Killer B Cells: Regulation of the Growth and Function of Fas Ligand-Expressing B Lymphocytes

by

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Dedication

To my mother, whose selflessness and determination still inspire.

And to Marianne, with my love and gratitude.

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Abstract

The immune system is a highly-specialized cellular network that must respond to a wide variety of microbial species while remaining tolerant of an organism's own cells and tissues. Loss of self-tolerance can lead to immune-mediated tissue destruction and disease, and therefore regulatory mechanisms have evolved that inhibit immune responses to self-antigens. The work presented in this dissertation sought to better understand the biology of B cells that express the death-inducing molecule Fas ligand (FasL), a potentially important regulatory B cell population. Conceptually, $FasL^+$ "killer" B cells are unique amongst regulatory B cells as they possess the potential for suppression that is both antigen-specific and permanent.

The experiments herein identified a novel antagonistic relationship between the type-2 cytokines interleukin-5 (IL-5) and IL-4 regarding their effects on $FasL^+$ B cell function. Treating murine B cells with IL-5 expanded a population of B cells with potent killing activity against $CD4^+$ T cells and that secreted the anti-inflammatory cytokine IL-10. In contrast, treatment with IL-4 inhibited both of these regulatory mechanisms in B cells. Therefore, drugs that activate pathways downstream of the IL-5 receptor or inhibit those downstream of the IL-4 receptor may lead to novel areas of drug discovery for the treatment of immune-mediated disorders.

Although $FasL^+$ B cells are rare *in vivo*, lymphoblastoid cell lines (LCLs) generated by transformation of human B cells with Epstein-Barr virus (EBV) showed robust expression of

intracellular FasL, suggesting that the EBV latency program in transformed B cells drives the production of FasL. LCLs also secreted $MHCII^{+}FasL^{+}$ exosomes that induced antigen specific apoptosis in $CD4^{+}$ T cells in two independent experimental designs. LCLs consequently represent a realistic source for immuosuppressive exosomes for therapeutic use in the treatment of human disease. This study has therefore set the groundwork for future investigations that may one day lead to powerful and novel therapeutic strategies.

Chapter 1

Introduction

1.1 The Adaptive Immune System and Self-Tolerance

Multicellular organisms exist in an environment teeming with microbial life, and therefore must possess the means to efficiently manage both harmful and innocuous microbes to maintain homeostasis. To accomplish this task, two central arms of the mammalian immune system have evolved that use distinct but complementary strategies to distinguish between "self" and "non-self."

The *innate* arm of the immune system, common to all vertebrates, recognizes molecular determinants essential to broad classes of microbes. These pathogen-associated molecular patterns (PAMPs) are recognized by germline-encoded receptors, allowing the innate arm to respond to microbes in a generic manner by recognizing common PAMPs, such as viral dsRNA or lipopolysaccharides of the bacterial outer membrane. Although the innate immune system

is efficient and effective, the exceptional variety and short life cycle of most microbial species tips the evolutionary arms race in their favor.

In jawed vertebrates, a second arm of the immune system has evolved which acts in concert with the innate arm [1]. Rather than relying on the recognition of PAMPs by invariant innate receptors, this *adaptive* arm generates a highly-diversified anticipatory repertoire of adaptive immune receptors in lymphocytes, the main effector cells of the adaptive arm. In response to infection, lymphocytes bearing receptors that recognize antigens specific to the invading pathogen clonally expand into a pool of effector cells. Upon resolution of the infection, some lymphocytes become long-lived memory cells capable of responding rapidly to a secondary infection from the same pathogen. This strategy employed by the adaptive arm allows an organism to respond to an incredibly large array of antigens, and theoretically results in a precisely-focused immune response targeted only to pathogenic microbes rather than self-tissues or commensal species.

As the lymphocyte repertoire is vast and randomly-generated, however, it is all-but-inevitable that lymphocytes recognizing self-antigens will arise in a given organism. Some estimates suggest that more than half of all antigen receptors generated *in vivo* display some level of reactivity with self-antigens [2]. Lymphocytes are therefore rigorously curated, and auto-reactive cells are deleted, anergized, or signaled to differentiate into immunosuppressive

regulatory lymphocytes (Figure 1-1). The end result of this selection process is an adaptive repertoire that is highly-diverse, but also *self-tolerant* – a state in which any aberrant immune response toward self-tissues is prevented from occurring or kept in check by regulatory mechanisms. Despite these processes, loss of self-tolerance occurs in a small but substantial number of people. Disorders thought to result from such breakdowns in self-tolerance are known as *autoimmune diseases*. The clinical manifestations of autoimmunity can vary greatly depending upon the self-antigens targeted, and although autoimmune diseases are individually rare, they collectively afflict more than 10 million people (1 in 31) in the United States [3]. The pathology associated with autoimmune diseases can be debilitating, painful, and life-threatening, and hence these diseases are the source of a great deal of human suffering.

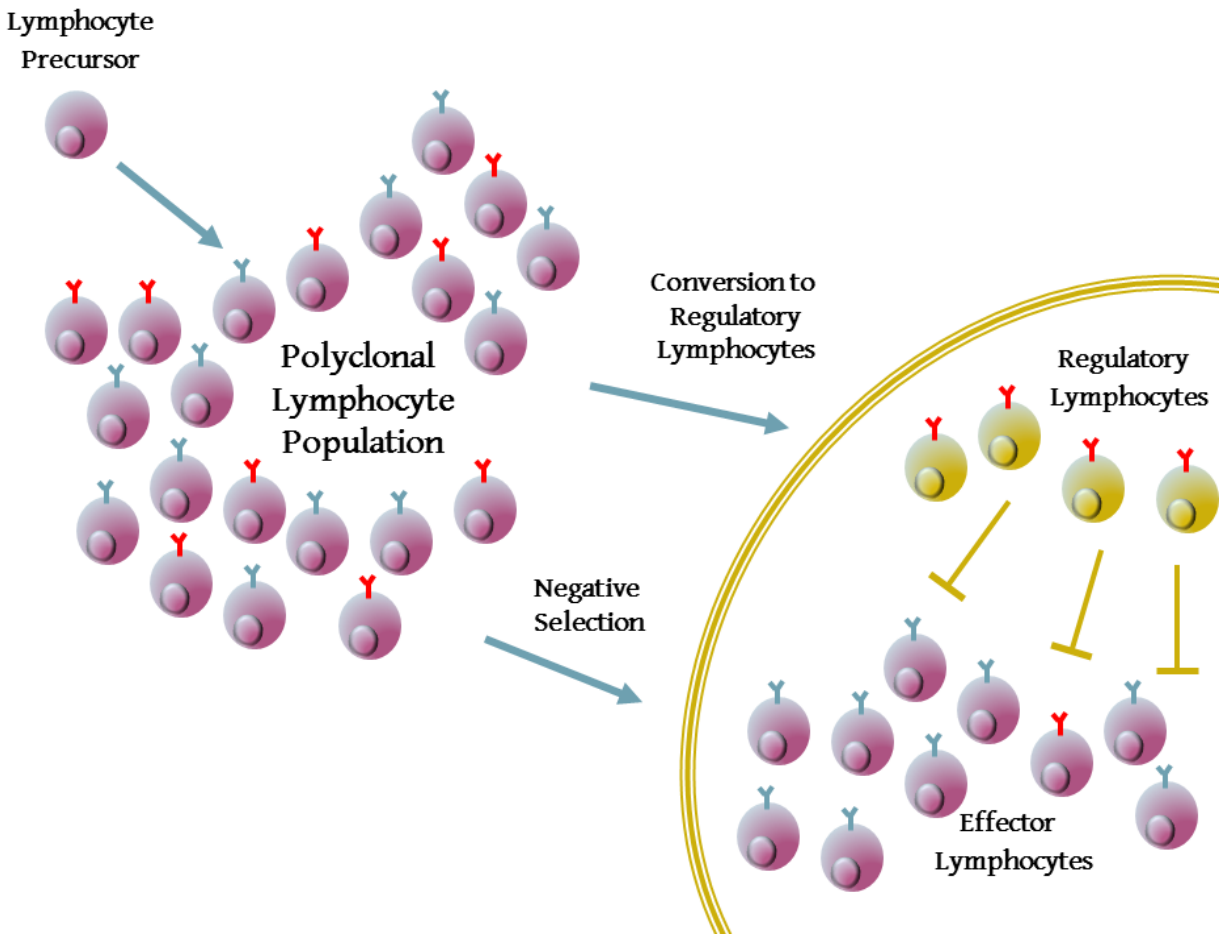

Figure 1-1: Curation of the lymphocyte repertoire.
Random rearrangement and mutation at the genetic loci encoding antigen receptors produces the highly-diverse anticipatory repertoire of lymphocytes, allowing the adaptive immune system to respond to essentially any antigen encountered. The random nature of rearrangement results in the production of self-reactive lymphocytes, which are either eliminated or inactivated through negative selection, or undergo differentiation to anti-inflammatory "regulatory" lymphocytes.

Therapies currently available for the treatment of autoimmune diseases act by general immune suppression and therefore affect protective as well as pathogenic immune responses. Side effects resulting from these broadly-immunosuppressive drugs include increased susceptibility to microbial infections and reduced cancer surveillance [4, 5]. Immunosuppressive drugs are expensive as well, and because most autoimmune diseases are chronic disorders requiring indefinite treatment, the expense of treating autoimmunity has become a large financial burden on the healthcare systems worldwide [6, 7]. There is therefore a great need for the development of therapeutic strategies for the treatment of autoimmunity superior to those currently available. A better understanding of how self-tolerance is maintained could lead to next-generation therapies that act by permanently suppressing responses specific to autoantigens, therefore leaving protective immune responses intact and eliminating the need for indefinite treatment.

1.2 B Lymphocytes

As the main effector cells of the humoral immune response, B cells are essential for host defense [8]. The defining feature common to all B cells is the expression of immunoglobulin, a glycoprotein complex comprised of two heavy chains (encoded by the IgH locus) and two light chains (encoded by either the Igκ or Igλ loci). Immunoglobulins contain two structural

regions: a divalent antigen binding region, and a constant region that mediates effector functions. B cells express immunoglobulins either as a membrane-bound component of the B cell receptor (BCR), or as secreted antibodies. The antigen-binding regions of immunoglobulins are encoded by numerous variable (V), diversity (D), and joining (J) segments separated by substantial genomic distances within the immunoglobulin loci. To produce a functional immunoglobulin, these segments are brought together by V(D)J recombination, a process whereby the intervening sequences are excised from the chromosome. As individual V, D, and J segments are selected stochastically and mutations from the germline sequence are frequently introduced during chromosome repair, this process generates extensive variability within the antigen-binding region. The result is an extremely diverse antibody repertoire, estimated in humans to be 10^{11} or greater [9].

B cells perform several essential immune functions beyond antibody production as well. Most cells of the B cell lineage are professional antigen presenting cells (APCs), capable of processing and presenting exogenous antigens to $CD4^+$ T cells via MHC class II (MHCII) molecules [10]. B cells preferentially activate T cells that share their antigen specificity, as BCR-mediated endocytosis allows them to efficiently process and present their cognate antigen to T cells [11, 12]. Through the secretion of polarizing cytokines, B cells can also affect the differentiation of T cells that they activate [13]. Therefore, B cells can directly influence T

cell-mediated immunity. Additionally, B cells can secrete an array of cytokines with local and systemic effects, and are essential for the proper development of lymphoid structures and mucosal barriers [14-16]. Because of these varied functions, B cells represent a unique and important functional nexus of the immune system and can influence nearly all aspects of an immune response.

Extensive heterogeneity exists within the B cell population, and various subsets of B cells have been defined based upon the correlation of surface markers with specific functions or developmental stages. Delineating B cells into subsets remains a controversial topic despite decades of study, as few subsets show absolute correlation with a given surface phenotype [17-19]. Despite this caveat, defining B cell subsets by this approach has proven useful in the study of nearly all aspects of B cell biology, and can provide important information regarding the lineage, stage of development, and activation status of a given B cell.

Two main B cell lineages have been described, each with distinct functions and phenotypes [20, 21]. *B-2 cells*, the most widely-studied lineage, are responsible for the precisely-targeted high affinity antibodies that arise against pathogen-associated antigens during an immune response. A second subset of *innate-like B cells* produces lower affinity poly-specific antibodies spontaneously or in response to stimuli associated with inflammation [22].

B-2 cells

One of the most remarkable features of the immune system is its ability to mount an antibody response against essentially any protein antigen, as well as other types of molecules. This feat is made possible by the highly-diversified anticipatory antibody repertoire generated by B-2 cells. V(D)J recombination occurs early in B-2 cell development and generates extensive junctional diversity within the immunoglobulin repertoire. This diversity is further enhanced by terminal deoxynucleotidyl transferase (TdT), an enzyme that adds random non-template nucleotides to DNA segments at the junction sites [23]. B-2 cells possessing a functional BCR after recombination are then screened for autoreactivity, and B-2 cells recognizing self-antigens either undergo apoptosis, become anergic, or initiate receptor editing to produce an antibody that is not self-reactive [24].

These early stages of B-2 cell development and selection occur in the bone marrow, where the cells of the B-2 lineage emerge from a pluripotent precursor population common to T cells and natural killer (NK) cells [25]. Immature B-2 cells then egress from the bone marrow into peripheral lymphoid organs via the blood stream [26]. B-2 cells are continuously produced throughout the life of an organism, as newly-differentiated cells from the bone marrow constantly replace dying B-2 cells in the periphery.

B-2 cells constitute the vast majority of B cells in peripheral lymphoid organs, where they undergo further maturation through several transitional stages [27]. Within these organs, B-2 cells reside mostly in the lymphocyte-dense follicles where they are supported by a network of follicular dendritic cells (FDC). The FDC network helps to capture and concentrate antigens from draining lymph or blood [28]. The follicle therefore represents a unique anatomical niche that increases interaction between B-2 cells and potential antigens.

Upon encountering their cognate antigen, B-2 cells within the follicle become activated and proliferate; this growing region of proliferating antigen-specific B-2 cells grows into a dynamic structure known as a germinal center [29]. Reciprocal interaction with $CD4^+$ T helper (T_H) cells occurs within germinal centers, as B-2 cells present antigen to follicular T cells which in turn provide survival and activation signals to B-2 cells. While germinal centers are initially pauciclonal, the repertoire within the germinal center is diversified by somatic hypermutation of the immunoglobulin loci [30]. Among hypermutated B-2 cells, those with higher affinity BCRs are more efficient at processing and presenting antigen, and therefore have an advantage in soliciting help from follicular T cells. As the germinal center reaction progresses, this selective pressure produces a pool of B-2 cells producing antibodies with progressively higher affinities [31]. Thus, the germinal center provides an environment which drives the emergence of B-2 cells producing antibodies with very high affinity for the cognate antigen. Cytokines

within the follicular microenvironment signal B-2 cells to undergo isotype switching, making B-2 cell-derived antibodies functionally diverse [32].

While the fate of most B-2 cells is to undergo apoptosis as the germinal center recedes, some germinal center B-2 cells will differentiate further. Plasma cells leave the germinal center and move to the bone marrow or the lamina propria of mucosa, where they secrete large amounts of high-affinity antibody. Some plasma cells can persist for long periods of time, providing continuous levels of protective antibodies. Other germinal center B-2 cells differentiate into memory B cells, which maintain surface immunoglobulin expression while secreting relatively small amounts of antibody. Memory B cells are long-lived and respond rapidly to future encounters from the same pathogen [33].

Innate-like B cells

According to the layered immune system hypothesis, cell populations arise evolutionarily and ontologically in order of increasing complexity [34]. Innate-like B cells, a subset comprised of B-1 cells and marginal zone (MZ) B cells, are thought to be the more primitive of the B cell layers and maintain some features of an ancestral B cell population [22]. Although their antibody response lacks much of the complexity of that of B-2 cells, innate-like B cells serve important non-redundant functions in host defense.

The first functional B cells to emerge during development, innate-like B cells arise before birth from a precursor population that emigrates from the bone marrow to the fetal liver [35]. Whereas the B-2 cell population requires constant replenishment, innate-like B cells develop early in life and persist into adulthood by self-renewal, with little replacement from the bone marrow [36]. Antibodies produced by innate-like B cells are secreted in the absence of antigenic stimulation and are therefore commonly referred to as "natural" antibodies. Production of natural antibodies begins before birth and is constitutive, and therefore natural antibodies provide a baseline level of antibody-mediated protection even in the absence of infection [37]. Innate-like B cells are found almost exclusively at sentinel locations within the body, with B-1 cells concentrating in tissues associated with mucosal barriers, and MZ B cells monitoring the contents of the blood stream in the marginal zone of the spleen. In these locations, innate-like B cells act as a "front-line" defense against pathogens, as they can rapidly respond to an infection by increasing their antibody production and releasing inflammatory cytokines. These functions help to limit microbial growth in the early stages of an infection before a full adaptive immune response can be generated.

Whereas the antibody repertoire of B-2 cells is vast, that of innate-like B cells is restricted and semi-invariant. Although the immunoglobulin loci of innate-like B cells undergo recombination, the recombined sequences are mostly unaltered from those found in the

germline and are restricted in their V_H gene usage. Antibodies secreted by innate-like B cells are frequently poly-reactive, binding many structurally-unrelated antigens with low affinity. This broad specificity makes natural antibodies effective in defense from a wide range of bacterial and viral pathogens [38-40]. Although MZ B cells play a role in shuttling antigens from the blood to follicles in the spleen, it is thought that innate-like B cells themselves do not participate in germinal center reactions nor undergo somatic hypermutation or affinity maturation [41, 42]. Therefore, while the repertoire of B-2 cells adapts in real time to produce antibodies against a specific pathogen, that of innate-like B cells is far less dynamic and has been selected over evolutionary time scales for broad specificity.

In addition to their broad reactivity with pathogen-associated antigens, natural antibodies frequently bind self-antigens as well. Prominent among the self-antigens bound by natural antibodies are apoptosis-associated determinants and oxidized lipids. These natural autoantibodies are thought to be adaptive rather than a consequence of incomplete negative selection, as innate-like B cells undergo a positive selection event based upon their reactivity with self-antigens [43, 44]. The function of these self-reactive natural antibodies will be discussed in detail later.

1.3 B cell Functions in the Pathogenesis of Autoimmune Disease

Autoimmune diseases are frequently associated with aberrant responses from both the humoral and cellular compartments, suggesting that both B cells and T cells play important roles in these diseases. For a variety of reasons, however, considerably more resources have gone toward the study of T cells in autoimmunity, especially in regard to so-called "T cell-mediated" diseases such as rheumatoid arthritis (RA), type 1 diabetes (T1D), and multiple sclerosis (MS). The incidence of many autoimmune diseases is strongly linked with specific human leukocyte antigen (HLA) class II haplotypes, suggesting that the nature of the $CD4^+$ T cell receptor repertoire is critical for the initiation of autoimmunity. In several animal disease models, adoptive transfer of activated antigen-specific T cells is sufficient to induce autoimmunity in recipient animals, and T cells are frequently found in inflamed organs such the synovium of RA patients [45-47]. T cells were therefore thought to be central to autoimmune pathogenesis, with B cells playing a secondary or indirect role in disease.

Over the past two decades, however, strong evidence has accumulated demonstrating that B cells are crucial players in the pathogenesis of many autoimmune disorders as well. In mice, the absence or depletion of B cells is protective in $CD4^+$ T cell-mediated autoimmune disease [48]. Production of the cytokine TNFα is a key contributor to inflammation in RA, and IgG immune complexes can drive TNFα production by binding to activating Fc receptors on

monocytes [49, 50]. These observations prompted clinical studies assessing the efficacy of B cell-depletion therapy in the treatment of autoimmunity in humans. The first such therapeutic to achieve approval was rituximab, a chimeric anti-CD20 antibody initially developed to treat B cell-derived lymphomas [51, 52]. Rituximab has achieved clinical success in the treatment of RA and some forms of vasculitis [51-55]. The efficacy of rituximab inspired a surge in research focusing on B cells in autoimmunity, and several other drugs targeting B cell functions have recently achieved approval or are the subject of ongoing clinical trials. The results of these studies have led to a greater appreciation for the complex role B cells have in autoimmunity, and it is now recognized that targeting the function of B cells can be a powerful therapeutic strategy.

Autoantibodies in Autoimmune Diseases

A hallmark feature of many autoimmune disorders is the presence of antibodies that bind to self-antigens. Autoantibodies against a wide variety of self-antigens have been described, including those targeting soluble proteins, intracellular and nuclear antigens, cell surface molecules, and components of the extra cellular matrix. The clinical effects of autoantibodies are generally a product of both the specific self-antigen recognized by the autoantibody, and its isotype.

Antibodies against self-antigens can mediate pathology by targeting an immune response toward self-antigens, often with devastating consequences. During a normal immune response, antibodies serve to identify invading pathogens and promote inflammation at the site of infection. Clearing the pathogen removes any antigen, and therefore antibodies no longer accumulate at the site of infection and inflammation subsides. Antibodies against self-antigens accumulate in locations with an abundance of self-antigen, leading to chronic inflammation and subsequent tissue damage. Autoantibodies are most commonly found as either IgM or IgG isotypes, both of which are capable of fixing complement [56]. Products of the complement cascade stimulate the release of pro-inflammatory cytokines and leukotrienes in local immune cells, and promote the recruitment of circulating effector cells. Autoantibodies also promote inflammation by binding to activating Fc receptors, as IgG autoantibodies binding to Fc receptors induces secretion of pro-inflammatory cytokines [49, 57]. Injection of autoantibodies is sufficient to induce onset of disease in some mouse models of autoimmunity, demonstrating that autoantibodies are capable of initiating immune-mediated tissue damage [58-60]. This ability of autoantibodies to induce inflammation is essential in the pathogenesis of collagen-induced arthritis, as mice lacking activating Fcγ receptors or the complement component C5 are resistant to disease despite high levels of circulating autoantibodies [61, 62].

Autoantibodies reactive against soluble self-antigens result in the formation of immune complexes in circulation. In the case of an abundant self-antigen, the rate of immune complex formation can overwhelm existing clearance mechanisms, leading to chronically high immune complex levels. Immune complexes are more likely to deposit in certain locations of the body and therefore accumulate in places such as small blood vessels and joints. This local buildup promotes inflammation, and the subsequent recruitment and activation of immune cells can result in substantial tissue damage. This mechanism is especially important in the pathogenesis of systemic lupus erythematosus (SLE), where immune complexes tend to accumulate in the blood vessels within glomeruli [63]. Over time, tissue damage resulting from this deposition can result in renal failure and death.

Complement-fixing autoantibodies recognizing cell surface antigens can promote the deposition of the membrane-attack complex on targeted cells. While most cells are resistant to lysis mediated by the membrane-attack complex, others can be depleted by this mechanism. Examples of such disorders include autoimmune thrombocytopenia and hemolytic anemia, diseases which are mediated by autoantibodies recognizing surface structures on platelets and erythrocytes, respectively [64, 65].

In some autoimmune disorders, autoantibodies mediate pathology independently of inflammation by binding to and modulating the activity of their target molecule. In Grave's

disease, autoantibodies activate the thyroid-stimulating hormone (TSH) receptor [66]. As a consequence, thyroid hormones are produced in excess, resulting in hyperthyroidism. Autoantibodies that inactivate acetylcholine receptors are thought to be responsible for development of myasthenia gravis, a potentially-fatal neuromuscular disorder [67]. In some patients with RA, autoantibodies that bind to and activate peptidylarginine deiminases (PADs) can be found [68]. PADs produce citrullinated proteins, a critical autoantigen in RA, and therefore PAD-activating autoantibodies may help to drive inflammation in RA by generating citrullinated autoantigens [68]. Disorders caused by antibodies against cytokines such as IFNγ, IL-6, and members of the IL-17 family have been identified as well, frequently resulting in impaired immune defense against specific types of microbes [69-71].

Antibody-Independent B cell Functions in Autoimmunity

There is strong evidence that the role of B cells in autoimmune disease extends beyond the production of autoantibodies. Rituximab, the most frequently-used B cell depletion therapy, does not deplete antibody-secreting plasma cells [72]. While a significant decline in autoantibody levels occurs after administration of rituximab, this decrease does not necessarily correlate with response to treatment in RA patients, and clinical improvement frequently precedes this reduction in autoantibody levels [73-75]. Additionally, although rituximab treatment is effective in treating MS, a drug targeting both B cells and plasma cells

exacerbated rather than alleviated symptoms in MS patients [76-78]. In a mouse model of SLE, mice with B cells incapable of secreting antibody still develop symptoms of nephritis [79]. These observations suggest that antibody-independent B cell functions are crucial in the propagation of autoimmune disease.

The reciprocal interaction between T cells and B cells is central to most adaptive immune responses, and as a consequence each is capable of modulating the activity of the other. B cells use BCR-mediated endocytosis to process and present their cognate antigen to $CD4^+$ T cells, preferentially activating T cells that share their specificity. An autoreactive B cell can therefore process and present self-antigens to T cells, thus driving autoimmunity in the cellular compartment. Importantly, although B cells recognize a specific epitope of a given antigen, the entirety of the antigen is endocytosed and processed for antigen presentation. As a result, B cells recognizing self-antigens present multiple epitopes to T cells, triggering diverse T cell responses to a single self-antigen [80]. Known as "epitope spreading," this phenomenon allows an autoimmune response to spread to targets other than the initial epitope as disease progresses [81]. This progressive diversification of the response to self-antigens is a feature common to many autoimmune diseases and usually correlates with increased severity of symptoms [82]. In some cases, epitope spreading can occur between two molecules if antigens are physically linked [83, 84].

