NEW MODES OF B LYMPHOCYTE REGULATION IN AUTOIMMUNE DISEASE

By

Christopher S. Wilson

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Microbiology and Immunology

January 31, 2019

Nashville, Tennessee

Approved By:

James W. Thomas, MD, Chair

Daniel J. Moore, MD, PhD, Thesis Advisor

Mark R. Boothby, MD, PhD

Jonathan M. Irish, PhD

William E. Russell, MD

DEDICATION

To my parents Brad and Anita Wilson,

Who gave me all the love, support, and example I needed

to stick this thing through

and to

My aunt, Bobbette Wilson and my brother, Clinton Wilson

Your fight inspired me to keep going

ACKNOWLEDGEMENTS

It's almost impossible to pinpoint the exact event that ensured my successful completion of my PhD. If I had to pick just one it would be choosing Dr. Daniel Moore's lab to rotate in and eventually join. It was not an easy task to convince Dan of my desire to pursue my PhD in immune tolerance of type 1 diabetes in his lab, but eventually he relented and I joined in the summer 2012. That singular event launched an amazing journey of discovery and enrichment, the likes of which I have never undertaken in my life. I am grateful for his mentorship and unwavering support throughout my PhD. Along this journey Dan has become a close mentor and a lifelong friend. Without his leadership and thoughtful training this work would not have been possible.

While the lab has changed in my tenure here, the group I started with will always hold a special place in my heart. Fellow graduate student Blair (gone off to pursue big science in Texas) was an integral part of my success. His motivation and passion drove me to be better. He was always willing to help and to listen to me vent. He became a true and dear friend who will remain important throughout my life and career. Andrew Marshall, was a crucial piece to our lab. Without him nothing could or would have happened. I am also indebted to the numerous collaborations, rotating students, and "science friends" who aided me every step of the way. I am also indebted to my committee who gave me invaluable advice and criticall insight of all my work contained herein.

Finally, I would like to thank my family. I don't know where I would be without the example and support. My father Brad Wilson, set a tireless examples of what hard work was capable of producing. Every morning he would get up and leave work at 4:30am (I think he still

does). I would cherish the days that I could ride alone with him and observe his work ethic, and people skills (and maybe eat a hot baloney sandwich at 7am). He also taught me to put others before myself. My mother imparted on me all her love and continually strove to make sure I was well-balance and grounded in my faith. From a young age my mother stayed home with me to foster early-education and creativity, something that has contributed to my continued success in my PhD. My family has been the driving reason for this PhD as we have been profoundly touched by T1D and autoimmune disease. I hope that this in some small way gives back a little of what it took from us and gives us hope towards the future.

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CHAPTER I

INTRODUCTION

Overview

The deleterious roles that B lymphocytes play in autoimmunity and regulation of immune tolerance are well documented in the literature. B lymphocytes are thought to mediate autoimmunity primarily through presentation of autoantigens to T lymphocytes. Targeting B lymphocytes by depletion clinically in autoimmune disease has been attempted with varying levels of success. The failure of current clinical approaches may be the result of a deficit in our understanding of the role of B lymphocytes in autoimmune disease. B lymphocytes possess multiple functions which, depending on context, may mediate distinct immunologic outcomes. The B lymphocyte compartment also contains diverse subsets that may be important for protection from disease. Further dissection of B lymphocyte responses in states of immune tolerance and autoimmunity will help guide future B lymphocyte therapies in autoimmune disease.

In this study I investigated two models of autoimmune disease with well-documented B lymphocyte involvement, Type 1 Diabetes (T1D) and Systemic Lupus Erythematous (SLE), in order to understand the roles of B lymphocytes in their etiology. In framing the key questions driving this dissertation I initially define the roles of B lymphocytes in immunity. Next, I will highlight ways in which B lymphocytes are known to regulate immunity. I will then define B lymphocyte defects that may predispose to failure of systeminc immune tolerance. Finally, I will summarize how my work revealed new opportunities to regulate the immune response through inducible B lymphocyte actions.

The Discovery of the B lymphocyte Lineage

In 1890 Emil von Behring and Shibasaburo Kitasato described circulating "antitoxins" against tetanus and diphtheria in rabbits[1]. These studies led to the first successful "passive immunity" therapy in humans. Emil von Behring was honored with the very first Nobel Prize for Medicine in 1901, (Kitasato was egregiously not awarded a Nobel prize, even though Behring and Kitasato worked together in Berlin to discover these antitoxins) thus spurring research to elucidate the cell types capable of such impressive protection from pathogenic toxins. Glick and Chang then determined that removal of the Bursa of Fabricius from birds led to an inability to mount an antibody response, eventually leading these antibody-producing cells to be referred to as "B" lymphocytes[2]. In 1965, Max Cooper and colleagues finally determined that B and T lymphocytes were separate cell lineages by studying lymphocyte development in chickens[3]. While the concept of antigen-specific immune responses was known before this, these studies set the stage for research to understand the cellular regulators of this process.

The Potential of B lymphocytes as Integrating Nodes for Immune Regulation

It is surprising that the B lymphocyte was defined as a distinct lineage only a little over 50 years ago, but in that time the amount of knowledge we have gained about its function in autoimmunity and normal immunity has increased exponentially. B lymphocytes are unique players in the adaptive immune response. Like their T lymphocyte counterparts, they develop an antigen-specific receptor that is screened through development and maturity for autoreactivity. Unlike T lymphocytes, B lymphocytes are unencumbered by the need to survey their epitope in the context of an MHC Class II or I molecules. Additionally B lymphocytes can recognize epitopes of any origin, while classical T lymphocyte epitopes must be proteinaceous. The BCR

has the capacity to recognize multople antigenic epitopes, take them up, and activate various effector programs. Their most recognized functions are the presentation of antigen to $CD4^+$ T lymphocytes and production and secretion of affinity-matured immunoglobulins. B lymphocytes can also guide the T lymphocyte response to antigen by providing or withholding costimulatory molecules such as LFA, CD80/CD86, CD40, CTLA-4, or secreted cytokines. While this process is by far the most well-studied of B lymphocytes, it is not the only response elicited by antigen recognition, a process that is regulated by the BCR along with other accessory molecules, complex downstream signaling pathways, and environmental and context-dependent signals.

Novel Properties of B lymphocyte Antigen Processing and Presentation

B lymphocytes possess antigen-specificity that make them unique among the milieu of Antigen Presenting Cells (APCs). Additionally, there exists multiple B lymphocyte subsets with unique properties and localization that can vastly alter immune responses, via distinct modes of antigen presentation and processing[4–7]. Antigen-specific presentation by B lymphocytes can enrich the presentation of certain epitopes on MHC Class II (increasing the potency of antigen-specific B lymphocytes 10^3-10^4 fold as stimulators of antigen-specific $CD4^+$ T lymphocytes over other antigen presenting cells)[8]. B lymphocytes possess other unique qualities that can further influence antigen presentation. Studies have demonstrated that strong BCR-binding to antigen may reduce the ability of cognate antigen be loaded into MHCs. This phenomenon could be related to the BCR-mediated protection of the antigen during processing by proteases in the endosome/lysosome, altering the types of peptides presented to both effector and regulatory T lymphocytes[9–12]. As stated, in preparation for surface expression of peptide in MHC Class II, peptides are cleaved in endosomal compartments by proteases[13]. In classical antigen

presentation, these cleaved products are then loaded into MHC class II. This loading process is regulated by MHC II-like proteins HLA-DO and HLA-DM[14–19]. HLA-DM dissociates the MHC II protein from Class II-associated invariant chain peptide (CLIP). HLA-DO regulates the ability of DM to remove CLIP from MHCII, thus enhancing the stringency of protein binding to MHCII. Interestingly the NOD mouse possesses MHC II variant IAg7 that is thought to bind more promiscuously to lower affinity peptides[20]. Overexpression of DO in NOD mice leads to prevention of diabetes, potentially related to altered auto-antigen presentation and reduced autoreactive $CD4^+$ T lymphocyte activation[20]. The HLA-DO protein is expressed most robustly in B lymphocytes and thymic epithelial cells, as compared to other antigen presenting cells (APCs), but the role of B lymphocytes in diabetes in prevention in this model remains unknown[20–23].

B lymphocytes, like all normal cells, also express MHC Class I and in certain instances can activate cytotoxic naïve $CD8^+$ T lymphocytes[24–26]. Of interest to autoimmunity, B lymphocytes can activate $CD8^+$ T lymphocytes in both T1D and SLE[27,28]. B lymphocytes also express non-classical MHCs with putative roles in immune regulation. B lymphocytes have been demonstrated to express non-classical MHC I Qa-1b, a molecule whose role in regulating the germinal center reaction and autoantibody production is becoming appreciated[29,30]. This MHC possesses a limited peptide repertoire that can interact with Natural Killer (NK) cells and inhibit their lytic capacity[31]. Qa-1b also affords the capacity for B lymphocytes to be surveyed by the CD8 T-regulatory system, important for modulation of the germinal center reaction[30]. Additionally, some subsets of B lymphocytes express increased levels of MHC I-like molecule CD1d. CD1d is restricted to presentation of lipid derived antigens to NK-T cells[32]. Interaction of these NK- T cells with B lymphocytes is important in both immune regulation and antibody

responses to bacterial wall components containing polysaccharides and lipids[33]. These studies demonstrate the capacity for B lymphocytes to modulate the function of multiple immune subsets through non-classical MHC interactions.

Outside of direct antigen processing and presentation, B lymphocytes have also been demonstrated to bind and enrich antigen that is then transferred to dendritic cells for antigen presentation[34]. Effective B lymphocyte responses require access to antigen in specialized follicles[34]. Marginal zone B lymphocytes exist on the margins of these follicles in the spleen. The follicles are where T-B lymphocyte interact to production of high-affinity class-switched antibody[34]. These marginal zone B lymphocytes, due to their anatomical location, have access to a myriad circulating antigens, and antigen bound immune complexes[35]. These marginal zone B lymphocytes capture antigen and traffic to the follicle to deposit the antigen with follicular dendritic cells to enhance the germinal center reaction. This shuttling is thought to occur independent of the BCR in part, due to increased expression of CD21 (a receptor able to capture complement coated antigens) on marginal zone B lymphocytes[34].

The Function of B lymphocyte Secreted Molecules in Shaping the Immune Response

The result of antigen presentation in B lymphocytes is the production of antibodies with unique immunoregulatory properties. Antibodies are a modified and secreted form of the BCR. These antibodies can mediate multiple immune processes based on the type and tissues in which they are secreted[36]. These functions are a direct effect of their isotype, which dictates the type of Fc receptors, and thus cell types these antibodies interact with[36]. The isotype is generated due to differential reocmbination of the heavy chain locus termed class-switch recombination. The type of isotype generated is dictated by strength of antigen binding, cognate cell help, and the local

cytokine milieu[36]. Outlined in Table 1 is the function and serum concentration of each isotype in humans. In the interest of brevity each isotype will not be discussed in detail, except to say that it is clear these Igs possess varied function that allow them to participate in multiple immune processes[36,37].

The production of particular antibody isotypes is modulated by cytokine secretion[36]. While these cytokines can be provided to B lymphocytes by other immune cells, B lymphocytes may also produce their own cytokines[38]. These cytokines can influence immune function and response of multiple cell types[38,39]. B lymphocytes have been demonstrated to drive Th1, Th2, or tolerogenic responses through differential secretion of cytokines[40–42]. (The role of B lymphocytes in mediating Th17 responses is less clear)[43]. Th1 responses are associated with intracellular pathogens and autoimmune responses such as Type 1 diabetes[44–47]. In this case B lymphocytes can produce IL12 and IFNγ, leading to the production of Th1 helper T lymphocytes[38]. Th1 cells produce more IFNγ driving B lymphocytes to secrete more IL-12 and IFNγ, enforcing the Th1 response[38,44]. Th2 responses are often elicited in the case of extracellular pathogens, in which B lymphocytes influence naïve T lymphocyte differentiation to Th2 by secretion of IL-4, IL-2, and IL-13[38,44]. These Th2 cells can then secrete IL-4 to drive B lymphocytes to perpetuate the Th2 response through additional IL-4 secretion[38,44]. Current research suggests that IL-10 and TGFB production by B lymphocytes can also inhibit these immune responses[38]. In the case of IL-10, this cytokine can enhance immunity in some instances[48,49]. Nevertheless, B lymphocytes possess the capacity to enhance or dampen immunity through cytokine secretion.

Mode of Action	IgM	IgD	IgG1	IgG2	IgG3	IgG4	IgA	IgE
Neutralization	+	-	++	++	++	++	++	-
Opsonization	-	-	+++	+*	++	+	+	-
Targeted for NK cell killing	-	-	++	-	++	-	-	-
Sensitization of mast cells	-	-	+	-	+	-	-	+++
Complement activation	+++	-	++	+	+++	-	+	-
Mean serum level (mg/ml)	1.5	.04	9	3	1	0.5	2.1	$3x10^{-5}$

Table 1.1 Mechanisms of actions of various immunoglobulin isotypes in humans.
"+"indicates increased activity. "-" indicates no activity. *This phenomenon is only observed in some Caucasians.

B lymphocytes Detect Microbial Components to Regulate Immunity

As can be appreciated thus far, B lymphocytes interact with a wide-range of immune responses, which they modulate qualitatively and may also regulate quantitatively These interactions are regulated by antigen recognition, cytokines, T lymphocyte help, and input from non-antigenic environmental cues like pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPS), toxins, and metabolites. Indeed, the commensal microbiome has an intricate relationship with B lymphocytes engaging in reciprocal regulation that alters B lymphocyte development and immune activation[48]. In order to sense microbial products, B lymphocytes express innate pathogen receptors such as nucleoside-oligomerization domain-like receptors (NLRs). These intracellular receptors recognize peptidoglycans from bacterial cell walls. Studies have demonstrated that NLR ligands enhance B lymphocyte proliferation, likely through increased activation of the inflammasome[50]. NLRs can cooperate with another class of innate-receptor, the Toll-like receptor (TLR), to provide maximal activation of B lymphocytes[51,52]. TLRs recognize various pathogenic components of viral and bacterial origin to enhance immune activation[50]. These class of receptors will be discussed in depth in Chapter II. Bacterial-derived toxins have been demonstrated to induce activation or depletion of B lymphocytes based on both the context and organism in which the B lymphocyte encounters this toxin[52,53]. This illustrates the capacity for B lymphocytes to integrate and direct antigen specific immune responses by integrating environmental cues into effector function.

B lymphocytes Possess the Capacity to Integrate and Direct Multiple Arms of the Immune System

While some of these functions have been demonstrated to occur in other immune cells (i.e. TLR signaling) and some have not (immunoglobulin production), B lymphocytes represent the only cell population to regulate immunity in complex ways and at the same time direct "antigen-specific" immune action. T lymphocytes certainly are important in regulation of antigen-specific immunity but are required to be localized to tissue or compartment in order to impact immune regulation where they are potentially impacted by the local metabolic and cytokine environment. B lymphocytes have the capacity to monitor circulating and local antigen and mediate immunity either at a distance or locally. These properties make B lymphocytes unique cells among the immune milieu.

Clues to B lymphocyte mediated regulation of immunity

B lymphocytes have the capacity to incite immunity through multiple pathways. It is also clear that B lymphocytes can regulate and suppress ongoing immunity. B lymphocyte directed therapy focuses on inhibiting the effector function of B lymphocyte without considering the therapy may eliminate regulatory roles of B lymphocytes. In this section I will highlight the ways in which B lymphocytes are known to mediate regulation. This thesis will revisit the dual function of B lymphocytes in inciting immunity as well as dampening immunity as I utilize therapeutics that I discover drive B lymphocyte mediated regulation.

