 day cultures of human B cells and

CD40L-Fb. After 5 days, cell counts were done to assess effects on B cell growth and culture supernatants were tested by ELISA for levels of IL-10. Table 3.1 lists the inhibitors, concentrations used, and their targets. Inhibitors against Btk, multiple JAK proteins, STAT5, and nuclear factor of activated T cells (NFAT) were used. NFAT has been shown to be upstream of IL-10 production from B cells and therefore was included although it is not specifically reported to be downstream of CD40 stimulation (Matsumoto et al. 2011). Of the inhibitors used, only ibrutinib (Btk) and Cyclosporin A (NFAT) reduced both cell counts after culture and levels of IL-10 production (Fig 3.4 A,B). Previously, our lab found that stimulation of murine B cells with CD40L and IL-5 increased their Fas Ligand (FasL) expression. To determine if any of these signaling proteins were involved in modulation of FasL expression in human B cells, cells were lysed after culture. Intracelluar FasL levels were tested; however, there were no significant differences (Fig 3.4C). In conclusion, CD40 ligation may activate Btk and NFAT to induce growth and IL-10 production from human B cells.

### 3.4.3 Signaling pathways involved in murine regulatory B cell growth and function

Similarly, murine splenic B cells were stimulated with CD40L and IL-5 (Fig 3.2) with and without inhibitors to investigate crucial signaling pathways for regulatory B cell growth and function. Inhibitors used and their cellular targets are listed in Table 3.1. Cell counts after culture suggested that all targeted signaling proteins are important for growth of B cells in this culture system (Fig3.5A,B). These include Btk, STAT5, and JAK1/2. Reduction of cell counts by JAK1-3 and TYK2 inhibition by tofacitinib and by pan JAK inhibitors were likely due to JAK1/2 inhibition as similar cell counts were found with specific inhibitors for JAK1/2. Levels of IL-10 secreted in the cultures were significantly reduced when activity of Btk, NFAT, or JAK was inhibited (Fig 3.5C, D). Interestingly, inhibition of JAK2 and STAT5 signaling significantly

increased IL-10 production in the culture (Fig 3.5C). Btk, JAK, NFAT signaling may be important for regulatory B cell growth and function. Additionally, JAK2 and STAT5 signaling may negatively regulate IL-10 production.

### 3.5 Discussion

The inhibitor data in this study are preliminary and will need to be further optimized to address short comings. Toxicity may play a role in the differences in IL-10 production. Optimization of dose and exposure time will be necessary to develop a non-toxic treatment while maintaining inhibitory activity. Since regulatory B cells make up a small portion of B cells in mice and humans, sorting the populations of interest before treatment is necessary to rule out effects due simply to the presence of other B cells. Additionally, these results will need to be validated through other techniques to confirm that differences were not due to off target affects of the inhibitors.

Previously, we have reported that CD40L stimulation induces IL-10 production from splenic $CD5^+$ murine B cells, which was inhibited in the presence of IL-4. Here we confirmed this antagonistic affect of IL-4 on human CD40L stimulated B cells, validating IL-4 and its subsequent signaling as potential targets for regulatory B cell modulation. Similarly, understanding signaling pathways crucial for induction of IL-10 production by B cells may also identify therapeutic targets.

Inhibitors for various signaling molecules downstream of CD40 stimulation were used to identify important pathways for B cell growth and IL-10 production. In both mice and humans the Bruton's tyrosine kinase (Btk) inhibitor, ibrutinib, significantly reduced B cell numbers and IL-10 production in our *in vitro* cultures. Ibrutinib is an irreversible Btk inhibitor that is currently used for the treatment of B cell malignancies (Advani et al. 2013, Herman et al. 2014). Btk is

downstream of B cell receptor (BCR) signaling which B cell malignancies are highly dependent on. Ibrutinib treatment results in high response rates and prolonged progression free survival (Byrd et al. 2015). In the treatment of malignant disease, short term response rates for preservation of life are of greater priority than long term effects on B cells. However it is important to understand these consequences when considering additional indications for this treatment.

Affects of Ibrutinib on B cells may lead to long term affects on host immunity and self tolerance. Patients treated with Ibrutinib have impaired replenishment of normal B cells likely due to the importance of Btk in B cell development in the bone marrow and periphery. X-linked agammaglobulinemia (XLA) patients have a mutated Btk (Tsukada et al. 2012). B cell development in these patients is arrested at the pre-B cell stage because they are unable to signal through the BCR, resulting in very low numbers of B cells in circulation and near absence of serum antibodies (Tsukada et al. 2012, Hendriks, Middendorp 2004, Vetrie et al. 2012). These patients have recurrent infections due to their lack of humoral immunity. Xid mice with a Btk loss of function mutation have a milder phenotype that reduces survival of immature transitional B cells in the periphery (Petro et al. 2002, Levine et al. 2000). B-1a cells are also highly dependent on Btk signaling and are absent in Xid mice (Khan et al. 1995). B-1a cell IgM and IL-10 production may play an important role in maintaining self tolerance by augmenting removal of apoptotic cells and debris to limit the potential for immune activation towards self (Rothstein et al. 2013). The importance of Btk for B cell development of B1 and B2 cells suggests that long term affects of Ibrutinib treatment could lead to B cell deficiencies, limited humoral responses, and emergence of autoimmunity. As evidence to this, patients treated with Ibrutinib have a high rate of serious adverse infectious events (Byrd et al. 2014). Long term studies have not been

done to assess risk of autoimmunity. While XLA patients and Xid mice do not have a high risk of spontaneous autoimmunity, death of cancerous B cells likely accompanying this treatment may increase this risk (Howard et al. 2006, Mason et al. 2017). In patients battling aggressive malignancies, these long term concerns are not priority however; recently there has been interest in utilizing Ibrutinib in allergic disease.

Btk is also a crucial signaling molecule for mast cell degranulation (Iyer et al. 2011). Ibrutinib treatment has been shown to inhibit mast cell and basophil activation in humans (Advani et al. 2013, MacGlashan et al. 2011, Chang et al. 2011). Treatment with Ibrutinib could suppress both life threatening anaphylaxis and other mast cell mediated diseases. Mast cells are long lived therefore, a lower concentration or few doses of ibrutinib may be sufficient for mast cell inhibition while possibly leaving the B cell compartment largely untouched. However, our studies suggest that this treatment could potentially lead to worse pathologies such as infectious disease and autoimmunity. Further dose optimization and investigation into long term effects of Btk inhibition are necessary before additional indications can be considered.