B cells can also influence a cellular immune response by the secretion of T cell polarizing cytokines. In experimental autoimmune encephalomyelitis (EAE), B cells were found to be the major producer of IL-6, a cytokine important for the function of T_H17 cells [85]. Additionally, while B cell depletion effectively treated disease in wild-type mice, depletion was ineffective in mice lacking IL-6 expression in B cells, suggesting that the therapeutic effect of B cell depletion was mediated by the loss of IL-6-expressing B cells [85]. Data from human subjects support this idea, as B cells from human patients with MS produced more IL-6 than healthy controls as well [85]. Remarkably, treatment of these patients with rituximab essentially "reset" their peripheral B cells, as those returning after depletion secreted IL-6 at levels equivalent to those of healthy controls [85]. The loss of IL-6-expressing B cells in both mice and human patients was associated with a reduction in T_H17 activity, suggesting that IL-6-expressing B cells are capable of inducing pathogenic T_H17 responses [85]. B cells capable of specifically supporting the production of either T_H1 or T_H2 cells have been identified as well, named B effector 1 (Be1) and B effector 2 (Be2) cells, respectively [13]. While these effector B cell subsets can skew a T cell response by secretion of polarizing cytokines, the importance of these cell types in autoimmunity has not thus far been evaluated.

B cells are frequently found at sites of autoimmune inflammation, such as the synovium of RA patients, the central nervous system of patients with MS, and the thyroid of some patients

with autoimmune thyroid disease [86-88]. In some cases, highly-organized lymphoid-like structures resembling follicles and germinal centers can be identified at these sites. In RA, the presence of ectopic germinal centers is associated with higher expression of inflammatory cytokines [89]. While there is some evidence that B cells within these ectopic germinal centers can undergo affinity maturation, their role in the production of high-affinity autoantibodies is still controversial [90]. The presence of B cells in the synovium, however, appears to be crucial to maintaining T cell activation, as the depletion of B cells significantly reduced T cell production of inflammatory cytokines in human synovium/severe combined immunodeficiency (SCID) mouse chimeras [91]. Synovial B cells have been reported to express a wide variety of inflammatory cytokines as well, including TNFα, IL-1α, IL-6, IL-12, and RANKL [92]. B cells therefore have multiple crucial roles in maintaining inflammation at sites of autoimmune attack.

1.4 Regulatory B cell Functions in Inflammatory Conditions

Although evidence for the existence of anti-inflammatory "regulatory" B cells was first reported almost 40 years ago, their immunosuppressive function has received significant attention only in the last 15 years [93]. Despite this, there is now clear evidence that B cells can modulate disease in many mouse models of autoimmune diseases, including MS [94, 95],

chronic intestinal inflammation [96], T1D [97, 98], SLE [99, 100], and RA [101]. B cells can mediate immune suppression through a variety of mechanisms, some of which are unique to B cells [102]. The major mechanisms of B cell-mediated immune suppression are discussed below.

Sialylated IgG

Despite the clear role of IgG in pathogen clearance, it has been known for decades that high doses of intravenous immunoglobulin (IVIg) therapy has an anti-inflammatory effect, and IVIg is now commonly administered to patients with inflammatory disorders such as idiopathic thrombocytopenic purpura [103, 104]. The high dose required for anti-inflammatory effects, however, suggested that only a minor fraction of IgG molecules possessed anti-inflammatory activity, and indeed a minor glycoform of IgG with terminal sialic acid additions has been identified that mediates anti-inflammatory effects at much lower doses [105]. Removing sialic acid residues from IVIg preparations severely reduced their immunosuppressive function, suggesting that sialylated IgG is the anti-inflammatory component of IVIg [105].

A family of Fcγ receptors are expressed on cells of both the innate and adaptive immune system that modulate the activity of a cell in response to binding IgG [106]. Both activating and inhibitory receptors exist within the Fcγ receptor family, and their relative levels of expression determine the activation threshold for a given cell [106]. The Fc portion of IgG

contains a single biantennary N-linked glycan that is required for binding Fcγ receptors, and whereas all IgG molecules appear to have a core heptasaccharide moiety comprised of N-acetylglucosamine (GlcNAc) and mannose at this site, more than 30 alternative glycoforms have been identified on naturally-occurring IgG molecules [107]. Relative to other glycoforms, sialylated IgG has reduced affinity for classical Fcγ receptors and increased ability to bind to an alternate C-type lectin receptor, SIGN-R1 [108].

Sialylated IgG mediates immune suppression through a novel innate immunosuppressive pathway using T_H2-related cytokines [108]. Splenic macrophages and dendritic cells expressing SIGN-R1 (or the human ortholog DC-SIGN) respond to Sialylated IgG by secreting IL-33, which in turn increases the number of circulating IL-4-producing basophils [109]. IL-4 produced by these activated basophils then acts on effector macrophages in the periphery, causing them to up-regulate the inhibitory FcγRIIB receptor and thus increasing the activation threshold of these cells and down-regulating their pro-inflammatory capacity [58].

IgG sialylation status may be an important factor in the pathogenesis or disease course of autoimmunity. Rheumatoid arthritis is associated with a decrease in galactosylated and sialylated serum IgG [107]. Additionally, it has been shown that autoantigen-specific IgG displays reduced sialylation, and this reduction was more pronounced at the site of active inflammation [110]. Despite this, remarkably little is known about signals that modulate the

glycosylation status of secreted antibodies. It appears that an active inflammatory response reduces the relative amount of sialylated IgG, although no mechanism for this reduction is currently known [105]. A recent study characterized the glycan profile of IgG secreted from human primary B cells in response to various pro-inflammatory cytokines and innate stimuli [111]. Somewhat surprisingly, it was found that many stimuli tested [including IL-4, IL-6, IL-17, TNFα, TGFβ, and LTα] had no measurable effect on IgG sialylation. Even more intriguingly, those pro-inflammatory stimuli that did have an effect [CpG, IL-21, IFN-γ, and all-trans retinoic acid) actually increased levels of sialylation and galactosylation rather than decreasing them. The phenotype or anatomic location of the B cells that secrete sialylated IgG is currently unknown. MZ B cells, however, may play a role in pathways downstream of sialylated IgG, as macrophages in the marginal zone do not express SIGN-R1 in mice lacking MZ B cells, and SIGN-R1^{+} macrophages transiently exit the marginal zone when MZ B cells are stimulated to do so [112].

Natural Antibodies

Natural antibodies secreted by innate-like B cells have multiple roles in maintaining immune homeostasis. Among these is aiding in the elimination of apoptotic cells, a key function of the immune system [113]. If this process is inhibited or overwhelmed, apoptotic cells are not removed before their plasma membrane loses integrity, resulting in the release of self-

antigens and inflammatory signals into the interstitial space. This in turn can lead to increased tissue destruction and induction or exacerbation of autoimmunity. The surface phenotype of cells undergoing apoptosis is altered from that of healthy cells by the presence of apoptosis-specific neo-epitopes, sometimes referred to as "eat-me" signals [114]. Apoptotic cells display "eat-me" signals on their cell surface, such as an enrichment of lipids containing phosphorylcholine [PC] or malondialdehyde [MDA]. The antibody repertoire of innate-like B cells is enriched for antibodies that bind these apoptosis-induced determinants, and their binding to apoptotic cells aids in their engulfment by phagocytic cells [115]. Natural antibodies also directly regulate the inflammatory capacity of APCs directly by inhibiting their maturation and reducing their expression of molecules involved in antigen presentation [116]. The administration of a model natural antibody *in vivo* has a general immunosuppressive effect, as mice receiving the antibody are protected from both collagen-induced arthritis and a passive transfer model of arthritis induced by anti-collagen IgG antibodies [116]. In addition to apoptotic cells, the lipid epitopes bound by natural antibodies are also found on the surface of bacteria such as *S. pneumoniae* and within oxidized LDL deposits found in atherosclerotic lesions [114]. In mouse models of atherosclerosis, natural antibodies reduce the severity of disease, and increasing levels of anti-PC natural antibodies by immunization led to a decrease in atherosclerotic lesion formation [117].

Circulating levels of natural antibodies are increased in mice that have received either large doses of apoptotic cells or have been immunized with pneumococcal extracts, suggesting that the presence of cognate antigen determines the level of natural antibody secretion. In some cases natural antibody responses to different antigens are coordinately regulated, as immunization with one B-1 cell-specific antigen, malondialdehyde-modified low density lipoprotein (MDA-LDL), resulted in an increase in natural antibodies recognizing another B-1 cell-specific antigen, phosphorylcholine [114]. Finally, T cell responses in mice immunized with natural antibody-binding antigens are dominated by the T_H2-type response even if the antigen is administered with T_H1-skewing adjuvant, suggesting that the selective expansion of T_H2 cells may be another regulatory mechanism of natural antibodies [117].

IL-10-secreting B cells

The most widely-studied and best-understood immunosuppressive mechanism in B cells is the secretion of IL-10, an important anti-inflammatory cytokine [118]. IL-10 has multifaceted effects on nearly all hematopoietic cells, but appears to act most directly on APCs and some T cell subsets [119]. In APCs, IL-10 inhibits the secretion of pro-inflammatory cytokines and inhibits antigen presentation [120-124]. IL-10 directly affects differentiated $CD4^+$ T cells as demonstrated in studies in which both T_H1 and T_H2 cells exhibited reduced proliferation and cytokine secretion in the presence of IL-10 [125, 126].

Although B cells had been known to secrete IL-10 for some time, only recently has the importance of B cell-derived IL-10 in immunosuppression been demonstrated definitively [95, 96, 127, 128]. A subset of IL-10-producing B cells in the gut-associated lymphoid tissue [GALT] was identified in 2002. These IL-10-producing B cells mediated suppression of intestinal inflammation in recipient animals upon transfer [96]. In the same year, a role for IL-10-producing splenic B cells in the regulation of autoimmunity was reported [95]. Subsequent work has demonstrated that B cell-derived IL-10 regulates autoimmunity in various other mouse models of autoimmune conditions including chronic intestinal inflammation, collagen-induced arthritis, T1D, and SLE [96, 97, 99-101]. Mice prone to spontaneous autoimmunity [NZB/W, NOD, and MRL/*lpr* mice] have increased numbers of IL-10-producing B cells relative to normal strains, whereas mice susceptible to induced autoimmunity [DBA/1, SJL] have lower basal numbers of IL-10-producing cells [129].

Splenic IL-10-producing B cells were initially reported to have a surface phenotype consistent with transitional 2-marginal zone precursor [T2-MZP] B cells, namely high expression of CD21, CD23, and IgM [130]. Later, a portion of splenic IL-10-producing B cells were shown to co-express CD5 and CD1d in addition to the previously described T2-MZP B cell markers, and $CD19^{+}CD5^{+}CD1d^{high}$ B cells were found to have IL-10-dependent immunosuppressive properties upon transfer into recipient animals [131]. More recently, the protein TIM-1 was reported to

identify 70% of IL-10-producing B cells, making this marker the most specific yet identified for IL-10-producing B cells [132]. Treatment of mice with an activating anti-TIM-1 antibody led to an increase in IL-10-producing TIM-1^{+} B cells and improved tolerance of an allogeneic tissue graft, suggesting that TIM-1 is of functional significance for IL-10-producing B cells as well.

Much controversy still exists regarding the relationship of IL-10-producing B cells to known B cell subsets. While IL-10-producing B cells are enriched in the T2-MZP and CD5^{+}CD1d^{hi} subsets, not all cells in these subsets express IL-10, suggesting that there is not an absolute correspondence between these B cell subsets and IL-10-producing B cells. The fact that many IL-10-producing B cells express CD5 suggests that they may also be related to the innate-like B cells, which is in accord with the known ability of B-1a cells to secrete large amounts of IL-10 [127]. Additionally, splenic IL-10-producing B cells are present at much higher frequencies in neonates than in older mice as is also true of B-1 cells [129]. Finally, while the CD5^{+}CD1d^{hi} B cell population is enriched for IL-10-producing B cells, only ~25% of IL-10-producing B cells are found in this population. Indeed, IL-10-producing B cells can be found among most major subsets of splenic B cells, albeit at a lower frequency than is seen among CD5^{+}CD1d^{high} B cells. Given that no set of surface markers identifies all IL-10-producing B cells, it is also possible that IL-10 production occurs in several B cell subsets depending upon differentiation or activation state.

While it is clear that IL-10 plays a major role in B cell-mediated immune suppression, it is not currently known if B cell-derived IL-10 acts exclusively on target cells or if the loss of IL-10 in B cells also leads to defects in other immunosuppressive functions. This is an important area of study, as IL-10 is a known growth factor for B cells and other suppressive mechanisms employed by B cells may work in synergy with IL-10 [133].

Fas Ligand-expressing Killer B cells

Interaction between Fas ligand (FasL) and its receptor Fas is critical for the maintenance of self-tolerance, as mice deficient in either of these molecules develop spontaneous systemic autoimmunity [134, 135]. Similarly, defects in Fas-mediated apoptosis are linked to autoimmune lymphoproliferative syndrome (ALPS) in humans [136]. Upon binding the Fas receptor (CD95), FasL induces apoptosis in target cells such as activated peripheral $CD4^+$ T cells [137].

B cells expressing FasL were initially reported following stimulation of murine B cells with mitogens [138]. Subsequently, several groups have reported FasL expression in B cells under a variety of conditions. Some forms of B cell-derived cancers in humans have been reported to express FasL, including multiple myeloma, B cell chronic lymphocytic leukemia, and large B cell lymphoma [139-141]. Expression of FasL by B cell-derived cancers is associated with increased T cell apoptosis [139, 140]. Viral infections have been reported to induce FasL

expression in B cells as well, and this has been proposed as mechanism of immune evasion employed by some viruses [142-144]. FasL-expressing B cells were induced by infection with the parasitic worm *S. mansoni* in mice, and their increased frequency coincided with greater levels of apoptosis in $CD4^+$ T cells [145]. Later, it was demonstrated that $CD5^+$ B cells isolated from schistosome infected mice could induce apoptosis in T cells, whereas $CD5^-$ B cells showed little killing activity [146].

There is also evidence that FasL-expressing B cells may play a role in the regulation of autoimmunity and maintaining self-tolerance. Activated B cells expressing FasL and TGFβ have been reported to delay the onset of diabetes in non-obese diabetic (NOD) mice, and the frequency of $FasL^+$ B cells is reduced in mice with severe autoimmune arthritis relative to those with mild or no arthritis [98, 147]. Mice with a B cell-specific loss of FasL spontaneously develop autoantibodies despite the fact that T cells in these animals are FasL-sufficient, demonstrating that B cell expression of FasL plays a role in maintaining immune homeostasis [148]. Bone marrow cells treated with the TLR-9 agonist CpG are enriched for B cells that express high levels of FasL and protect NOD mice from type 1 diabetes upon adoptive transfer [149]. Additionally, these CpG-elicited $FasL^+$ B cells induced FasL-mediated apoptosis in $CD4^+$ T cells *in vitro* and showed no evidence of increased IL-10 secretion [149]. B cells from Fas-deficient MRL/lpr mice also express high levels of FasL, and kill Fas-susceptible target cells

with an efficiency similar to that of NK cells [150]. In a male-to-female skin graft model, transfer of B cells from wild-type males prior to skin grafting induced tolerance in female recipients, whereas FasL-deficient B cells were unable to transfer tolerance [151].

Conceptually, $FasL^+$ B cells are unique amongst regulatory B cell populations as they possess the potential for suppression that is both *antigen specific*, since only $CD4^+$ T cells recognizing antigens presented by $FasL^+$ B cells are targeted, and *permanent*, as FasL signaling results in cell death rather than suppression (Figure 1-2) [152].

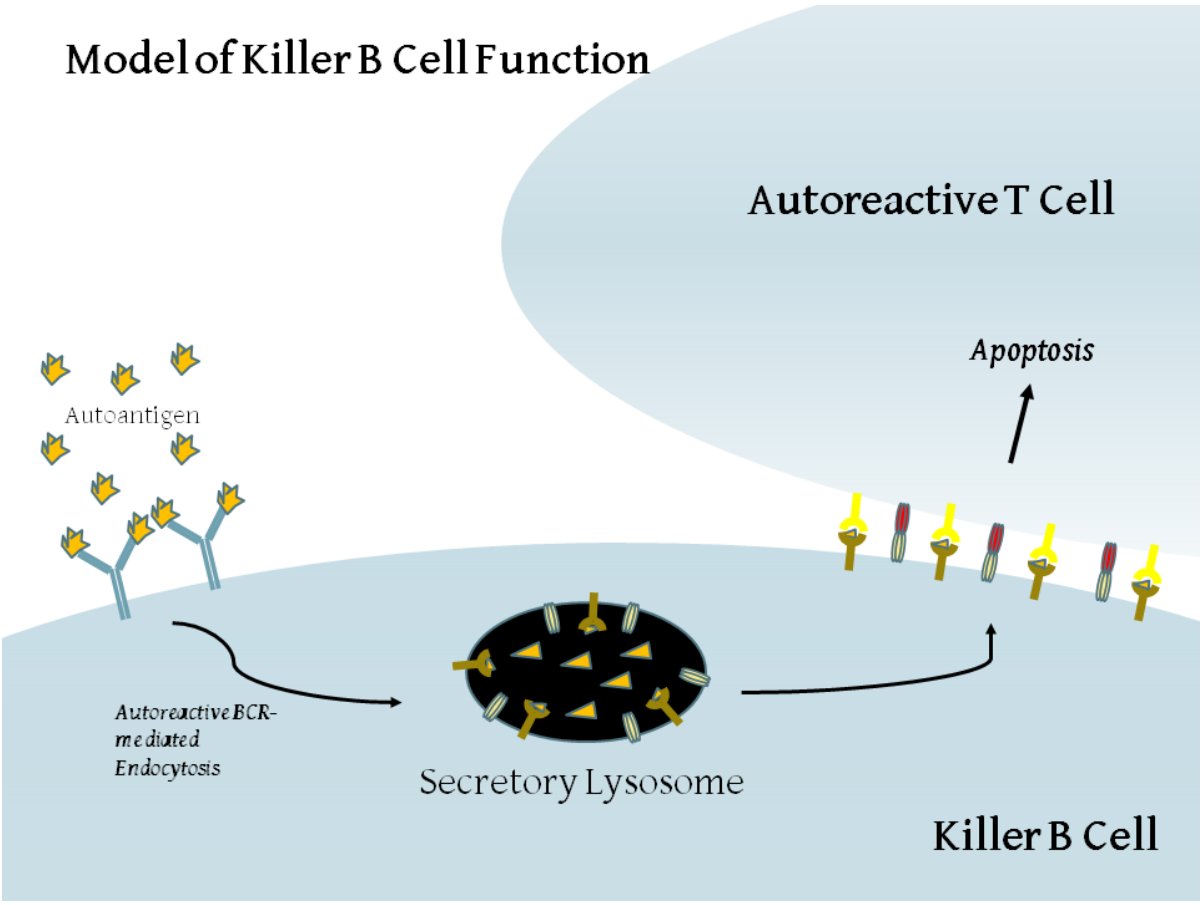

Figure 1-2: Model of killer B cell function.
In our model of killer B cells, antigens are taken up and concentrated in the secretory lysosome through BCR-mediated endocytosis. MHCII molecules loaded with the antigen are expressed at the surface along with FasL. $CD4^+$ T cells recognizing the antigen are then killed by Fas-mediated apoptosis.

1.5 Fas Ligand Function and Regulation

FasL (CD178) and its receptor Fas (CD95) are transmembrane proteins and members of the tumor necrosis factor (TNF) and TNF receptor superfamilies, respectively. Mutations in the genes coding for either Fas or FasL in humans can result in autoimmune lymphoproliferative syndrome, a monogenic disease characterized by lymphadenopathy, splenomegaly, and autoimmune disease [136]. Mice bearing similar mutations develop a homologous disorder that presents with lymphadenopathy, production of autoantibodies, and age-related lupus-like symptoms [134, 135]. Therefore, the Fas/FasL signaling axis is critical for maintaining immune homeostasis and self-tolerance.

FasL is the only known ligand for the pro-apoptotic surface receptor Fas, and as a potent inducer of apoptosis its expression is restricted to a few cell types and is highly regulated. [153, 154] In locations of immune privilege (e.g., eyes, testes), several cell types constitutively express FasL mRNA and display FasL protein on their plasma membrane to eliminate any immune cells that may enter their compartment. In contrast, FasL expression and trafficking is inducible in NK cells and $CD8^+$ cytotoxic T cells (CTLs), as these cells sequester FasL intracellularly and translocate it to the immunological synapse only upon contact with a target cell [155, 156]. FasL, like other TNF-related ligands, functions as a homotrimer. Once on the surface of cells, the extracellular portion of FasL is cleaved by metalloproteases and the soluble

form of FasL is released [157]. This soluble form, however, is relatively unstable in trimeric form and therefore shows greatly-diminished apoptosis-inducing activity. After cleavage of the extracellular portion, the intracellular portion is also released from the membrane and translocates to the nucleus [158]. The functional consequences of this nuclear translocation are currently unknown.

The main function of Fas/FasL signaling is the induction of apoptosis in the Fas-bearing cell [137]. Upon binding FasL, Fas recruits the adapter Fas-associated protein with death domain (FADD), which in turn recruits caspase-8 (FLICE) to the receptor complex to form the death-inducing signaling complex (DISC). Autocatalysis activates and releases caspase-8 to cleave and activate effector caspases, ultimately resulting in cell death. This pathway is inhibited by cellular FLICE-like inhibitory protein (cFLIP), an inactive caspase-8 analog that competes with caspase-8 for incorporation in the DISC. An alternative pathway downstream of the DISC exists in some cell types whereby the pro-apoptotic molecule Bid is activated by caspase-8 [159]. Once activated, Bid initiates the release of cytochrome c from mitochondria, allowing activation of caspase-9 through formation of the apoptosome. Activated caspase-9 can then initiate apoptosis by activating effector caspases such as caspase-3.

T cells are thought to be the most important *in vivo* targets for FasL-mediated killing, as the transfer of Fas-deficient T cells from *lpr* mice into wild type animals is sufficient to induce

lymphoproliferative symptoms in recipients [160]. Fas is found in abundance on the surface of activated $CD4^+$ T cells, and most thymocytes [137]. Despite high expression in thymocytes, Fas-mediated cell death does not appear to be important for negative selection [161].

Nearly all studies of FasL transcription and trafficking in hematopoietic cells have been performed using CTLs, so very little is known about the regulation of FasL in B cells. Through this work in CTLs, FasL expression is known to be controlled by a variety of transcription factors [154, 162]. In T cells, signaling through the TCR leads to transcription of FasL via several parallel pathways, including calcineurin-mediated activation of the NFAT family of transcription factors, PKCΘ-mediated activation of NF-kB, and MAPK pathway-mediated activation of the AP-1 transcription factor complex [162]. Additionally, interferon regulatory factors (IRFs), transcription factors that induce the production of interferons in response to viral detection, appear to synergize with TCR-mediated signaling to increase FasL expression in CTLs [163]. Post-transcriptional regulation of FasL has been reported as well, specifically in the form of AKT-mediated up-regulation of miR-21, which appears to directly target the 3'UTR of FasL mRNA [164].

In hematopoietic cells, however, post-translational regulation appears to be the most important level of organization for proper function of FasL. In the absence of post-translational modifications, FasL is sorted through the Golgi to the plasma membrane [165]. In

hematopoietic cells, however, FasL is sorted via phosphorylation and mono-ubiquitylation to an intracellular structure known as the secretory lysosome (SL) [165]. The SL is functionally and morphologically similar to multi-vesicular bodies (MVBs) of the late endosome, and serves as a point of intersection between the endocytic and exocytic pathways. In this compartment FasL is sorted on to intraluminal vesicles within the limiting membrane of the SL known as exosomes [166]. When the limiting membrane fuses with the plasma membrane during exocytosis, the FasL-bearing exosomes are expelled into the extracellular space. This allows for FasL to be released in its membrane-bound form which displays much greater efficacy in inducing apoptosis than the soluble form shed from the plasma membrane.

1.6 Exosomes and Immune Regulation

Exosomes are extracellular vesicles approximately 50-90nm in diameter that are secreted by a variety of immune cells and cell lines. In APCs exosomes originate from the same intracellular compartment where newly-synthesized and recycled MHCII molecules are loaded with peptides derived from endocytosed proteins [167]. Exosomes derived from APCs therefore contain peptide-loaded MHCII and co-stimulatory molecules, and are capable of activating antigen-specific T cells [167]. Unlike other types of APCs, B cells can target antigens to their SL

via BCR-mediated endocytosis, thus allowing them to more efficiently load their cognate antigen in MHCII molecules bound to exosomes [168].

The existence of immunosuppressive APC-derived exosomes has been established as well. Stimulation of murine bone marrow-derived dendritic cells (BMDCs) with rIL10 resulted in the production of exosomes capable of suppressing an immune response *in vivo* [169]. Additionally, BMDCs transfected with a vector expressing the FasL gene produced $MHCII^{+}FasL^{+}$ exosomes that were able to suppress an immune response *in vivo* [170]. Importantly, the suppression mediated by the $MHCII^{+}FasL^{+}$ exosomes was antigen-specific and FasL-dependent. Later, naturally-occurring $MHCII^{+}FasL^{+}$ exosomes were isolated from the blood of immunized mice, confirming the production of $MHCII^{+}FasL^{+}$ exosomes by non-transformed cells [171]. These endogenously-produced $MHCII^{+}FasL^{+}$ exosomes also demonstrated antigen-specific immune suppression upon transfer to recipient mice. Currently the cell type responsible for producing $MHCII^{+}FasL^{+}$ exosomes remains unclear, although based upon the presence of CD11b, macrophages and B cells (specifically B-1 cells) are likely candidates.