Foundational Studies in Breg Biology

Almost 9 years after the discovery of the B lymphocyte lineage, B lymphocyte involvement in suppression of delayed-hypersensitivity in guinea pigs was described(1965)[54]. However, the "Regulatory B cell" moniker would not emerge for another 30 years. Charles Janeway was the first to establish a role for B lymphocyte regulation in experimental autoimmune encephalomyelitis (EAE)[54]. This was found to be dependent on Interleukin-10 (IL-10), as mice with loss of IL-10 in B lymphocytes did not recover. Bregs were then, somewhat surprisingly, found to play a role in colitis when a T cell receptor alpha knockout mouse was crossed onto B lymphocyte deficient mice developed more aggressive colitis compared to B lymphocyte replete control mice[54]. This phenomenon was again associated with IL-10 producing B lymphocytes, but this time included a role for CD1d upregulation in gut-associated lymphoid tissues[54]. This foundational research led to a study of classical Bregs with suppressive capacity. Unfortunately, it is unclear if these so-called "Bregs" mediate tolerance in stringent models due to a lack of evidence of antigen specificity. Additionally, its primary effector cytokine, IL-10, is counterregulatory in certain instances, highlighting the need for investigation into additional modalities of B lymphocyte regulation[48,49]. As highlighted above B lymphocytes have the capacity to mediate immunity via multiple mechanisms. While the majority of these actions appear to activate immunity, there are specific instances in which these processes can mediate immune dampening and regulation.

B lymphocytes Mediate T lymphocyte Suppression Through Cell-Cell Adhesion Molecules

B-T lymphocyte MHC interactions often lead to mutual licensing of these cells to produce high-affinity class-switched antibodies or gain effector activity against target antigens,

respectively. A basic tenet of this interaction is the need for costimulation for naïve T lymphocytes to become fully activated[55]. T lymphocyte recognizing antigen in the context of the MHC that do not receive co-stimulation through CD80/CD86 become anergized (or nonresponsive)[56,57]. It has been demonstrated that resting or naïve B lymphocytes induce tolerance due to poor capacity to provide costimulation[58,59]. In a classic experiment carried out in the lab of Polly Matzinger, it was demonstrated that transfusion of resting B lymphocytes from a male mouse into a female mouse specifically tolerized these mice to the H-Y antigen, as measured by cytotoxic killing assay. Transfer of total male splenocytes to a female mouse did not offer the same protection, indicating a special tolerogenic function of B lymphocytes over other APCs[59]. In contrast studies in humans have demonstrated that activated $CD25^{+}$ peripheral B lymphocytes induce anergy and apoptosis in certain subsets of activated T lymphocytes, in a IL-2 dependent but CD95 independent fashion[60]. These data indicate the potential for B lymphocytes to regulate the activation of both naïve T lymphocytes and activated T lymphocytes, possibly through distinct mechanisms.

Central to the function of B lymphocytes in normal immunity is their participation in the germinal center (GC) response, and mounting evidence suggests that enhanced GC activity is associated with multiple autoimmune diseases[61–63]. $PD\text{-}L1^{Hi}$ B lymphocytes have been demonstrated to interact with Tfh cells to limit the germinal center response[64]. Programmed-death ligand 1 is responsible for mediating apoptosis of PD-1 receptor expressing target cells[65]. Transfer of $PD\text{-}L1^{Hi}$ B lymphocytes ameliorated of EAE with decreased pathology and marked decrease in Tfh cells. This effect was independent of both IL-10 and Treg action[64]. Interestingly, these Bregs were resistant to anti-CD20 depletion leading to a mode by which anti-CD20 could select for B regulation over B effector function[64].

B lymphocytes Regulate Intracellular and Extracellular Metabolites to Impact Immunity

Studies of GC B lymphocytes illustrates that B lymphocytes have the capacity to respond to a wide array of metabolic conditions. The GC has been demonstrated to be an area of hypoxia that requires adaptive metabolic programs[66–68]. In the GC induction of Hif1a promotes survival of B lymphocytes in this environment. Additionally, studies of B lymphocyte cancers and B lymphocytes in solid tumors illustrate they can survive and modulate metabolites in such a way to impact the immunity of other cell subsets[69,70]. The importance of cellular metabolism in dictating appropriate and robust immune responses is gaining increasing acknowledgment in clinical treatment of infection and autoimmunity.

While B lymphocytes have the capacity to alter intracellular metabolism to impact immune function, they likewise can alter the availability of extracellular metabolites. Adenosine, a purine nucleoside, is a powerful immunosuppressant whose mode of regulation will be discussed in more detail in Chapter V. B lymphocytes creation of adenosine is mediated by the breakdown of ATP by two ectonucleases, CD39 and CD73[71,72]. Both CD39 and CD73 are expressed on B lymphocytes at baseline leading to the production of adenosine that regulates B lymphocyte responses in an autocrine fashion. In activated B lymphocytes CD73 is downregulated preventing the conversion of ATP to adenosine, thus rendering 5-AMP. This metabolite was shown to inhibit $CD8^+$ and $CD4^+$ T lymphocyte proliferation and activation, possibly through activation of AMPK[72]. This mechanism of suppression may be especially important in areas of increased extracellular ATP, such as has been described at sites of inflammation[73,74].

In humans, a subset of Bregs has been demonstrated to modulate T lymphocyte metabolism through the secretion of indoleamine 2,3-dioxygenase (IDO)[75]. IDO is a heme containing enzyme that catabolizes the breakdown of tryptophan. The mechanism of action of tryptophan breakdown in immune suppression is incompletely understood but has been related to the capacity to induce Tregs in T lymphocytes interacting with Bregs[75,76]. It has also been demonstrated that breakdown of tryptophan suppresses effector T lymphocyte function[77,78]. Researchers have speculated this relates to reduced availability of this essential amino acid to T lymphocytes, thus blunting metabolism. More likely catabolites of IDO (such as kynurenines) activate caspase 8 thus leaving T lymphocytes sensitized to apoptosis[78]. The capacity for B lymphocytes to regulate immune metabolism to induce immune suppression demonstrates the importance of proper metabolism in effector and regulatory cell function.

Allotransplant Reveals Modes of B lymphocyte Regulation

One of the strongest bodies of evidence of the collaborative effect of B and T lymphocyte interactions is in models of allograft rejection. Multiple studies have indicated a deleterious role for B lymphocytes in both priming T lymphocytes and antibody-mediated graft rejection[79–84]. However, studies have pointed to an emerging regulatory role for B lymphocytes in human transplant. Studies of human spontaneous kidney graft acceptance, observed in patients who stopped taking immunosuppressives due to non-compliance or intolerance to side effects, is associated with a unique B lymphocyte signature[79]. Little direct evidence demonstrates the capacity of these human Bregs; however mouse transplant studies clearly indicate a regulatory role for B lymphocytes. One such transplant tolerance regime, utilizing tolerizing agent anti-CD45RB, demonstrated that B lymphocytes play a crucial role in cardiac allograft tolerance[85–88].

Indeed, anti-CD45RB robustly modulates B lymphocytes through increases in adhesion molecule ICAM and MHC II. This B lymphocyte mediated tolerance induction was dependent on CD40-CD40L and LFA-ICAM interactions, reinforcing the important of cell-cell interactions to drive tolerance[88]. In direct opposition to current Breg literature, IL-10 actually plays a deleterious role in tolerance induction by anti-CD45RB. These data indicate that induction of true immune tolerance by B lymphocytes is not regulated by IL-10 production[48]. Interestingly, anti-CD45RB also differentially modulates splenic and bone marrow B lymphocyte compartments in non-autoimmune prone B6 and autoimmune prone NOD mice. Autoimmune mice possessed abnormal mobilization of marginal zone and mature recirculating cells after anti-CD45RB therapy (data not shown). Both of these populations have been associated with B lymphocyte regulation as well as autoimmune pathology, indicating abnormal B lymphocyte regulation may be important in tolerance resistance. This point will be emphasized and discussed in Chapter IV. While it was unclear in these initial studies what immune subsets B lymphocytes interacted with during anti-CD45RB treatment, tolerance requires Tregs, making these cells a likely target for B lymphocytes.

Naïve B lymphocytes Induce Conventional and Unconventional Tregs

Interactions between B lymphocytes and Tregs have been of interest in recent literature. The possibility for antigen-specific enrichment and presentation by B lymphocytes to Tregs to expand these cells or to naïve T lymphocytes to induce these cells to become Tregs represents an exciting therapeutic opportunity. As previous studies demonstrated that naïve B lymphocytes induce anergy or apoptosis in naïve T lymphocytes, researchers question whether these same naïve B lymphocytes could drive naïve T lymphocytes to a regulatory phenotype. Researchers

indeed found that naïve B lymphocytes induced regulatory T lymphocytes that were Foxp3$^-$ and mediated inhibition via a contact dependent mechanism utilizing LAG-3, and CTLA-4[89–91]. These so-called Treg-of-B have been utilized to mediate immune suppression in multiple models. Models of self-antigen presentation on B lymphocytes or DCs demonstrated that splenic B lymphocytes were able to induce Foxp3$^+$ T regs in the periphery while DC presentation of self-antigen led to anergy in T lymphocytes interacting with them[92]. These studies illustrate an important role for B lymphocytes in mediating peripheral tolerance through manipulation of Tregs.

Thymic B lymphocytes Play Important Roles in Selection of Effector and Regulatory T lymphocytes

Activity of peripheral Tregs prevents autoreactive T lymphocytes from becoming activated, while thymic selection prevents this process from becoming overwhelmed by an abundance of autoreactive T lymphocytes. T lymphocytes develop in the thymus where they are educated on peripheral antigens, via enforced expression of autoimmune regulator protein (AIRE) in thymic medullary epithelial cells, leading to culling of autoreactive T lymphocytes[92]. In fact loss of functional AIRE in humans and mice leads to Autoimmune Polyendocrinopathy Syndrome type 1[93,94]. Interestingly, B lymphocytes express AIRE and are especially potent at deleting autoreactive T lymphocytes[95]. These B lymphocytes appear to be activated by CD40-CD40L interactions with cognate CD4$^+$ T lymphocytes. This activation leads to expression of AIRE, as well as antibody class-switching, and upregulation of MHC II[95]. The impact of these B lymphocytes in normal immune regulation are just recently coming to the forefront.

While many T lymphocytes of an autoreactive specificity are deleted in the thymus, at the same time some developing $CD4^+$ lymphocytes of autoreactive specificities are selected to become $Foxp3^+$, $Helios^+$, $CD25^+$ natural Tregs (nTregs). These Tregs traffic to the periphery to mediate immune regulation. The thymus is divided into a cortex, where cortical epithelial cells, thymic macrophages, and dendritic cells mediate positive selection, and a medulla, where medullary epithelial cells and dendritic cells enforce negative selection. Interestingly, loss of the medulla space (via mTEC depletion by Relb knockout) did not reduce total $CD4^+$ or $CD8^+$ T lymphocyte output but completely blunted $Foxp3^+$ Treg development[95]. This illustrated a crucial role for the medulla in Treg development.

Studies of other thymic B lymphocyte subsets reveals that $CD19^+$ IgM^+ B lymphocytes cluster near the medullary border[96]. It was discovered that in models of BAFF overexpression that these B lymphocytes were essential for enhanced generation of Tregs[96]. These data illustrate the possibility that, as demonstrated in peripheral models of B-T interactions, activated B lymphocytes may mediate deletion while naïve B lymphocytes foster generation of Tregs in the thymus. Very little is known about the phenotype or origin of the thymic B lymphocyte compartment. Conflicting studies report these cells develop in the thymus, while some studies state they recirculate from the spleen and bone marrow[97,98]. Regardless of the origin it is of interest to identify the function and phenotype of these B lymphocytes. The capacity to shape T lymphocyte selection through capture and enrichment of rare antigens or acquisition of neoantigens, represents a dynamic way for the immune system to respond to an ever-changing self and pathogenic antigen milieu.

Secreted Immunoglobulins Possess Varied and Unique Regulatory Properties

A discussion of B lymphocyte modes of regulation would be incomplete without discussing briefly the role of secreted antibody in immune regulation. As illustrated in Table 1 the istoypes have varied roles in immune function. Here I will highlight just the functions that prevent robust and deleterious immune responses. IgG is the most abundant and widely known induced Ig from the B lymphocyte[36,99]. These secreted molecules carry out effector function to mediate pathogen neutralization and clearance. IgGs can also suppress immunity through interactions with immunoinhibitory receptor Fcγr2b, a low affinity receptor for IgG associated with an immunoreceptor tyrosine-based inhibitory motif (ITIM)[100,101]. This inhibitory receptor is predominantly expressed on myeloid cells and B lymphocytes[102]. It downregulates the antibody response and in some cases mediates apoptosis of bound cells. IgG is the main component of clinical IV-Ig therapy that provides an immunosuppressive effect in autoimmune diseases such as myasthenia gravis[103,104].

Maintenance of protective mucosal integrity is important to prevent initiation of potentially harmful immune responses. As already discussed the microbiota is important for immune development, but is at the same time tightly regulated to prevent opportunistic infections. Early in life transfer of maternal IgAs from mother to child helps establish the commensal microbiome[105,106]. This may impact lifelong immune development as the immune system develops permanent characteristics early in life that may predispose to autoimmunity or maintain proper regulation. Production of IgA from B lymphocytes residing in Gut-Associated Lymphoid Tissue (GALT) regulates the mucosal border and commensal and pathogenic microbiome through both antigen specific and FC dependent interactions[107,108]. Mice models have demonstrated that IgA production from B lymphocytes helps alleviate dextran-sulfate

induced colitis[109]. Humans with selective IgA deficiency are more susceptible to infection, autoimmune disease, allergy, and asthma, indicating the important regulatory properties of IgA[105].

Proper immune homeostasis is important for normal immunity and prevention of autoimmune disease. Homeostatic immune defects are characteristic of autoimmune diseases like T1D and SLE[110,111]. Secreted IgM is the most abundant non-induced circulating IgM[112]. This Ig is known to be important as first line of defense against pathogens, through binding and activating complement[113]. At the same time the IgM repertoire has demonstrated a relative high abundance of autoreactivity[114]. This autoreactivity is thought to contribute to tissue homeostasis by aiding in binding and clearance of necrotic or apoptotic cells[113]. This clearance is thought to prevent persistence of auto-antigens that could initiate sterile inflammation and autoimmunity. Mice deficient in secreted IgM demonstrate an autoimmune phenotype.[115,116] This is thought to be due to Fc mediated interactions with the cognate IgM-Fc receptor TOSO (Fcmr)[117]. The underpinnings of this system will be discussed in greater detail in Chapter IV.

The Role of B lymphocytes in T1D and SLE

The importance of B lymphocytes in T1D and SLE is clear, but their functions and defects in each disease are distinct. In this section I will highlight the B lymphocyte defects in T1D and SLE to set the stage for my thesis work. These foundational studies in B lymphocyte biology during autoimmunity informed the molecular pathways I target to influence B lymphocyte function in this thesis.

B cell selection and development in T1D and SLE

B lymphocyte development begins in the bone marrow, where approximately 70% of B lymphocytes generated are of an autoreactive specificity[118]. They then undergo receptor editing or deletion. The surviving cells emerge to the spleen to complete development. Once matured, approximately 25% of the naïve mature B lymphocyte population demonstrates hallmarks of receptor editing[118]. The autoreactive cells that persists after development are thought to undergo anergy, in healthy people, rendering them nonresponsive to their cognate antigen[118]. Recent studies of insulin-reactive B lymphocytes indicated that prediabetic and new-onset T1D patients possessed fewer anergic insulin-reactive B lymphocytes as compared to healthy controls or first-degree relatives, indicating loss of B lymphocyte anergy may be important in T1D pathology[118]. The role of B lymphocyte anergy is SLE is largely unknown.

B lymphocyte development has been studied extensively in the NOD mouse, which is the preclinical model of T1D, and humans with T1D[28,32,119–124]. Studies in mice and humans with T1D demonstrate a reduction in receptor editing in the mature population of B lymphocytes[125,126]. Additional studies, conducted by the Thomas lab at Vanderbilt, indicate insulin-reactive cells emerge in the bone marrow at a greater frequency in NOD mice than in their nonautoimmune counterparts[127]. The role of central tolerance remains less well defined in SLE; studies in humans do not indicate any abnormal Ig gene skewing or usage[128]. Additional research in B lymphocyte selection needs to be undertaken to fully understand the developmental abnormalities in SLE.