Regulatory B cells are known to play a protective role in autoimmunity and in the development of tolerance after transplantation (Mauri, Menon 2015). High numbers of IL-10 producing regulatory B cells correlates with better disease control in both autoimmunity and transplantation (Mauri, Bosma 2012). Cyclosporin A is an immunosuppressant that has been widely used in the treatment of autoimmunity and prevention of graft rejection. Cyclosporin A treatment has been shown by us and others to negatively affect regulatory B cell numbers and IL-10 production *in vitro* (Matsumoto et al. 2011). Additionally, Cyclosporin A treatment in non transplant and transplant patients significantly reduced regulatory B cell numbers and IL-10 production (Tebbe et al. 2016). The irony that treatment for autoimmunity and transplant

rejection inhibits regulatory cells that could contribute to tolerance, illustrates the necessity of expanding investigations to include regulatory cell populations.

Intracellular levels of FasL in human B cells were determined after culture with and without inhibitors and no differences were observed. However, it is unclear if stimulation with CD40L affects FasL levels compared to unstimulated human B cells. FasL is stably stored intracellularly and only made *de novo* following release of stores (Lettau et al. 2008). Therefore, this experimental set up may not induce release of intracellular FasL stores and *de novo* expression. Additionally, the signaling pathways inhibited may simply not be involved in FasL modulation resulting in no differences compared to DMSO or untreated control.

In our studies with murine B cells stimulated with CD40L and IL-5, inhibition of JAK2 and STAT5 increased IL-10 production although cell numbers were reduced. Since these cultures were done with total splenic B cells and not sorted $CD5^+$ B cells, the IL-10 producers, effects observed with inhibition of these signaling proteins may be confounded by the presence of the non IL-10 producing cells. To this affect, in a single experiment with sorted $CD5^+$ B cells, JAK2 and STAT5 inhibition did not reduce growth or enhance IL-10 production (data not shown) suggesting effects of JAK2 and STAT5 inhibition in the total splenic cultures were not due to modulation of regulatory B cells themselves. Interestingly, this also suggests that murine regulatory $CD5^+$ B cells may signal IL-5 in a unique pathway compared to other B cells (Takatsu 1998). Investigating this signaling pathway could identify therapeutic targets inherently specific for regulatory B cells.

Regulatory B cells are now recognized as important immune modulators that can contribute to maintaining and inducing tolerance. Ibrutinib and Cyclosporin A are two examples of currently used therapeutics that negatively affect regulatory B cell growth and function.

Further investigation into the affects of therapeutics on regulatory B cells is necessary moving forward as we continue to elucidate the roles of these B cells in multiple disease contexts. Understanding how currently approved therapeutics affect regulatory B cells will lead to better understanding of their biology and, possibly new indications to modulate regulatory B cells.

Further studies are necessary to confirm the findings reported here. In addition to optimizing the inhibitor concentrations, we would also use silencing RNAs to knockdown expression of the signaling molecules of interest to confirm their role in the induction of IL-10 production. These signaling molecules would be knocked down preceding stimulation to induce IL-10 production. While knockdown studies have been done in B cells, maintaining viable B cells without introducing stimulations that would affect their IL-10 production would be difficult. In order to eliminate this issue, using B cells from mice deficient in certain signaling molecules may be more feasible to interrogate their role in the induction of IL-10 production. B1 cells are highly dependent on IL-5 and mice that are IL-5R$^{-/-}$ have reduced numbers of B1 cells (Yoshida et al. 1996). In light of this, using B cells from deficient mice would only be feasible to interrogate signaling pathways downstream of CD40L but not IL-5.

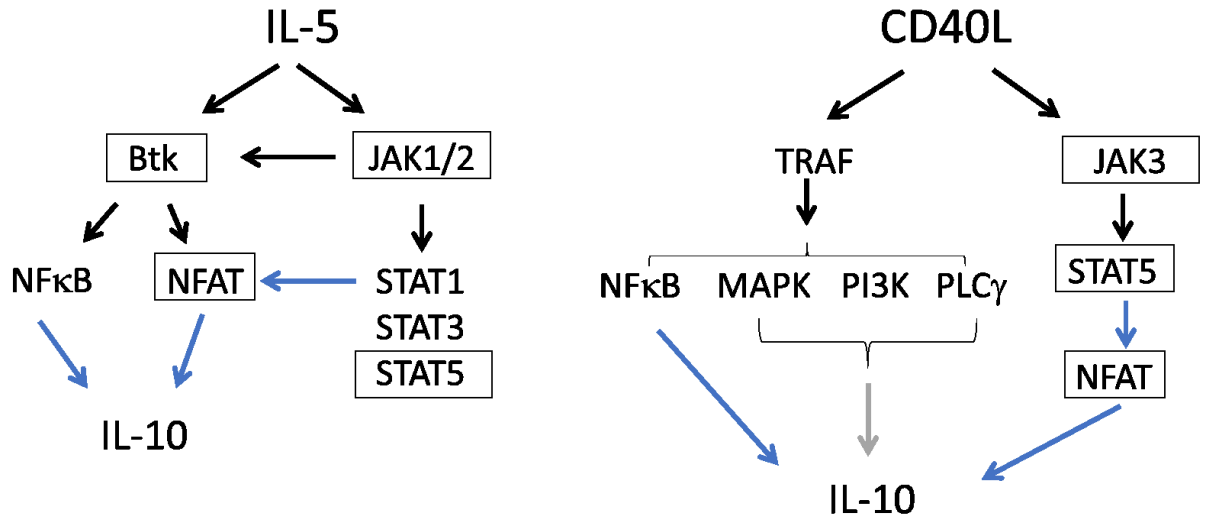



**Figure 3. 1 Signaling pathways downstream of IL-5 and CD40L stimulation**

Signaling downstream of IL-5 and CD40L stimulation are summarized above. The black arrows indicate known pathways. The blue arrows indicate pathways that are known but, not described downstream of the indicated stimulation (IL-5 or CD40L). The gray arrow indicates a potential pathway that has not been described. The boxes indicate signaling proteins that were inhibited in this study.