Chapter 2

Interleukin-5 supports the expansion of FasL-expressing B cells that induce antigen-specific apoptosis in CD4$^+$ T cells and secrete IL-10

2.1 Summary

The experiments described in this chapter were performed with the goal of identifying factors important for the growth or function of FasL$^+$ killer B cells. To this end, we initially sought to better characterize the surface phenotype of splenic FasL$^+$ B cells, hypothesizing that this knowledge might provide insight into their biology. Although work from our laboratory had previously demonstrated that FasL$^+$ B cells were CD5$^+$, a more thorough analysis was required to determine the relationship of FasL$^+$ B cells to various known subsets of B cells, including at least two IL-10-producing B cells identified by others [130, 131, 146, 147]. The results of these experiments showed that FasL$^+$ B cells shared some phenotypic characteristics with the so-called "B10" subset – a population of IL-10-producing B cells first identified by the Tedder

laboratory [131]. Given these similarities, we attempted to determine the degree of overlap between FasL$^+$ B cells and IL-10-producing B cells. Although we did identify a very rare population of FasL$^+$IL-10$^+$ B cells, IL-10 production was not more common among FasL$^+$ B cells than FasL$^-$ B cells despite their shared enrichment in a common B cell subset.

Following this phenotypic characterization, we analyzed global mRNA expression in a subset of B cells that is enriched for both FasL$^+$ B cells and IL-10-producing B cells by microarray. The results of this microarray showed that two components of the IL-5 receptor were expressed at higher levels in the B cell population enriched for regulatory cells, implicating IL-5 as a potentially-important cytokine for regulatory B cell function. Data presented herein confirmed this finding, demonstrating that IL-5 can indeed expand a population of B cells that are enriched for FasL$^+$ B cells and capable of IL-10 secretion. B cells stimulated with IL-5 displayed potent killing activity against CD4$^+$ T cells, inducing apoptosis in targets cells in a FasL-dependent and antigen-specific manner.

Finally, we performed experiments to explore the effects of cytokines frequently associated with IL-5 on regulatory B cell activity. Somewhat unexpectedly, the results of these experiments revealed a novel antagonistic relationship between the type-2 cytokines IL-4 and IL-5 in their effects on regulatory/killer B cells *in vitro*. Specifically, IL-4 dominantly inhibited

both the enhanced killing activity of B cells as well as the enhanced secretion of IL-10 mediated by IL-5.

2.2 Methods

Mice: All protocols involving animals were approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA). Wild-type DBA/1LacJ mice were obtained from our breeding colony at the University of Michigan or purchased from Jackson Laboratories. The cII-TCR transgenic mice were generated on the DBA/1LacJ background and recognize an immunodominant epitope of type 2 collagen ($cII_{259-272}$) when presented by the MHC class II I-A^q molecule expressed in DBA/1 mice [147]. The presence of the transgene was confirmed by polymerase chain reaction genotyping in all animals.

Flow Cytometry: Fluorochrome-conjugated antibodies and isotype controls were obtained from BD Biosciences (anti-CD19-APC, anti-CD19-PECy7, anti-B220-PECy7, anti-CD93-FITC, anti-CD23-FITC, anti-IgM-PECy7, anti-CD21-PE, anti-CD24-PE, anti-CD5-APC, anti-CD1d-FITC, anti-CD125-PE, anti-FasL-PE, anti-FasL-Biotin) or Biolegend (anti-CD3-APC, anti-CD9-FITC). Cells were incubated with anti-CD16/CD32 Fc Block (BD Biosciences) prior to staining and analyzed on an Accuri C6 flow cytometer. In all analyses, propidium iodide was used to exclude dead cells and

doublets were excluded by gating based on forward scatter height and area channels. Data were analyzed using Cytobank web-based software [172] or FlowJo v7.6.5 (Tree Star, Inc.).

Fluorescence-Activated Cell Sorting: Splenocytes from naïve mice were stained with anti-CD19-APC and anti-FasL-PE (BD Biosciences). $CD19^{+}FasL^{-}$ and $CD19^{+}FasL^{+}$ populations were then isolated using a FACSAria II (BD Biosciences).

Identifying IL-10-producing B Cells: $FasL^{-}$ and $FasL^{+}$ B cell populations obtained by FACS were cultured for five hours with 50 ng/mL phorbol myristate acetate (PMA), 1 µg/mL ionomycin, and 5 µg/mL lipopolysaccharide (LPS) in the presence of monensin (GolgiStop, BD Biosciences). B cells were then fixed (4% paraformaldehyde) and permeabilized (0.5% Saponin, 0.2% BSA, 0.1% sodium azide in phosphate buffered saline) prior to staining with anti-IL10-FITC or isotype control antibody (BD Biosciences). No cells staining positive for IL-10 were detected in cultures without stimulation or in B cells treated with monensin in the absence of PMA/ionomycin/LPS stimulation (data not shown).

B cell Isolation and Culture: B lymphocytes were positively-selected from single-cell splenocyte suspensions using anti-CD19-coated magnetic beads (Miltenyi Biotec). Isolated B cell populations were routinely >95% pure. Mouse CD40L-transduced NIH-3T3 fibroblasts (a gift from Dr. Kevin McDonagh) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, penicillin/streptomycin, and L-glutamine [173]. For B cell

stimulation cultures, $CD19^+$ cells were added to a confluent layer of irradiated (30 Gy) CD40L-expressing fibroblasts in 24- or 96-well flat-bottomed culture plates in the presence or absence of 50 ng/mL recombinant mouse IL-5 and/or IL-4 (Peprotech Inc.). All stimulations were performed in DMEM supplemented with 10% fetal calf serum, penicillin/streptomycin, L-glutamine, 50 µg/mL transferrin, and 5 µg/mL insulin. After 5 days in culture, supernatants and B cells were collected for further study. Viable cells were enumerated using trypan blue exclusion.

Proliferation Assay: B cells were cultured with irradiated CD40L-expressing fibroblasts in the presence or absence of IL-5. After 4 days, ^{3}H-thymidine was added (5 µCi final concentration) and cells were harvested after 18 hours in culture. Radioactivity was assessed by a scintillation counter. Control wells containing CD40L-fibroblasts alone or B cells cultured on non-transduced NIH-3T3 fibroblasts showed no detectable proliferation in response to IL-5 (data not shown).

Immunoblotting: Cell lysates from equal numbers of freshly-isolated $CD19^+$ cells and B cells stimulated for five days with CD40L-expressing fibroblasts and cytokines were separated by SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked and incubated with polyclonal rabbit anti-FasL IgG (Millipore) or anti-β-Actin (Cell Signaling). Antibody binding

was detected with an anti-rabbit IgG-HRP secondary antibody (Cell Signaling) and ECL reagent (Thermo Scientific).

Apoptosis Assay: Splenocytes were harvested from cII-TCR transgenic mice and stimulated with 2 μg/mL Concanavalin A for three days. $CD4^+$ T cells were then negatively selected by magnetic activated cell sorting (MACS) and cultured with freshly-isolated $CD19^+$ B cells or B cells stimulated for 5 days on CD40L-expressing fibroblasts with or without IL-5. $CD4^+$ T cell populations were >90% $CD4^+$ after negative selection. Apoptosis assays were performed in RPMI supplemented with 10% fetal calf serum, penicillin/streptomycin, L-glutamine, HEPES, sodium pyruvate, and β-Mercaptoethanol. After 18 hours of co-culture (1:1 effector-to-target ratio), cells were collected and stained with anti-CD4-PE, Annexin V-FITC (BD Biosciences), and propidium iodide. Prior to co-culture with $CD4^+$ T cells, some B cells were incubated with 10 μg/mL anti-FasL blocking antibody (R&D Systems) or an isotype control antibody for one hour and washed. To assess FasL-mediated "fratricide" between $CD4^+$ T cells, control wells with T cells alone contained these antibodies for the full duration of culture. Apoptosis was measured by flow cytometry as the frequency of Annexin V^+ cells within the $CD4^+$ gated population.

Enzyme-linked Immunosorbent Assay (ELISA): B cells were cultured on CD40L-expressing fibroblasts for five days in the presence or absence of IL-5. Equal concentrations(7.5×10^5 cells/mL) of stimulated B cells and freshly-isolated $CD19^+$ B cells were then cultured in 96-well

flat-bottomed plates with either no further stimulation, or with PMA (50 ng/mL) and ionomycin (1 μg/mL). Supernatants were collected after 24 hours, and IL-10 was quantified by ELISA following manufacturer's protocols (R&D Systems).

Statistics: The Student's t test was used to compare experimental groups using GraphPad Prism 5.0 software. Levels of significance are denoted throughout as follows: *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. All graphs depict the mean ± SEM of replicate conditions/animals.

2.3 Results

Frequency and surface phenotype of FasL$^+$ B cells

It is well-established that the Fas/FasL signaling axis is critical for the maintenance of self-tolerance, as mice bearing natural mutations in genes coding for either of these molecules develop spontaneous systemic autoimmunity [134, 135]. Cells expressing FasL are therefore potentially important in maintaining immune homeostasis and ultimately self-tolerance. To determine the cellular composition of FasL$^+$ splenocytes, we examined FasL$^+$ and FasL$^-$ splenocytes in naïve DBA/1 mice by flow cytometry. The majority of FasL-expressing spleoncytes (~60%) showed high surface expression of the B cell markers B220, CD19, and IgM

(Figures 2-1A and 2-1B). Most of the remaining FasL$^+$ splenocytes were CD3$^+$ T cells, although a few apparent non-lymphoid cells showed some expression of FasL as well. We found similar results upon examining splenocytes from naïve CBA, C57BL/6, and Balb/c mice housed under identical conditions in our mouse colony (data not shown). B cells, therefore, appear to be the predominant FasL-expressing splenocyte in naïve mice.

The B cell population in peripheral organs is comprised of functionally and developmentally distinct subsets that can be identified in part by differential expression of surface markers. Among these subsets, IL-10-producing B cells have been reported to be enriched in both the CD5$^+$CD1d^{high} (B10 cells) and CD23$^+$CD21^{high} transitional 2-marginal zone precursor (T2-MZP) B cell subsets, although most IL-10-secreting B cells are distributed among other B cell subsets [130-132]. Previous work showed that FasL$^+$ B cells were enriched among CD5$^+$ B cells, but had not further characterized their surface phenotype [146]. We therefore compared surface expression of several surface markers in FasL$^+$ and FasL$^-$ B cells to understand how FasL$^+$ B cells relate to previously-identified B cell subsets. CD19$^+$FasL$^+$ B cells displayed a modest but statistically significant enhancement in surface expression of CD5, CD1d, IgM, and CD24 than CD19$^+$FasL$^-$ B cells (Figure 2-2). Surface levels of CD9, CD93, CD21 and CD23 were similar in both FasL$^+$ and FasL$^-$ B cells.

To determine if $CD5^{+}CD1d^{high}$ B cells were more frequent among the $FasL^{+}$ B cells, we stained $FasL^{+}$ and $FasL^{-}$ B cells for surface expression of CD5 and CD1d. These experiments showed that $CD5^{+}CD1d^{high}$ B cells were indeed more frequent among $FasL^{+}$ B cells than $FasL^{-}$ B cells (Figures 2-3A and 2-3B). Reciprocally, the $CD5^{+}CD1d^{high}$ B cell subset had a nearly four-fold enrichment for $FasL^{+}$ B cells (Figure 2-3C). Although $FasL^{+}$ B cells were enriched among $CD5^{+}CD1d^{high}$ subset cells, most $FasL^{+}$ B cells were not within this subset, indicating that, much like IL-10-producing B cells, $FasL^{+}$ B cells are not found exclusively in a single B cell subset. A similar experiment comparing expression of CD23 and CD21 failed to find evidence of an increase in the frequency of $CD23^{+}CD21^{high}$ T2-MZP B cells among the constitutively $FasL^{+}$ B cells relative to $FasL^{-}$ B cells (Figure 2-4A and 2-4B).

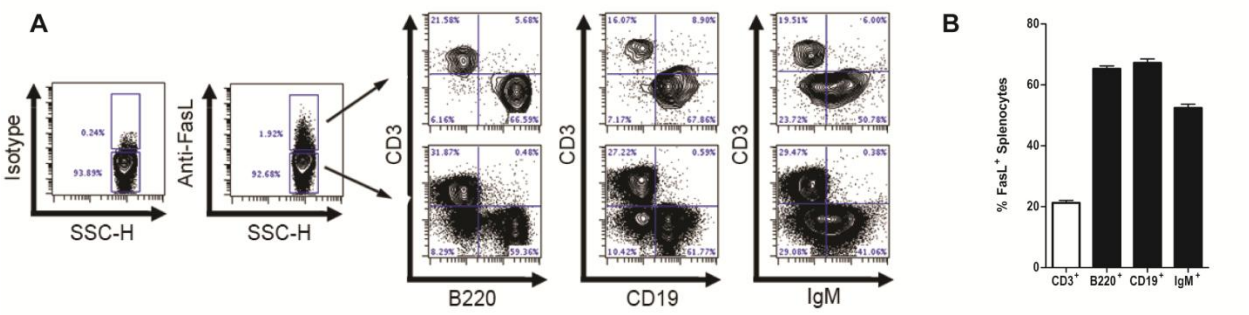

Figure 2-1: The majority of FasL$^+$ splenocytes are B cells.
Splenocytes from naïve mice were stained for surface expression of FasL and the cellular composition of FasL$^-$ and FasL$^+$ subsets was examined by flow cytometry. (**A**) Representative dot plots of splenocytes stained with a T cell marker (CD3) and three independent B cell markers (B220, CD19, and IgM). (**B**) The frequency (mean ± SEM) of cells staining positive for the markers presented in (A) among FasL$^+$ splenocytes. Data are representative of more than 5 independent experiments with at least three animals per experiment.

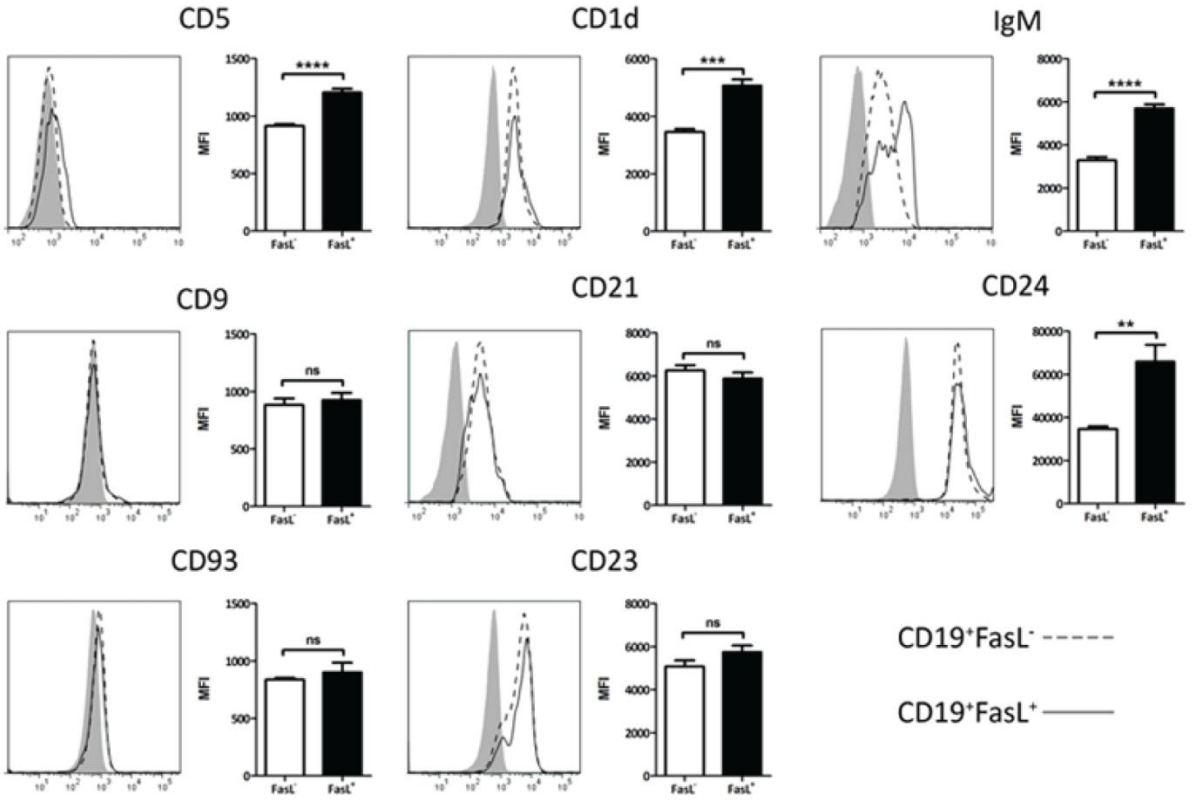

Figure 2-2: FasL$^+$ B cells have higher expression of CD5, CD1d, IgM, and CD24 than FasL$^-$ B cells.

Representative histograms showing relative surface expression of markers for B cell subsets in CD19$^+$FasL$^-$ and CD19$^+$FasL$^+$ B cells. Grey histograms represent isotype control antibody staining. Bar graphs depict the average median fluorescence intensity for cells obtained from replicate animals. **p<0.01, ***p<0.001, and ****p<0.0001

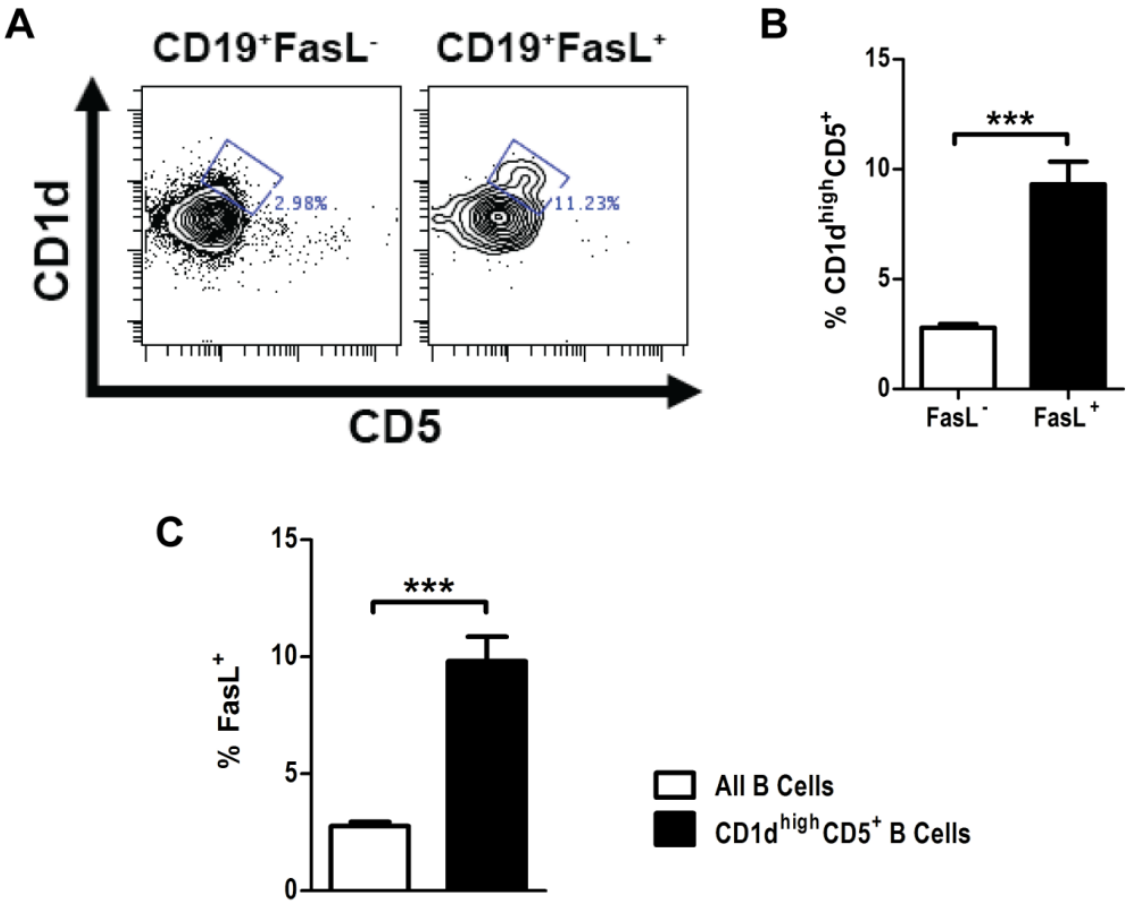

Figure 2-3: FasL$^+$ B cells are enriched in the CD5$^+$CD1d^{high} B cell subset.
(**A**) FasL$^-$ and FasL$^+$ B cells were stained for co-expression of CD5 and CD1d. (**B**) The frequency of CD5$^+$CD1d^{high} cells (mean ± SEM) among FasL$^-$ and FasL$^+$ B cells in replicate animals was measured. (**C**) Reciprocally, the frequency of FasL$^+$ cells among all B cells and CD5$^+$CD1d^{high} B cells was measured. Data are representative of four independent experiments using at least 3 animals per experiment. ***p<0.001

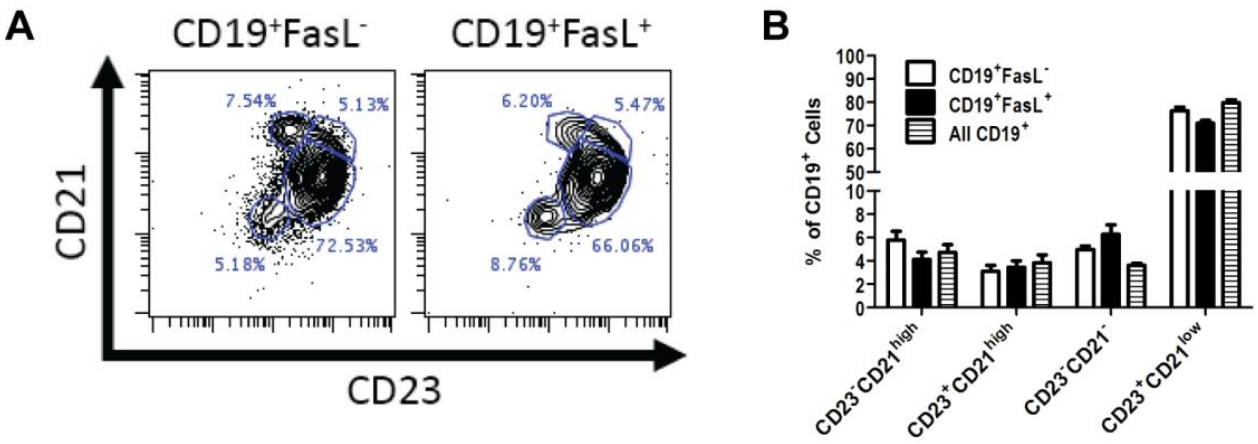

Figure 2-4: CD23^{+}CD21^{high} B cells are not enriched among FasL^{+} B cells.
(**A**) FasL^{-} and FasL^{+} B cells were stained for co-expression of CD23 and CD21. (**B**) The frequency of B cell populations as gated in (A) among FasL^{-} and FasL^{+} B cells in replicate animals was measured. (mean ± SEM)

FasL$^+$IL-10$^+$ B cells are extremely rare in naïve mice

As FasL$^+$ B cells shared some phenotypic similarities with the B10 cells, we next sought to determine whether these were discrete or overlapping cell populations by co-staining for both immunosuppressive molecules. Although FasL$^+$ B cells can be readily identified without further stimulation, IL-10-producing B cells cannot be identified directly without several hours of *in vitro* stimulation with PMA/ionomycin/LPS in the presence of a secretion-blocking compound such as monensin [174]. Using previously-published methods for detecting IL-10-producing B cells, we initially attempted to stain for both surface FasL and intracellular IL-10 in the same B cells [175]. Unfortunately, the culture and fixation protocols necessary to identify IL-10-producing B cells all-but eliminated FasL staining (Figure 2-5). Whether this result is due to the loss of FasL expression in FasL$^+$ B cells or their death in culture is still an open question. Regardless, a consequence of this phenomenon is that any experimental protocols requiring identification of FasL$^+$ B cells must be completed within 2-4 hours of isolation.

Taking an alternative approach, we sorted splenocytes into CD19$^+$FasL$^-$ and CD19$^+$FasL$^+$ subsets by FACS immediately after isolation from the spleen. This allowed us to identify and isolate FasL$^+$ B cells within two hours of isolation - before FasL$^+$ B cells are no longer detectable among splenocytes. After sorting, FasL$^+$ and FasL$^-$ B cells were cultured for 5 hours with PMA/ionomycin/LPS in the presence of monensin, and subsequently fixed and stained for

intracellular IL-10. We found that IL-10-producing B cells were present at similar frequencies among both FasL$^+$ and FasL$^-$ B cells (Figures 2-6A and 2-6B). While the majority of FasL$^+$ B cells do not produce detectable IL-10 under these conditions, ~4% of FasL$^+$ B cells produced IL-10 upon *ex vivo* stimulation, suggesting that a rare subset of FasL$^+$ B cells can express both immunosuppressive molecules and therefore may be capable of using multiple means of immune suppression. The FasL$^+$ B cells that do secrete IL-10, however, are extremely rare, making up <0.1% of all splenic B cells (~2% of B cells are FasL$^+$, and ~4% of FasL$^+$ B cells produce IL-10 after *ex vivo* stimulation).