Once these immature B lymphocytes emerge to the periphery, they enter the transitional B lymphocyte compartment. This developmental stage is important for further B lymphocyte selection, as a very small percentage of B lymphocytes survive this developmental stage. In T1D

the transitional B lymphocyte population is abnormal and thought to be a subset important in loss of B lymphocyte tolerance[129,130]. In NOD mice, there is a robust loss of this compartment as the mice age, this is thought to lead to the survival of autoreactive clones to maturity[129]. Patients with SLE also demonstrate abnormal transitional B lymphocyte dynamics[131,132]. While there doesn't appear to be loss of this compartment, enhanced BCR and Toll-like receptor (TLR) signaling seems to lead to survival of autoreactive cells[133]. Indeed the transitional compartment has been shown to be modulated by BAFF, TLR, and autoreactive T lymphocyte help, all factors implicated in both T1D and SLE[133–135].

The Role of Mature B lymphocytes in T1D and Lupus

Once transitional B lymphocytes pass selection, they are recruited into two splenic populations of mature B lymphocytes, follicular and marginal zone B lymphocytes. Follicular B lymphocytes are the subset most commonly associated with generation of high-affinity class-switched antibody, or autoantibody in autoimmune disease[136]. In order to induce high-affinity antibody responses, follicular B lymphocytes enter the germinal center reaction. Both T1D and SLE exhibit abnormal germinal center reactions. In SLE there is an increase in frequency of germinal centers, characterized by increased numbers of T-follicular helper (Tfh) cells and autoreactive germinal center B lymphocytes[137,138]. In NOD the role of the germinal center reaction is not well understood. While the expansion of GCs is not as apparent in NOD mice, there is establishment of abnormal tertiary lymphoid structures in the pancreas,that contain GC-like B lymphocytes[139]. The result of these interactions is the production of anti-islet and anti-nuclear antibodies in T1D and SLE, respectively.

Developing B lymphocytes can also enter the marginal zone (MZ) compartment once they reach maturity. This compartment is expanded in NOD mice and thought to traffic to the pancreatic lymph node to facilitate disease[124,140]. While the MZ is not expanded in mouse models of SLE, it has been demonstrated to contribute to disease by inappropriate infiltration of the follicular zone and interaction with $CD4^+$ T lymphocytes[141]. While the antigen specificity of the marginal zone is somewhat restricted, their role in autoimmunity has largely been attributed not directly to their antigen-presentation via MHCs but due to their ability to capture complement bound autoantigen and transfer it to the follicular zone[34]. Similar in antigen specificity and function but distinct in anatomical location and development, B1 B lymphocytes also have roles in T1D and Lupus. These B lymphocytes can be found in small numbers in the spleen, but the majority can be found in the peritoneal cavity[34]. The B1 cell subset phenotypically overlap with B lymphocytes that possess regulatory capacity in other models[142]. Studies in the NOD mouse demonstrated that depletion of B lymphocytes from the peritoneal cavity, via injection of distilled water, prevented these cells from trafficking to the pancreas and prevented disease[142]. Studies in SLE demonstrated an expansion of B1 B lymphocytes and presence of these cells in the kidney of humans[143]. The pathologic role of these B1 cells, most often associated with immune regulation, in T1D and SLE may indicate a breakdown in Breg function.

Autoantibodies in T1D and SLE

The role of autoantibodies in T1D and SLE is complex. In NOD mice there seems to be no role for secreted islet-directed antibody in beta cell damage[144,145]. The importance of anti-islet directed immunoglobulin appears to be related to their capacity to capture and concentrate islet-

antigen in B lymphocytes via their membrane-bound form[146,147]. Some studies suggest that maternal antibodies play a role in transfer of disease to progeny, but additional studies do not support this hypothesis[148,149]. In SLE autoantibody deposition in the kidney leads to complement activation and hallmark glomerulonephritis[150]. Interestingly, in mouse models of lupus and T1D that possesed B lymphocytes unable to secrete antibody still developed disease and exhibit increase mortality[144,150]. These data point to additional roles for B lymphocytes in SLE outside of antibody production.

Clinical evaluation of autoantibody titers in patients with T1D and SLE do not offer clarity on this matter. In T1D, autoantibodies are an excellent predictor of future risk of developing beta cell loss and insulin dependence. In fact, patients who possess 2 islet autoantibodies in the absence of hyperglycemia, are now diagnosed with Stage I T1D, with a inevitability they will experience beta cell loss if they live long enough[151]. As SLE is a relapsing and remitting disease, the interpretation of the presence of autoantibody is somewhat more complex. In a cohort of SLE patients it was demonstrated that ~10% possessed autoantibodies but no active disease[151]. Additionally of this 10%, half did not develop relapsing disease in a 17 year follow-up study[151]. Analysis of SLE patients with nephritis (confirmed by renal biopsy) and SLE patients without nephritis revealed 32% of patients with nephritis did not have circulating anti-dsDNA antibodies. Conversely, 50% of patients without nephritis possessed anti-dsDNA antibodies[151]. While autoantibody production is clearly an important indicator of disease in T1D and SLE, it is most likely a result of T-B lymphocyte collaborations that facilitate autoimmune damage by a myriad of other modalities. **Table 1.2** illustrates the roles of B lymphocytes in both T1D and SLE.

Overview and Significance of Research

The previous discussion illustrates the importance of B lymphocytes in T1D and SLE. The integral role these lymphocytes play in autoimmunity is clear in a general sense, but their specific role(s) are not fully defined. In T1D B lymphocytes are generally thought to be required for autoantigen presentation that incites rapid anti-islet immunity. Genetic models that lack B lymphocytes seem to bear this hypothesis out[122]. However, depletion of Tregs from B lymphocyte deficient NOD mice renders these normally resistant mice diabetic, indicating B lymphocytes may play a crucial role in modulating immune-regulatory cells[151]. These data indicate that inappropriate B lymphocyte regulation erodes tolerance, thus driving autoimmunity indirectly. Armed with the knowledge that B lymphocyte mediated regulation is a dynamic and diverse process, I sought to understand the processes, context, and signaling molecules that drives B lymphocyte mediated regulation in autoimmune disease. Ultimately, I identified key B regulatory mechanisms that may improve outcomes in patients with autoimmunity and reveal new pathways that dictate immune tolerance.

In Chapter II, I utilize TLR stimulation as a surrogate for environmental input to demonstrate how certain environmental signals can facilitate B lymphocyte regulation to prevent diabetes in NOD mice. I then chose to assess whether intervening on antigen specific BCR signaling could induce B lymphocyte regulation in NOD mice. In this study I demonstrated that targeting c-Abl with imatinib induced a B lymphocyte mediated regulatory pathway fostered by beta cell specific ROS-scavenging in islets of NOD mice and diabetes reversal. While these two studies demonstrated B lymphocyte centric modes of B lymphocyte regulation, I wanted to determine whethere correcting B lymphocyte homeostasis defects, characteristic of T1D, would restore B lymphocyte regulation .

Function	Type 1 Diabetes	SLE
Central Tolerance	Appears to be defective	Unknown
Peripheral Tolerance-Selection	Loss of transitional zone correlating with poor negative selection	TLR mediated survival of autoreactive cells
Peripheral Tolerance-Anergy	Evidence demonstrates abnormal insulin specific B lymphocyte anergy in humans	Unknown
Marginal Zone B lymphocytes	Expanded and thought to invade pancreatic lymph nodes to incite autoimmunity	Thought to carry autoantigen to follicles
Follicular/GC B lymphocytes	Spontaneous GCs in spleen and tertiary structures	Increased numbers of GC
B1 B lymphocytes	Depletion leads to protection from diabetes	Expansion correlates with disease in humans
Autoantibodies	Highly predictive of disease but not directly pathologic	Somewhat predictive of disease and thought to play a role directly in SLE pathology

Table 1.2 The role of B lymphocyte defects in T1D and SLE

In Chapter IV, I determined that IgM from a healthy donor mouse reversed disease in NOD mice and normalized both B and T lymphocyte abnormalities in NOD mice and human immune cells. In Chapter V, I demonstrate that B-lymphocyte dependent anti-CD45RB was unable to drive immune tolerance in SLE unless T lymphocyte metabolism was inhibited prior to therapy. This demonstrates that no matter what B lymphocyte regulation is utilized in autoimmunity, it will be important to determine whether target cells are resistant or amenable to this regulatory mechanism. Overall, I demonstrate novel modes of B lymphocyte regulation mediated by divergent pathways in autoimmune disease, with the potential to prevent and reverse disease in humans. Portions of work in Chapter II were previously published by me in *J. Diabetes*, portions of Chapter IV and V were published by me in *Diabetes and The Americsan Journal of Transplantation*[32,152,153].

CHAPTER II

B LYMPHOCYTES PREVENT DIABETES IN NOD MICE FOLLOWING TRIF-MEDIATED TLR STIMULATION

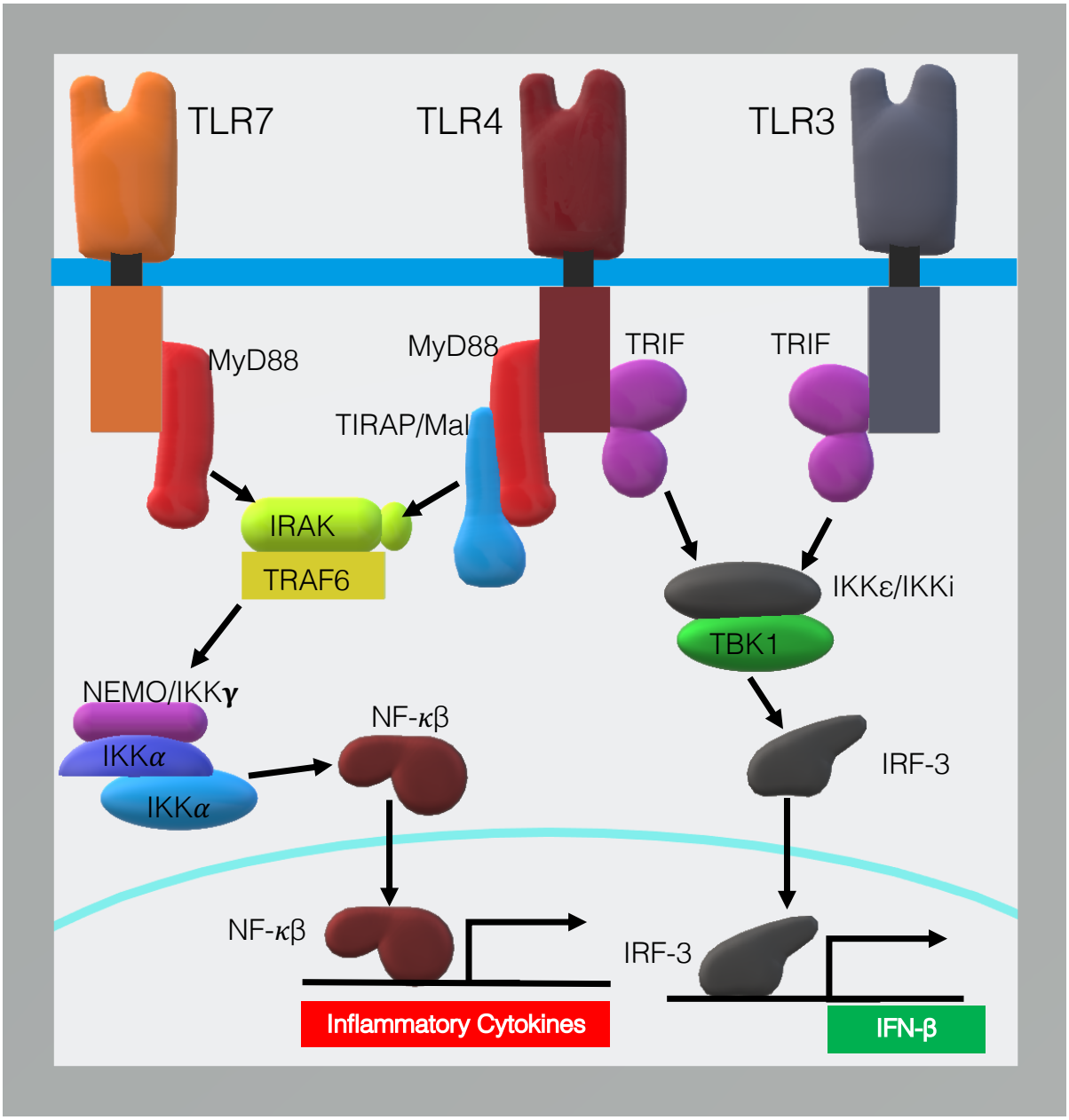
Scientific Goal

Studies in twins with T1D and even in NOD mice, which are highly inbred, demonstrate variable onset of disease indicating a contribution of the environment[154,155]. The contribution of the environment is further supported by the rising rate of T1D in many industrialized countries[156,157]. One explanation for this increase in T1D occurrence relates to the relatively recent advent of antibiotics and vaccines that have altered the pathogenic and commensal load in children. This "hygiene hypothesis" has been supported in NOD mice but a clear cellular target for this modulation has not been identified[155]. Many immune cells have the potential to interact with environmental factors but the central role of B lymphocyte in T1D make them a likely target of environmental modulation. Toll-like receptors (TLR) recognize conserved viral and microbial epitopes that, when bound, drive signaling pathways that shape the immune response. In Chapter II I investigate the hypothesis that TLR signaling induces B lymphocyte mediated regulation to prevent diabetes onset. This chapter addresses longstanding questions about the responsiveness of B lymphocytes to TLR signals and how B lymphocytes serve as a bridge between environmental signals and adaptive immune responses in NOD mice.

Introduction

The development of overt tissue autoimmunity in T1D is dependent on B lymphocytes[122]. In T1D it is clear that deleterious interactions occur between B and T lymphocytes that drive autoimmunity and beta cell destruction; less well understood is whether certain other B lymphocyte interactions can lead to disease prevention. Interestingly, others have demonstrated that priming B lymphocytes with activating signals can alter disease pathology, effectively preventing disease in NOD mice, and indicating the potential for regulatory capacity of B lymphocytes in T1D[158,159]. Furthermore, these data demonstrate the context in which B lymphocytes interact with other immune cell subsets dictates whether a response is regulatory or deleterious. This model led me to investigate the conditions that may favor B lymphocyte mediated immune regulation over autoreactive immune activation in NOD mice.

Mounting evidence in T1D indicates an influence of the viral and microbial flora on the time to disease[155,160,161]. Work has gone into detecting infectious events that incite autoimmune attack in T1D but only a few correlations have been found, with no solid evidence of a common inciting event[162–167]. Conversely, mice and human studies have indicated that the natural bacterial flora shapes the immune system and prevents disease[160,161,168–171]. While there is little understanding of the cellular players in this prevention, the TLR receptor system has been identified as a key target in this process[171–175]. The TLR system is composed of multiple receptors that detect conserved pathogen associated molecular patterns (PAMPs) and signal through three primary signaling pathways illustrated in **Figure 2.1**. In NOD mice knockout of TLR signaling molecule MyD88 leads to protection from diabetes[172]. This protection is



abrogated in the same mice when grown in a germ-free environment indicating in the absence of MyD88 other microbial signaling contributes to disease protection[172].

Figure 2.1 Representative signaling used by the TLR system. While not all TLRs are represented here, this cartoon illustrates the 3 primary pathways and the transcriptional changes regulated by each. (Adapted from Takeda et al., *Seminars in Immunology*, 2004)[176].

Researchers have knocked out other components of the TLR system with variable effects on disease in NOD mice but have consistently demonstrated a role for the microbial flora in disease progression (**Table 2.1**)[172–175]. As genetic models indicate a strong contribution of the TLR signaling system to T1D onset, researchers have utilized TLR signaling agonists to understand their influence on disease. Surprisingly, TLR agonists tested thus far facilitate disease protection in NOD mice with exception of TLR9 agonist CpG ODN in a transgenic model of diabetes (**Table 2.2**)[159,173,175,177–180]. Nevertheless, the cellular mechanisms that mediate the effects of TLR stimulation on the course of diabetes remain poorly understood. As B lymphocytes have the capacity to express TLRs and at the same time present and enrich specific antigens and secrete immunomodulatory molecules, I hypothesized they are a target for mediating prevention of diabetes by TLR agonists.