Btk, Bruton's tyrosine kinase,

TRAF, tumor necrosis factor receptor associated factors

MAPK, mitogen activated protein kinases

PI3K, phosphoinositide 3-kinase

PLCg, phopholipase Cg

JAK, Janus family kinase

STAT, signal transducer and activator of transcription

NFAT, nuclear factor of activated T cells

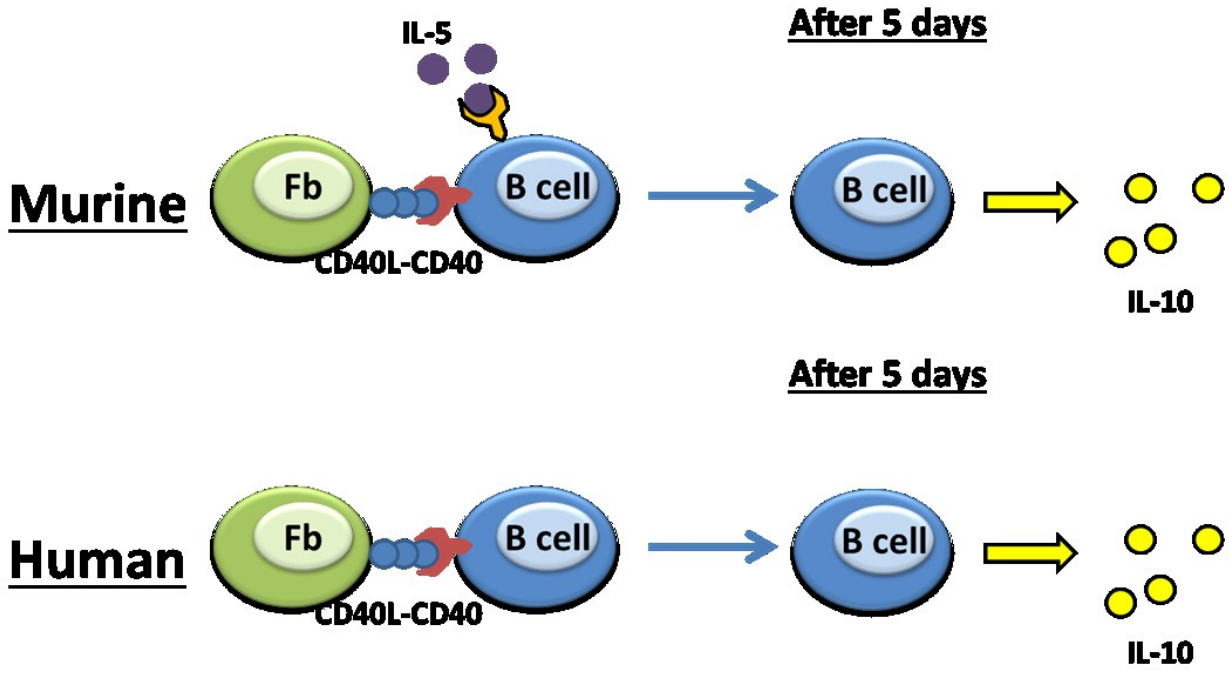



**Figure 3. 2 Differences in IL-10 stimulation for murine and human B cells**

Murine B cells were cultured for 5 days with CD40L expressing fibroblasts in the presence of IL-5 to induce detectable levels of IL-10 secretion. Human B cells only need 5 days of CD40L stimulation to induce IL-10 production. The addition of IL-5 to human B cell cultures did not enhance IL-10 production. Inhibitors were added at the beginning of these cultures to assess effects on IL-10 production, growth, and FasL expression.

| Inhibitor | Target | Concentration | Reference |
|---|---|---|---|
| **Ibrutinib** | **Btk** | **1uM** | (Dubovsky et al. 2014) |
| **Tofacitinib** | **JAK1,3,2 and TYK2** | **100nM** | (Wang et al. 2014b) |
| **JAK2 inhibitor** | **JAK2** | **100nM** | (Wang et al. 2014b) |
| **Ruxolitinib** | **JAK1/2** | **100nM** | (Appelmann et al. 2015) |
| **PAN JAK inhibitor** | **JAK1,3,2 and TYK2** | **100nM** | (Appelmann et al. 2015) |
| **STAT5 inhibitor** | **STAT5** | **50uM** | (Schafranek et al. 2015) |
| **Cyclosporin A** | **NFAT** | **250ng/ml** | (Han et al. 2010) |

**Table 3. 1 Inhibitors used to investigate signaling pathways downstream of CD40L & IL-5.**

Inhibitors used in the murine and human studies are listed in this table along with their targets and the published concentrations used.