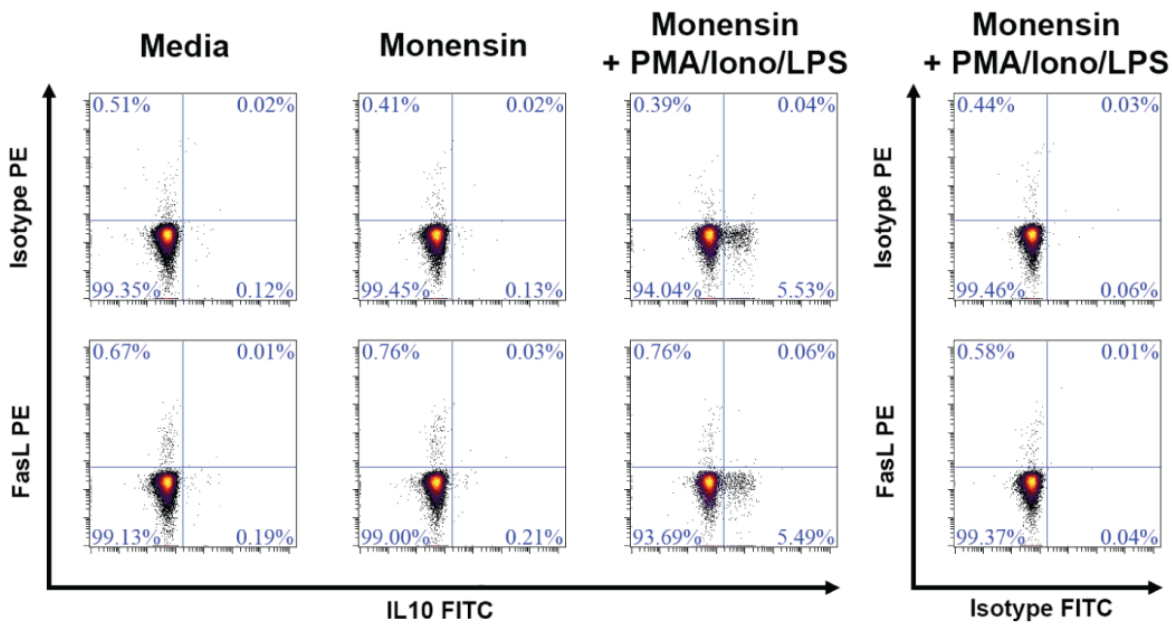

Figure 2-5: Staining protocols for identifying IL-10-expressing B cells by intracellular staining significantly reduce FasL staining.

Short-term culture reduces FasL surface staining in B cells. Splenocytes from naïve mice were cultured for 6 hours with PMA (50 ng/mL), ionomycin (1 µg/mL) and LPS (5 µg/mL), stained with anti-CD19 and anti-FasL (or isotype control), then fixed and stained for intracellular IL-10. $CD19^+$ cells were assayed for surface expression of FasL and intracellular IL-10.

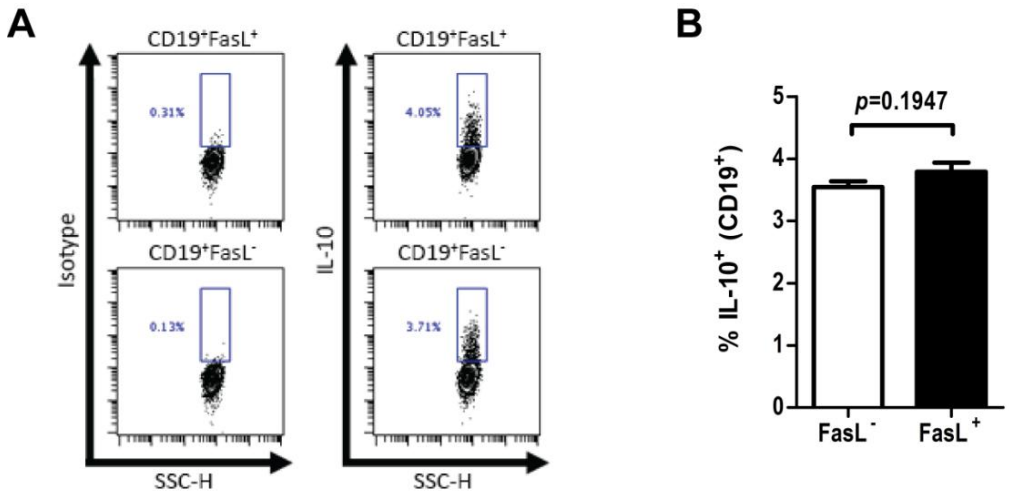

Figure 2-6: IL-10-producing B cells are not enriched in the FasL$^+$ subset.
FasL$^-$ and FasL$^+$ splenic B cells from naïve mice were sorted by FACS and stimulated with PMA, ionomycin, and LPS in the presence of monensin for five hours. (**A**) Following stimulation, FasL$^-$ and FasL$^+$ B cells were fixed and stained for intracellular IL-10. (**B**) Frequency of IL-10-producing B cells (mean ± SEM) among CD19$^+$FasL$^-$ and CD19$^+$FasL$^+$ subsets.

FasL$^+$ B cells have higher expression of the IL-5 receptor

The CD5$^+$CD1d^{high} B cell subset is enriched for both FasL$^+$ and IL-10-producing B cells, two subsets of B cells with potential regulatory function. Knowledge of the factors responsible for the growth or function of regulatory B cells might help identify molecular pathways with therapeutic potential for further study. To identify such factors, we sorted splenic B cells from naïve DBA/1 mice into CD5$^+$CD1d^{high} and CD5$^-$CD1d^{high} subsets by FACS and subsequently used microarrays to compare global gene expression between these sorted populations. These data are available from the NCBI GEO depository (Accession number GSE46245). Among the most differentially-expressed genes were those encoding two components of the IL-5 receptor: the alpha chain (*Il5ra)* and the beta chain *(Csf2rb*). Both genes were expressed at greater than three-fold higher levels in CD5$^+$CD1d^{high} B cells (data not shown). We therefore assessed surface expression of the alpha chain of the IL-5R (CD125) on FasL$^+$ and FasL$^-$ B cells by flow cytometry and observed that FasL$^+$ B cells displayed higher CD125 expression than FasL$^-$ B cells (Figures 2-7A and 2-7B). With a nearly two-fold difference in median fluorescence intensity, CD125 was the most differentially-expressed surface marker between FasL$^+$ and FasL$^-$ B cells of all tested in this study (Figure 2-7C).

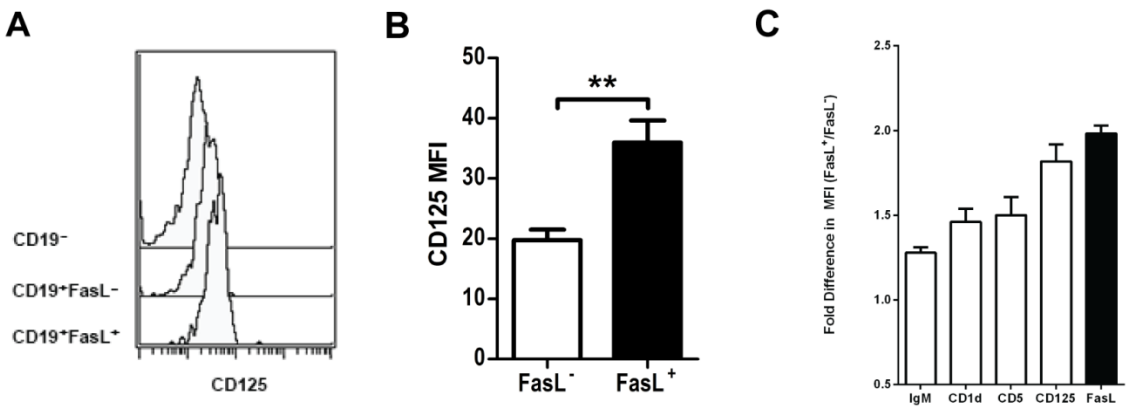

Figure 2-7: FasL⁺ B cells express higher levels of the IL-5 receptor than FasL⁻ B cells.
(**A**) Histograms comparing surface expression of CD125 in CD19⁻, CD19⁺FasL⁻ and CD19⁺FasL⁺ cells. (**B**) Quantification of median fluorescence intensity of CD125 expression in CD19⁺FasL⁻ and CD19⁺FasL⁺ B cells in replicate animals (mean ± SEM). (**C**) Fold difference in surface expression for B cell markers between CD19⁺FasL⁺ and CD19⁺FasL⁻ B cells (mean ± SEM). The data for FasL (black bar) were obtained using a second anti-FasL antibody conjugated to a different fluorochrome than that used to select CD19⁺FasL⁺ cells. Data are representative of more than three independent experiments $^{**}p<0.01$

IL-5 and CD40L stimulation expands B cells enriched for FasL expression

As FasL$^+$ B cells showed higher expression of the IL-5 receptor, we hypothesized that IL-5 might be important for the survival or growth of these cells and therefore set out to test the effects of IL-5 on B cells *in vitro*. B cell survival is generally low *in vitro*, however, and as a result several techniques have been developed to improve the viability and yield of B cells in culture. Most of these techniques involve stimulation of B cells, either with mitogenic stimuli such as LPS, or with co-stimulatory molecules such as CD40 ligand (CD40L). Interpretation of experiments performed using these stimuli must be careful to take their effects into consideration; on the other hand, however, experiments performed in their absence have caveats as well, as B cells cultured without stimulation have low viability and die quickly in culture. We chose to evaluate the effects of IL-5 and other cytokines on B cells stimulated with CD40L as this is a physiologically relevant stimulus that B cells are likely to encounter *in vivo*. To provide this stimulation, we adapted a technique used for the maintenance of human B cells for culture of murine B cells. As outlined in Figure 2-8A, CD19$^+$ B cells from naïve mice were isolated and cultured with a monolayer of irradiated NIH-3T3 fibroblasts stably expressing a vector encoding murine CD40 ligand (CD40L Fb). Culturing CD19$^+$ B cells on CD40L Fb allowed for maintenance of B cell cultures for up to 14 days and was dependent upon CD40L expression, as

control fibroblasts without the CD40L expression vector did not increase B cell survival above that of B cells cultured in medium alone (data not shown).

Using this culture system, we investigated the effects of adding recombinant murine IL-5 to cultures of $CD19^+$ B cells. As seen in Figure 2-8B, the addition of IL-5 to $CD19^+$ B cells cultured with CD40L Fb led to a dose-dependent increase in the number of B cells recovered after five days. At high concentrations of IL-5 (100 ng/mL), more B cells were collected after five days in culture than had been added, demonstrating that IL-5-induced proliferation of B cells. We confirmed increased proliferation in B cells treated with IL-5 by assessing ^{3}H-thymidine incorporation after 5 days in culture (Figure 2-8C). Therefore, IL-5 promotes the growth and proliferation of B cells stimulated with CD40L.

Next, we compared B cells cultured with IL-5 and CD40L to freshly-isolated $CD19^+$ B cells for surface expression of FasL and markers associated with $FasL^+$ B cells *in vivo*. B cells stimulated with IL-5 and CD40L showed higher surface expression of FasL, CD5, and CD1d (Figure 2-9A and 2-9B), and were significantly enriched for $FasL^+$ B cells (Figure 2-9C). Immunoblotting for FasL confirmed this result, as IL-5-stimulated B cells had readily-detectable FasL protein whereas FasL was not detectable in an equivalent number of freshly-isolated $CD19^+$ B cells (Figure 2-9D). Interestingly, the small residual population of living B cells remaining after five days of culture

with CD40L Fb in the absence of IL-5 also displayed increased surface expression of FasL, CD5, and CD1d relative to freshly-isolated B cells (Figure 2-9B).

Finally, we sought to assess the independent effects of CD40L and IL-5 on FasL expression in B cells. As primary $CD19^{+}$ B cells survive poorly in the absence of CD40L stimulation, we instead used a mouse B cell-derived hybridoma cell line that grows independently of CD40 stimulation. By immunoblot, we found that IL-5 alone led to an increase in FasL expression, as did the co-culture of B cells with CD40L Fbs (Figure 2-10). Stimulation with both CD40L and IL-5 led to a cooperative effect, as indicated by increased FasL protein relative to either stimulation condition individually (Figure 5F). From these data, we conclude that IL-5 is a growth factor for FasL+ B cells and that both IL-5 and CD40L independently up regulate levels of FasL protein in B cells.

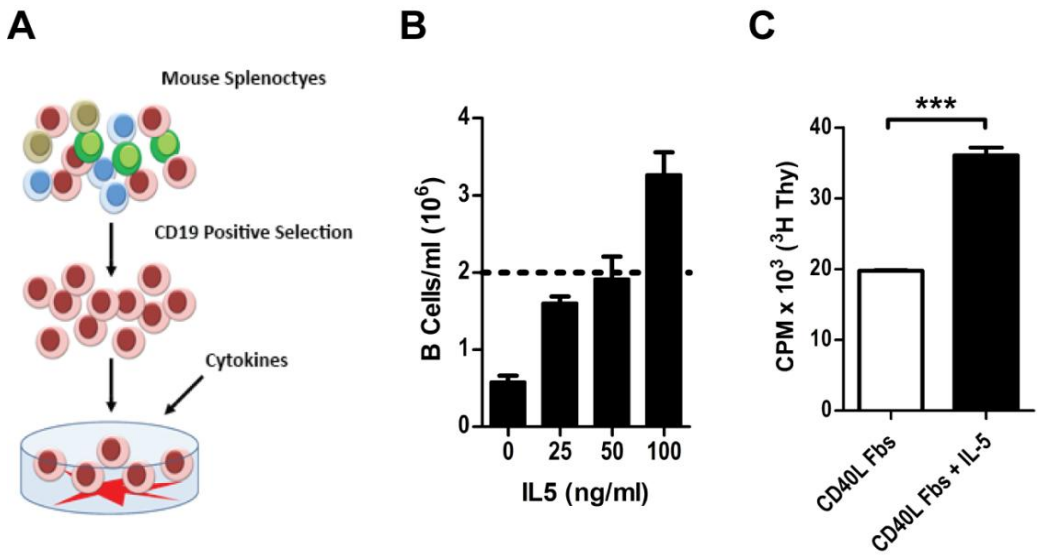

Figure 2-8: IL-5 and CD40L stimulation induce B cell proliferation.
(**A**) Diagram of B cell co-culture experiments with CD40L-expressing fibroblasts for study of the effects of IL-5 on B cell growth and function. (**B**) $CD19^+$ B cells were cultured as illustrated in (A) and harvested after five days. Viable B cells recovered from culture were quantified from replicate samples using a hemocytometer and trypan blue exclusion. The dotted line represents the concentration of B cells at the beginning of the experiment. (**C**) Proliferation of B cells cultured with CD40L-expressing fibroblasts in the presence or absence of IL-5 was assessed by the incorporation of ^{3}H-thymidine.

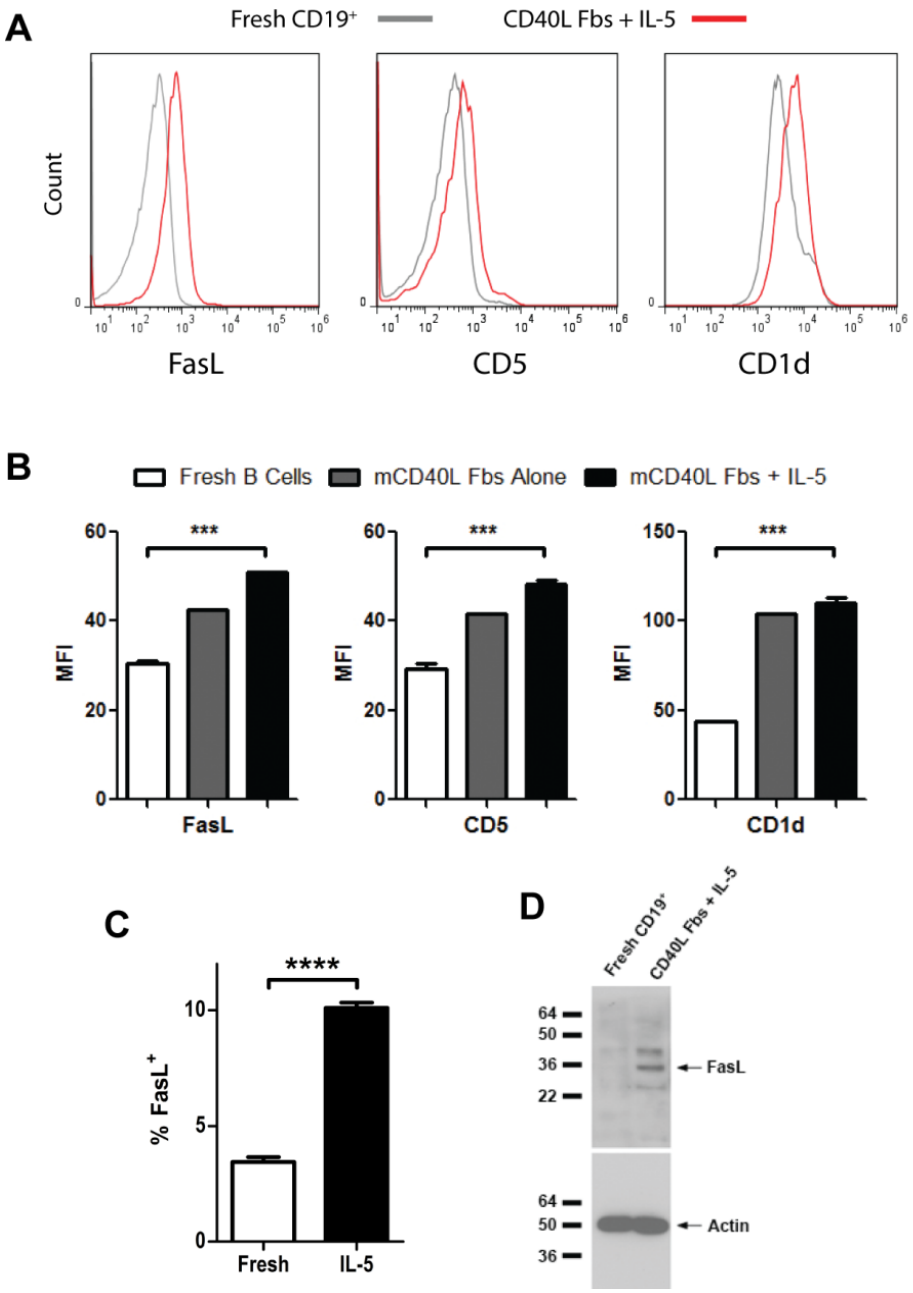

Figure 2-9: B cells stimulated with IL-5 and CD40L express higher levels of FasL, CD5, and CD1d.

(**A**) B cells stimulated with CD40L in the presence or absence of IL-5 were stained for FasL, CD5, and CD1d and compared with freshly-isolated $CD19^+$ B cells and B cells. (**B**) Median fluorescence intensity of surface markers stained as in (A) on freshly-isolated $CD19^+$ B cells, B cells stimulated with CD40L, and B cells stimulated with CD40L and IL-5. (**C**) The frequency of $FasL^+$ B cells among freshly-isolated $CD19^+$ B cells and B cells stimulated with CD40L and IL-5 was measured by flow cytometry as in (D). (**D**) Cell lysates from equal numbers of freshly-isolated $CD19^+$ B cells and IL-5-stimulated B cells were probed by immunoblot for FasL and β-Actin. Data are representative of more than four independent experiments. ***p<0.001, ****p<0.0001

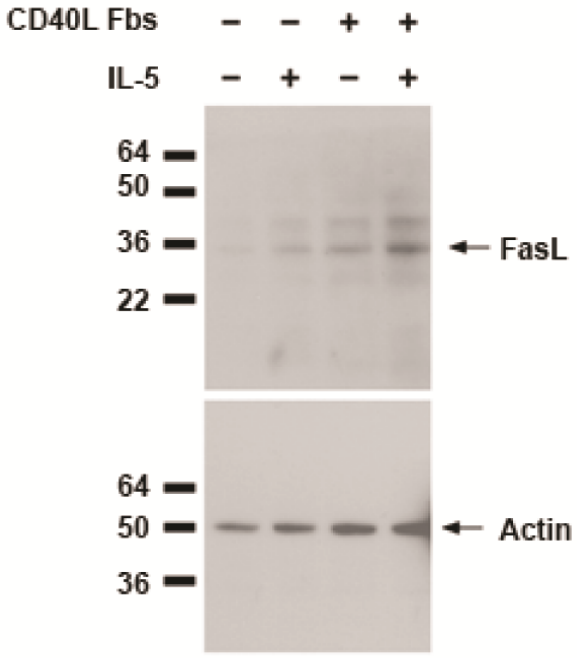

Figure 2-10: CD40L and IL-5 have additive effects on FasL levels in a B cell-derived cell line.
The murine B cell-derived hybridoma cell line CIIC1 was cultured for two days with CD40L-expressing fibroblasts, IL-5 (50 ng/mL), or both CD40L-expressing fibroblasts and IL-5. Cell lysates from each condition were then probed for FasL and β-Actin proteins by immunoblot as in Figure 5G of the main text. The CIIC1 hybridoma was generated by fusing the Ag8.653 myeloma cell line with a B cell from a DBA/1 mouse immunized with chick type 2 collagen emulsified in completes Freund's adjuvant.

B cells stimulated with IL-5 and CD40L induce antigen-specific apoptosis in CD4⁺ T cells

Although several types of cells are susceptible to FasL-mediated killing, this mechanism is especially important in preventing pathologic proliferation and activation of T cells. We therefore chose $CD4^+$ T cells to serve as target cells in experiments testing the killing activity of stimulated B cells. While naïve $CD4^+$ T cells are relatively protected from FasL-mediated killing, these same cells readily undergo apoptosis in response to Fas signaling after activation. To obtain activated, FasL-susceptible $CD4^+$ T cells, splenocytes from cII-TCR transgenic (cII-TCR Tg) mice were cultured with Concanavalin A. After three days, $CD4^+$ T cells were negatively-selected from splenocyte cultures by MACS. T cells from these mice are heavily-enriched for transgenic expression of a MHC class II-restricted TCR specific for an immunodominant peptide of type-II collagen. APCs from MHCII-matched mice are able to present this peptide, and therefore the addition of this peptide to culture results in antigen-specific interactions between cII-TCR Tg T cells and APCs.

Activated $CD4^+$ T cells were co-cultured overnight (~18 hours) at a 1:1 ratio with B cells, and apoptosis in T cells was assessed by Annexin-V/propidium iodide staining. In the absence of cognate peptide, B cells stimulated with CD40L alone or in conjunction with IL-5 induced slightly higher levels of apoptosis than were induced by fresh $CD19^+$ B cells (Figures 2-11A and 2-11B). In the presence of cognate peptide, however, IL-5-stimulated B cells induced

significantly higher levels of apoptosis in $CD4^+$ T cells than either freshly-isolated $CD19^+$ B cells or B cells stimulated with CD40L alone (Figures 2-11A and 2-11B). Therefore, B cells stimulated with IL-5 and CD40L display potent antigen-specific killing activity. B cells stimulated with CD40L alone also induced antigen-specific apoptosis in T cells, but had significantly weaker killing activity relative to that of B cells stimulated with IL-5 and CD40L (Figures 2-11A and 2-11B).

To test the role of B cell-derived FasL in this killing activity, we pre-treated B cells for one hour with an anti-FasL blocking antibody or isotype control antibody prior to co-culture with $CD4^+$ T cells. Blocking FasL on B cells significantly inhibited the antigen-specific killing function of CD40L–stimulated and IL-5/CD40L-stimulated B cells (Figures 2-11A and 2-11B). As activated $CD4^+$ T cells can express FasL, it is important to evaluate the extent of FasL-mediated "fratricide" between $CD4^+$ T cells in these experiments. The level of apoptosis in T cells cultured in the absence of B cells was not above baseline, and the presence of anti-FasL antibody for the duration of the experiment did not reduce apoptosis (Figures 2-11A and 2-11B).

Taken together, these results demonstrate that B cells stimulated with CD40L can induce antigen-specific and FasL-dependent cell death in cognate T cells, and that IL-5 strongly enhances this killer B cell activity.

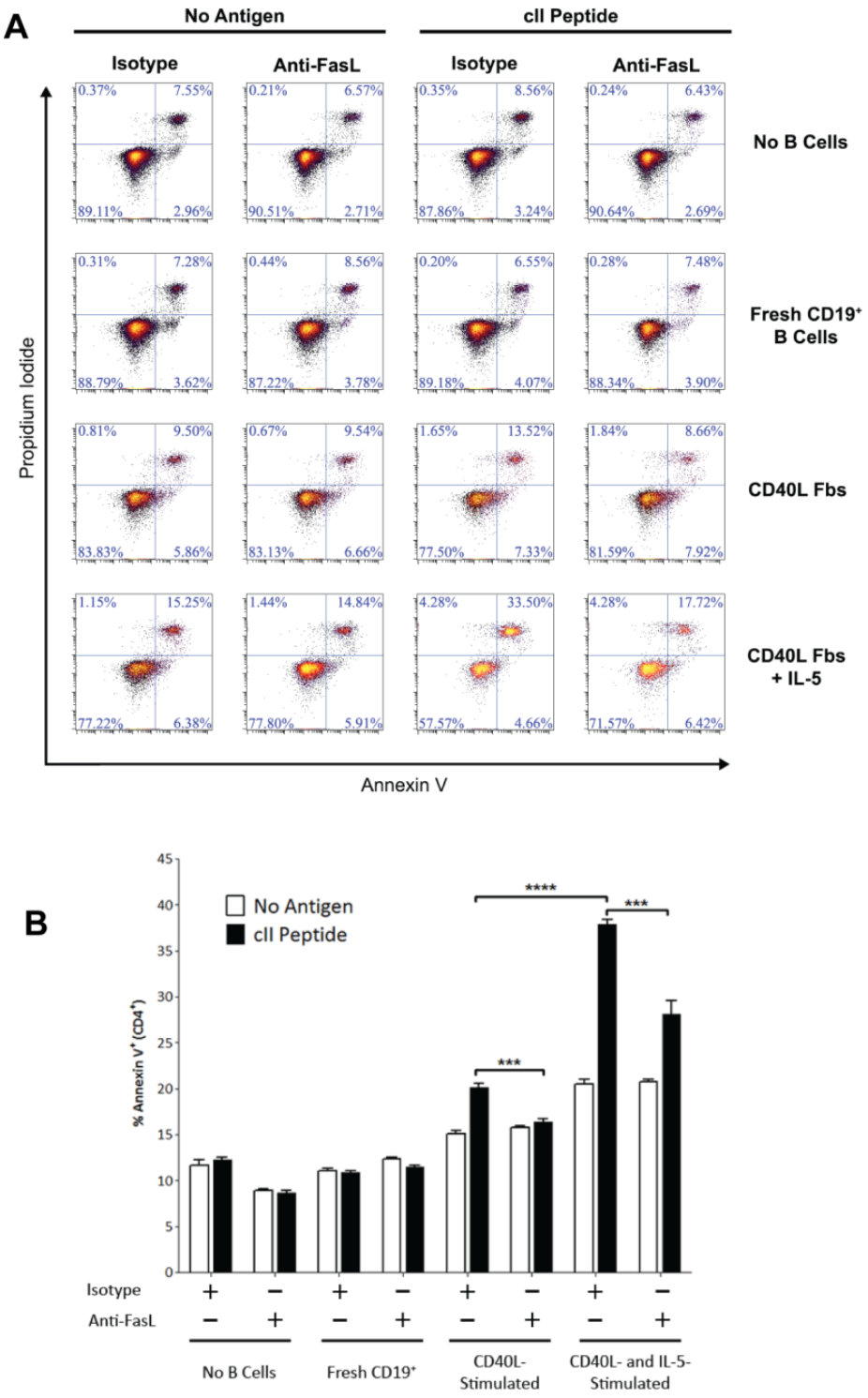

Figure 2-11: B cells stimulated with IL-5 and CD40L induce antigen-specific apoptosis in CD4⁺ T cells.