B lymphocytes exist as a heterogeneous population of cells with different function and speculated relevance to diabetes progression[124,181–183]. B lymphocytes develop in the bone marrow and emigrate to the spleen where they complete their development into two primary B lymphocyte subsets—follicular and marginal zone B lymphocytes. While follicular B cells are the primary participants in T lymphocyte interactions that lead to the production of high-affinity class-switched autoantibodies indicative of T1D, the marginal zone B lymphocyte compartment is expanded and thought also to contribute to disease in NOD mice[124]. The marginal zone cell subset has, in other models, been demonstrated to possess potent immunoregulatory capacity, a process that is potentially deficient in T1D[124,184]. In keeping with the hypothesis that interactions with environmental determinants influence disease outcome in T1D, B lymphocytes of the

marginal zone are uniquely positioned anatomically to sample and quickly respond to antigens returned via blood circulation[185,186]. In some studies, marginal zone B lymphocytes demonstrate

Gene Knockout	Effect on Disease in NOD mice	Effect on disease in germ free NOD mice
Myd88	Disease protection	No effect
Tlr2	Disease attenuation	No effect
Tlr3	No effect	No data
Tlr4	Disease acceleration	No effect
Tlr9	Disease prevention	No data

Table 2.1. Targeting toll-like receptors in NOD mice modulates disease. Genetic ablation of Toll-like receptors or signaling component Myd88 led to prevention of disease in NOD mice. This effect appears to depend on the microbiome, as knockout of these genes in germ free mice has no impact on disease.

TLR Ligand	Target TLR	Effect on disease
Poly I:C	TLR3	Disease protection
Zymosan P40 (Klebsiella pneumoniae) Pam3Cys lipopeptide Pam3CSK4	TLR2	Disease protection
LPS	TLR4	Disease Protection
Resiquimod (R8484)	TLR7	Disease Protection
Empty plasmid DNA or CpG ODNs	TLR9	Disease protection
CpG ODNs	TLR9	Activation of cytotoxic T cells in NOD 8.3 TCR transgenic mice

Table 2.2. Administration of TLR ligands in NOD mice impacts disease. Administration of certain TLR ligands has disease protective effects in NOD mice. The lone exception is the administration of CpG ODN in TCR transgenic mice on an NOD background, which accelerates disease.

robust responses to TLR stimulation, indicating increased TLR signaling component expression[33,187]. Furthermore, marginal zone B lymphocytes are also thought to possess antigen specificity to bacterial wall components increasing the likelihood of TLR interactions in these cell types[187].

The critical role of marginal zone B lymphocytes in T1D and their enhanced capacity to respond to environmental cues make them likely to serve as an integrating node for environmental input during diabetes progression. Nonetheless, in this study, I took an unbiased and detailed approach to assessing B lymphocyte responses to TLR engagement in NOD mice in order to gain a complete understanding of this system. I find that B lymphocytes express increased levels of TLR response proteins but are not hyper-responsive to TLR engagement. Instead, I find that marginal zone B lymphocytes are highly sensitive to depletion following MyD88-independent/TRIF signaling by TLR3 *ex vivo*. *In vivo* NOD, but not B6 B lymphocytes, resist mobilization by TLR3 ligation and fail to egress from the spleen. This failure to mobilize was linked to a defect in actin mobilization in NOD B lymphocytes. This defect allows for enhanced interactions between CD1d+ marginal zone B lymphocytes and DX5+ NK cells leading to diabetes protection.

NOD B lymphocytes express higher levels of TLR-sensing molecules

To understand the capacity of B lymphocytes from NOD mice to responds to TLR agonists, I assessed expression of TLR-interacting proteins by flow cytometry on B6 and NOD B lymphocytes (**Figure 2.2**). I noted increased expression of CD180 and MD-1 (positive regulators

of LPS/TLR-4 signaling) as well as CD9 (a negative regulator of LPS/TLR-4 signaling). This increase could be attributed to the expansion of marginal zone B lymphocytes, characteristic of NOD mice. All NOD B lymphocytes demonstrated an increase in CD284/TLR4. CD274/PD-L1/B7-H1 was also increased in NOD B lymphocytes, a molecule that has been associated with the response to TLR3 signaling[187]. Strikingly, all NOD B lymphocytes demonstrated a dramatic increase in CD36, which has been linked to signaling in toll-like receptors 2, 4, and 6[188]. This increased expression of TLR molecules in NOD B lymphocytes could lead to enhanced activation of B lymphocytes or alternatively provide heightened induction of TLR-induced immunoregulation in NOD mice.

NOD B lymphocytes are not hyper-responsive to TLR engagement despite increased baseline activation

Having determined that NOD B lymphocytes express increased levels of several proteins involved in TLR activation, I anticipated that the cells may be hyper-responsive to TLR engagement. As illustrated in **Figure 2.1**, TLRs signal through TRIF and MyD88 mediated pathways. In order to get coverage from all signaling molecules and understand the complete response of B lymphocytes in NOD mice to TLR signaling, I utilized multiple TLR ligands. I stimulated cells with LPS which targets TLR4 and relies on Mal, MyD88, TRAM, and TRIF signaling. I also stimulated with polyIC which targets TLR3 and relies on TRIF signaling. Finally, I stimulated with imiquimod which targets TLR7 and relies exclusively on MyD88. Whole splenocytes from B6 or prediabetic NOD mice were cultured overnight in the presence of LPS, polyIC, or imiquimod at varying concentrations to assess the dynamic range of the B

lymphocyte response. A whole splenocyte system was selected to best model the *in vivo* environment where multiple cellular interactions would govern the overall response. After 18hrs

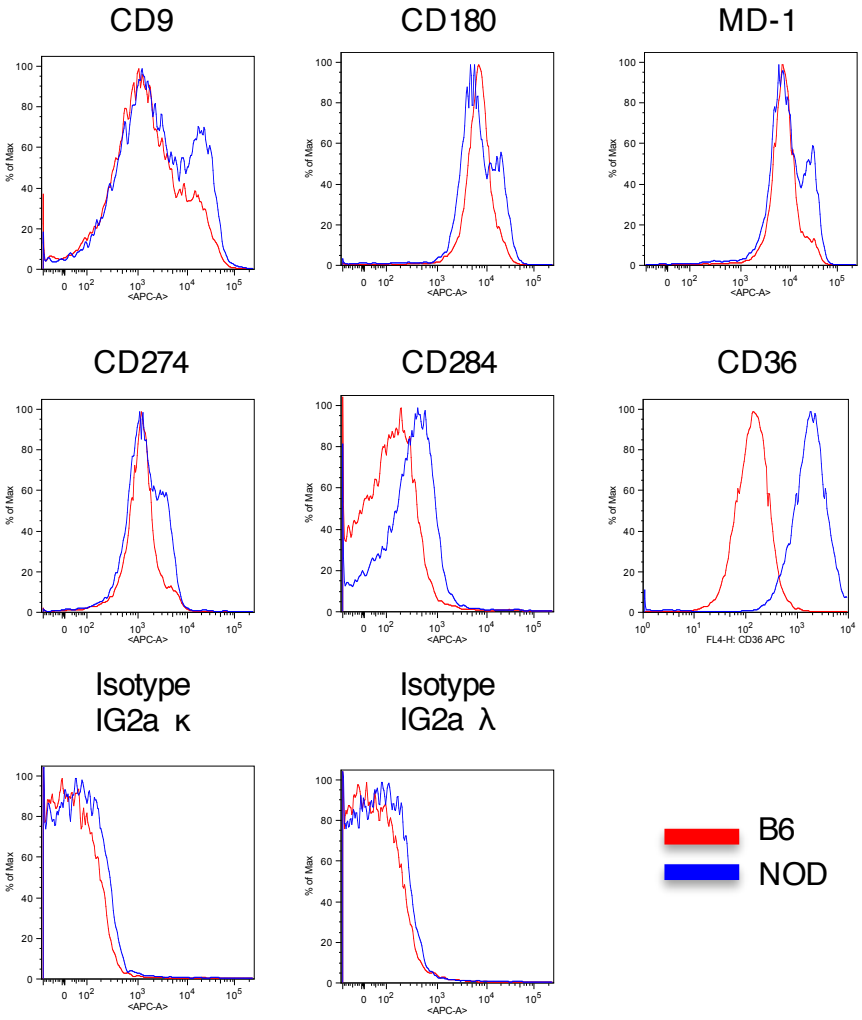


Figure 2.2. Upregulation of TLR interacting receptors in NOD B lymphocytes. Splenocytes were isolated from pre-diabetic 9-11 week old female NOD mice and matched B6 controls. Cells were immediately stained with B220, CD21, CD23, and the indicated surface marker in the diagram. Overlays of B220+ splenocytes are shown with NOD B cells indicated in blue and B6 in red. NOD mice show an increase in a high expressing population for CD9 (tetraspanin family), CD180 (TLR4/LPS co-receptor, pairs with MD-1), MD-1 (TLR4/LPS co-receptor, paris with CD180), and CD274 (PD-L1). The entire NOD B lymphocyte population shows increased surface expression of CD284 (TLR4) and CD36 (thrombospondin receptor/fatty acid translocase) as indicated by a right shift in the NOD histogram. No differences were seen in the isotype control stains, all of which were clearly negative. The same results were obtained by staining and gating on CD19+ or IgM+ cells. Comparison of staining between CD180, MD-1, CD274, and CD9 was performed based on determination of percent positive cells; differences were significantly different between all groups ($p<0.0001$, ANOVA followed by post test, $n>3$ for all). For CD36 and TLR4, comparison was made based on the population MFI and was also statistically significant ($p<0.0001$ for CD36 and $p<0.05$ for TLR4, t-test, $n>3$ for both). The figure is representative of at least two independent experiments in all cases.

of culture, cells were harvested and analyzed by flow cytometry for expression of CD69, CD80 (B7.1), and CD40. The results, shown in **Figure 2.3**, indicated no difference in terms of minimal ligand sensitivity or maximal response but instead indicate that the major difference in B lymphocyte activation is measurable prior to exogenous TLR stimuli.

TLR3 signaling targets Marginal Zone B lymphocytes ex vivo

Although NOD B lymphocytes as a whole did not exhibit enhanced sensitivity to TLR ligation, overexpression of certain TLR molecules in NOD mice is associated with marginal zone B lymphocytes and suggested that this subset may have a differential response to TLR signaling[188]. Further B lymphocyte subset analysis of data in **Figure 2.3** did not reveal any differences in activation within the B lymphocytes subsets as compared to B6 (not shown). However, I hypothesized that TLR ligation that alters the marginal zone in comparison to follicular B lymphocytes may enhance the protective capacity of marginal zone cells. To assess this possibility *ex vivo* whole splenocytes were cultured overnight in the presence of varying concentrations of LPS, polyIC, and imiquimod. As seen in **Figure 2.4**, LPS and imiquimod (both utilizing MyD88) induced a relative increase in marginal zone B lymphocytes as compared to no stimulus. In contrast polyIC induced a relative loss of marginal zone cells in both strains. This phenotype could be exacerbated as marginal zone loss was apparent even at baseline in NOD mice. The trends were similar in NOD and B6 B lymphocytes and were dose responsive, as shown in **Figure 2.4**. Follicular B lymphocytes presented an opposite trend at baseline in which follicular B lymphocytes were maintained at baseline in NOD. B6 follicular B lymphocytes

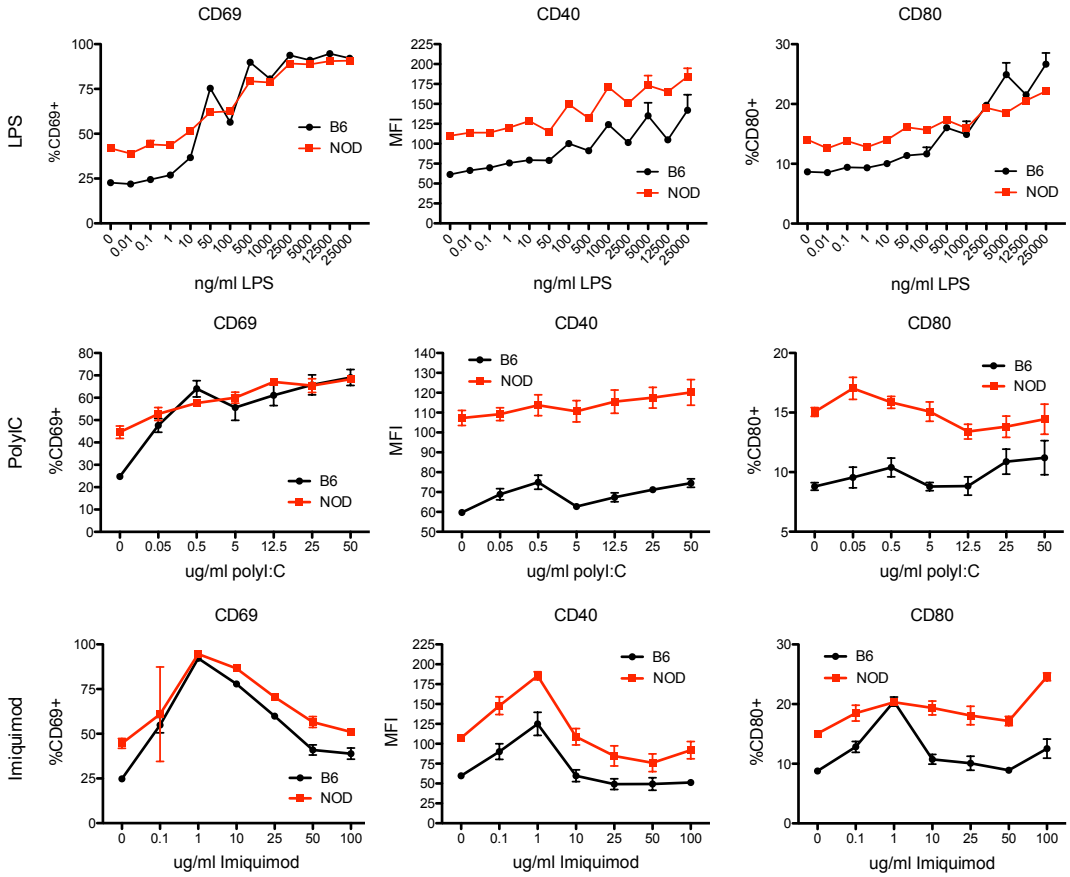


Figure 2.3. The dynamic response of B lymphocytes to TLR engagement. Splenocytes were isolated from pre-diabetic female NOD mice and matched B6 controls. Cells were cultured overnight in the presence of TLR agonists including LPS (TLR4), polyIC (TLR3) and imiquimod (TLR7). After overnight culture, cells were harvested, stained with B220, CD40, CD69, and CD80. Data is demonstrated as percent positive cells for CD69 and CD80. For CD40, the population MFI is shown as all B cells were positive. Measurement of all markers shows greater staining in NOD B lymphocytes at baseline as compared to B6. Following stimulation, B6 B lymphocytes largely achieve the same maximal activation as measured for NOD. In the case of CD40 surface expression, the baseline difference appears maintained throughout a range of stimulation conditions, which follow identical dynamics between the strains. For other comparisons, the baseline difference is the largest difference observed. For all comparisons shown, the responses of the two strains are statistically different as assessed by ANOVA ($p<0.01$ for all); this difference is largely attributable to the baseline difference, which is lost at higher levels of stimulation particularly for CD69. Data are obtained from 3 separate experiments; n=6 for all doses demonstrated.

demonstrated improved maintenance after TLR stimulation with LPS or polyIC while NOD mice remained unchanged. Increasing doses of MyD88 dependent imiquimod significantly decreased the maintenance of follicular B lymphocytes in NOD mice.