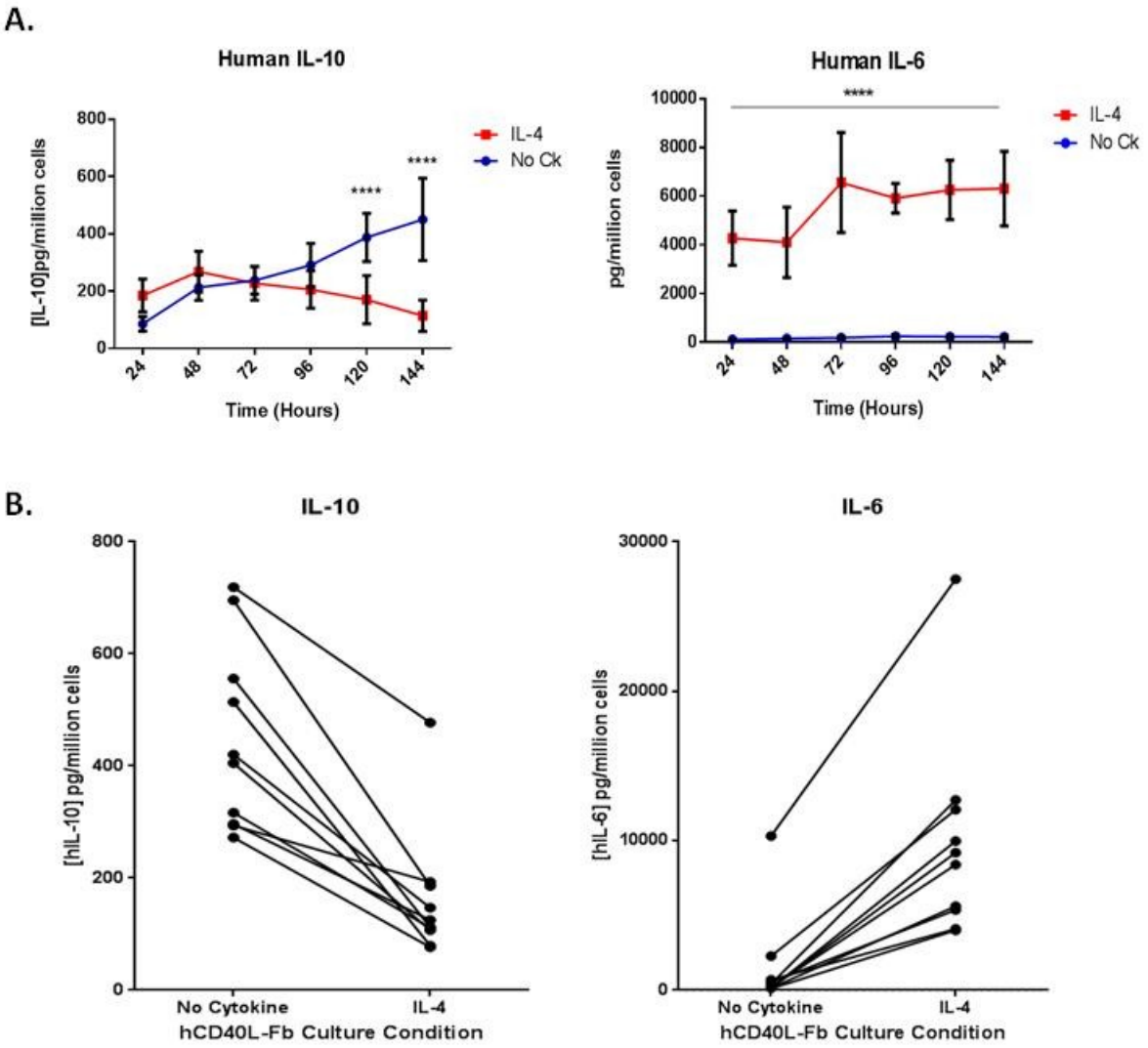



**Figure 3. 3 Human B cell IL-10 and IL-6 production are consistently differentially regulated by IL-4**

$CD19^+$ B cells were purified from the peripheral blood of healthy human donors were cultured for 6 days with irradiated hCD40L-Fb ± 50 ng/mL rhIL-4. (A) Supernatant was collected every 24 hours to determine the levels of IL-10 and IL-6 production in the using ELISA. Error bars are mean $\pm$ SD Supernatants were collected from triplicate wells and analyzed for the presence of IL-10 and IL-6 by ELISA. (B) Supernatants were collected on day 6 for ELISA. Each symbol represents the mean cytokine concentration for a single subject with the diagonal lines connecting the values with or without IL-4 for each individual.

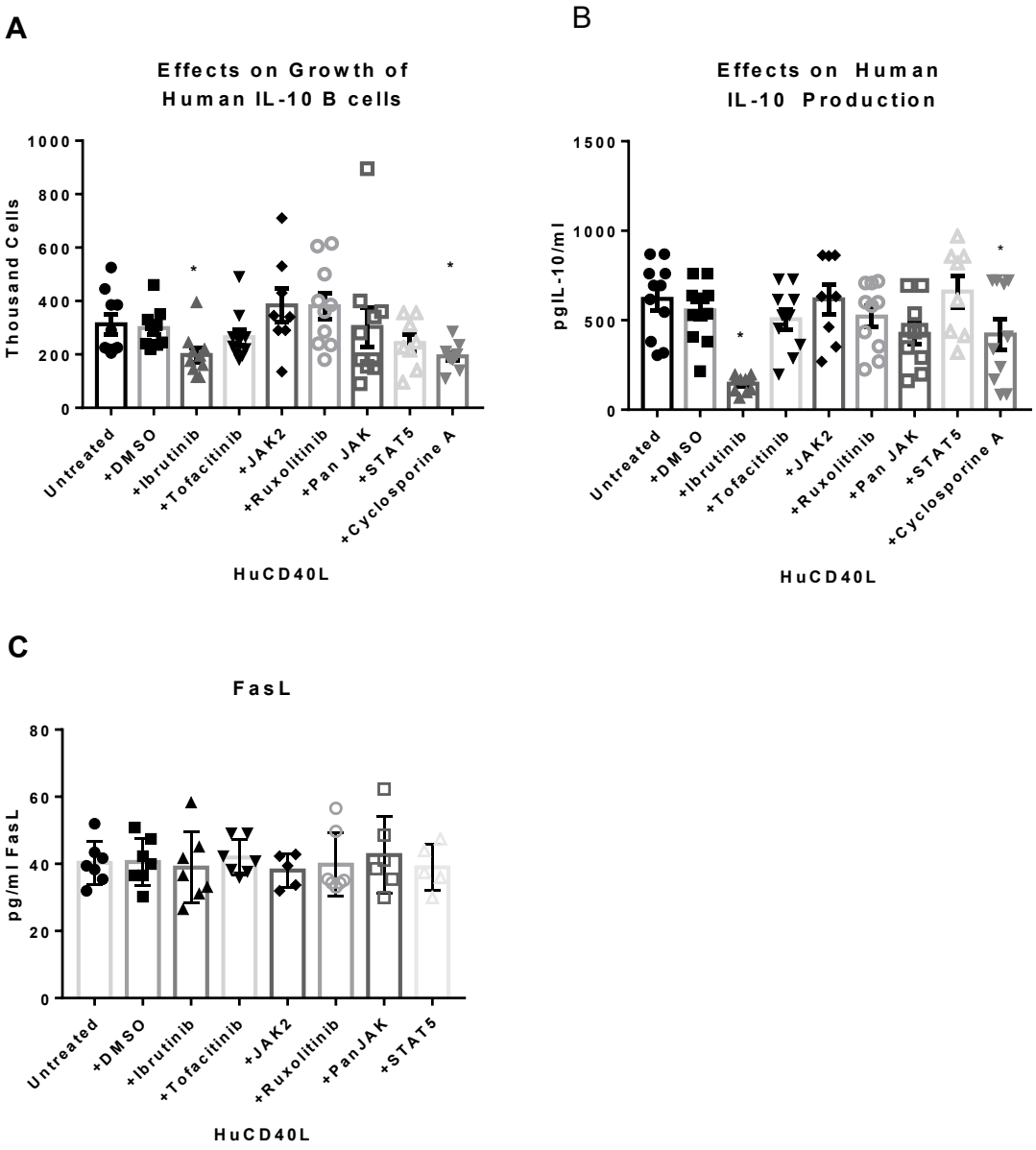



**Figure 3. 4 Crucial pathways in CD40L stimulation of human regulatory B cells**

Filters from the American Red Cross were used to obtain human B lymphocytes from healthy individuals. B cells were isolated using Ficoll Histopaque 1077 and magnetic bead separation. The B cells were cultured with HuCD40L transfected NIH3T3 fibroblasts. Inhibitors were added at the beginning of the culture. After 5 days the cells were counted **(A)** and the supernatant was collect for detection of IL-10 by ELISA **(B)**. **(C)** Cells were lysed after counting for detection of intracellular FasL. Data is displayed as standard error of the mean and significance was determined by two tailed ttest compared to the untreated control. Each point is a single person.