Splenocytes were isolated from naive cII-TCR transgenic mice and stimulated *in vitro* with Concanavalin A. After three days in culture, CD4⁺ T cells were negatively-selected by MACS and co-cultured with freshly-isolated CD19⁺ splenic B cells or stimulated B cells at a 1:1 ratio.

(*legend continued on next page*)

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Figure 2-11: B cells stimulated with IL-5 and CD40L induce antigen-specific apoptosis in $CD4^+$ T cells.

B cells were incubated with anti-FasL blocking antibody or an isotype control antibody for 1 hour and washed with PBS prior to co-culture with $CD4^+$ T cells. In wells with no B cells, anti-FasL antibody or isotype control antibody were directly added to culture wells to assess FasL-mediated "fratricide" between $CD4^+$ T cells. Apoptosis was measured at 18 hours by flow cytometry as the percentage of $CD4^+$cells that were positive for Annexin V staining. (**A**) Representative plots of gated $CD4^+$ T cells for Annexin V and propidium iodide following co-culture with B cells. (**B**) Quantification (mean ± SEM) from a single experiment as described in (A). Data are representative of four independent experiments. ***p<0.001 and ****p<0.0001

IL-5 enhances secretion of IL-10 in CD40L-stimulated B cells

Our initial microarray results demonstrated that expression of the IL-5 receptor was higher in $CD5^{+}CD1d^{high}$ B cells, a population which is enriched for both $FasL^{+}$ B cells and IL-10-producing B cells. As IL-5 enhanced FasL-mediated killing by B cells, we therefore hypothesized it might also enhance IL-10 secretion. Using the same system outlined in Figure 2-8A, we cultured B cells with CD40L Fb in the presence or absence of IL-5 for five days. B cells were then harvested, washed, and re-cultured at equal concentrations along with freshly-isolated $CD19^{+}$ B cells. After 24 hours, culture supernatants were collected and IL-10 secretion was measured by ELISA. As seen in Figure 2-12A, only B cells stimulated with IL-5 and CD40L produced detectable amounts of IL-10, whereas IL-10 secretion was not detectable under these conditions from B cells stimulated by CD40L alone or from freshly-isolated B cells. In response to stimulation with PMA and ionomycin, B cells stimulated with CD40L alone secreted IL-10, and this secretion was greatly enhanced by the addition of IL-5 (Figure 2-12B). IL-5 therefore enhances secretion of IL-10 in B cells.

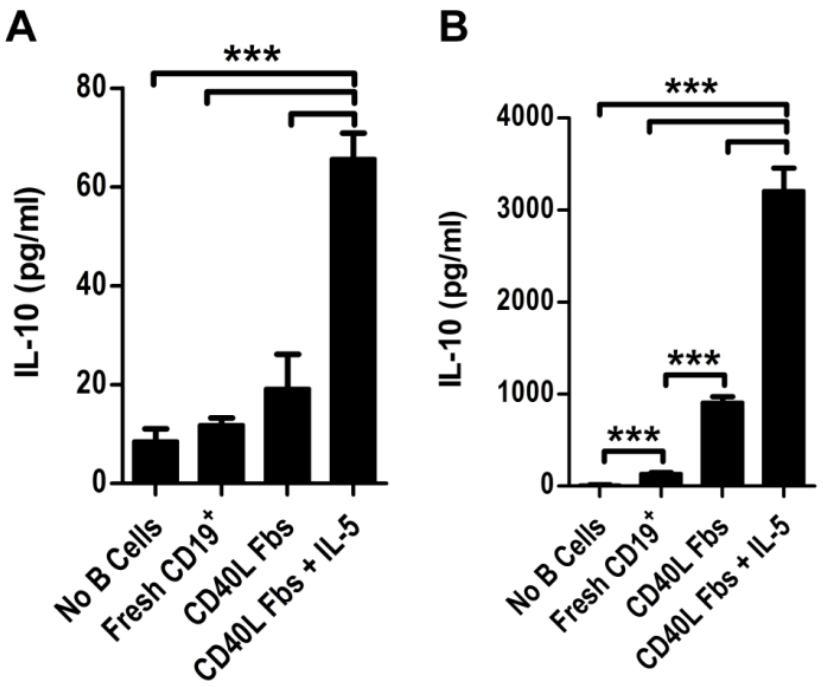


Figure 2-12: IL-5 enhances secretion of IL-10 in CD40L-stimulated B cells.
$CD19^+$ B cells were isolated by MACS from naïve mice and cultured for five days with CD40L-expressing fibroblasts in the presence or absence of IL-5. B cells were then harvested, washed in PBS, and cultured overnight at equivalent cell concentrations in the presence or absence of stimulation with PMA and ionomycin. IL-10 in the culture supernatants was measured by ELISA. (**A**) IL-10 secretion from B cells after overnight culture with no further stimulation. (**B**) IL-10 secretion from B cells after overnight culture with PMA and ionomycin. (mean ± SEM). ***p<0.001

IL-5-mediated induction of killer B cell function, but not FasL expression, is inhibited by IL-4

IL-5 is most closely-associated with type-2 immunity, a stereotyped immune response that coordinates the action of many cell types in response to large multicellular pathogens such as parasitic worms. Previous work had shown that $FasL^+$ B cells were more frequent in mice infected with the parasitic worm *S. mansoni* and those with chronic asthma, two conditions characterized by high levels of type-2 cytokines. We therefore hypothesized that IL-4, the hallmark cytokine of the type-2 response, might have an enhancing effect on the IL-5-mediated killer B cell program.

To test this, we assessed the apoptosis-inducing capacity of B cells cultured with CD40L Fb and IL-4, IL-5, or both cytokines simultaneously as in Figure 2-8A. B cells stimulated with IL-4 and CD40L did not display any detectable killing activity, in contrast to the measurable antigen-specific apoptosis induced by CD40L stimulation alone and the robust killing displayed by B cells stimulated with IL-5 and CD40L Fb. (Figure 2-13A). When both IL-4 and IL-5 were present in B cell cultures, the resulting cells again displayed no detectable killing activity (Figure 2-13A). These data therefore suggest that, rather than enhancing the effects of IL-5, IL-4 instead strongly inhibited the killing function induced by IL-5 in B cells.

The simplest mechanistic explanation for this inhibition of killing activity would be a reduction in FasL expression in B cells stimulated with IL-4. Somewhat unexpectedly, we

instead found essentially equivalent amounts of FasL protein (on a per cell basis) in all B cells stimulated with CD40L, regardless of the addition of cytokines (Figure 2-13B). Therefore, IL-4 must inhibit killer B cell activity through a mechanism other than a reduction in FasL expression.

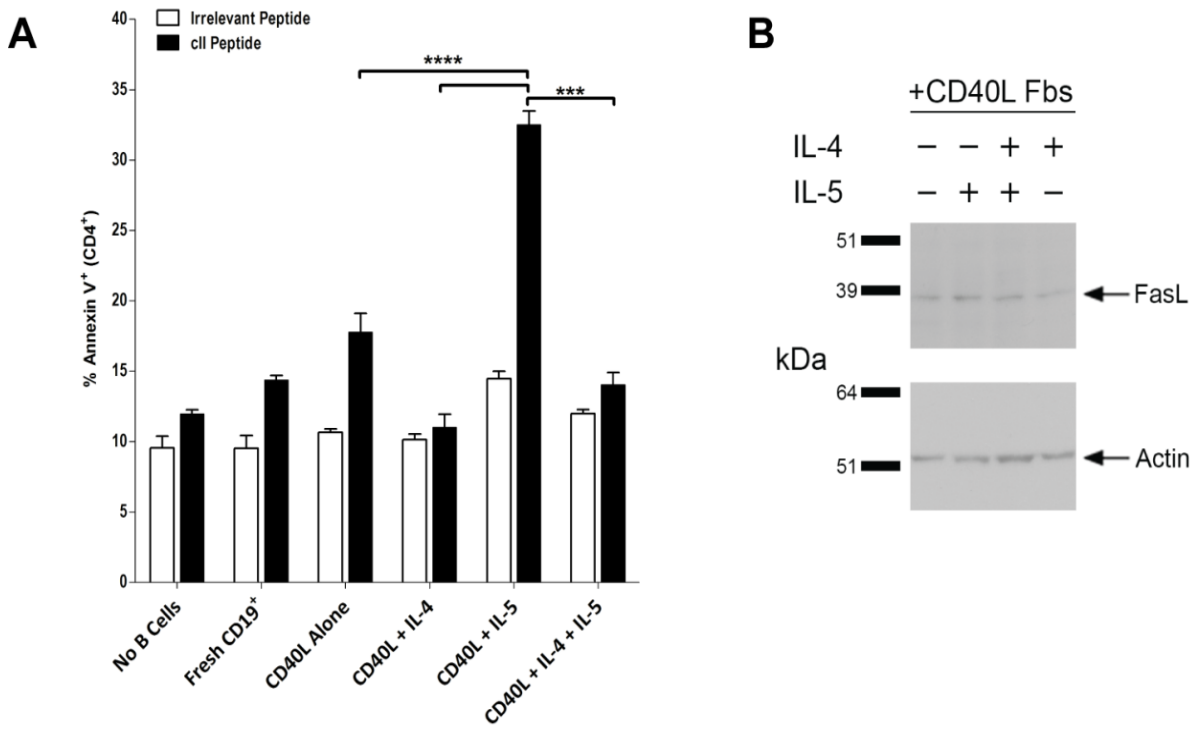


Figure 2-13: IL-5-mediated induction of killer B cell function, but not FasL expression, is inhibited by IL-4.

$CD19^+$ B cells from naïve mice were cultured for five days with IL-4, IL-5, or both cytokines concurrently. (**A**) The ability of B cells stimulated under these conditions to induce apoptosis in activated $CD4^+$ T cells was then assessed as in Figure 6. Apoptotic cells were identified and enumerated (mean ± SEM) on the basis of positive staining for Annexin V by flow cytometry. (**B**) B cells were cultured with CD40L-expressing fibroblasts in the presence or absence of IL-4 and IL-5, or in the presence of both cytokines. Cells were harvested after 5 days in culture and cell lysates were probed for FasL and B-Actin proteins as in Figure 5G. ***p<0.001 and ****p<0.0001

IL-4 dominantly inhibits IL-5-mediated enhancement of IL-10 secretion B cells

The ability of IL-4 to inhibit IL-5-induced killer B cell activity prompted us to assess its effect on IL-5-induced IL-10 secretion. To this end, B cells were cultured with CD40L Fb and increasing concentrations of IL-5 in the presence or absence of IL-4. B cells were harvested after five days in culture, washed, counted, and re-cultured at equivalent cell concentrations. After 18 hours, culture supernatants were collected and IL-10 concentration was assessed by ELISA as in Figure 2-11. As we saw previously, the addition of IL-5 to B cells stimulated with CD40L enhanced secretion of IL-10 (Figure 2-14). In the presence of IL-4, however, no enhancement of IL-10 secretion was mediated by IL-5 (Figure 2-14). This inhibition appears quite robust, as low concentrations of IL-4 (10 ng/mL) were able to inhibit the effects of high concentrations of IL-5 (up to 80 ng/mL).

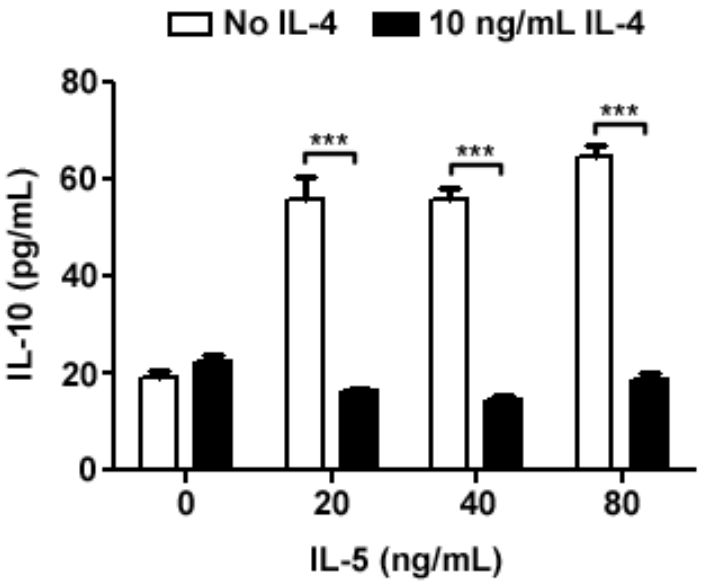


Figure 2-14: IL-4 dominantly inhibits IL-5-mediated enhancement of IL-10 secretion B cells. $CD19^+$ B cells were isolated by MACS from naïve mice and cultured for five days with CD40L-expressing fibroblasts with increasing concentrations of IL-5 in the presence or absence of 10 ng/mL IL-4. B cells were then harvested, washed in PBS, and cultured overnight at equivalent cell concentrations. IL-10 in the culture supernatants was measured by ELISA. Data are representative of more than 5 individual experiments.

2.4 Discussion

The Surface Phentoype of FasL$^+$ B Cells

The Fas/FasL signaling axis is essential for maintaining self-tolerance, and therefore cells expressing high levels of FasL are likely to be critical for this function. Among splenocytes in naïve mice, B cells are the predominant FasL$^+$ cell population. FasL$^+$ APCs have demonstrated efficacy in establishing tolerance upon adoptive transfer, as both endogenous unaltered B cells and APCs engineered to express FasL can induce antigen-specific tolerance [151, 176, 177]. In light of these studies, further examination of endogenous FasL$^+$ APCs is warranted to understand their importance in maintaining immune homeostasis and self-tolerance.

The surface phenotype of FasL$^+$ B cells bears some similarity to that reported for the B10 subset of IL-10-producing B cells [131]. Specifically, FasL$^+$ B cells are moderately enriched in the CD5$^+$CD1d^{high} B cell subset, but only a minority of FasL$^+$ B cells is found within this B cell subset. IL-10-producing B cells are also enriched among CD5$^+$CD1d^{high} B cells, but only ~25% of IL-10-producing B cells reside in the CD5$^+$CD1d^{high} subset [131, 132]. Despite their shared enrichment in the CD5$^+$CD1d^{high} B cell subset, IL-10-producing cells are not more frequent among FasL$^+$ B cells than FasL$^-$ B cells. The ~4% of FasL$^+$ B cells that do secrete IL-10, however, might represent a multi-potent population of killer/regulatory B cells. In naïve mice, these multi-potent suppressor B cells are extremely rare, making up <0.1% of all splenic B cells (~2% of B cells are

FasL$^+$, and ~4% of FasL$^+$ B cells produce IL-10 after *ex vivo* stimulation). Although FasL$^+$IL-10$^+$ B cells might be more frequent in animals mounting an active immune response, their extreme paucity in naïve mice has made further characterizing these cells technically difficult.

FasL$^+$ B cells also share some phenotypic characteristics with B-1a cells (CD5$^+$IgM^{high}) and MZ B cells (CD1d^{high}). These innate-like B cell subsets share functional similarities [22, 37], and arise from a common precursor population distinct from that of follicular B-2 cells [20, 178]. As discussed earlier, innate-like B cells are the main source of natural anti-inflammatory IgM antibodies that help maintain immune homeostasis in a variety of ways [113, 115, 179]. Currently, only circumstantial evidence supports a link between regulatory B cells and innate-like B cells, and more study is required to determine the relationship between these cell types.

IL-5 is a Growth Factor for Regulatory B Cells

Increasing the frequency or activity of regulatory immune cell populations is an as-yet unrealized potential therapeutic strategy for combating inflammatory conditions such as autoimmune diseases. The initial knowledge required to develop a therapy using such a strategy is a thorough understanding of the factors important for the growth or function of a given regulatory cell population. The work presented in this chapter demonstrates that B cells stimulated with IL-5 secrete the anti-inflammatory cytokine IL-10 and can induce FasL-mediated apoptosis in CD4$^+$ T cells. This finding is consistent with previous studies

demonstrating that FasL$^+$ B cells were increased in mice infected with *S. mansoni* and in a mouse model of chronic allergen-induced asthma, two conditions characterized by an increase in T cell activation and production of IL-5 and other type 2 cytokines [146, 180].

It is important to note that the effects of IL-5 on the immune system are pleiotropic, as both pro- and anti-inflammatory effects have been reported. IL-5 has a role in generating optimal antibody responses, as mice in which B cells are unable to respond to IL-5 signaling are defective in antibody-secreting plasma cell differentiation and have reduced levels of circulating antibodies [181]. Accordingly, over-expression of IL-5 results in increased antibody levels and hypereosinophilia, and mice deficient in IL-5 receptor signaling have reduced levels of antibodies [156, 182]. In contrast, IL-5 is a critical survival factor for peritoneal B-1 cells, and administration of IL-5 leads to an increase in secretion of anti-inflammatory natural antibodies by B-1 cells [117, 183]. In a rat model of multiple sclerosis, IL-5 administration had therapeutic effects, acting at least in part by inducing FoxP3$^+$ regulatory T cells [184]. Further work is needed in examining the role of IL-5 in autoimmunity, however, as mice deficient in IL-5 appeared to have a similar clinical course of EAE [185]. None-the-less, because of its ability to induce at least three mechanisms of immune suppression in B cells, the IL-5/IL-5 receptor axis makes for an intriguing target for therapeutic intervention in pathogenic inflammatory conditions.

Although the *in vivo* target(s) of FasL$^+$ B cells remains to be determined, we show here that IL-5 stimulated FasL$^+$ B cells induce apoptosis in activated CD4$^+$ T cells. This killing function was greatly increased upon the addition of antigen, suggesting that FasL$^+$ B cells preferentially target T cells based upon antigen specificity. The potency of this killing effect is especially noteworthy, as an effector-to-target ratio 10-20 fold lower than usually required to measure cytotoxic activity resulted in significant levels of apoptosis in CD4$^+$ T cells. Negative selection is thought to eliminate T cells which respond to self-antigens, however some autoreactive T cells are none-the-less present among peripheral T cells [186]. As innate-like B cells frequently possess self-reactive BCRs, the antigens presented by FasL$^+$ B cells would be expected to be enriched for self-antigens due to BCR-mediated endocytosis and processing of host-derived molecules [22]. FasL$^+$ B cells may therefore play a role in maintaining self-tolerance by eliminating CD4$^+$ T cells recognizing self-antigens that escape negative selection in the thymus. While it has been reported that mice with a targeted deletion of the *Fasl* gene in B cells show symptoms of autoimmunity, further work is needed to evaluate the importance of this mechanism in animal models of autoimmunity [148].

IL-4 Dominantly Inhibits IL-5-induced Regulatory Mechanisms in B Cells

While B cells treated with IL-5 were enriched for B cells with regulatory functions, this enrichment was completely blocked by the addition of IL-4. Intriguingly, despite the vast

difference in their killing activity there is no consistent difference in FasL protein levels (on a per cell basis) between B cells stimulated with IL-5 and CD40L and those stimulated with IL-4, IL-5, and CD40L (Figure 2-12B). Thus, IL-4 must inhibit the FasL-mediated killing function induced by IL-5 through other mechanisms. These data are in agreement with previous findings regarding induction of $FasL^+$ B cells by IL-4 and IL-10 in the schistosome granuloma model [146]. While perhaps unexpected, this result is consistent with what is known regarding the complex and unusual regulation of FasL. Killer cells such as cytotoxic T cells and NK cells keep most FasL protein sequestered in the secretory lysosome, an endosomal-like intracellular compartment [28, 156]. FasL is then moved to the cell surface in response to signals received upon encounter with an appropriate target cell. There are several nodes of regulation in this process upon which IL-4 may act, such as preventing the phosphorylation events required for the transport of FasL to the secretory lysosome, inhibiting the movement of the secretory lysosome to the cell surface, or reducing the signals received from target cells that result in mobilization of secretory lysosome granules to the cell surface [165]. This result also suggests a prominent role for CD40 signaling in FasL induction in B cells, as even the small population of B cells that survived with only CD40L stimulation expressed FasL and had measurable FasL-mediated killer function. Similar effects of CD40 ligation on FasL expression have been noted in mouse Langerhans cells, human hepatocytes and ovarian cancer cells [187-189]. Additionally,

CD40 signaling results in down-regulation of BCL6, a transcription factor that competes with STAT3 for DNA binding sites [187, 188]. CD40 signaling therefore enhances STAT3 binding and facilitates the downstream effects of cytokines that signal through STAT3 by reducing levels of BCL6. The IL-5 receptor has been shown to activate STAT5, a transcription factor which shares many binding sites with STAT3 throughout the genome [165]. Therefore, it is possible that CD40 signaling acts in synergy with IL-5-receptor signaling to induce a regulatory phenotype in B cells. Using a B cell-derived cell line, we found that both CD40L and IL-5 stimulation individually induced FasL expression in an additive manner, suggesting that these two pathways might work in concert to increase FasL expression in B cells (Figure 2-13).

It has been suggested that, rather than existing as a homogenous population, regulatory B cells can arise from both B-1 and B-2 cells [190]. In this case, it is likely that different growth factors would differentially act on regulatory B cells from these different lineages. Recently the cytokine IL-21 was identified as a growth factor for IL-10-producing B cells using a similar culture system as we have used in this study [191]. As IL-21 is associated with the growth of follicular B cells, it is possible that IL-21 drives the growth of B-2-derived regulatory B cells, while IL-5 induces the growth of B-1-derived regulatory B cells. Given their differing niches, regulatory B cells derived from B-2 cells might be more effective in inhibiting autoimmunity in the peripheral lymphoid organs, whereas B-1-derived regulatory cells might be more effective

at mucosal sites. More work is required, however, to clarify the differences and similarities of regulatory B cells stimulated by IL-21 or IL-5, as these two types of regulatory B cell might also target different cells *in vivo*.

As B cells stimulated with IL-5 and CD40L secrete IL-10 and are potent inducers of apoptosis in activated T cells, we have made several attempts to assess their immunosuppressive function *in vivo* by adoptive transfer in a collagen-induced arthritis model. Our results thus far have failed to clearly define an effect for these transferred cells, as mice receiving untreated B cells or PBS have a similar disease course as those receiving IL-5-stimulated B cells (data not shown). B cells stimulated with IL-5 and CD40L, although enriched for B cells with regulatory function, still display heterogeneity. Given the reported pleiotropic effects of IL-5 on B cells outlined above, it is possible that IL-5 and CD40L stimulation results in expansion of a population of cells that is enriched for regulatory B cells but not exclusively immunosuppressive in nature. Recently-published data reporting the strong effects of IL-21 on regulatory B cell growth showed a similar outcome, as *in vitro* treatment of $CD19^{+}$ B cells with IL-21 led to enrichment of IL-10-producing B cells, but IL-10-producing B cells still comprised only a minority of B cells in culture [191]. *In vivo* suppression of autoimmunity was only demonstrated by IL-21-stimulated B cells that had been sorted from a heterogeneous population after culture. It is also possible that, as mentioned above, B-1-derived regulatory B

cells exert their immunosuppressive effects at locations distinct from those of B-2-derived regulatory B cells, and therefore the function of these cells would be better assessed in a different model system. Further work is therefore warranted to definitively demonstrate a role for IL-5 in the generation of immunosuppressive B cells *in vivo*, and this is an important caveat to this study.

In summary, the data presented herein characterize the phenotype of $FasL^+$ B cells and demonstrate that $FasL^+$ B cells are enriched in the $CD5^+CD1d^{high}$ B cells subset. A portion of $FasL^+$ B cells are multi-potent as they express IL-10 in addition to FasL, but these $FasL^+IL\text{-}10^+$ B cells are extremely rare in naïve mice, representing <0.1% of all splenic B cells. Additionally, we show here for the first time that IL-5 is an inducer or enhancer of at least two immunosuppressive mechanisms in regulatory B cells, as treating splenic B cells with IL-5 and CD40L expanded a population of B cells with potent FasL-mediated T cell-killing activity, and IL-5 enhanced IL-10 secretion from CD40L-stimulated B cells. Finally, IL-4 strongly inhibited the B cell killing activity mediated by IL-5, suggesting that cross-talk between these type-2 cytokines might be important in modulating the activity of regulatory B cells (Figure 2-15). Understanding the specifics of this IL-4/IL-5 axis in regards to regulatory B cells could provide new targets for the design of therapeutic strategies for patients with autoimmune disorders or other inflammatory conditions.