In vivo treatment with polyIC prevents diabetes but does not deplete marginal zone B lymphocytes in NOD mice

As polyIC robustly targeted marginal zone lymphocytes in NOD mice, I hypothesized that utilizing this therapy *in vivo* may prevent diabetes by restoring immune regulatory capacity in this cell subset. 9 week old female NOD mice were injected with 100μg of polyIC daily for 9 days and followed for the development of diabetes. This treatment significantly protected against diabetes (**Figure 2.5A**). Analysis of the spleens of an additional group of mice, at the end of the treatment regimen, revealed that the marginal zone in B6 mice was depleted but remained intact in NOD mice (as identified by CD21 and CD23; the same results were obtained with CD1d /IgD staining Figure 2.4B and not shown).

Retention of marginal zone B lymphocytes after polyIC in NOD mice is due to a defect in actin mobilization leading to local induction of diabetes protective DX5+ NK cells

The differential response of marginal zone B lymphocytes in NOD and B6 mice was an interesting phenomenon and I sought to dissect the mechanisms involved in this process. While I did not directly assess the fate of marginal zone B lymphocytes in B6 mice, I entertained the hypothesis that these cells were either mobilized or depleted by polyIC therapy. Activation of marginal zone B lymphocytes by MyD88-dependent TLR ligands leads to mobilization of these cells by modulation of S1P family of sphingosine receptors[189]. However, this mechanism is not

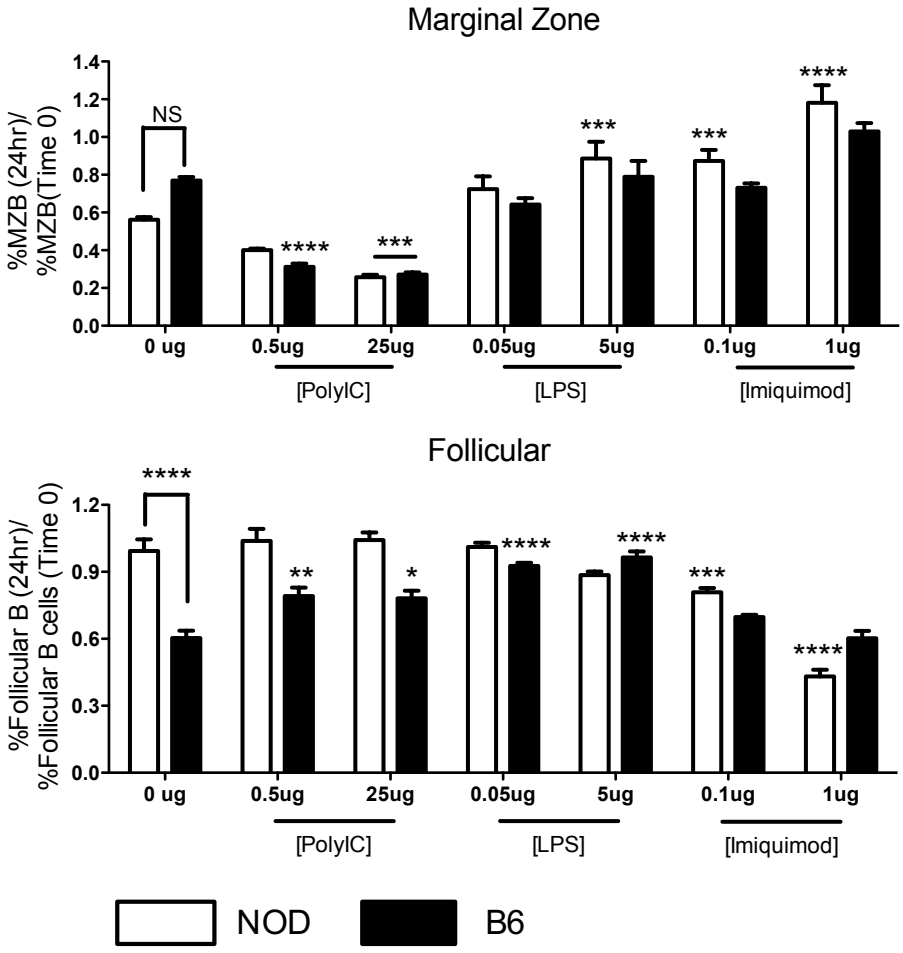


Figure 2.4. The differential response of marginal zone B cells to MyD88 dependent and independent TLR pathways. Splenocytes were isolated from 9-11 week old, pre-diabetic female NOD mice and age-matched B6 controls. The baseline percentage of marginal zone and follicular B cells were obtained by staining with B220, CD19, CD21, and CD23. Cells were cultured overnight in the presence of the indicated concentration of LPS, imiquimod, or polyIC. After culture, cells were again stained and the percentage of follicular and marginal zone B cells calculated. The graphs display the ratio of the percent cell type identified at the end of culture to that present at the beginning. A ratio of 1 indicates no change in the relative cell proportion in the culture. Examining the unstimulated condition, marginal zone B cells stability is similar between strains in overnight culture whereas NOD follicular zone B cells are relatively better retained. Stimulation with LPS, which depends on all major TLR adaptors, shows increased retention of the marginal zone phenotype in NOD mice in a dose responsive pattern. B6 mice show a modest effect in the marginal zone and show significantly improved maintenance of follicular B cells. The pattern is similar with imiquimod, which depends only on MyD88, as NOD marginal zone B cells are more persistent in culture following increased stimulation; conversely follicular zone B cells are decreased by increasing imiquimod concentration. PolyIC, which depends upon TRIF, shows an opposite effect in the marginal zone, where both NOD and B6 cells are diminished by treatment. PolyIC shows little effect on the follicular zone. Data are from 3 separate experiments. (n=9 per strain; p<*0.05,**0.01,***0.001,****0.0001, ANOVA followed by Bonferroni post-test). Statistics are shown comparing NOD to B6 at baseline; other comparisons are for the stimulated condition for the given strain to its own baseline.

used by polyIC that instead relies on upregulation of CD69[187]. Analysis of *in vivo* stimulated B lymphocytes demonstrated the same degree of CD69 upregulation between responding NOD and B6 B lymphocytes, indicating activation was not a likely mechanism (not shown).

Regardless of the mechanism that leads to the loss of marginal zone cells, the actin cytoskeleton plays an important downstream role by allowing cellular mobility and by transducing strength of signal[190,191]. To assess actin dynamics in B lymphocytes, I utilized hydrogen peroxide as a well-described, potent stimulus of actin reorganization[192]. I elected to measure actin dynamics by flow cytometry so that the dynamics of the marginal zone could be directly assessed by co-staining for marginal zone B cell markers. The cytoskeletal response was measured by staining with rhodamine phalloidin, which selectively binds to F-actin network but cannot bind to unassembled G-actin. Activation by peroxide for 10 minutes revealed a robust decrease (>20 fold, **Figure 2.6A black histogram**) in B6 mice. This change in rhodamine phalloidin binding suggested dynamic reorganization of the actin cytoskeleton in the non-autoimmune B6 strain. NOD mice showed virtually no response to this stimulus as indicated (**Fig 2.5A red histogram**). Previous exposure to polyIC by intraperitoneal injection before peroxide activation resulted in a decrease in actin mobilization in both strains. However, there was still a 10-fold decrease in rhodamine staining in B6 B lymphocytes whereas changes in the NOD actin network were almost completely abrogated by this intervention (**Figure 2.5B**). To further validate this measure of cytoskeletal dynamics, I performed a time course to determine whether the response gradually returned to baseline over 40 minutes; at no time did the NOD response rise above 5 fold and it was never greater than B6 (Figure 2.5C). The response was dependent on proximal signaling molecules as pre-incubation with dasatanib or the Src family kinase inhibitor Src-I1 largely abolished the response (**Figure 2.5D**). The NOD network was,

however, capable of de-polymerization as incubation with latrunculin B, which induces complete F-actin de-polymerization, resulted in identical responses between NOD and B6.

As marginal zone B lymphocytes were targeted by TLR3 agonist polyIC, activated, and retained in their anatomical niche in NOD mice, I attempted to understand how these cells may offer diabetes protection. PolyIC has been demonstrated to offer diabetes protection via induction of protective NK cells[192]. CD1d, a non-classical MHC capable of interacting with NK cells, is a defining marker of marginal zone B lymphocytes, making them well-suited to interact with these cells[192]. I hypothesized that B lymphocytes may be important in inducing diabetes protective NK cells.

To investigate this interaction, NOD WT and B lymphocyte deficient NOD mice (NODμMT) mice were treated with a 9 day course of polyIC. At the end of the treatment, DX5+ cells, which include NK cells, were obtained from these mice. One million of these cells were isolated from spleens and transferred into immunodeficient NOD scid recipients along with 20 million cells from a NOD WT diabetic donor; NOD scid mice were also given diabetic splenocytes alone as a control for diabetes onset in this model (Figure 2.5E). DX5+ cells derived from polyIC-treated, WT NOD donors prolonged time to diabetes and prevented diabetes in some recipients. DX5+ cells from NODμMT mice treated with polyIC did not offer any protection from disease indicating activation of B lymphocyte protection in the spleen by TLR3 ligation, most likely due to the action of marginal zone B lymphocytes.

Discussion

Mounting evidence indicates that environmental factors modulate diabetes pathogenesis in humans and animal models, potentially serving as a rheostat over the background of genetic

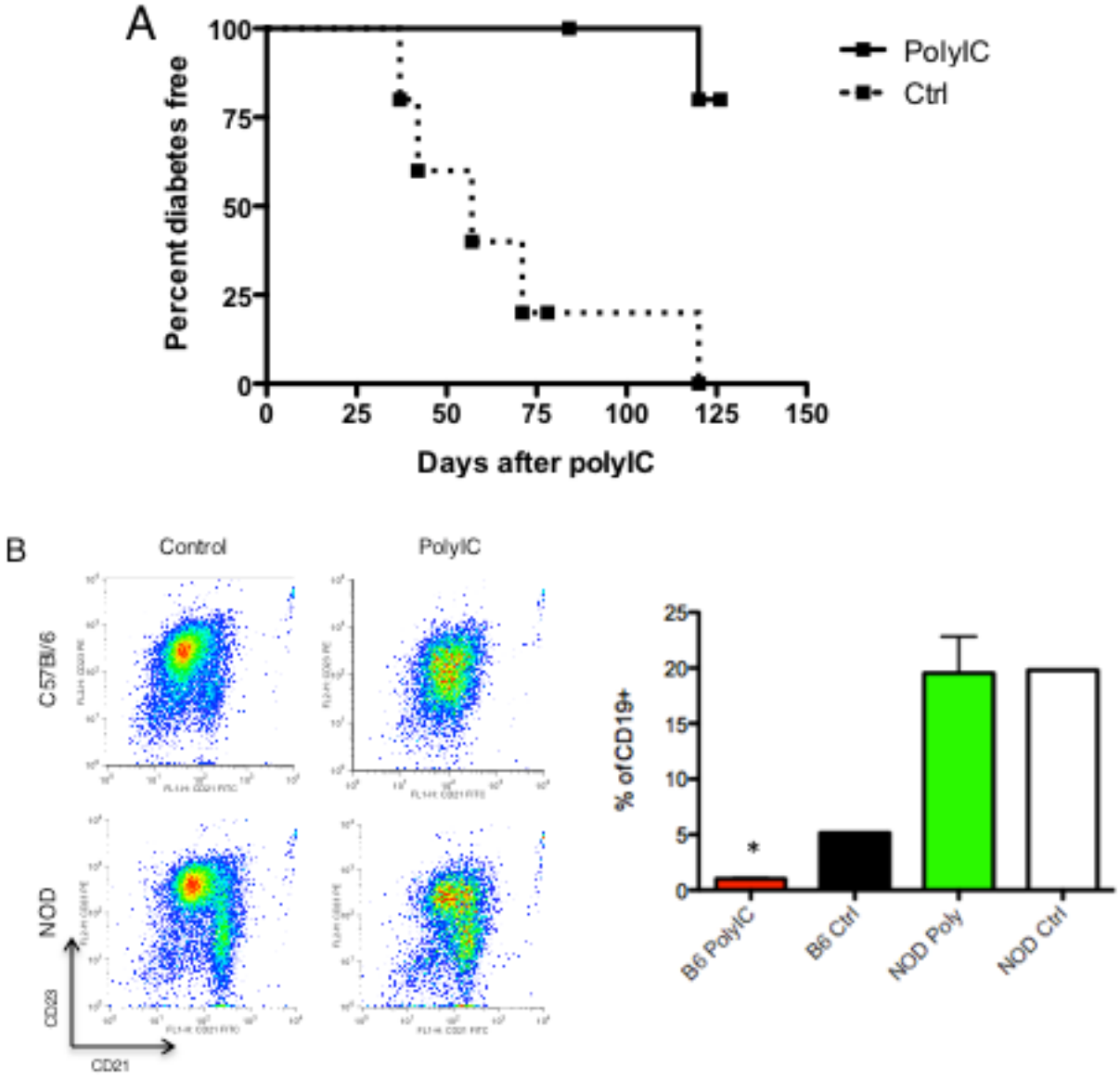


Figure 2.5. PolyIC injection prevents Type 1 diabetes but does not deplete the marginal zone in vivo A). 9-10 week old female NOD mice were given daily injections of 100ug polyIC for 9 days. Mice were followed for the development of diabetes. PolyIC treated mice demonstrated significant diabetes protection (**p<0.0001, log-rank). N=10 in each group. B) After a 9 day course of 100ug polyIC injections, spleens were obtained from NOD and B6 mice and analyzed for marginal zone B cells by staining with B220, CD19, CD21, and CD23. B6 B cells showed an expected decrease in the presence of marginal zone B cells as compared to baseline (*p<0.05 ANOVA, followed by Bonferroni post-test). Unexpectedly, no change was identified in the marginal zone compartment in NOD B cells. Similar results were obtained when staining with CD1d and IgD was used instead. Panel B is representative of 5 different experiments and combined results are shown in the histogram.

predisposition. My findings indicate that B lymphocytes may be one target of modulation by environmental signals via the TLR system. Interestingly, previous studies suggested that NOD B lymphocytes are hyper-responsive to TLR innate signaling, and much focus has been placed on the ability of TLR signals to break tolerance in B lymphocytes[193–195]. My characterization of surface markers expressed by NOD B lymphocytes also supported their potential for excessive responsiveness to TLR activation, in particular LPS/TLR4. However, my dose-response study did not reveal hyper-responsiveness by B lymphocytes. Although the baseline activation was elevated in NOD mice, hyper-responsiveness should be reflected either as an ability to respond to lower amounts of stimulus or the capacity to more rapidly reach a maximal response (steeper does-response curve). NOD B lymphocytes when stimulated through all major TLR pathways did not show increased sensitivity or a proportionally greater response at any concentration as compared to B6. TLR ligands play a poorly understood role in B lymphocyte development. Some studies have demonstrated positive effects on survival of autoreactive cells and others have demonstrated the converse[52,196]. Nevertheless, the role TLR signaling plays on B lymphocyte development, while outside of the scope of these studies, cannot be ruled out as a factor in alteration of B lymphocyte development in Type 1 Diabetes and other autoimmune diseases. Additionally, developmental responses to TLR signaling may drive the differences in baseline activation in mature NOD B lymphocyte subsets we noted in this study. While some TLR associated signaling molecules (CD284 and CD36) were upregulated on all B lymphocytes, increases in other molecules were related to the expansion of marginal zone B lymphocytes in NOD mice. While work in T1D relates marginal zone expansion to pathogenic capacity, others have suggested the marginal zone contains regulatory cell subsets[197]. Our data suggests that treatment with polyIC also induces regulatory capacity in this cell subset.