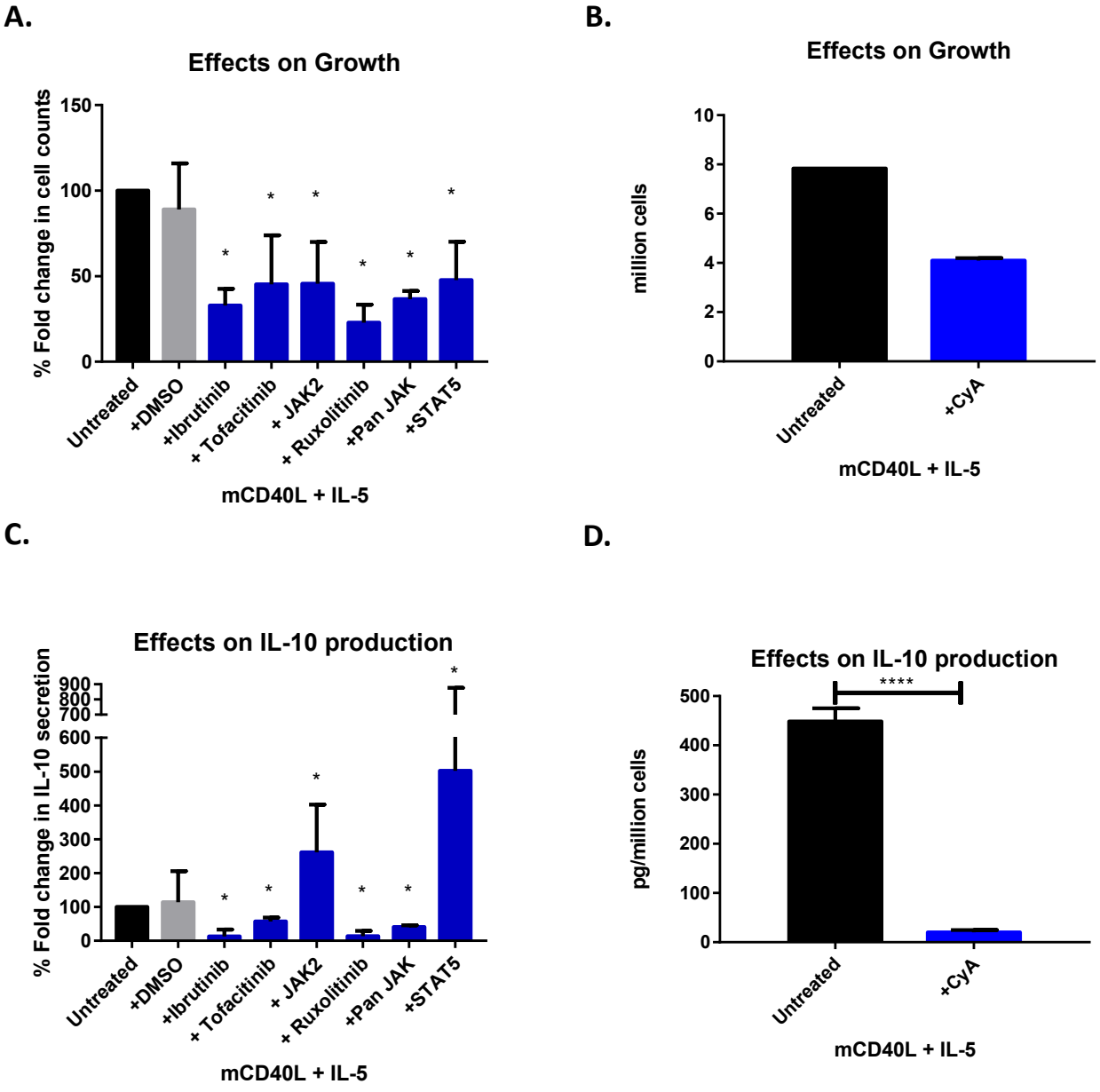



**Figure 3. 5 Crucial pathways in CD40L/IL-5 stimulation of murine regulatory B cells**

Splenic B cells were isolated from Balb/C mice by magnetic separation and cultured with mCD40L and IL-5, inhibitors were added at the start of the culture After 5 days the cells were counted **(A and B)** and supernatant was collect for detection of pgIL-10/ml by ELISA **(C and D)** All graphs were normalized to the untreated control expressed as percent fold change. Data is mean+ SEM and statistical significance was determined by two tailed ttest. n=3 or a single experiment.

# CHAPTER 4

## Discussion

Little is known about the effects of $T_H2$ cytokines on regulatory B cells. In multiple studies of patients with allergic asthma and in other allergic diseases, IL-10 producing regulatory B cells are reduced compared to healthy individuals (van der Vlugt et al. 2014, Kamekura et al. 2015, Noh et al. 2010, Lee et al. 2011). These studies suggest that during allergic inflammation regulatory B cell expansion and function may be limited by the cytokine milieu. Cytokines associated with allergic $T_H2$ responses include IL-4, IL-5, and IL-13. Limited investigation into the effects of these cytokines on regulatory B cells has been published. This dissertation aimed to understand how regulatory B cells may be functionally and numerically reduced during allergic responses and whether $T_H2$ cytokines could influence this deficiency.

We described a novel stimulation for expansion of a regulatory B cell subset proven to be suppressive and protective in an allergic airway disease model. The work presented here builds on a previous finding from the lab that IL-5 and CD40L stimulation of B cells leads to elevated levels of IL-10 detected in culture supernatants (Klinker et al. 2013). It was further determined that CD40L stimulation induced IL-10 production from $CD5^+$ B-1a cells and found IL-5 may be more important as a growth factor. In addition to stimuli that enhanced regulatory function, IL-4 negatively regulated IL-10 production and its enhancement by IL-5. Adoptive transfer of IL-5

and CD40L activated B cells proved protective in a mouse model of allergic airway disease dependent on their production of IL-10.