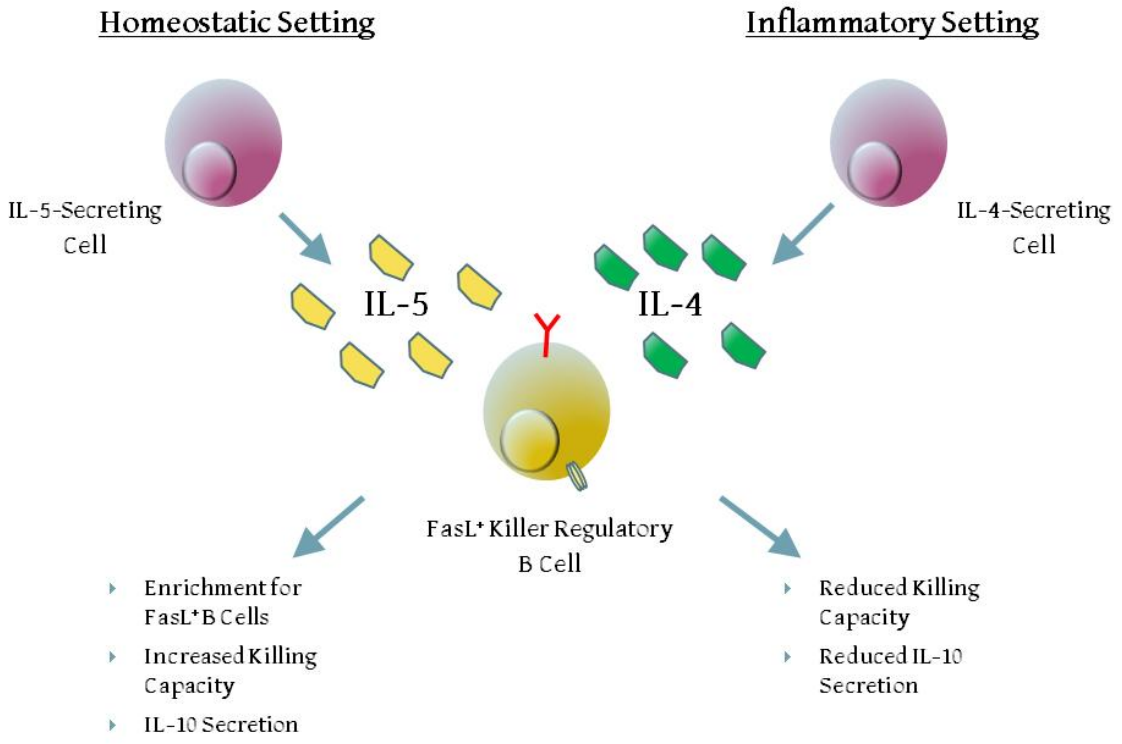


Figure 2-15: IL-4 and IL-5 have antagonistic effects on regulatory B cell function.
B cells stimulated with IL-5 are enriched for FasL+ B cells, have enhanced antigen-specific T cell killing activity, and secrete IL-10. In the presence of IL-4, these effects of IL-5 are completely eliminated.

2.5 Future Directions

What signaling pathways downstream of IL-5 are important for inducing regulatory B cell function?

The identification of IL-5 as a novel inducer of regulatory B cell function provides a new signaling axis to target with therapeutics. IL-5 binding to its receptor initiates activation of several signaling pathways (Figure 2-16). The Janus kinases (JAK) JAK1 and JAK2 associate with the receptor complex, and in turn activate the signal transducer and activator of transcription (STAT) molecules STAT1 and STAT5. Colonel Bruton's tyrosine kinase (Btk), a molecule important in the function of most B cells, is also activated by IL-5-receptor signaling. Compounds that inhibit the function of either JAKs or Btk are currently available, some of which are the subject of ongoing clinical trials. Using these compounds, it is possible to assess the respective importance of JAKs and Btk in mediating the effects of IL-5 on B cells *in vitro*. Treating animals with these compounds is feasible as well, although it is difficult to definitively demonstrate the mechanism of action in these experiments as JAKs and Btk are activated by several receptors and are present in many types of cells.

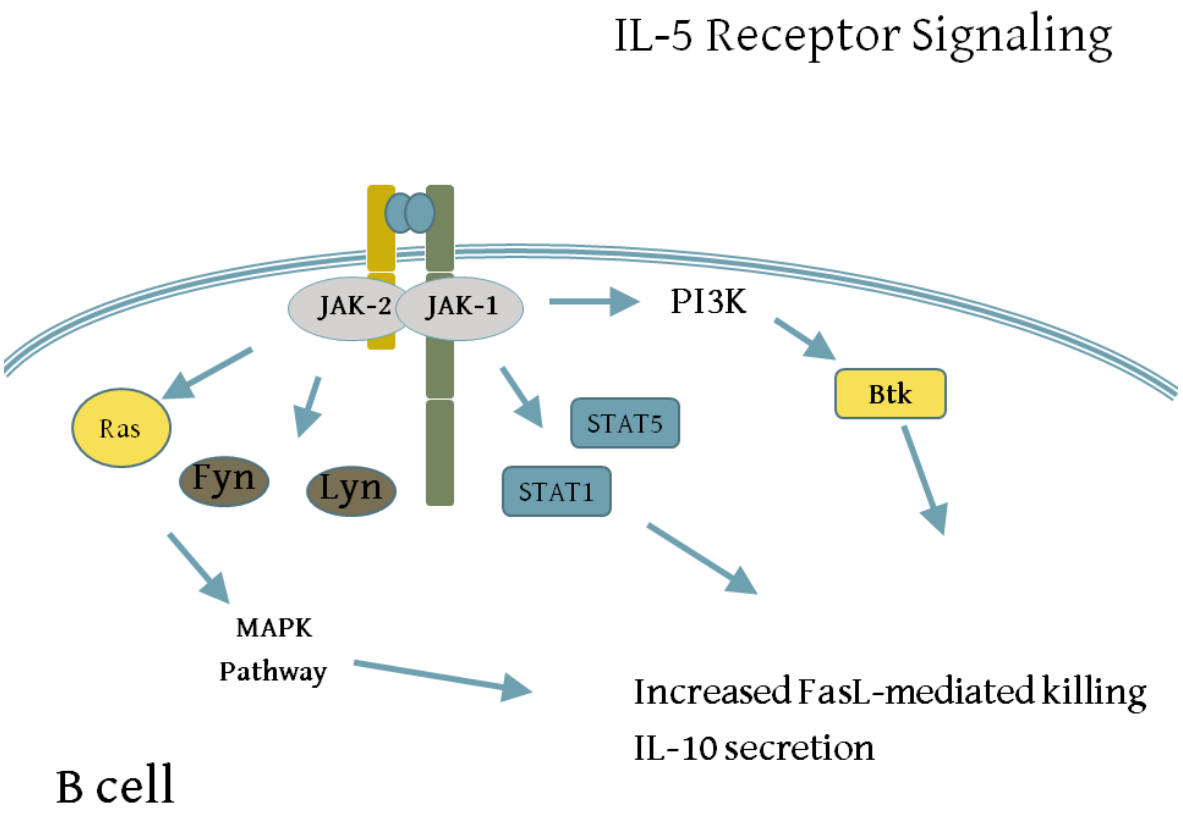


Figure 2-16: Summary of signaling molecules downstream of the IL-5-receptor.
The IL-5 receptor activates multiple signaling pathways, including a JAK-STAT pathway consisting of JAK1 and JAK2 signaling through STAT1/STAT5. Additionally, signaling through PI3K leads to the activation of Btk in response to IL-5 receptor signaling.

How does IL-4 inhibit the effects of IL-5?

The IL-5-mediated enhancement of regulatory B cell function was dominantly inhibited by IL-4, suggesting that cross-talk between these type-2 cytokines is important in modulating the activity of regulatory B cells. How IL-4 mediates this inhibition remains unknown. We found that levels of FasL in B cells treated with IL-5 alone were essentially equal on a per cell basis with those treated with both IL-5 and IL-4, despite the drastic difference in the killing activity between these cells. This suggests that IL-4 interrupts the actions of IL-5 through a mechanism unrelated to the production of FasL protein.

As mentioned above, post-transcriptional regulation is important for the proper trafficking of FasL protein. IL-4 can affect this proper trafficking at several nodes in the regulatory circuit. First IL-4 might inhibit the movement of FasL to the secretory lysosome, and therefore FasL is improperly sorted or degraded. To investigate this possibility, fluorescent microscopy can be used to determine the intracellular location of FasL in B cells treated with IL-5 alone or in conjunction with IL-4. This can be done by staining with fluorescently-labeled antibodies to FasL and markers specific for subcellular compartments (such as CD63 for the secretory lysosome), or making expression vectors for FasL fusion proteins with fluorescent labels. Once FasL reaches the cell surface, it is cleaved and inactivated by metalloproteases, and it is also possible that IL-4 up-regulates the activity of FasL-cleaving proteases. Therefore if IL-4 acts

through this mechanism, B cells stimulated with IL-4 should display better killing function in the presence of protease inhibitors. In T cells and NK cells, signals from the target cell initiate the movement of FasL from the secretory lysosome to the cell surface. While it is still unknown if a similar mechanism exists in B cells, the antigen-specificity of B cell-mediated killing suggest that interaction with the target cell can enhance killing. Therefore IL-4 could potentially inhibit the killing efficiency of B cells by inhibiting the signal received by the B cell to initiate FasL mobilization.

The relevance of this IL-4/IL-5 antagonism in vivo remains to be investigated as well. While expression of IL-4 and IL-5 is coordinately regulated in Th2 cells, other cell types secrete IL-5 and little or no IL-4. Among these cells are recently-identified innate lymphoid cells [192-194]. These cells are the major producers of IL-5 under homeostatic conditions, and while present but infrequent in the spleen, they are heavily enriched in locations where immunoregulation is essential for maintaining tolerance to innocuous environmental antigens and commensal microbes, such as lymphoid structures associated with the lung and intestine. One role for these innate IL-5-producing lymphoid cells might therefore be to help preserve immune homeostasis at these sites through IL-5-mediated induction of regulatory B cell functions. It has also been reported that as murine Th2 cells are activated over time they transition from producing both IL-4 and IL-5 to producing exclusively IL-5 [195]. This is consistent with our

previous finding that mice chronically infected with *S. mansoni* have increased numbers of FasL$^+$ B cells [145], and suggests a model in which the return to homeostasis at the cessation of type 2 responses includes IL-5-mediated induction of regulatory B cells.

Are B cells stimulated with IL-5 heterogeneous?

It still remains to be demonstrated that B cells stimulated with IL-5 are indeed regulatory *in vivo*, as our experiments in the collagen-induced arthritis model were inconclusive. The outcomes of adoptive transfer experiments can be affected by many variables, and this is true for even well-established regulatory populations such as natural regulatory T cells (nTregs). While nTregs can prevent collagen-induced arthritis (CIA) in DBA/1 mice, they fail to do so in another model of adjuvant-induced arthritis in BALB/c mice [2,3]. Additionally, adoptive transfer of nTregs can prevent CIA if transferred before disease onset, but no suppression is observed if the transfer occurs after disease has been established [4]. Therefore, although adoptive transfer experiments are sometimes capable of establishing the regulatory function of a cell population, a negative result in a single disease model does not preclude a suppressive function.

The apparent lack immunosuppressive function *in vivo* may be in part the result of heterogeneity among IL-5-stimulated B cells. As outlined above, there is good evidence that IL-5 has both pro- and anti-inflammatory effects on B cells, and therefore stimulation with IL-5 is

likely to result in a population of cells that, while *enriched* for regulatory B cells, are not *exclusively* immunosuppressive in nature. Recently-published data from the Tedder lab reporting the strong effects of IL-21 on regulatory B cell growth showed a similar outcome, as *in vitro* treatment of $CD19^+$ B cells with IL-21 led to enrichment of IL-10-producing B cells, but IL-10-producing B cells still comprised only a minority of B cells in culture [191]. *In vivo* suppression by B cells was only demonstrated in this study by sorting immunosuppressive B cells from the heterogeneous IL-21-stimulated B cell cultures. Whether immunosuppressive B cells can be identified among IL-5-stimulated cells by a difference in surface marker expression has not been thoroughly investigated, and remains an important outstanding question.

Chapter 3

B Cell-Derived Lymphoblastoid Cell Lines Constitutively Produce Fas Ligand and Secrete MHCII$^+$FasL$^+$ Killer Exosomes

3.1 Summary

To identify a cell line suitable for the study of FasL in B cells, we screened a panel of B cell-derived cell lines for FasL expression, including more than 20 independent lymphoblastoid cell lines (LCLs). Unexpectedly, we found that all LCLs tested for this study showed robust expression of FasL as determined by immunoblot. In contrast to endogenous murine FasL$^+$ B cells, LCLs maintained nearly all of their FasL protein intracellularly, with little or no surface FasL detectable by flow cytometry.

As FasL can be secreted on exosomes by NK cells and CTLs, we then tested for the presence of FasL in LCL-derived exosomes. By immunoblot, we confirmed that both FasL and MHCII proteins were present in exosomes secreted by LCLs. Using a bead-based exosome capture assay, we used anti-MHCII-coated polystyrene beads to capture $MHCII^+$ exosomes, and found evidence for the presence of FasL among $MHCII^+$ exosomes by flow cytometry, indicating that LCLs can produce $MHCII^+FasL^+$ exosomes.

To test the apoptosis-inducing ability of LCL-derived exosomes, we used two independent experimental approaches. First, we stimulated T cells with staphylococcal enterotoxin A (SEA) in the presence of exosomes, and assessed apoptosis among $CD4^+$ T cells. In the presence of SEA, exosomes derived from an autologous LCL induced significant levels of apoptosis. Secondly, we stimulated PBMCs from another donor with an immunodominant peptide of tetanus toxoid (TT). After activation and enrichment for T cells reacting to TT peptide, we isolated $CD4^+$ T cells and cultured them overnight with autologous LCLs in the presence or absence of TT peptide. As expected, we found that exosomes loaded with TT peptide were able to induce significant levels of apoptosis among activated $CD4^+CD62L^-$ T cells, and this killing was partially blocked by the addition of an anti-FasL blocking antibody.

Finally, we attempted to isolate B cell-derived exosomes from the spleens of mice. To do so, we used antibody-coated polystyrene beads to bind exosomes with B cell-specific markers and

stained them for FasL. We found that FasL was present on exosomes bound by anti-IgM-coated beads, suggesting that $IgM^{+}FasL^{+}$ exosomes are present in the mouse spleen.

3.2 Methods

Preparation of Peripheral Blood Mononuclear Cells: All donors provided informed consent prior to their participation in this study. Blood was obtained by venipuncture and collected into syringes containing sodium heparin. Following a 1:1 dilution with un-supplemented RPMI 1640, blood was gently layered onto Histopaque-1077 (Sigma Aldrich) in 50 mL centrifuge tubes. Buffy coats containing peripheral blood mononuclear cells were collected from tubes following centrifugation at 1,200 x g for 30 minutes at 20°C.

Cell Lines: Lymphoblastoid cell lines were prepared according to established techniques for the transformation of B cells by Epstein-Barr virus [196]. Cell lines used were derived from either healthy donors and generated in our laboratory, or were from a collection of LCLs derived from monozygotic twin pairs discordant for rheumatoid arthritis (a kind gift from Dr. Joseph Holoshitz, University of Michigan). LCLs were maintained in RPMI 1640 media supplemented with 20% FBS, 2% L-glutamine, 1% penicillin/streptomycin, 1% non-essential amino acids, and 1% sodium pyruvate. Most cell lines were kept in culture continuously for longer than 2

months with no detectable changes in growth, viability, or experimental results. Once or twice a week, LCL cultures were split 1:3, and kept in a 37°C 5% CO_2 incubator.

Immunoblotting: LCLs and LCL-derived exosomes were lysed in SDS buffer prior to separation by SDS-PAGE and transfer to a PVDF membrane. Membranes were blocked and incubated with polyclonal rabbit anti-FasL IgG (Cell Signaling), mouse anti-HLA-DR (Abcam, clone TAL 14.1) or polyclonal rabbit anti-β-Actin (Cell Signaling). Antibody binding was detected with an anti-rabbit or anti-mouse IgG-HRP secondary antibody (Cell Signaling) and ECL reagent (Thermo Scientific).

Flow Cytometry: PE-conjugated anti-FasL and isotype control antibody was obtained from Biolegend (clone NOK-1). LCLs were incubated with anti-CD16/CD32 Fc Block (BD Biosciences) prior to staining and analyzed on a Beckman Coulter FC500 flow cytometer. For intracellular staining, LCLs were fixed for 20 minutes at room temperature in 4% PFA, washed three times with PBS, and permeabilized with 0.5% saponin buffer prior to staining with anti-FasL. For apoptosis staining, Annexin-V-FITC (eBioscience) was used to identify cells in early apoptosis and propidium iodide was used to identify dead cells. Data were analyzed using Cytobank web-based software [197] or FlowJo v7.6.5 (Tree Star, Inc.).

Exosome Isolation and Preparation From LCL Culture Supernatants: Exosome-free FBS was produced by centrifuging FBS overnight at 100,000 x g to remove any bovine-derived exosomes. Culture

supernatants from LCL cultures were spun at 500 x g for 10 minutes to remove cells, followed by a spin at 10,000 x g for at least 1 hour to remove large cellular debris and microparticles. Exosomes were obtained by centrifugation at 100,000 x g for 1-4 hours. The resulting exosome pellets were diluted once with PBS prior to another 100,000 x g centrifugation, after which pellets were re-suspended in a small volume of PBS. Protein concentration was used as a proxy measure for the amount of exosomes in a given re-suspension, and was determined by BCA assay. In some cases, supernatant from bulk cultures of LCLs were concentrated using a 100 kDa filter prior to exosome isolation by centrifugation.

Density Gradient Centrifugation: Serial dilutions of iodixanol (OptiPrep; Sigma-Aldrich) were prepared with PBS, with densities ranging from 1.03 g/mL to 1.27 g/mL. One milliliter of each density fraction was added sequentially to an ultracentrifuge tube so as to maintain a discontinuous gradient. A sample of LCL-derived exosomes in solution was placed on top of the density gradient and centrifuged at 100,000 x g for 1 hour. Layers were then removed to separate tubes and diluted in PBS, and diluted fractions were centrifuged in individual tubes overnight at 100,000 x g. Pellets from each density fraction were lysed in an equal amount of SDS buffer, and interrogated for FasL and MHCII by immunoblot.

Exosome Bead Capture Experiments: Polystyrene beads (~6.7 μm diameter) coated with streptavidin were obtained from Corpuscular or Spherotech. Beads were coated for 1 hour at

20°C with biotinylated antibodies against human HLA-DR (Biolegend, clone L243), mouse IgM (BD Biosciences, clone R6-60.2), mouse CD19 (BD Biosciences, clone 1D3), or appropriate isotype controls. After washing, antibody-coated beads were incubated for several hours with exosomes at 4°C with gentle agitation. Unbound exosomes were then washed away, and bead-bound exosomes were stained for FasL and subsequently analyzed on a Beckman Coulter FC500 flow cytometer.

Exosome-induced Apoptosis Assays (SEA): $CD4^+$ T cells were isolated from whole blood by RosetteSep Human $CD4^+$ T cell Enrichment Cocktail (Stem Cell Technologies) and stimulated for 6 days with Staphylococcal enterotoxin A (10 ng/mL; Sigma-Aldrich) in the presence or absence of exosomes from an autologous LCL. T cells were then harvested and apoptosis was assessed by Annexin-V/propidium iodide staining among $CD4^+$ T cells.

Exosome-induced Apoptosis Assays (TT peptide): PBMCs were isolated from whole blood and stimulated for 12 days with an immunodominant peptide of tetanus toxoid (10 μg/mL). This donor had received a scheduled booster vaccination against tetanus within 2 months of these experiments. $CD4^+$ T cells were separated from PBMC cultures by negative selection by MACS (Miltenyi Biotec) and cultured overnight with exosomes derived from an autologous LCL in the presence or absence of the stimulating peptide. The activity of FasL was blocked in culture by

the addition of anti-FasL antibody (Biolegend; clone NOK-1). Apoptosis was assessed in T cell by Annexin-V/propidium iodide staining among activated $CD4^{+}CD62L^{-}$ T cells.

Mice: All protocols involving animals were approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA). Mice expressing the human MHCII gene, DR4-AE_0 transgenic mice (C57BL/6 background), were obtained from our breeding colony at the University of Michigan. The presence of the transgene was confirmed by polymerase chain reaction genotyping in all animals.

3.3 Results

A high frequency of LCLs constitutively produce intracellular FasL protein

To study the regulation and trafficking of FasL in B cells, we obtained several B cell-derived cell lines to screen for FasL expression. Among the cell lines tested were several lymphoblastoid cells lines generated by transformation of human peripheral blood B cells with an attenuated form of the Epstein-Barr virus (EBV). By immunoblotting for FasL, we found that all LCLs tested (>20 independent lines) displayed robust and constitutive expression of FasL protein (Figure 3-1A). Expression of FasL in other types of B cell-derived cell lines such as human B cell lymphomas was sporadic (data not shown). We therefore conclude that a high frequency of

cell lines made by transformation of human B cells with EBV constitutively produce FasL protein.

We next sought to determine the cellular localization of FasL in LCLs by flow cytometry. To this end we stained the surface of LCL cells with anti-FasL or an appropriate isotype control antibody. Somewhat surprisingly, we found little or no detectable FasL on the surface of all LCLs tested (Figure 3-1B). After fixation and permeabilization, however, we were able to detect intracellular FasL in all LCLs tested (Figure 3-1B). It therefore appears that while LCLs constitutively produce FasL protein, very little FasL is present on the cell surface under normal conditions.

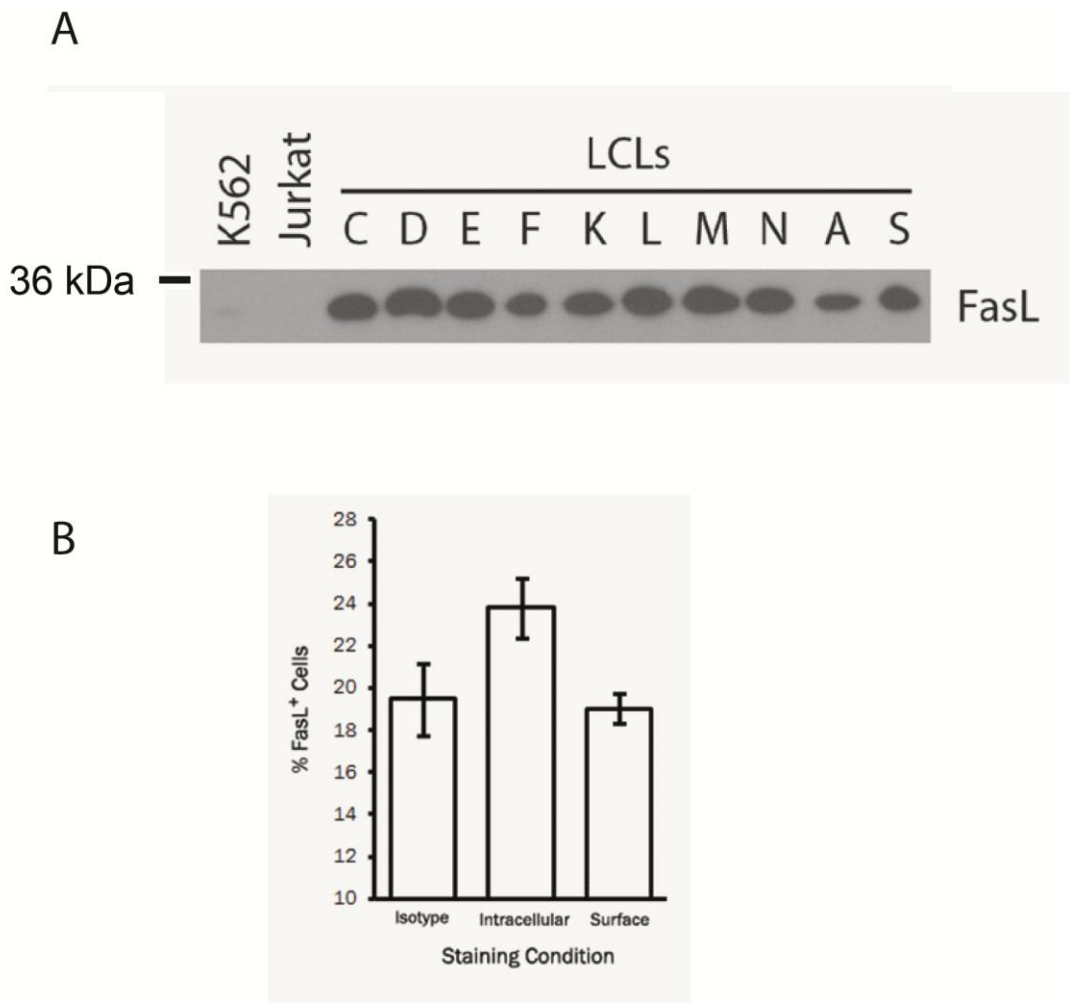


Figure 3-1: A high frequency of LCLs constitutively-produce intracellular FasL protein.
(A) Equivalent numbers of LCLs and control cell lines were lysed and probed for expression of FasL protein by immunoblot. **(B)** LCLs were fixed and stained for surface FasL, or permeabilized with saponin buffer for intracellular FasL staining by flow cytometry. Results are representative of at least three LCLs tested.

LCLs secrete exosomes containing FasL and MHC class II

Lymphoblastoid cell lines are known to spontaneously secrete exosomes, but there are no reports of LCL-derived exosomes containing FasL [167]. Additionally, the secretory lysosome is the default destination for FasL in cells which possess this compartment [155]. We therefore hypothesized that as LCLs express robust amounts of FasL, this FasL is likely to be sorted to the secretory lysosome and secreted on exosomes. To test this hypothesis, we collected supernatants from several independent LCLs and isolated exosomes from these supernatants using ultracentrifugation. Briefly, cells and large debris were removed from supernatants by centrifugation at 500 x g and 10,000 x g, respectively. To pellet exosomes, the cleared supernatants were spun between at 100,000 x g for 1-4 hours. The resulting exosome pellets were lysed with SDS buffer and probed for expression of FasL and HLA-DR by immunoblot. FasL was detectable in the exosome fraction from all LCLs tested (Figure 3-2A). We also confirmed that MHCII molecules were present in LCL-derived exosomes, as we found abundant HLA-DR in the exosome pellets as well (Figure 3-2A).