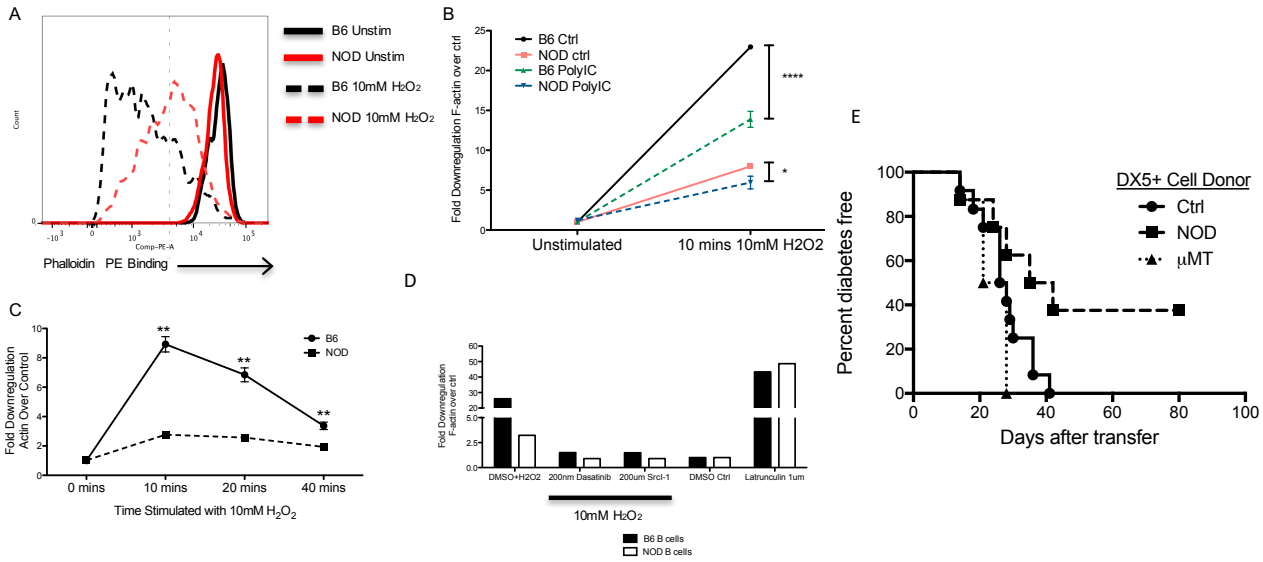


Figure 2.6. Attenuated actin cytoskeletal dynamics in NOD B lymphocytes. A) Splenocytes from pre-diabetic NOD and matched B6 mice were stimulated for 10 minutes with 10mM hydrogen peroxide (H_2O_2). F-actin depolymerization was measured by flow cytometry in B220 positive cells by co-staining with rhodamine-phalloidin. A representative diagram of the actin response is shown. At baseline, phalloidin staining is consistent between strains. Following stimulation, B6 lymphocytes show a significantly more dynamic response of the actin network as indicated by a greater left shift. B) The response of the actin cytoskeleton is presented as a fold decrease in phalloidin staining compared to baseline. The effect of polyIC was assessed by injection of the mice with 100ug of polyIC 6h prior to the activation assay. Marginal zone B cells (identified by CD21/23) in B6 mice show an over 25-fold reduction in staining compared to a maximal 8-fold reduction in NOD mice. Exposure to polyIC decreases this response in both strains. However, the B6 response remains significantly greater than NOD and the NOD response is reduced to nearly baseline levels ($p<*0.05$ or ****0.0001, ANOVA followed by Bonferroni post-test). C) The kinetics of the response were also assessed. Stimulation with hydrogen peroxide results in maximal response at 10 minutes which returns to near baseline in B6 mice by 40 minutes. NOD B lymphocytes show similar kinetics with severely reduced amplitude (**$p<0.005$, ANOVA followed by post-test). (D). The measured actin response is regulated by normal signaling pathways. Addition of the broad kinase inhibitor dasatanib or by the more selective SRC family inhibitor Src-Inhibitor I prevents the depolymerizing response induced by H_2O_2 to nearly baseline levels as compared to control. Addition of the actin depolymerizing toxin latrunculin shows the capacity for NOD B lymphocytes to demonstrate complete de-polymerization. Data shown are representative of at least three independent experiments. DX5+ cells were isolated from either polyIC treated NOD mice (n=8, squares) or polyIC treated B cell deficient NOD.mMT mice (n=8, triangles). In treated mice, 100ug polyIC was given daily for 9 days. 1 million DX5+ cells were transferred along with 20 million splenocytes from a diabetic donor into NOD/scid recipients. The time to diabetes was also compared to control NOD mice that received diabetic splenocytes alone (n=12, circles). Blood sugars were followed twice weekly until diabetes developed. Cells from NOD mice protected against diabetes development (*$p<0.05$ NOD/polyIC vs Ctrl) whereas the same cells from B cell deficient donors lost their capacity to prevent diabetes transfer (**$p<.05$ vs NOD/polyIC, log rank). Data are from two separate experiments.

This finding may indicate that enhanced marginal zone expansion is partially a homeostatic response to hypofunctional regulatory cells in this subset in NOD mice. Alternatively, but not mutually exclusive, is the possibility that elevated signaling via NFkB may mediate inappropriate expansion and function of marginal zone B lymphocytes. Indeed, I discovered that TLR signaling mediated by MyD88 and its downstream target NFkB drove marginal zone expansion while TRIF-dependent/MyD88-independent signaling diminished marginal zone B lymphocytes. NFkB signaling is known to be abnormal in NOD mice and humans with T1D, and targeting this pathway diminishes marginal zone B lymphocyte development and prevents disease[198–201]. My work demonstrates that when TRIF mediated signaling predominates, immune regulation is restored in NOD mice and diabetes is prevented. Studies have demonstrated that TRIF transcriptional target IRF-3 has the capacity to inhibit NFkB activity directly[202]. This could potentially provide an explanation for the diabetes protection of MyD88 knockout NOD mice, in which TRIF signaling predominates in response to TLR stimulation, from diabetes only when housed in conditions in which TLR stimulation can occur (i.e. not germ-free).

In keeping with these data and previous reports, I treated NOD mice with a 9-day course of polyIC and achieved disease protection. PolyIC mediated robust depletion of marginal zone B lymphocytes in B6 mice but not in NOD mice. While I did not track the fate of these cells, I hypothesized that cell death or splenic egress, as reported by others in response to TLR signaling, may account for loss of marginal zone in B6 mice. While *ex vivo* data suggested marginal zone cell loss in response to TLR3 signaling, this observation was not recapitulated in NOD mice *in vivo*, where other factors may impart resistance to polyIC induced signaling, such as BAFF.

In the absence of any clear difference in the well-described effectors of marginal zone B cell mobilization, I considered that successful mobilization would require engagement and re-organization of the actin cytoskeleton. The regulation of the actin cytoskeleton is now appreciated to regulate B lymphocyte signaling and other functions[203,204]. Direct de-polymerization of the actin cytoskeleton, even in the absence of other known signals, activates B lymphocytes[191]. Moreover, actin-mediated mobility of BCRs is critical for their clustering and activation[191]. Once activated, cytoskeletal dynamics are also required for cell migration; this process has been well described in T lymphocytes where interactions between CD43 and the ezrin-radixin-moesin system are required[191]. Interestingly, I have also identified a slight upregulation in CD43 in NOD B lymphocytes, but the biologic significance is unknown at this time (not shown). This abnormal regulation of cytoskeletal dynamics may also account for the increased dwell time of NOD marginal zone B cells in the spleen, a reported phenotype in this autoimmune background[191].

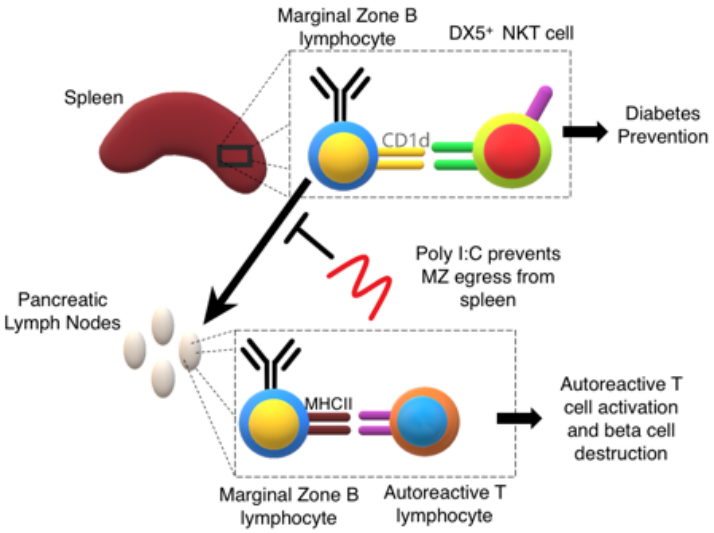
I elected to assess actin cytoskeletal dynamics with a potent stimulus that results in dramatic rearrangement of the actin cytoskeleton. In addition, stimulation with hydrogen peroxide likely provides the signals that are derived from superoxides *in vivo*, which may act as a "Signal 3" in lymphocyte activation and which are required in diabetes pathogenesis[205,206]. Activation by this potent stimulus in B6 B cells lead to a rapid decrease in polymerized F-actin as measured by phalloidin binding. This mobilization was not seen in NOD B lymphocytes at any concentration tested or at any time. PolyIC stimulation reduced F-actin mobilization in both NOD and B6 mice; however, in B6 mice even after this reduction, the overall decrease in F-actin remained considerable. In NOD mice, the modest activation seen in control conditions was almost completely eliminated by polyIC.

With the emerging role of the cytoskeleton in the control of lymphocyte activation, the consequences of abnormal regulation of polymerization and de-polymerization events may be numerous. Importantly, the actin cytoskeleton has been implicated in determining the strength-of-signal detected by the B cell receptor, and inhibition of depolymerization may be another mechanism to inhibit elevated NFkB in NOD mice[191,207]. In B lymphocytes, actin engagement is also required for the turnover of B cell receptors in the process of antigen capture[208]. The relative requirement for actin dynamics in B cell activation vs antigen uptake is not known. Altered cytoskeletal dynamics could lead to a B lymphocyte in which full cell activation is limited (anergy) while antigen uptake and presentation is preserved. This "split tolerance" phenomenon has been previously described in autoreactive B lymphocytes in which an apparent anergy is induced while antigen presentation and T cell activation continues to proceed efficiently[146,209]. More importantly the contribution of the actin cytoskeleton in impeding B lymphocyte mediated immune regulation remains completely unknown.

I considered that the retention of cells with marginal zone markers in the spleen following polyIC may lead to B lymphocyte mediated regulation as markers of the marginal zone also include B lymphocytes with suspected regulatory activity. Previous studies of polyIC mediated diabetes protection suggested a role for NK-like cells[210]. I therefore investigated the induction of regulatory capacity in DX5+ cells in both B cell sufficient and B lymphocyte deficient animals as it has remained technically impossible to obtain large numbers of viable marginal zone B cells by conventional sorting techniques for direct assessment of this hypothesis. Although our diabetes protective effect was modest, this effect relied on the presence of B lymphocytes in the spleen. Whether other cell types may be activated to promote tolerance was not investigated. Nonetheless, stable immune regulation and prevention of autoimmunity

requires interactions between a number of cell types with both innate and adaptive immune properties. The role of B lymphocytes as organizers of this protection demands continued investigation. Molecular mechanisms of this phenomenon were outside of the scope of this study but have been largely dissected elsewhere. In these studies, it has been demonstrated that NK-like cells (DX5+) lead to a skewing of cells to a Th2 phenotype that is dependent on TGFB signaling[210]. Skewing an immune response to Th2 in T1D is known as "immune diversion" and whether it represents bona fide tolerance to islet antigens is still debated, but may represent a "practical prevention" or prope-tolerance scenario.

Overall, I describe the specific interaction between B lymphocyte subsets and TLR signals in NOD and B6 mice. Unexpectedly, I determined that MyD88-independent TLR signaling results in diminution of marginal zone B lymphocytes in *ex vivo* studies in NOD and B6 mice. *In vivo*, this situation is more complex where I determined for the first time that NOD B lymphocytes have a substantial defect in mobilization of the actin cytoskeleton, a defect that may underlie unexplained aspects of B lymphocytes biology in NOD mice. In addition, other inter-cellular and stromal interactions may promote further differences in the handling of TLR signals by lymphocytes in NOD and B6 mice. Furthermore, I have defined how abnormal signaling may contribute to defective immune regulation by marginal zone B lymphocytes further elucidating their role in T1D. Understanding the regulation of these signaling processes presents new opportunities to halt the relentless progression of autoimmune disease by coupling receptor activation to the desired lymphocyte response by regulating the B lymphocyte response to favor immune regulation over immune activation and autoimmune damage (as illustrated in the model Figure 2.8). The following chapters will revisit these themes as well as determine other ways in which B lymphocytes in an autoimmune setting can promote immune regulation



Key Findings

-NOD mice express enhanced levels of TLR-related signaling molecules yet are not hyperresponsive to TLR stimulation.
-TLR3 signaling preferentially targets marginal zone B lymphocytes.
-In vivo treatment depletes marginal zone B lymphocytes in B6 mice but they are retained in NOD mice, a phenotype related to altered actin dynamics.
-Improper actin dynamics leads to activated marginal zone B lymphocytes that promote NK cell mediated protection from diabetes.

Future Aims

-Define the regulatory marginal zone subsets that contribute to NK cell mediated diabetes protection.
-Understand the molecular defects that prevent adequate immune mediated regulation from the marginal zone B lymphocyte compartment in diabetes.
-Determine whether polyIC therapy induces true immune tolerance in NOD mice by testing in islet transplant models.
-Assess whether slow progressors or first degree relatives of patients with T1D demonstrate activation of TLR signaling pathways in B lymphocytes.
.

and tolerance induction.

Figure 2.8 Model for prevention of diabetes by poly I:C in NOD mice, key findings and future aims

CHAPTER III

IMATINIB INCREASES ANTIOXIDANT CAPACITY IN B LYMPHOCYTES TO REVERSE T1D

Scientific Goal

In the previous chapter, I demonstrated B lymphocyte signaling via the TLR system induces a previously unknown regulatory capacity in B lymphocytes from NOD mice. While the TLR system is an important immunologic signaling pathway, B cell receptor (BCR) signaling couples antigen recognition directly to B lymphocyte function through complex signaling pathways. Genetic or therapeutic manipulation of signaling proteins in the BCR signaling pathway has led to both prevention and induction of autoimmunity, highlighting the importance of this pathway in autoimmunity. In Chapter III I investigate the hypothesis BCR mediated signaling in NOD and nonautoimmune C57Bl6/J (B6) connects to distinct pathways that can be targeted for beta cell protection in NOD mice. This chapter will demonstrate that signaling capacity among the B lymphocyte subsets in NOD and C57Bl6/J mice is very similar in all but a handful of pathways. These pathways can be targeted via small molecule therapy and when targeted reverse diabetes by induction of a previously undescribed B lymphocyte-mediated immunoregulatory mechanism, involving redox regulation.