Our results define a novel antagonistic role for IL-4 in regulatory B cell biology. In our study, we investigated the effects of IL-4 on IL-10 production from two of the most utilized stimulations, CD40L and LPS. IL-4 inhibitory effects on IL-10 were recapitulated in both culture systems, giving greater merit to its possible influence *in vivo*. The effects of IL-4 on regulatory B cell expansion and function implicate this $T_H2$ cytokine in the deficiency of regulatory B cells in allergic diseases (van der Vlugt et al. 2014, Kamekura et al. 2015, Noh et al. 2010, Lee et al. 2011). From these findings we suggest that during allergic inflammation, regulatory B cells expansion and function may be limited due to the presence of IL-4 allowing for continuation of allergic responses instead of the induction of tolerance. Our results provide a rationale for targeting IL-4 in the treatment of asthma and other $T_H2$ mediated diseases. Targeting IL-4 may reduce $T_H2$ inflammation while supporting regulatory B cell growth and function due to the presence of IL-5 and sources of CD40L during $T_H2$ response. Regulatory B cells can suppress multiple T helper responses, $T_H1$, $T_H2$, and $T_H17$, found in asthmatic patients (Wesolowska-Andersen, Seibold 2015). While blockade of $T_H2$ cytokines would potentially only benefit $T_H2$ subsets of patients, enhancement of regulatory B cells that suppress multiple T helper subtypes may allow for a more comprehensive therapy.

### 4.1 Understanding the influence of IL-4 on regulatory B cells

Recently dupilumab, a humanized mAb against the IL-4Rα chain, a shared component of both the IL-4 and IL-13 receptor, has been approved for treatment of atopic dermatitis. Dupilumab has also shown great promise in clinical trials for treatment of asthma, reducing

exacerbations, asthma symptoms, and exhaled nitric oxide while increasing $FEV_1$ and lung function (Walsh 2017). In contrast to subset specific protection afforded by antibodies targeting IL-5 and IL-5Rα, dupilumab was effective in all subgroups of patients with moderate to severe asthma. The broad protection throughout subsets of patients seen with dupilumab treatment could potentially be due to enhancement of regulatory B cells in the absence of IL-4 signaling. Treatment of allergic patients with dupilumab, the IL-4Rα blocking antibody, affords a unique opportunity to investigate the effects of blocking IL-4 signaling on regulatory B cell populations in humans. By assessing regulatory B cells frequencies and IL-10 production before and after treatment with dupilumab, we could determine the effectiveness of IL-4 as a target for regulatory B cell modulation. We hypothesize that dupilumab treatment would lead to enhanced frequencies and IL-10 production from B cells, supporting our *in vitro* studies. In these studies, we would be limited to B cells present in the blood and in the broncoalveolar lavage fluid. However, it is unclear whether changes in regulatory B cells in these locations will reflect B cells that would contribute to limiting allergic inflammation.

To address whether dupilumab could enhance regulatory B cells within the lungs, which may not be reflected in the BAL or blood of the patients, we could utilize our mouse model of AAD and treat with mouse IL-4Rα blocking antibody. Using an IL-10 reporter mouse, we could easily determine if this treatment enhances IL-10 producing B cells within the lungs. Additionally, a comparison of the frequency of regulatory B cells in the blood, BAL, and lungs could be made to assess the relevance of changes seen in these locations and protection.

Lastly, studies of patients with atopic dermatitis, an inflammatory skin disease, would allow direct access to the site of inflammation. B1 cells are present in the skin therefore, skin biopsies of atopic dermatitis patients would allow assessment of dupilumab's effects on

regulatory B cells at the site of inflammation (Geherin et al. 2016). Additionally, these studies would also allow a comparison between changes in regulatory B cell frequencies in the blood and the site of inflammation. These studies could establish the value of IL-4 as a therapeutic target for regulatory B cell modulation and elucidate protective mechanisms of dupilumab treatment.

Along these lines, polymorphisms in the IL-4Rα gene, some of which can result in enhanced and/or sustained signaling, are associated with the development of atopic disease in humans (Tachdjian et al. 2009, Stephenson et al. 2004, Ford et al. 2009). We hypothesize that IL-4Rα polymorphisms that enhance and/or prolong IL-4 signaling may lead to reduced IL-10 production from human B cells conferring increased susceptibility to developing allergic diseases. It would be interesting to determine whether production of IL-10 from human B1 cells are affected by these polymorphisms. Human B1 cells can spontaneously produce IL-10 *ex vivo* and can be further stimulated to enhance IL-10 production. This allows investigation into the differences in homeostatic and stimulated regulatory B cell activity between polymorphisms. Lastly, these polymorphisms can result in varying strengths of IL-4 signaling (Wallis et al. 2011). In chapter 3, we found that IL-4 consistantly reduced IL-10 production and there were differences in the degree of reduction between individuals. We suggest the strength of signaling associated with different polymorphisms may also be associated with the degree of IL-10 inhibition in our B cell cultures. Understanding how IL-4Rα polymorphisms affect regulatory B cell function could give insight into the mechanism behind its association with allergic disease and autoimmunity (Tachdjian et al. 2009, Wallis et al. 2011).

Finally, while we have shown IL-4 negatively affects IL-10 production from B cells *in vitro* confirmation *in vivo* is necessary. By transferring BALB/c or STAT6$^{-/-}$ B cells into our

AAD model, we expect STAT6$^{-/-}$ B cells IL-10 production not be limit by the presence of IL-4 providing enhanced protection. To determine if IL-4 affects IL-10 production from endogenous B cells, irradiated mice could be reconstituted with BALB/c bone marrow and BALB/c or STAT6$^{-/-}$ x IL10 reporter B1 progenitors. Following induction of AAD, B cells from the lungs, BAL, and MLN could be assessed by flow cytometry to investigate differences in the frequency of IL-10$^{+}$ B1 cells. We expect more STAT6$^{-/-}$ B cells would be secreting IL-10 compared to WT since they would not be inhibited by IL-4.