The pellet obtained by centrifuging supernatants at 100,000 x g can potentially be contaminated with large soluble complexes or other types of cell debris. It has been reported that exosomes have a characteristic density distinct from other membrane fragments or microparticles [167]. We therefore spun re-suspended LCL-derived exosomes through a

discontinuous density gradient made by serial dilution of iodixanol in PBS. After spinning for 1 hour at 100,000 x g, each layer was harvested and diluted in PBS, and centrifuged again at 100,000 x g overnight. The resulting pellets were then lysed in SDS buffer and probed for the presence of FasL by immunoblot as in Figure 3-1A. FasL protein was detected only the fraction with a density of 1.16 g/mL (Figure 3-2B), a density indicative of exosomes [167]. Taken together, these data demonstrate that exosomes containing FasL are secreted by LCLs.

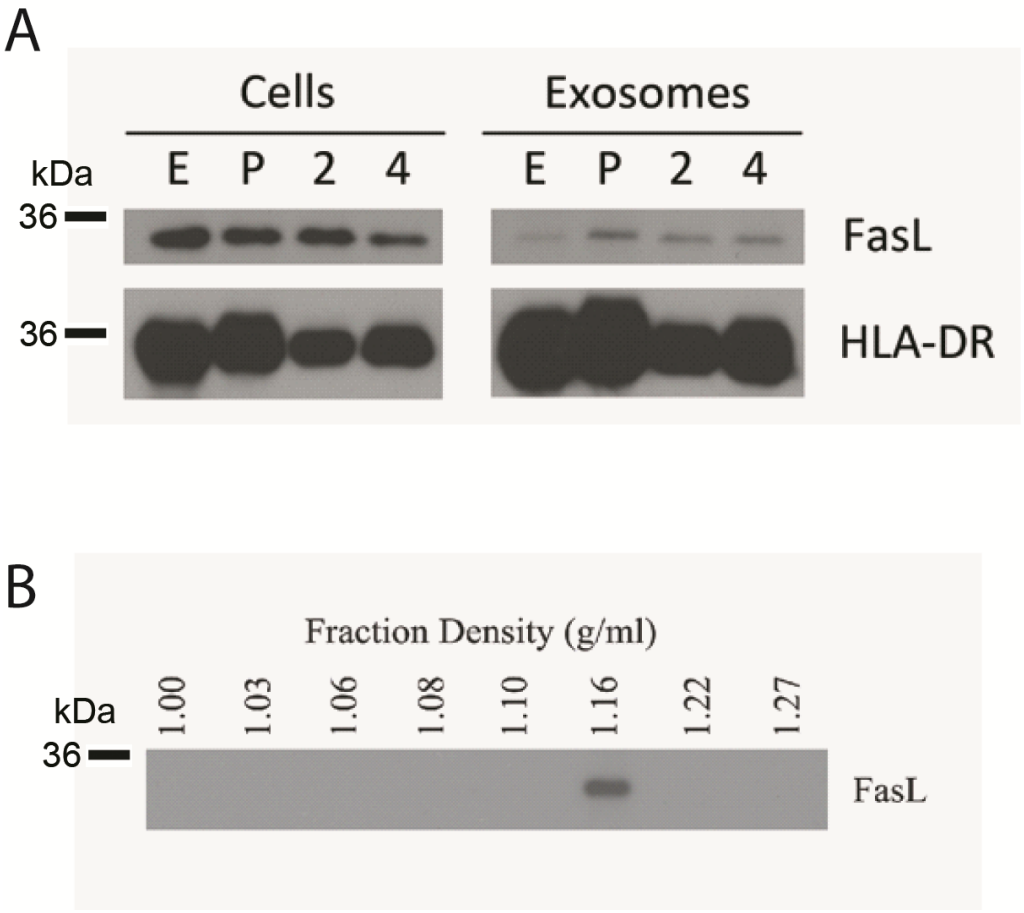


Figure 3-2: LCLs secrete exosomes containing FasL and MHC class II.
(A) Cell lysates and spontaneously-secreted exosomes were collected from four LCLs (lines E, P, 2, and 4) and probed for the presence of FasL and HLA-DR by immunoblot. **(B)** Exosomes from a representative LCL were floated onto a discontinuous density gradient of iodixanol solution and spun for 1 hour at 100,000 x g. Individual layers were removed and diluted with PBS, followed by an overnight centrifugation at 100,000 x g. The resulting pellets were lysed with SDS buffer and probed for FasL by immunoblot.

Double-positive FasL$^+$MHCII$^+$ exosomes are present among LCL-derived exosomes

Our previous experiments demonstrated that both FasL and MHCII protein were present in exosomes secreted by LCLs. While both proteins are present on exosomes, it is not clear from these data whether individual exosomes possess both FasL and MHCII (FasL$^+$MHCII$^+$), or whether FasL and MHCII are present on distinct subsets of exosomes. Exosomes are too small to be accurately detected by flow cytometry, and so must be linked in aggregate to larger beads for flow cytometric analysis. We therefore developed an assay to capture exosomes on an antibody-coated bead and stain the captured exosomes with fluorescently-conjugated antibodies (Figure 3-3). Polystyrene beads coated with streptavidin were incubated with a biotinylated antibody and washed several times. Antibody-coated beads were then incubated with exosomes, and after washing away excess exosomes, those bound to the beads were stained with anti-FasL (Figure 3-3).

To test for the co-localization of MHCII and FasL into the same exosomes, we harvested exosomes from unstimulated and PMA/ionomycin-stimulated LCL culture supernatants and concentrated them by centrifugation. Exosomes were then incubated with beads coated with anti-MHCII antibody, and stained for various markers. As can be seen in Figure 3-4A, exosomes bound to anti-MHCII-coated beads stained positive for the presence of FasL, suggesting that FasL and MHCII are found on the same LCL-derived exosomes. Stimulation with

PMA/ionomycin increased the amount of FasL detected on $MHCII^+$ exosomes in both LCLs tested (Figure 3-4A). Cells from these experiments were also lysed and probed for FasL and, and both cell lines increased FasL production in response to PMA/ionomycin stimulation MHCII (Figure 3-4B). Therefore, LCLs secrete $MHCII^+FasL^+$ exosomes, and both the production of FasL and the release of $MHCII^+FasL^+$ exosomes can be increased by stimulation with PMA/ionomycin.

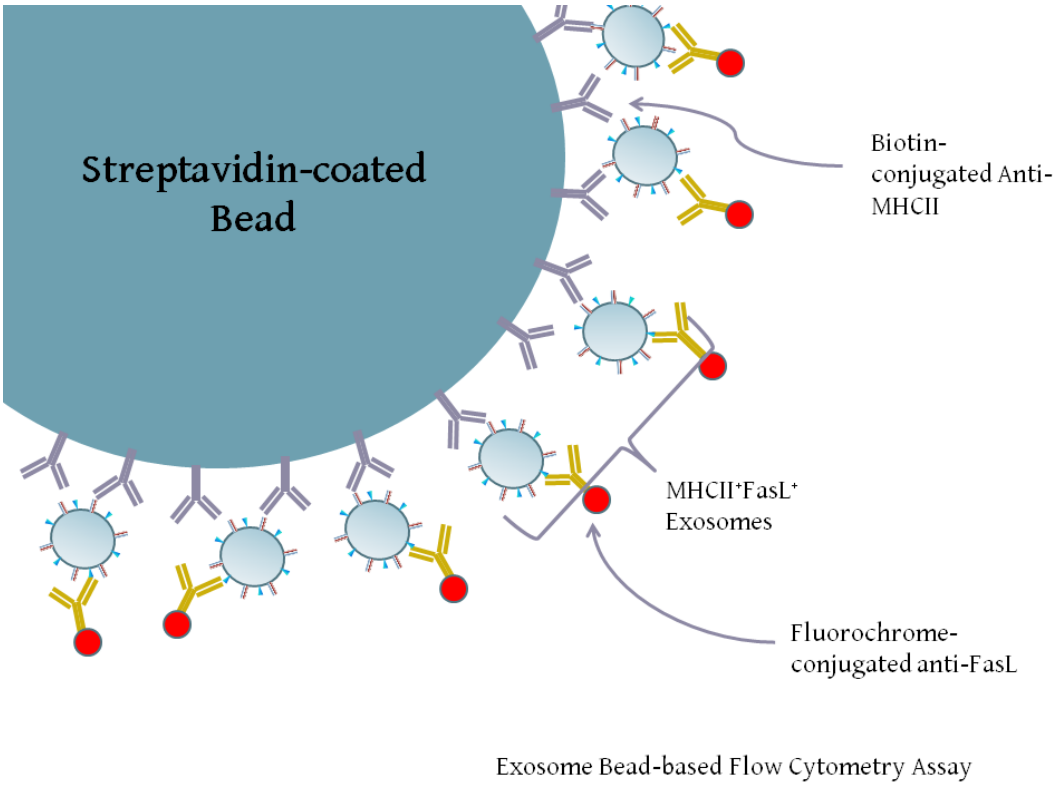
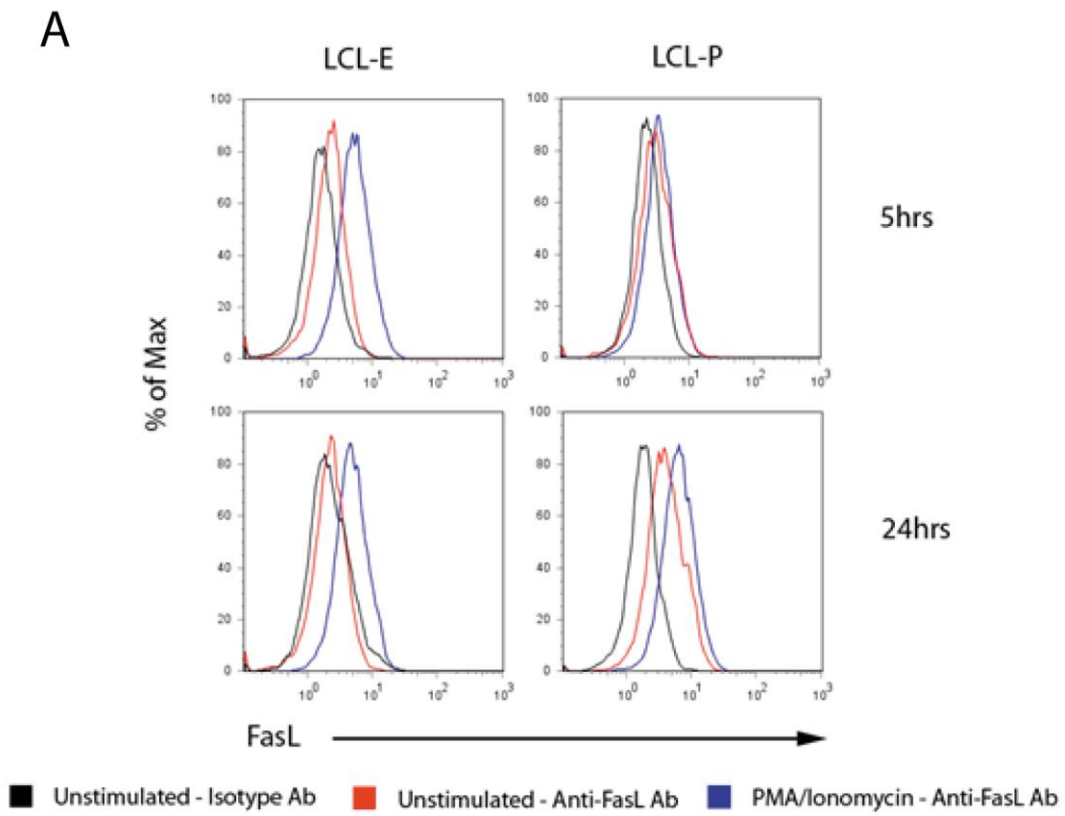


Figure 3-3: Diagram of exosome-bead capture experiments.
Exosomes are too small to be accurately detected by flow cytometers, and so must be linked in aggregate to larger beads for flow cytometric analysis. Polystyrene beads coated with streptavidin were incubated with biotin-conjugated anti-HLA-DR or an isotype control antibody. Beads were then washed and incubated with gentle agitation for several hours with LCL-derived exosomes. Beads were washed and stained with PE-conjugated anti-FasL or an appropriate isotype antibody. Positive staining for FasL indicates the presence of $MHCII^{+}FasL^{+}$ exosomes.



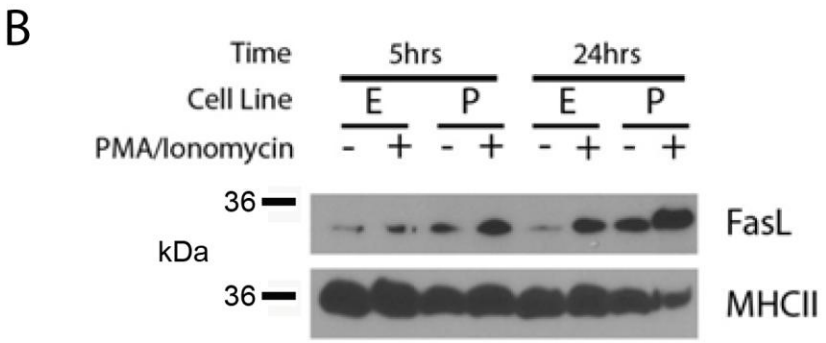


Figure 3-4: LCLs secrete MHCII$^+$FasL$^+$ exosomes.
(A) Two LCLs were placed into fresh exosome-free media in the presence or absence of PMA/ionomycin. After 5 hours (top panels) or 24 hours (bottom panels), exosomes were isolated from culture supernatants as described in the materials section. Purified exosomes were then incubated with anti-HLA-DR-coated beads as described in Figure 3-3. Beads were washed and stained with either anti-FasL or an isotype control antibody. Beads coated with an appropriate isotype control antibody did not display any FasL staining (data not shown). **(B)** Cells from above experiment were harvested and probed for FasL and MHCII (HLA-DR) by immunoblot.

LCL-derived exosomes can induce apoptosis in autologous CD4⁺ T cells

As LCL-derived exosomes contained MHCII⁺FasL⁺ exosomes, we hypothesized that activated CD4⁺ T cells would be susceptible to exosome-induced apoptosis. To test this hypothesis, we obtained peripheral blood mononuclear cells (PBMCs) from a donor from whom we had previously made an LCL. Using autologous T cells prevents the induction of allogeneic interactions between the LCL-derived exosomes and the CD4⁺ T cells of the donor. LCL-derived exosomes were isolated from unstimulated LCL culture supernatant as described above and cultured with fresh CD4⁺ T cells in the presence or absence of super-antigen (SEA). After six days in culture, we assessed apoptosis in CD4⁺ T cells by Annexin-V/propidium iodide staining. In the absence of SEA, LCL-derived exosomes produced a modest increase in apoptosis in CD4⁺ T cells (Figure 3-5). In contrast, in the presence of SEA exosomes induced significant levels of apoptosis in CD4⁺ T cells (Figure 3-5). Similar results were obtained using an LCL and CD4⁺ T cells from a second independent donor (data not shown). As SEA facilitates interaction between MHCII molecules on exosomes and the TCR on a subset of CD4⁺ T cells, this suggests that LCL-derived exosomes can mediate antigen-specific killing of CD4⁺ T cells.

T cells specific for a nominal antigen are rare among peripheral CD4⁺ T cells, and therefore testing the capacity of LCL-derived exosomes to induce antigen-specific apoptosis requires prior activation to enrich for antigen-specific T cells. We obtained PBMCs from a healthy

subject who had recently received a scheduled booster vaccination against tetanus. PBMCs from this donor were cultured with an immunodominant peptide of tetanus toxoid (TT) to enrich the $CD4^+$ T cell pool for cell specific to this antigen. After 12 days in culture, $CD4^+$ T cells were isolated by negative selection and incubated overnight with autologous LCL-derived exosomes in the presence or absence of the TT peptide. Apoptosis was assessed by Annexin-V/propidium iodide staining among activated T cells ($CD4^+CD62L^-$). LCL-derived exosomes in the presence of TT peptide induced significant levels of apoptosis in $CD4^+$ $CD62L^-$ T cells (Figure 3-6). While exosomes in absence of TT peptide also induced apoptosis above baseline, this difference did not reach significance (Figure 3-6). Similarly, the addition of a blocking anti-FasL antibody to culture with exosomes and TT peptide inhibited exosome-induced apoptosis, but again this difference did not reach statistical significance. (Figure 3-6). Taken together, these data suggest that LCL-derived exosomes can induce antigen-specific apoptosis in activated T cells.

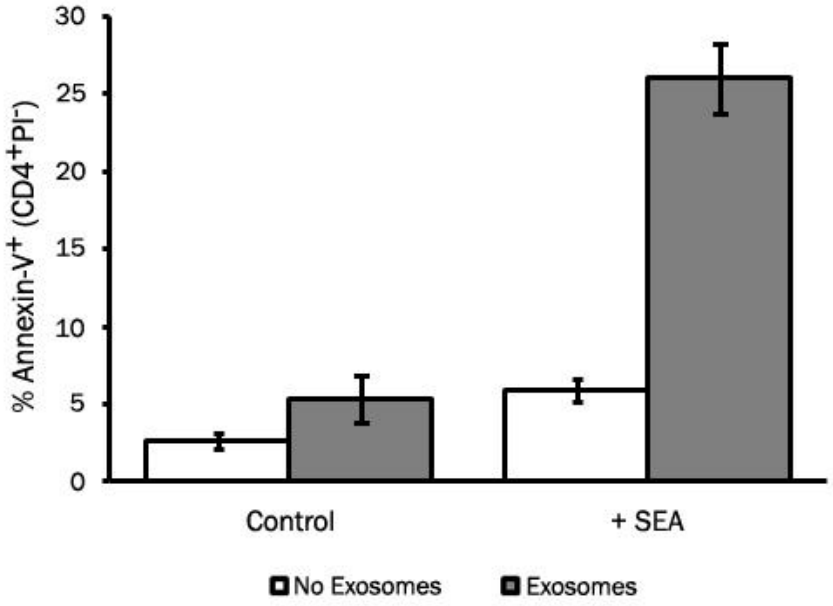


Figure 3-5: LCL-derived exosomes can induce apoptosis in autologous $CD4^+$ T cells.
$CD4^+$ T cells were harvested from a repeat donor from whom we had previously generated an LCL. $CD4^+$ T cells were incubated with exosomes in the presence or absence of super-antigen (SEA). After 6 days in culture PBMCs were harvested, and apoptosis was assessed in $CD4^+$ T cells by Annexin-V/propidium iodide staining. Data are representative of at least 3 independent experiments using two distinct donors.

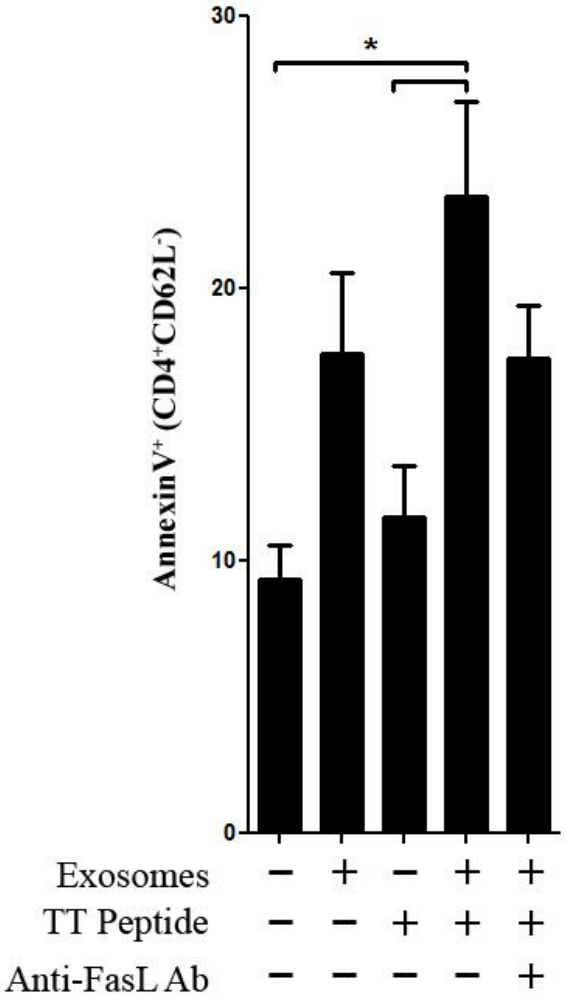


Figure 3-6: Antigen-specific killing with TT peptide.
Peripheral blood mononuclear cells were harvested from a repeat donor from whom we had previously generated an LCL. This donor had received a scheduled vaccination against tetanus less than one month prior. PBMCs were cultured in the presence of an immunodominant peptide of the tetanus toxoid for 12 days. $CD4^+$ T cells were then isolated by negative selection and cultured overnight with or without autologous LCL-derived exosomes and in the presence or absence of the tetanus toxoid peptide. Apoptosis in activated $CD4^+CD62L^-$ T cells was assessed by Annexin-V/propidium iodide staining. *$p<0.05$

IgM$^+$FasL$^+$ exosomes can be isolated from splenic tissue homogenates

The previous experiments demonstrate that B cell-derived cell lines can produce FasL$^+$ exosomes, but whether this occurs *in vivo* is still an open question. Exosomes containing FasL are present but rare in human plasma under normal conditions[198, 199]. We therefore returned to the mouse to look for endogenous B cell-derived exosomes containing FasL. To assay for the presence of FasL$^+$ exosomes generated by splenic B cells, we harvested spleens from HLA-DR4-transgenic mice. Spleens were disrupted in PBS, and cells and cellular debris were removed by centrifugation. The exosome fraction was obtained by centrifugation of the remaining supernatant at 100,000 x g. Exosomes were then incubated with beads coated with antibodies for B cell-specific markers based on the hypothesis that B cell-derived exosomes may have B cell-specific molecules. These exosome-coated beads were than assayed for FasL by flow cytometry. As seen in Figure 3-7, no FasL was detected on beads coated with anti-CD19, but FasL was detected on beads coated with anti-IgM. This result suggests that IgM$^+$FasL$^+$ exosomes exist in the spleen, and based on the presence of IgM are likely to be B cell-derived. No MHCII was detected on either of the beads (data not shown). Although there are important caveats to these results, takent together they suggest that IgM$^+$FasL$^+$ exosomes produced by B cells are present in the spleen.

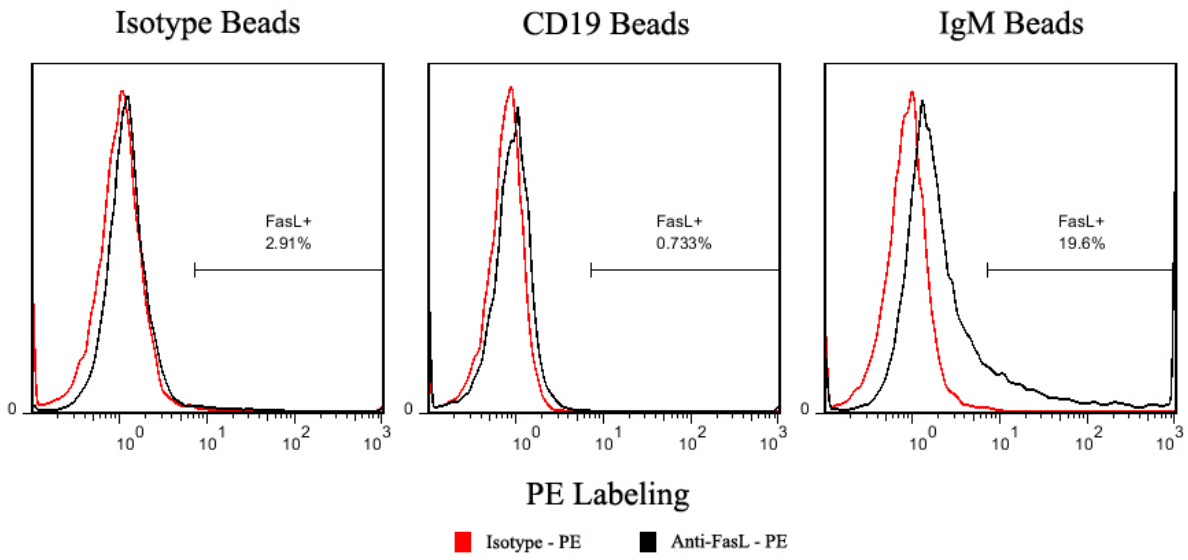


Figure 3-7: IgM$^+$FasL$^+$ exosomes can be isolated from splenic tissue homogenates.
Spleens from wild-type naïve mice were removed, disrupted in PBS, and passed through a cell strainer. Splenocytes and large debris were then removed by centrifugation at 500 x g and 10,000 x g, respectively. The resulting supernatant was centrifuged at 100,000 x g for 4 hours and diluted with PBS prior to a second spin at 100,000 x g. Exosomal pellets were then re-suspended in PBS and incubated with beads coated with anti-CD19, anti-IgM, or an isotype control antibody. Exosome-coated beads were stained with anti-FasL or an isotype control antibody and staining was quantified by flow cytometry. Isotype staining is depicted by red histograms, and anti-FasL staining by black histograms.

3.4 Discussion

A high frequency of LCLs express FasL

B cells expressing FasL are relatively infrequent under most conditions, and we therefore assumed at the outset of this project that FasL expression among B cell-derived cell lines would be rare as well. While FasL expression was indeed infrequent among cell lines derived from B cell cancers, we found that FasL protein was present in cell lysates from all lymphoblastoid cell lines tested in this study. This result was somewhat surprising as LCLs are reportedly susceptible to FasL-induced apoptosis, and LCLs have been used extensively as APCs for activating T cells [200-203]. These conflicting results can be explained in part by the fact that unlike endogenous $FasL^+$ B cells in mice, FasL protein is undetectable on the surface of LCLs. Therefore, although LCLs produce FasL, this intracellular sequestration makes it unavailable for inducing apoptosis in target cells.