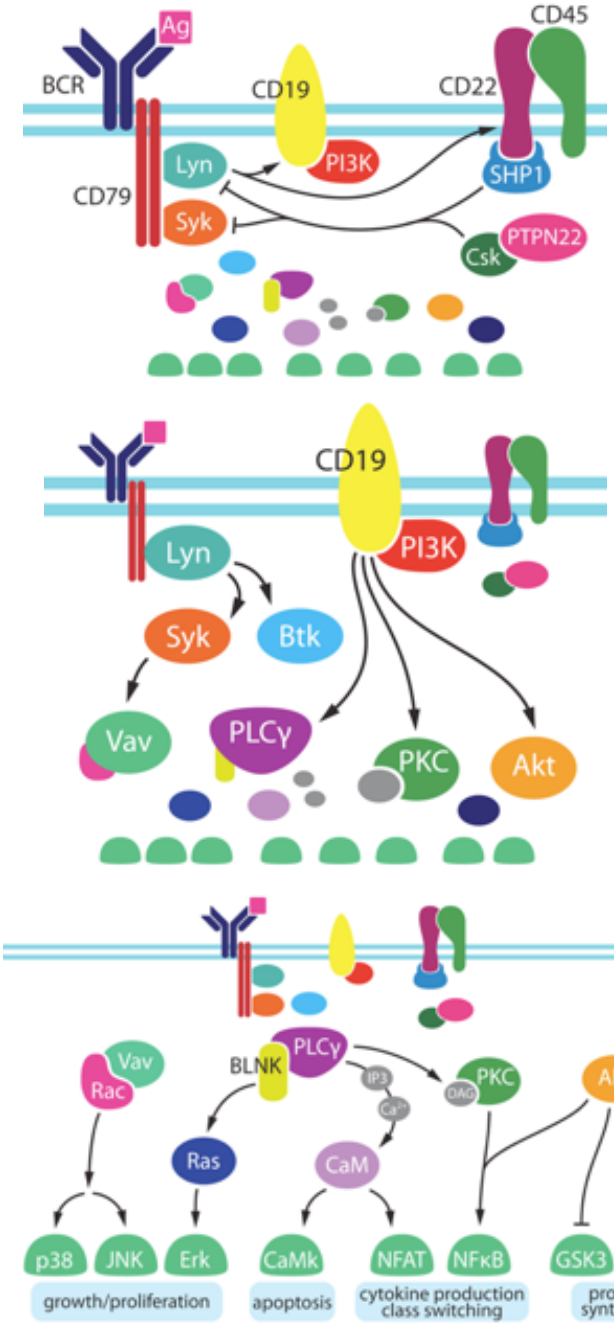
Introduction

Abnormal B lymphocyte function has been implicated in driving disease in NOD mice and humans with T1D. I as well as others have demonstrated that B lymphocytes can, under certain circumstances, mediate immune regulation[32]. While I have shown TLR activation leads to

protection from diabetes, others have shown that stimulation of B lymphocytes via the B cell receptor (BCR) can lead to immune protection[158]. These data indicate a role for BCR signaling in B lymphocyte mediated regulation.

B lymphocytes couple antigen recognition to effector function via a complex signaling cascade (**illustrated partially in Figure 3.1**)[211]. This signaling is initiated by antigen binding and conformational alteration of the BCR by either crosslinking or altering of the molecular weight of the BCR (in the case of non-crosslinking antigens) leading to localization of these signaling complexes to lipid rafts[212]. These lipid rafts are rich in Src-Family and other kinases that phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) in CD79, creating docking sites for other kinases that mediate downstream signaling[213–215]. At the same time counterregulatory proteins, termed phosphatases, alter the activity of kinases and other phosphorylated proteins to modulate the B lymphocyte response in both duration and intensity[216]. The affinity, proximity, and activation status of these phosphatases plays an important role in their ability to modulate the B lymphocyte response. While little is known about the particular signaling pathways that govern Breg development, there is much known about the signaling pathways important for B lymphocyte development and how antigen responses shape these pathways to drive B lymphocyte tolerance.

As mentioned previously, the BCR transduces signal into the cell via recognition of an antigen. Antigen specificity is determined through random genetic recombination and governed by central and peripheral selection mechanisms to ensure proper mature B lymphocyte responses to autoantigens (i.e. deletion, anergy, or secondary immune receptor rearrangement).



Proximal Signaling

Antigen biding leads to the phosphorylation of BCR coreceptors CD79a/b via Src Family Kinases (SFKs) (primarily Lyn in B lymphocytes). This leads to docking of additional SFKs and Syk. The drives phosphorylation of CD19 creating a docking site for PI3K, and CD22 leading to recruitment of SHP-1, a phosphatase and negative regulator of BCR signaling. Proximity of phosphatases CD45 and PTPN22-Csk regulate the quality and strength of BCR signaling.

Intermediate Signaling

Phosphorylation proceeds from proximal signaling molecules to phosphorylation of integrating nodes of BCR signaling. CD19 and PI3K lead to activation of PKC and AKT pathways, as well in aiding in part in the activation of PLC**γ**. Lyn and Syk activity target integrating signaling via Vav and Btk activation.

Distal Signaling

Vav/Rac activity leads to activation of p38 and JNK. While PLC**γ**/BLNK drive Ras and ERK signaling, important for B lymphocyte survival. PLC**γ** also induces calcium flux via generation of IP3. Calcium flux from the ER leads to activation of CamK and NFAT. DAG is also generated by PLC**γ** leading to PKC activation and ultimate NFkB activity, a protein whose activity is elevated in NOD mice. Finally AKT activation via PI3K leads to metabolic changes through its action of mTOR, GSK3B and S6.

Figure 3.1. B lymphocyte signaling. This cartoon partially illustrates the complex signaling pathways that couple antigen recognition to transcriptional activity in B lymphocytes.

In autoimmune diseases, such as T1D, there is an apparent breakdown in the ability to functionally silence autoreactive B lymphocytes leading to persistence and activation of these cells[119,121,127,129,130,146,181,217]. This process is governed by the intensity and duration of B lymphocyte signaling during development[211,218].

During early B lymphocyte development (**Illustrated in Figure 3.2**), rearrangement and transport of the immunoreceptor to the surface induces a low-grade signal to indicate the assembly of a functional immune receptor[219]. This stimulus leads to the subsequent inhibition of further genetic recombination. After this event, B lymphocytes begin development as immature cells that test the affinity of their BCR against the circulating antigenic milieu to determine the autoreactive potential of the newly assembled BCR. During development, BCR mediated signaling results in different outcomes based on the developmental stage. While tonic BCR signaling is still necessary for B lymphocyte survival, a strong signal from antigenic engagement leads to further rearrangement of the second light chain locus, allowing the developing cell another opportunity to generate a non-autoreactive specificity[220,221]. After assembly of this new BCR, a second antigenic recognition leads to apoptosis or anergy of the offending lymphocyte.

These previous steps occur in the bone marrow, which is analogous to the thymus for T lymphocyte development. Unlike T lymphocytes, B lymphocytes exit their niche of primary development in an immature state, and traffic to the spleen as transitional 1 B lymphocytes (T1). During this time they undergo a similar selection processes in the antigenically diverse splenic architecture[222]. The reason for this extra-peripheral selection step is unknown but may be due to lack of an AIRE mediated process that would increase exposure to a diverse antigenic repertoire in the bone marrow.

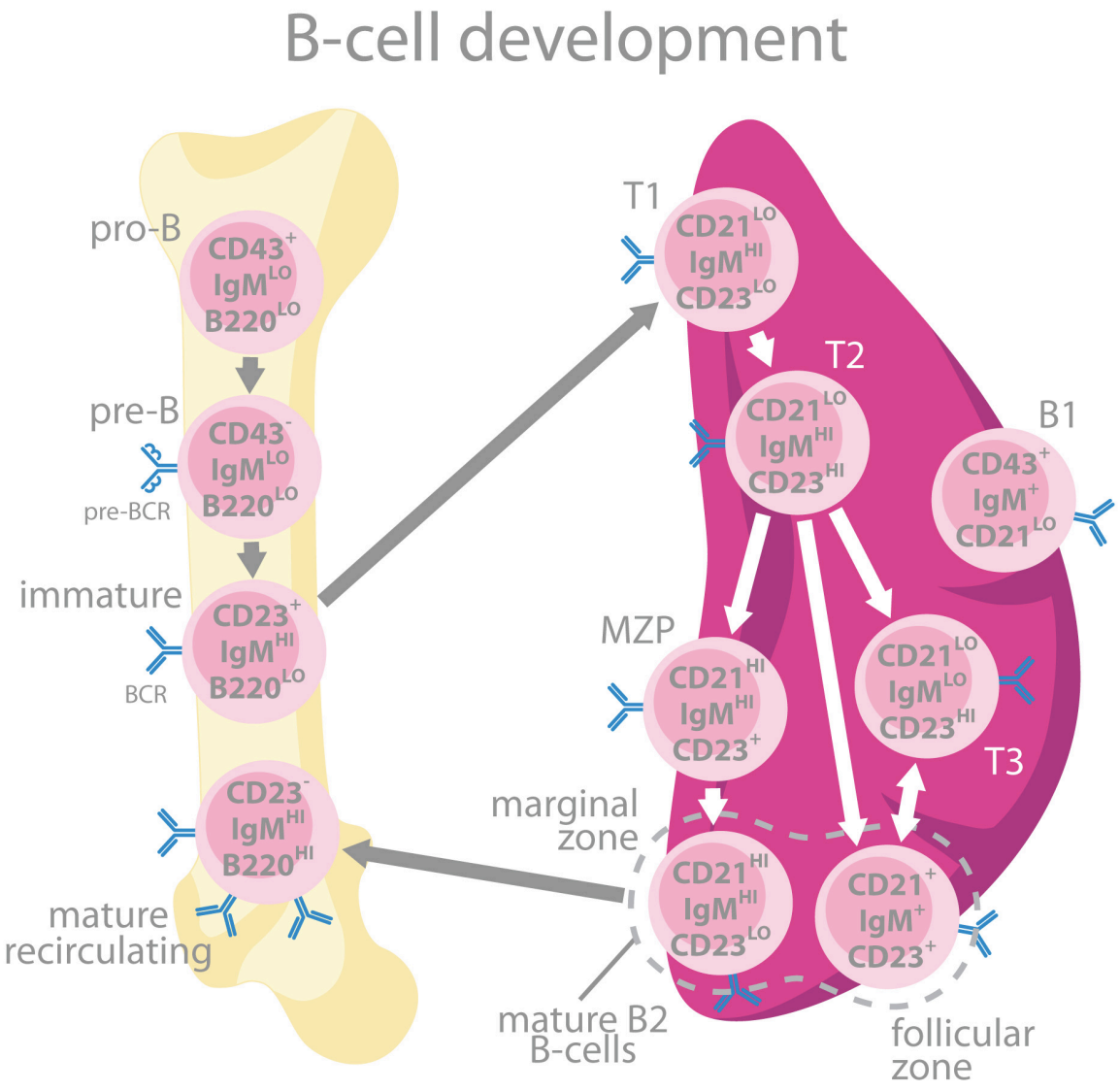


Figure 3.2. B lymphocyte Development. This figure depicts the steps of B lymphocyte development and maturation (excluding GC and memory B lymphocytes). B lymphocytes begin their life in the bone marrow where they are subjected to negative and positive selection. Once they emerge to the periphery they undergo additional selection in the transitional zone before committing to follicular or marginal zone phenotypes. Mature naïve B lymphocytes can recirculate to the bone marrow. Additionally B1 B lymphocytes are present in small numbers in the spleen and are thought to arise from a separate lineage. The majority of B1 B lymphocytes are found in the peritoneal and pleural cavities. The role of BCR mediated signaling is distinct in each case and can be differentially modulated by extrinsic factors to facilitate survival, activation, or apoptosis.

Only a very small fraction of the B lymphocytes that enter the transitional compartment emerge as mature B lymphocytes, indicating this is an extremely important stage for culling autoreactive cells. The T1 stage in T1D is of particular interest as this portion of B lymphocyte development is diminished in NOD mice[111,121]. This loss is thought to contribute to the emergence of autoreactive B lymphocytes into maturity.

During the T1 stage strong BCR signaling leads to apoptosis or anergy of autoreactive cells (some evidence indicates T1 B lymphocytes have the opportunity to activate RAG-1 to rearrange the immunoreceptor if they have not previously rearranged their second light chain in the bone marrow)[222,223]. The grade of T1 B lymphocyte response to antigenic stimulation is influenced by signaling input from TLRs, BAFF signaling, and T lymphocyte help; these events can foster survival of autoreactive B lymphocytes even on a nonautoimmune background.[194] The influence of each of these processes in T1D has been investigated in NOD mice, and they have been shown to be important for B lymphocyte development, but it has been unclear whether they play an additional deleterious role in autoreactive lymphocyte survival.

Once B lymphocytes transit the T1 stage, they enter the transitional 2 stage (T2), where the outcome of BCR signaling again changes. The mechanism for this switch in the outcome of BCR signaling is thought to be related to the generation of Diacylglycerol (DAG) at the T2 stage leading to the activation of NFkB signaling, thus promoting survival[222]. From this developmental stage these cells can enter the marginal or follicular developmental pathway. This decision is thought to be dictated partially by strength of BCR signaling with lymphocytes receiving a strong signal being recruited to the follicular zone and those receiving a reduced BCR signal entering the marginal zone[224]. The reason for this developmental decision is unknown but may be related to the need for T lymphocyte help in the follicular zone and the T-independent manner

in which marginal zone B lymphocytes become activated. This signaling decision is also influenced by input from other signaling systems that will not be discussed here[225,226]. Once these cells reach maturity, they are maintained by low-grade tonic BCR signaling, with robust antigenic signaling leading to activation[221].

While much is understood about the interplay between BCR signaling and B lymphocyte development the signaling pathways needed for Breg development are largely unknown. This is partially due to the apparent confusion of the phenotypic definition of Bregs, of which IL-10 is the most consistently associated phenotypic marker. The inability to define a consistent subset will continue to impede the dissection of the developmental pathways that imprint regulatory capacity on developing B lymphocytes. Several studies utilizing knockouts of phosphatases in models of B lymphocyte mediated regulation demonstrate that loss of phosphatase activity leads to a dramatic increase in IL-10 secreting "Bregs"[227]. In autoimmune disease immune phosphatase activity appears to be enhanced leading to a potential modality by which Breg activity could be diminished. Several phosphatases and phosphatase docking molecules, like SHP-1, CD22, PTPN22, and CD45 have been implicated in autoimmunity and have been targeted to modulate immune tolerance in B lymphocytes[228–231]. Regardless of the mechanism, I hypothesized that analysis of the signaling capacity of B lymphocytes in NOD mice as compared to B6 would demonstrate how a similar stimulus yields different outcomes, including regulation.

In this study, I utilize phosphoflow cytometry to interrogate B lymphocyte signaling across splenic development in NOD and B6 mice. I determine that NOD and B6 B lymphocytes possess similar signaling capacity across most B lymphocyte subsets, save a handful of proximal signaling molecules. The most distinctive among these pathways was c-Abl-kinase, whose importance in hematologic malignancies is widely studied[232]. This kinase has been targeted for

diabetes reversal in NOD mice and humans with T1D, with Gleevec (Imatinib)[233]. As such, I hypothesized that B lymphocytes may be targeted and required for diabetes reversal by imatinib. I determined that B lymphocytes were required for disease reversal in NOD mice treated with imatinib. In the presence of B lymphocytes, reversal was linked to an increase in serum insulin concentration, but not an increase in islet beta cell mass or proliferation. However, improved beta cell function was reflected by a partial recovery of MafA transcription factor expression, a reactive oxygen species (ROS) sensitive marker of beta cells that is required for adult beta cell function[234]. Imatinib treatment was found to increase superoxide dismutase (SOD) activity in B lymphocytes, improving ROS handling in NOD islets. This study reveals a signaling pathway that induces a novel and context specific form of B lymphocyte regulation in autoimmune disease.

Phosphoflow signaling analysis reveals defects in c-Abl signaling pathways in NOD B lymphocytes

In order to define pathways important in B lymphocyte mediated regulation of T1D, I utilized BCR stimulation with anti-IgM (Fab2) and phosphoflow cytometry. Phosphoflow cytometry couples extracellular subsetting antibodies with intracellular antibodies against phosphorylated components of the BCR signaling pathway (**Proteins assessed and data analysis scheme illustrated in Figure 3.3A and B**). The phosphorylation was compared over a time course, with the MFI of each phospho-antibody being utilized to calculate the area under the curve (AUC), serving as the readout in each B lymphocyte subset (**Illustrated in 3.3B**). The signaling profile for each protein within its distinct cell subset was graphed on a scatter-plot depicting differences between NOD and B6 responses (**Figure 3.3C**). Similarly regulated

proteins inside each subset would fall along the diagonal line, while those differentially regulated would fall off the line. The data demonstrated a reduction in SFK signaling in NOD B lymphocytes. This was an unsurprising finding as polymorphisms in SFK targeting phosphatase PTPN22 (PEP or Lyp in mice) that disrupt interactions with membrane localizing protein Csk and enhance its function are highly associated with abnormal immune function and T1D specifically. I could however not detect defects of downstream SFK targets CD79a, CD19, Syk, or BTK (**Figure 3.3C**). As differential SFK phosphorylation did not change the phosphorylation of downstream targets, it was unclear if this differential phosphorylation had an impact on B lymphocyte function. I also detected alterations in phosphorylation of c-Abl in my initial screen (**Figure 3.3C**). Confirmatory experiments revealed defects reduction in c-Abl signaling was localized to transitional 1 (T1) and 2 (T2) B lymphocytes in NOD mice (**Figure 3.3D**). Analysis of c-Abl target Crk-like (Crkl) revealed the phosphorylation of this protein was also reduced. Interestingly the follicular zone of B lymphocytes also possessed reduced c-Abl activity in both B6 and NOD mice (**Figure 3.3E**). C-abl inhibitor imatinib has been used to reverse diabetes in NOD mice, and is presently being evaluated in an ongoing phase 2 clinical trial in early onset T1D patients (NCT01781975)[233]. Although the mechanism of action is still unclear, imatinib appears to act by reducing endoplasmic reticulum stress levels in islet beta cells, which are proposed to exist in the islet under hyperglycemic and immune-infiltrating conditions. Specifically, the c-Abl tyrosine kinase promotes beta cell ER stress by enhancing IRE1-a activity, which drives the unfolded protein response leading to ER stress and ultimately beta cell death [235–239]. Although inhibiting c-Abl signaling through imatinib therapy leads to reduced beta cell stress, no immune requirement has been identified for successful treatment.