## 4.2 Potential influence of IL-5 and IL-4 in B-1a cell functional dichotomy

### 4.2.1 Functional Dichotomy of B-1a cells

B-1a cells play important roles in both host defense and maintenance of tolerance. B-1a are primarily known for their production of natural antibodies that act as one of the body's first defenses against infection (Briles et al. 1981). However, recent studies suggest an additional housekeeping role for B-1a cells. In addition to specificity for microbes, B1 cells BCRs have self-reactivity and potentially allergen-reactivity (Shaw et al. 2003). For example, they recognize phosphorylcholine on apoptotic cell membranes, oxidized lipids, and phosphatidylcholine on senescent red blood cell membranes (Pennell et al. 1989b, Hardy et al. 1989). B-1a cell recognition of self-antigens may support tolerance by accelerating elimination of apoptotic cells and debris by increasing phagocytosis and limiting phagocyte activation (Chen et al. 2009). In support of this concept, mice that lack secreted natural IgM are prone to development of autoimmunity (Boes et al. 2000). Additionally, natural IgM also disposes of pathogenic lipids such as oxidized low density lipoproteins, protecting against the development of atherosclerosis (Soto et al. 2009, Kyaw et al. 2011). Beyond their self-reactivity, B-1a cells are also potent antigen presenting cells, comparable to dendritic cells, and are able to interact with and influence

T cell activation (Mohan et al. 1998, Sato et al. 2004, Zhong et al. 2007a). Lastly, as has been covered in this dissertation, B-1a cells are able to produce anti-inflammatory cytokines and limit disease severity. The auto-reactivity, antigen presentation abilities, and regulatory functions position B-1a cells as a potentially crucial cell type in maintaining immune tolerance.

### 4.2.2 ILC2 cells may direct B-1a cell functional dichotomy

Insight into the regulation of B-1a cells lie in their homeostatic niche. B1 cells present in the fluid phase of the serous cavities are homeostatically maintained in fat-associated lymphoid clusters (FALCs) located in the peritoneal, pleural, and pericardial cavities and in milk spots in the omentum (Cruz-Migoni, Caamano 2016, Meza-Perez, Randall 2017, Moro et al. 2010, Benezech et al. 2015). These structures are rich in IL-33 produced by the stromal cells which can stimulate group 2 innate lymphoid cells (ILC2) and enhance their homeostatic production of IL-5 (Nussbaum et al. 2013, Halim et al. 2012). ILC2s are lineage negative lymphocytes that constitutively produce IL-5 and can produce high levels of $T_H2$ cytokines (IL-5, IL-13, and IL-4) upon activation. ILC2s are the dominant source of homeostatic IL-5 in multiple tissues including the lung, intestine, skin, uterus, heart, brain, and kidney but, do not produce IL-4 at homeostasis (Nussbaum et al. 2013). IL-5 is important for B-1a cell IgM secretion, homeostatic self-renewal, and proliferation (Moon et al. 2004, Moro et al. 2010, Jackson-Jones et al. 2016). Upon stimulation, B-1a cells are recruited to FALCs by CXCL13 and this migration is required for natural antibody secretion and body cavity immunity (Ansel, Harris & Cyster 2002). The FALCs bring B-1a cells and ILC2s together however, the importance of their interaction for activation of B-1a functional dichotomy is still uncertain (Jackson-Jones et al. 2016). However, ILC2s have been shown to interact with B1 cells. *In vitro*, ILC2s from the lungs of naïve mice induced proliferation of and IgM secretion from B1 cells dependent on ILC2 derived IL-5 (Drake et al.

2016). Additionally, *in vivo* ILC2s enhanced antigen specific IgM secretion from B-1 cells in response to the T independent antigen NP-Ficoll. ILC2s secretion of IL-5 and IL-4 after activation and their enhancement of B-1 cell IgM secretion suggests ILC2s may play a role in directing activation of B-1 cells for both immunity and tolerance.

The migration of B-1 cells to the FALCs after stimulation suggests the signals received in the FALC will steer their response (Ha et al. 2006). ILC2 constitutively produce IL-5 which is enhanced in response to IL-33 (Nussbaum et al. 2013). Activation of ILC2s requires additional stimulation beyond IL-33, such as IL-2, to induce proliferation and secretion of IL-13 and IL-4 (Mirchandani et al. 2014, Oliphant et al. 2014, Klein Wolterink et al. 2012, Wilhelm et al. 2011, KleinJan et al. 2014). Changes in cytokine production of ILC2, from purely IL-5 to IL-5 and IL-4 after activation, may be a central determinant of whether B-1 cells respond to antigen in a proinflammatory or tolerogenic fashion. From our studies we found that IL-5 supported IL-10 production while IL-4 blocked IL-10 production from B-1a cell induced by LPS or CD40L. We hypothesize that during homeostasis, in the absence of immune activation, B-1 cells that are stimulated by innocuous antigen home to the FALC and encounter inactive ILC2s that produce IL-5 but no IL-4. Additionally, they could also encounter inactive tissue resident ILC2. Stimulation by inactive ILC2s could lead to increased IL-10 production and IgM secretion from B-1a cells supporting a tolerogenic response instead of proinflammatory immune activation. In support of this, B-1a cells from the spleen and peritoneum produce IL-10 in response to apoptotic cells (AC) *in vitro* and are the major IL-$10^+$ B cell *in vivo* after AC injection (Miles et al. 2018). In AC injected mice, only IL-$10^+$ B cells secreted IgM with specificities similar to those found in the serum. IgM from IL-$10^+$ B cells preferentially bound AC compared to IL-$10^-$ B cells, enhancing phagocytosis by bone marrow derived dendritic cells and blocking their TNF-

α production. Additionally, B-1a cells stimulated with AC induced IL-$10^{+}CD4^{+}$ T cells *in vitro* (Miles et al. 2018). In response to antigen stimulation in the absence of immune activation, B-1a cells are constitutively producing IL-10 and secreting IgM. Interactions with inactive IL-5 producing ILC2s likely supports expansion of the stimulated B-1a cells and supports their housekeeping role.

If immune activation accompanies B-1a cell antigen stimulation, ILC2s may induce a proinflammatory response from the B-1a cells. Immune activation can provide the additional signals, such as IL-2, for activation of ILC2s and their production of IL-4. We have shown IL-4 can negatively affect IL-10 production from B-1a cells but, other studies have shown that it can enhance IgM secretion. IL-4 inhibition of IL-10 production and enhancement of IgM secretion from B-1a cells would allow for better host defense and immune activation. Upon infection, activated B-1a cells will migrate to the spleen and secrete increased amounts of IgM into circulation during the early stages of infection to limit the risk of sepsis (Jackson-Jones, Benezech 2017). During infection B-1a cells can also become innate response activators that migrate to the spleen and produce GM-CSF in an autocrine loop to enhance their IgM secretion (Rauch et al. 2012). These innate response activators also migrate into the lung parenchyma during pneumonia and secrete IgM (Weber et al. 2014). During respiratory tract infection with influenza, B-1a cells migrate to the lymph nodes, spleen, and mediastinal and pericardial FALCs to secrete protective IgM (Jackson-Jones, Benezech 2017, Waffarn et al. 2015, Yang et al. 2007). From these studies we suggest that there are differences in the response of B-1a cells depending on the activation status of ILC2s. Immune activation may induce IL-4 secretion by ILC2s and lead to enhanced IgM secretion, inhibition of IL-10 secretion, and production of proinflammatory cytokines from B-1a cells augmenting their role in host defense.