Lymphoblastoid cell lines are generated by infection with the Epstein-Barr virus, a γ-1 herpesvirus with oncogenic properties in humans [204]. The virus persists in most infected individuals in latently-infected circulating memory B cells [205, 206].Greater than 90% of adults are infected with EBV, and although clinical manifestations of infection are generally rare, the transforming properties of the virus can lead to B cell-derived malignancies such as Burkitt's and Hodgkin lymphomas [207, 208]. LCLs generated by infection with EBV maintain a

latent viral growth program, expressing at least 8 proteins from the viral genome [207]. Among these proteins is latent membrane protein 1 (LMP1), a functional mimic of CD40 [209]. Signaling of LMP1 differs from that mediated by CD40, as LMP1 signaling is constitutive rather than ligand-dependent. Therefore LCLs are essentially in a state of constant CD40 stimulation. We previously observed that mouse B cells stimulated with CD40L express higher levels of FasL (Chapter 2), and therefore the CD40-mimicry of LMP1 might potentially explain the constitutive production of FasL in LCLs. As mentioned previously, stimulation with CD40L has been reported to induce FasL expression in other types of cells as well [187-189].

While most people are first exposed to EBV in infancy, those infected later in life can develop acute infectious mononucleosis (AIM) [210]. At the height of acute infection, T cells are susceptible to Fas-mediated apoptosis, and *in vitro* infection of PBMCs with EBV leads to elevated levels of FasL on the surface of B cells [142]. Inducing surface expression of FasL in B cells may therefore be a means of immune evasion employed by EBV during the lytic cycle [211]. Importantly, FasL localization appears to differ between the lytic and latent cycles, as FasL in LCLs is intracellular whereas FasL can be found on the surface of B cells during acute infection [142]. As the virus transitions into a latent state and settles into homeostasis with the host immune system, infected B cells may maintain FasL production but cease transporting it to the cell surface.

LCLs produce MHCII$^+$FasL$^+$ exosomes

Immunosuppressive exosomes are an emerging concept with potentially immense utility for immunotherapy. Exosomes produced by dendritic cells genetically modified to produce FasL suppressed induction of collagen-induced arthritis at least as efficiently as intact FasL-expressing dendritic cells [170]. Endogenous MHCII$^+$FasL$^+$ exosomes isolated from murine plasma have immunosuppressive properties, and were able to induce acquired tolerance in recipient animals upon adoptive transfer [171]. Although apparently more rare than in mice, MHCII$^+$FasL$^+$ exosomes have been identified in human plasma as well, and showed FasL-dependent suppressive activity against CD4$^+$ T cells [199]. For the treatment of human autoimmune diseases, exosomes may represent a safer alternative to regulatory cells for immunotherapy because the phenotype of exosomes is static, whereas regulatory cells can potentially differentiate into effector cells after transfer [212]. Although this technique has promise, much further work to better understand the effects of exosome-mediated immunotherapy before it can become a viable treatment.

We demonstrate here that LCLs constitutively produce MHCII$^+$FasL$^+$ exosomes with apoptosis-inducing activity against CD4$^+$ T cells. Although FasL can be detected in LCL-derived exosomes under normal conditions, stimulation with PMA/ionomycin increased both the amount of MHCII$^+$FasL$^+$ exosomes secreted and FasL production in LCLs. Additionally, while FasL is

abundant in LCL cell lysates, it is relatively difficult to detect in exosomes. These results would suggest that although constitutively-produced exosomes have T cell killing activity, enriching LCL-derived exosomes for FasL may produce more potent immunosuppressive exosomes. Additionally, LCLs may prove useful in identifying biologically relevant stimuli that increase release of immunosuppressive exosomes in B cells. Finally, a blocking anti-FasL antibody only partially inhibited exosome-mediate apoptosis, suggesting that other molecules capable of inducing apoptosis in T cells may be present on or in exosomes.

IgM$^+$FasL$^+$ exosomes are present in vivo

Although MHCII$^+$FasL$^+$ exosomes have been identified in both mouse and man, the cells responsible for producing these exosomes remain unknown [171]. The surface phenotype of exosomes can differ from that of its parent cell, and therefore determining the cellular source of exosomes based upon the proteins they contain is not straightforward. Importantly, only a subset of surface proteins is also found in the secretory lysosome, and as a result surface markers specific for a given cell type might not be present on exosomes produced by those cells. In our attempts to find B cell-derived exosomes by antibody-coated bead capture, we chose to target potential exosomal proteins produced only by B cells. We found that beads coated with anti-IgM were able to bind exosomes with FasL, suggesting that B cell-derived FasL$^+$ exosomes are present in the mouse spleen. It is important to note that the specificity of

the IgM bound to the bead is unknown, and therefore the possibility remains that FasL$^+$ exosomes detected are bound indirectly by the specificity of IgM. Natural IgM antibodies are known to bind to phospholipid determinants present on both apoptotic cells and exosomes, and so this is an important caveat to these results [113, 213].

3.5 Future Directions

How does EBV transformation induce FasL expression in LCLs?

All LCLs tested in this study robustly produced intracellular FasL, suggesting that the latency program of EBV drives production of FasL. The mechanism responsible for this phenomenon remains unclear, and is an important remaining question. LCLs express at least 8 proteins from the EBV episome, including 6 nuclear antigens and 3 membrane antigens [207].The viral protein LMP1 is among these viral proteins, and constitutively mimics the signals produced by CD40. This tonic signaling from LMP1 may therefore mediate FasL expression in LCLs, as stimulation with CD40L led to an increase in FasL production in mouse B cells. LMP1 is essential for both B cell transformation continued proliferation of LCLs, and so testing this hypothesis using a LMP1-deficent virus is not feasible [214, 215]. The C-terminal of LMP1 contains several activating regions which associate with many of the signaling adapters shared with CD40, including TNF receptor associated factors (TRAFs) and TNF receptor associated

death domain protein (TRADD) [216-218]. B cell lines deficient in several of the TRAF molecules exist, and examining these lines for expression of FasL may provide insight into the signaling events downstream of both CD40 and LMP1 that induce production of FasL [219]. An important difference between FasL production in CD40-stimulated B cells and LCLs is localization, as LCLs do not have surface expression of FasL. In this regard, differences in the signaling pathways downstream of CD40 and LMP1 may provide mechanistic insight. One such difference is the use of TRAF2, as CD40 signaling is largely eliminated by the loss TRAF2, but the effects of LMP1 are largely unaffected by TRAF2 deficiency [220, 221]. Signals mediated by TRAF2, therefore, may promote the translocation of FasL from intracellular stores to the cell surface.

Do B cells produce FasL$^+$ exosomes in vivo*?*

While we have presented suggestive evidence that B cells produce FasL$^+$ exosomes *in vivo*, much more study is required to definitively demonstrate this phenomenon. MHCII$^+$FasL$^+$ exosomes are present in the plasma of mice, and therefore examining the plasma of mice genetically deficient in B cells for the presence of MHCII$^+$FasL$^+$ exosomes is a straightforward way to test this hypothesis. It is important to note, however, that mice lacking B cells during development have other potentially-confounding abnormalities, including decreased numbers of thymic and splenic T cells, and impaired organization and development of secondary lymphoid organs [15, 222, 223]. Mice transiently depleted of B cells may therefore be a more

relevant subject for these experiments. If available, mice with a B cell-specific FasL deficiency could be used to test this hypothesis as well. If regulatory B cells are indeed the producers of immunosuppressive exosomes, than transgenic animals with increased frequencies of regulatory B cells, such CD19-transgenic mice, would be expected to have higher concentrations of $MHCII^{+}FasL^{+}$ plasma exosomes [131].

Can LCL-derived exosomes be used therapeutically?

Exosome immunotherapy is an intriguing concept because $MHCII^{+}FasL^{+}$ exosomes have demonstrated permanent and precisely focused suppression of an antigen-specific immune response in mouse models [170, 171]. Regulatory cells adoptively transferred can behave unpredictably after transfer, or even differentiate into effector cells [212]. Additionally, some immunotherapies require transfection with adenoviral vectors that can potentially increase the risk of developing cancer in recipients. Exosomes, in contrast, can be isolated reliably and are therefore cell-free, and do not alter their phenotype in response to different immunogenic stimuli.

Producing $MHCII^{+}FasL^{+}$ exosomes to treat an individual with autoimmunity requires that the exosomes be MHC matched with the recipient, and the only feasible way to ensure this match is to use an individual's own cells to produce the exosomes. Transformation of B cells with EBV is reliable, simple, and does not require cell sorting. The genome of EBV is maintained in

proliferating LCLs as a large episome (~167kb), and techniques for engineering recombinant EBV are well-established [224, 225]. For autoimmune diseases for which an autoantigen has been identified, a recombinant EBV can be produced containing the coding sequence for the relevant autoantigen fused to a lysosomal sorting sequence. Proteins containing this sequence are actively sorted to the secretory lysosomal compartment where they are processed and presented on MHCII molecules [226]. Thus, $MHCII^{+}FasL^{+}$ exosomes produced by such a LCL would present various epitopes of the autoantigen (Figure 3-8). The large size of the viral genome allows for the addition of other transgenes as well, such a region containing the FasL gene under a strong, ubiquitous promoter to ensure robust production of FasL. The resulting exosomes can be harvested under sterile conditions by centrifugation or filtration, and either frozen for future use or administered directly to the patient (Figure 3-8). Similar strategies could be developed for inducing allogeneic tolerance in transplant recipients as well.

While there are clear advantages to this technique, some potential limitations are worth discussing. First, this therapy would only be effective in the treatment for autoimmune diseases for which the autoantigen(s) responsible for activating T cells had been identified. Additionally, most autoimmune diseases eventually involve reactivity to multiple antigens, and so eliminating the $CD4^{+}$ T cell response to only a single antigen may not be sufficient to abrogate symptoms [82]. It is also important to consider the effects of such a therapy on the

ability of the recipient to control latent EBV infections, as greater than 90% of patients are likely to harbor endogenous virus, and T cell immunity appears to be crucial for controlling this latent infection [227]. While $CD8^+$ T cells among PBMCs are relatively resistant to FasL-mediated apoptosis, it may still be necessary to reduce MHC class I levels in LCL-derived exosomes to prevent them from interacting with $CD8^+$ T cells after transfer. This could be accomplished by including a shRNA targeting a gene important for MHC class I expression in the recombinant EBV genome, such as transporter associated with antigen processing (TAP) or β_2 microglobulin [228]. Such a strategy would reduce the amount of MHC class I on exosomes, and therefore reduce the likelihood that LCL-derived exosomes would deplete EBV-specific $CD8^+$ T cells in recipients.

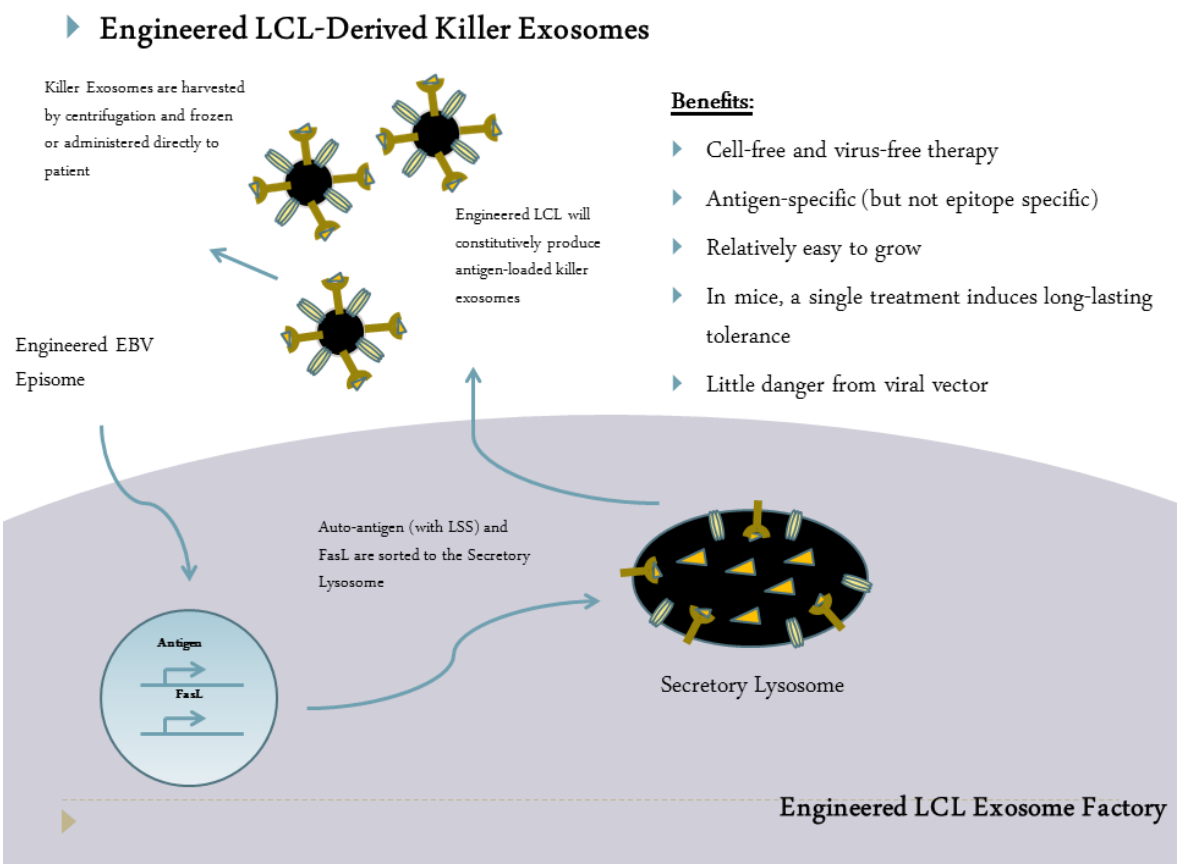


Figure 3-8: Engineered LCL-derived killer exosomes.
Because of its large size, the EBV genome can be engineered to express various recombinant genes. Hypothetically, a recombinant virus could be produced that would contain the gene coding for a relevant autoantigen linked to a lysosomal sorting sequence. This fusion protein would be sorted to the lysosomal compartment where it would be processed for antigen presentation. The engineered LCL therefore secretes $MHCII^{+}FasL^{+}$ exosomes presenting various peptides derived from the autoantigen. The engineered virus would be used to transform a patient's own B cells, and therefore the exosomes produced by this LCL would be MHC matched with the recipient.

Chapter 4

Conclusions

4.1 Summary of Findings

Regulatory lymphocytes play an essential role in maintaining immune homeostasis and self-tolerance. A subset of B cells expresses the apoptosis-inducing molecule FasL and is thought have a role in immune regulation through the killing of autoreactive $CD4^+$ T cells (Figure 1-2). This mechanism of immune suppression is unique among regulatory lymphocytes populations in that it is both antigen specific and permanent. Despite this, relatively little was known about the regulation of $FasL^+$ B cell activity or growth prior to the commencement of this study. The experiments presented in the preceding chapters were performed to gain insight into the biology of $FasL^+$ B cells, with the ultimate goal of finding molecular targets that could be

manipulated pharmaceutically to modulate their activity in patients with a variety of inflammatory disorders.

Various functionally and developmentally distinct subsets of B cells have been identified that can be distinguished by differential expression of cell surface markers. To better understand how $FasL^+$ B cells relate to other B cell subsets, an extensive phenotypic analysis was performed comparing the levels of various surface markers in $FasL^+$ and $FasL^-$ B cells (Figure 2-2). Although the surface phenotype of $FasL^+$ B cells was heterogeneous, these cells were enriched in the $IgM^{high}CD5^+CD1d^{high}$ B cell subset previously reported to contain a higher frequency of B cells producing IL-10 (Figure 2-3). A rare population of B cells expressing IL-10 was present among $FasL^+$ B cells, but most $FasL^+$ B cells did not produce IL-10 (Figure 2-5).

An analysis of global gene expression found that genes encoding components of the receptor for the cytokine IL-5 were expressed at three-fold higher levels in a B cell population enriched for $FasL^+$ B cells, suggesting that IL-5 might be important for the growth or function of these cells. Surface expression of the IL-5 receptor was indeed higher on $FasL^+$ B cells than on $FasL^-$ B cells, and treating B cells with IL-5 and CD40L resulted in the expansion of a population enriched for $FasL^+$ B cells (Figures 2-8, 2-9). B cells stimulated with IL-5 and CD40L were potent inducers of apoptosis in activated primary $CD4^+$ T cells, and this killing function was antigen specific and dependent upon FasL (Figure 2-11). IL-5 also enhanced secretion of the anti-

inflammatory cytokine IL-10 in B cells stimulated with CD40L (Figure 2-12). Collectively, these data identify IL-5 as a novel inducer of regulatory B cell function, capable of inducing or enhancing at least two distinct regulatory mechanisms. Another type-2 cytokine, IL-4, had an opposing effect on regulatory B cell activity, as B cells treated with IL-4 had reduced killing activity and did not increase IL-10 production in response to stimulation with IL-5 (Figure 2-13 and 2-14). These results demonstrate for the first time an antagonistic relationship between IL-4 and IL-5 regarding the function of regulatory B cells.

Although $FasL^+$ B cells are rare *in vivo*, LCLs derived from human B cells showed robust expression of FasL protein (Figure 3-1), suggesting the latency program initiated by EBV in transformed B cells drives the production of FasL. Although FasL was not detected on the surface of LCLs, both MHCII and FasL were present in exosomes isolated from LCL culture supernatant (Figure 3-2). A bead-based exosome capture assay showed that $MHCII^+FasL^+$ exosomes are present among those secreted by LCLs, suggesting that LCL-derived exosomes may be able to induce antigen specific apoptosis in $CD4^+$ T cells (Figure 3-4). This ability was confirmed by two independent experimental approaches using autologous T cells, showing for the first time that LCLs secrete exosomes capable of antigen-specific immune suppression (Figure 3-5 and 3-6). To test for the presence of $FasL^+$ B cell-derived exosomes *in vivo*, a bead-based exosome capture assay was used to isolate exosomes containing B cell-specific markers

and determine if FasL was present among these B cell-derived exosomes. The results of these experiments suggested that $IgM^{+}FasL^{+}$ exosomes are present in the mouse spleen, and given the presence of IgM on these exosomes, they are likely to be derived from B cells. Taken together, these results suggest that B cells may be capable of mediating immune suppression systemically through the secretion of $FasL^{+}$ exosomes.

4.2 Future Directions

Therapeutic potential

Prior to the commencement of this study, relatively little was known about the regulation of killer B cell activity and growth. The experiments presented in chapter 2 identified two novel regulators of $FasL^{+}$ B cells. The cytokine IL-5 increased both the killing function and secretion of IL-10 in B cells (Figure 2-11, 2-12). Additionally, it has been reported that IL-5 increases secretion of anti-inflammatory natural antibodies in B cells [117, 229]. Therefore, targeting pathways downstream of the IL-5 receptor may lead to powerful therapeutics that modulate multiple immunosuppressive mechanisms of regulatory B cells. Drugs that activate pathways downstream of the IL-5 receptor could be of potential use in the treatment of inflammatory conditions such as autoimmune diseases and in patients receiving tissue or organ transplants [80, 230].

Inhibiting these pathways may prove therapeutic as well, as the activity of regulatory B cells can be detrimental during an immune response to cancer [231, 232]. Additionally, some B cell-derived cancers express FasL, and this expression is more common in aggressive forms of disease [140, 233]. *In vitro*, some B cell-derived cancers are capable of inducing FasL-mediated apoptosis, suggesting that FasL expression by B cell-derived cancer may be a mechanism for inhibiting cancer-specific immune responses [140]. In multiple myeloma, FasL expression increases at later stages of disease, and is associated with more severe anemia and bone erosion [234]. These more severe complications are thought to be due in part to FasL-mediated apoptosis of erthyroblasts and osteoblasts, respectively [234]. Drugs that inhibit pathways downstream of the IL-5 receptor may therefore have two mechanisms of action in cancer – reducing the detrimental activity of non-transformed regulatory B cells, and inhibiting FasL expression in B cell-derived cancer cells. Much further work is required, however, to identify specific molecular targets and develop appropriate drugs to modulate their activities.

As demonstrated in chapter 2, IL-4 treatment of B cells severely inhibited the regulatory function of B cells, although it is not yet clear how this effect is mediated (Figure 2-11 and 2-12). Increasing the activity of the pathways downstream of the IL-4 receptor may therefore also be therapeutically relevant in the treatment of cancers derived from B cells. Identifying the precise molecular mediators in these pathways is an important next step toward realizing

this therapeutic potential. It should be noted that IL-4 has wide-ranging effects on many types of cells, including T cells and conventional B cells [235]. Additionally, a T_H2-polarized immune response is thought to promote tumor growth in some contexts, whereas a T_H1-polarized response is often associated with tumor rejection [236]. It will therefore be especially important to find a molecular target that is specific to B cells for this strategy to work therapeutically.

In chapter 3, data were presented demonstrating that a high frequency of human B cells transformed into LCLs by EBV constitutively produce FasL protein (Figure 3-1). Additionally, all LCLs tested in this study produced $MHCII^+FasL^+$ exosomes that were capable of inducing apoptosis in $CD4^+$ T cells (Figure 3-5, 3-6). Other groups have reported that administering $MHCII^+FasL^+$ exosomes to mice results in long-lived antigen specific tolerance [170, 171]. Generating LCLs from peripheral blood B cells is a widely-practiced and relatively simple process, requiring only minimal laboratory labor and reagents. Therefore, LCL-derived exosomes are a potential source of MHC-matched immunosuppressive exosomes that could realistically be used therapeutically in humans. Much further work is required for this to become a reality, however. An essential first step would be to test the efficacy and toxicity of LCL-derived exosomes in a mouse model. One difficulty in performing these experiments is that LCLs cannot be produced from mouse B cells. Alternatively, using humanized mice that

express a single common human MHCII protein, such as HLA-DR4, could be used for these experiments. Because HLA-DR4 is a common haplotype, obtaining an LCL from an individual homozygous for this haplotype would not be prohibitive. Experiments could then be performed by injecting HLA-DR4-transgenic mice with LCL-derived exosomes from an HLA-DR4$^+$ LCL, taking advantage of the ability of human FasL to signal through mouse Fas [243]. Using such an experimental system, it would be possible to directly test the immunosuppressive qualities of LCL-derived exosomes *in vivo*, and techniques for improving their efficacy could be optimized prior to clinical trials. Although this therapeutic strategy is unorthodox and requires further research, its potential as a safer and more effective alternative to cellular immunotherapy make it worth pursuing.

The role of FasL$^+$ B cells in the immune system

The work presented in this dissertation was performed under the hypothesis that the role of FasL$^+$ B cells *in vivo* is to kill autoreactive CD4$^+$ T cells and thereby help to maintain self-tolerance (Figure 1-2). In support of this hypothesis, FasL$^+$ B cells generated by stimulation with CD40L and IL-5 were potent inducers of apoptosis in activated T cells *in vitro*, and this killing activity was greatly increased upon the addition of the relevant T cell antigen to culture (Figure 2-11). Although these *in vitro* data show that FasL$^+$ B cells have the potential to kill CD4$^+$ T cells, it has not been definitively shown that this occurs *in vivo*. It is therefore prudent to

consider the possibility that FasL$^+$ B cells may have functions other than maintaining self-tolerance.

Among these alternative functions could be the control of cell populations other than CD4$^+$ T cells. In humans, neutrophils are present in the marginal zone of the spleen and interact with MZ B cells [237]. As FasL$^+$ B cells are phenotypically similar to MZ B cells (Figure 2-2), it is possible that FasL$^+$ B cells reside in the marginal zone where they could interact with Fas-susceptible neutrophils [238]. In this context, the function of FasL$^+$ B cells may be to prevent neutrophils from entering other compartments of the spleen, or to prevent the production of neutrophil extracellular traps by ensuring that neutrophils undergo apoptosis instead. Another potential function of FasL$^+$ B cells is the direct killing of malignant cells, as some types of cancers are susceptible to apoptosis induced by death ligands such as FasL [239]. Infiltrating B cells can be found in many types of tumors, although presently their function at these sites is not known [240]. Finally, although the most well-known consequence of Fas signaling is cell death, there are reports of Fas signaling mediating activation rather than apoptosis [241, 242]. The effects of Fas signaling appear to differ depending upon the context in which the signal is received, and therefore FasL$^+$ B cells may serve to activate T cells under certain conditions rather than kill them.

These alternative functions are not mutually exclusive, and it remains possible that FasL$^+$ B cells can have different functions in different immunological contexts. Given the phenotypic heterogeneity displayed by FasL$^+$ B cells (Figure 2-2), this population may indeed be comprised of distinct subsets with differing functional roles or cellular targets. Further work is required to determine the true function(s) of FasL$^+$ B cells *in vivo*, and many of these potential roles could be investigated using mice genetically deficient in FasL in B cells only. Such mice have not been systemically investigated, and should be the subject of future studies.

4.3 Concluding Remarks

The work described in this dissertation sought to provide novel insights into the regulation and function of FasL$^+$ killer B cells, a potentially important regulatory B cell population. The results of these experiments have identified biologically relevant stimuli that positively and negatively regulate the activity of FasL$^+$ B cells. The molecular mediators downstream of these stimuli are novel targets for pharmaceutical intervention in a variety of disorders, including autoimmune diseases and cancer. Additionally, this work describes for the first time the production of immunosuppressive exosomes by B cells. The use of B cell-derived exosomes for immunotherapy as described herein is a novel concept, and has several advantages over cell-based immunotherapy. These experiments have therefore set the groundwork for future studies that may eventually lead to powerful and novel therapeutic strategies.

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