### 4.2.3 NKT cells may influence activation ILC2s

Natural killer (NK) T cells may also influence B-1a cell activity indirectly through their effects on ILC2s. NKT cells during the recovery phase of influenza infection were found to produce IL-33 and induce IL-5 production from ILC2s (Gorski, Hahn & Braciale 2013). ILC2 can contribute directly to tissue repair and culmination of viral responses, however, we suggest that this stimulation may also result in activation of tolerigenic B-1a cell functions (Monticelli et al. 2011). Secretion of IgM and IL-10 by B-1a cells during the recovery phase could contribute to the return to homeostasis and prevent activation against self through elimination of dead and damaged cells and suppression of phagocyte activation (Chen et al. 2009). Conversely, NKT cells are also involved in inflammation induced formation of new FALCs. Following inflammation, FALC formation was found to be dependent on NKT cell activation and partially on production of IL-4 (Benezech et al. 2015). Involvement of NKT cells in FALC formation and their production of IL-4 would position them to influence B-1a cell activation towards a proinflammatory response.

In conclusion, we suggest a model in which NKT cells may also be involved in stimulating ILC2 cytokine production that influence inflammatory and tolerigenic activation of B-1a cells (Fig 5.1). Further investigation into B-1a cell biology is necessary to understand their dichotomous roles in immunity and the central cellular and molecular determinants that control it.

Lastly, *in vitro* co-cultures could be used to assess the ability of ILC2s to steer responses of B1a cells. Culturing B-1a cells with activated or inactive ILC2 and apoptotic cells, we could assess differences in IgM secretion and IL-10 production from the B cells. Activated ILC2 cells can produce IL-4 in addition to IL-5 while inactive ILC2 only produce IL-5. Using blocking

antibodies for IL-4 and IL-5 we could determine the effects of these cytokines on IgM and IL-10 production from the B cells. The interaction between these cells types could be also assessed *in vivo* by apoptotic cell or bacterial injection to induce these interactions. *In vivo* imaging of FALCs or milky spots could be used to visualize interactions between ILC2 and B cells and subsequent production of IL-10. Transgenic mice allowing for fluorescent labeling of ILC2, B cells, and IL-10 would be necessary to visualize these interactions live. Through these studies we could determine if ILC2 cells may be involved in modulating B cell functions dependent on their cytokine production.

Regulatory B cells are now well appreciated immunomodulatory cells. Interest in these cells has exploded in the last two decades, leading to their presence and regulatory roles being reported in countless human diseases and their accompanying murine models. Our work has identified potential targets for regulatory B cell modulation and a theory on the regulation of B-1 cells by ILC2s and NKT cells. This interaction may prove to be central in controlling immune responses elicited by B cells to evoke a protection against infection or maintenance of peripheral tolerance. While many questions still remain, there have been significant advancements in the understanding of regulatory B cell biology and their importance in human health. The field of regulatory B cells is still full of questions and avenues of pursuit, promising exciting discoveries and potential therapies.

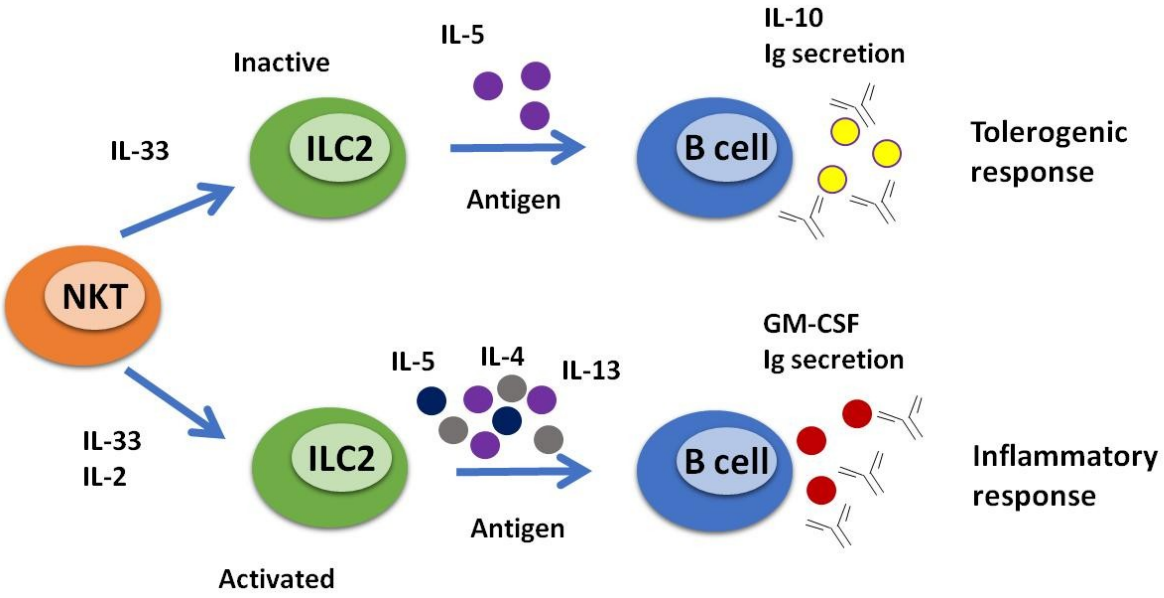



**Figure 4.1 Model of IL-4 and IL-5 regulation of B-1a cells**

We suggest a model in which depending on the activation status of the NKT cell they can secrete IL-33 stimulating IL-5 production from ILC2 but not activation. B-1a cells that encounter the IL-5 and antigen are induced to secrete IL-10 and Ig contributing to a tolerogenic response that may help limit responses to self antigens. Activated NKT cells could secrete costimulatory cytokines such as IL-2 in addition to IL-33 leading to activation of ILC2 and their secretion of IL-5, IL-4, and IL-13. Stimulation of B-1a cells with antigen in the presence of IL-4 may lead to secretion of proinflammatory cytokines and Ig, contributing to an inflammatory response necessary for host defense. These interactions may occur in the FALC and in the tissue as NKT cells, ILC2, and B-1a cells can all be found in both location.

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