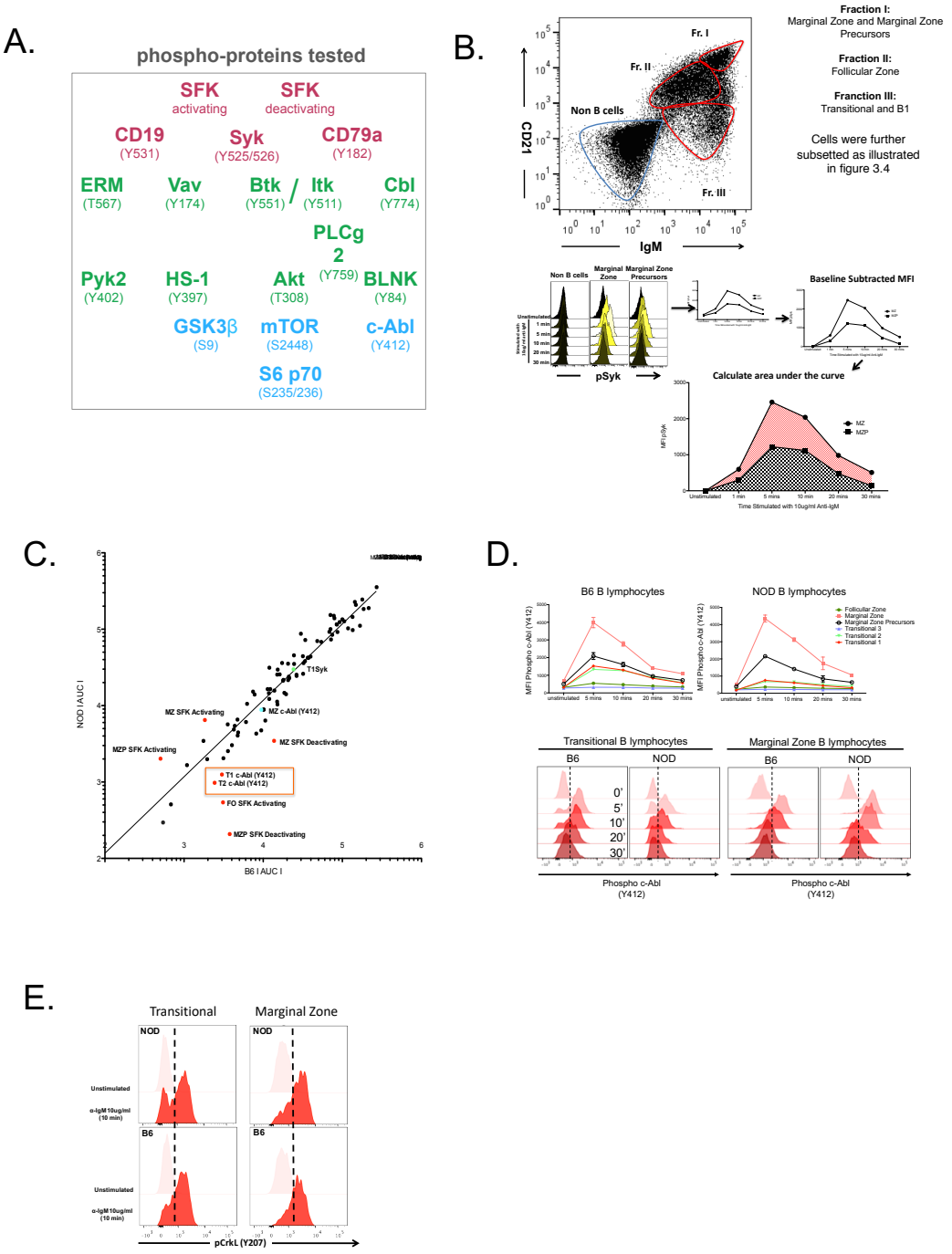


Figure 3.3. B lymphocyte signaling analysis reveals defective c-Abl signaling in NOD mice. A. A list of the phosphorylated protein residues I analyzed via phosphoflow. B. Phosphoflow analysis scheme. B lymphocytes were stimulated with anti-IgM (10ug/ml). These cells were subsetted via flow cytometry as illustrated. The area of the curve of each signaling protein was calculated for each B lymphocyte subset. This data was plotted and compared in a pair-wise fashion. C. Pair-wise comparison revealed only SFK and c-Abl signaling were differentially regulated in NOD B lymphocytes. No other defects in SFK signaling pathways were detected (representative data of at least 9 technical repeates. D. Confirmatory c-abl signaling analysis revealed transitional B lymphocytes in NOD mice possessed reduced c-Abl signaling as compard to B6 controls. (n=3) E. Phosphorylation of c-Abl downstream target Crkl revealed a reduction in phosphorylation only in transitional B lymphocytes form NOD mice, while the marginal zone in NOD and B6 mice demonstrated similar signaling capacity (representative of at least 3 experimental repeats).

As imatinib exhibits enhanced preference and inhibitory potency against the inactive form of c-Abl [240], I hypothesized that NOD B lymphocytes may be especially sensitive to imatinib therapy due to reduced activity in c-Abl pathways. Imatinib targets B lymphocytes in vitro and in vivo in NOD miceTo investigate the hypothesis that imatinib targets B lymphocytes in T1D, I analyzed splenocytes isolated from imatinib-treated and untreated prediabetic NOD mice. These mice were treated with imatinib mesylate (1.5mg/mouse) or saline for 7 days via intraperitoneal injection. A significant decrease in total splenocyte numbers was found in imatinib-treated mice, with B lymphocytes accounting for the majority of the lost cell numbers (Figure 3.4A). In contrast, no significant effect on T lymphocytes was observed, in agreement with previous reports (Figure 3.4B) [233,241]. Additionally, imatinib treatment led to preferential apoptosis of NOD B lymphocytes in *ex vivo* assays, as measure by Cleaved-Caspase 3 and Cleaved-PARP (Figure 3.4C). Having identified that c-Abl signaling was lowest in follicular and transitional subsets, I hypothesized that these subsets would be preferentially depleted by imatinib therapy in NOD mice. To assess this, I utilized flow cytometry to analyze the B lymphocyte compartment before and after imatinib therapy. Analysis of mature B lymphocytes revealed a preferential depletion of follicular B lymphocytes while marginal zone and its precursor population remained intact (Figures 3.4D, E, and G). Immature splenic B lymphocytes demonstrated a preferential loss of transitional 1 and transitional 2 B lymphocytes while transitional 3 B lymphocytes, largely thought to be an anergic population of B lymphocytes, were not impacted (Figures 3.4D, F, and H). In agreement with our signaling data, only those cells with the lowest c-Abl signaling capacity were robustly depleted (compare Figure 3.3G to Fig 3.4G and H).

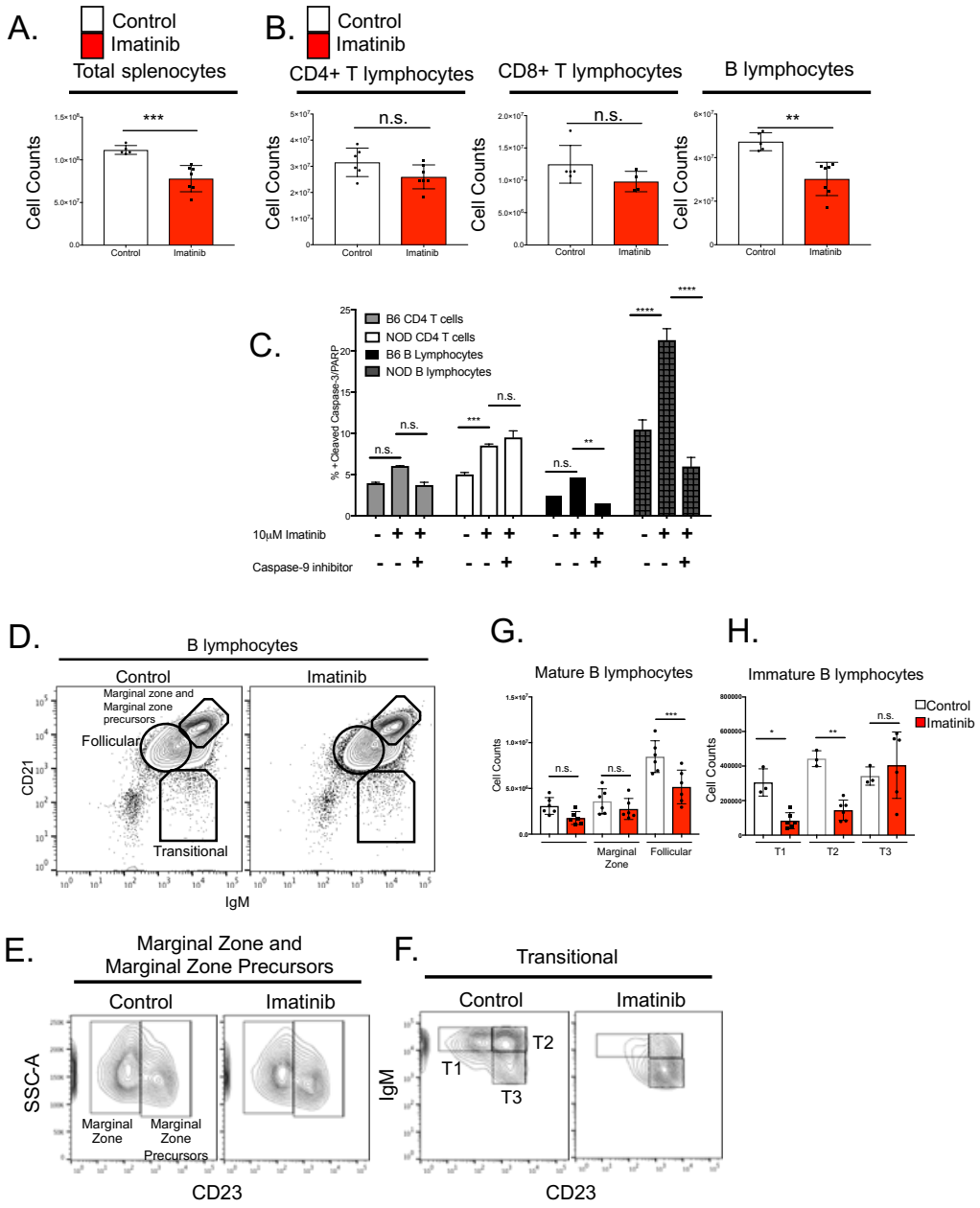


Figure 3.3 B lymphocytes are sensitive to depletion by c-Abl inhibitor imatinib. A) Analysis of splenocytes revealed a reduction in total splenocyte numbers in NOD mice following imatinib (***p = 0.022). B) B lymphocytes were preferentially depleted in NOD mice (p = 0.041) as compared to T lymphocytes (p = 0.071). C) Total splenocytes were plated in DMEM supplemented with 10% FCS and Pen/Strep. Imatinib was added to these cells to a final concentration of 10uM. Apoptosis was analyzed by co-staining of cleaved-PARP and caspase-3. B lymphocytes from NOD mice demonstrated enhanced sensitivity to imatinib-induced apoptosis. Imatinib-induced apoptosis was inhibited by Caspase-9 inhibitor (Z-LEHD-FMK). ****, $p < 0.0001$; ***, $p < 0.0003$; **, $p < 0.008$,; n.s. = not significant D) Subset analysis of B lymphocytes revealed that among mature B lymphocytes, follicular B lymphocytes (***p = 0.0006) were depleted. E) Marginal zone (n.s. p = 0.28) and marginal zone precursors (n.s. p = 0.65) remained intact. F) Immature B lymphocytes were also depleted, including preferential depletion of T1 (*p = 0.022) and T2 (**p = 0.002) subsets but not anergic T3 (n.s. p = 0.80) B lymphocytes. This is quantified in G) and H).

Nonetheless, depletion was incomplete and I hypothesized the remaining B lymphocytes may have developed resistance to imatinib therapy, a phenomenon characteristic of immune cells treated with imatinib in cancer studies [242–244]. These alterations in B lymphocyte function could impact betacell health in the islet as B lymphocytes represent the mose prominent component of the cellular islet infiltrate in NOD mice [245].

B lymphocytes are required for reversal of T1D by imatinib

Because imatinib only partially depleted the B lymphocyte population, I considered that its protective effect resulted from either depleting auto-aggressive B lymphocytes or by enriching B lymphocytes with a beneficial regulatory function. Consequently, I evaluated the ability of imatinib to reverse diabetes in an animal devoid of B lymphocytes. As our initial studies demonstrated robust B lymphocyte effects with i.p. injection of imatinib mesylate, resuspended in saline, I chose to continue with this therapeutic approach. Previously published reversal studies in NOD mice utilized Gleevec crushed, suspended in peanut oil, and delivered by oral gavage. I first established that injected imatinib possessed the same capacity to reverse disease (**Figure 3.5A**). I demonstrated that roughly 63% of diabetic NOD mice treated with imatinib via i.p. delivery had their diabetes reversed.

As NOD mice devoid of B lymphocytes do not develop disease, I developed a strategy to induce diabetes and to test the effectiveness of imatinib in B lymphocyte deficient models of diabetes [246]. I utilized anti-CD25 (PC61) or cyclophosphamide, as these methods have been consistently shown to break immune tolerance to islet beta cells and induce diabetes in an immune dependent manner [247,248]. Of the B lymphocyte deficient NOD μMT mice rendered

diabetic, only one demonstrated reversal of disease with imatinib, while B lymphocyte sufficient NOD WT mice had ~50% reversal **(Figure 3.5B).**

To eliminate the possibility that genetic or developmental abnormalities of the NOD μMT contributed to imatinib mediated diabetes reversal, I utilized an immune cell transfer model of diabetes induction. In this model splenocytes were removed from a diabetic NOD WT donor mouse and depleted of all B lymphocytes. The B lymphocyte negative fraction was then transferred into NODRag1$^{-/-}$ mice, which lack an adaptive immune system and develop diabetes only when donor NOD immune cells are transferred (**Figure 3.5C).** Once 2 consecutive daily blood glucose readings of >200 mg/dl mice were measured following splenocyte transfer, imatinib therapy (1.5mg/mouse) or imatinib with the transfer of 20x10^6 B lymphocytes from prediabetic NOD mice was administered intraperitoneally. As I found that normalization of blood glucose occurred very early in NOD WT mice reversed by imatinib **(Figure 3.5A)**, I assessed diabetes reversal after 4 days on therapy. Only mice that received B lymphocytes reversed diabetes; none of the imatinib-treated mice without B lymphocytes demonstrated diabetes reversal **(Figure 3.5D, E, and F)**. This surprising finding led me to hypothesize that the remaining B lymphocytes were acquiring a regulatory capacity that boosted or restored beta cell function.

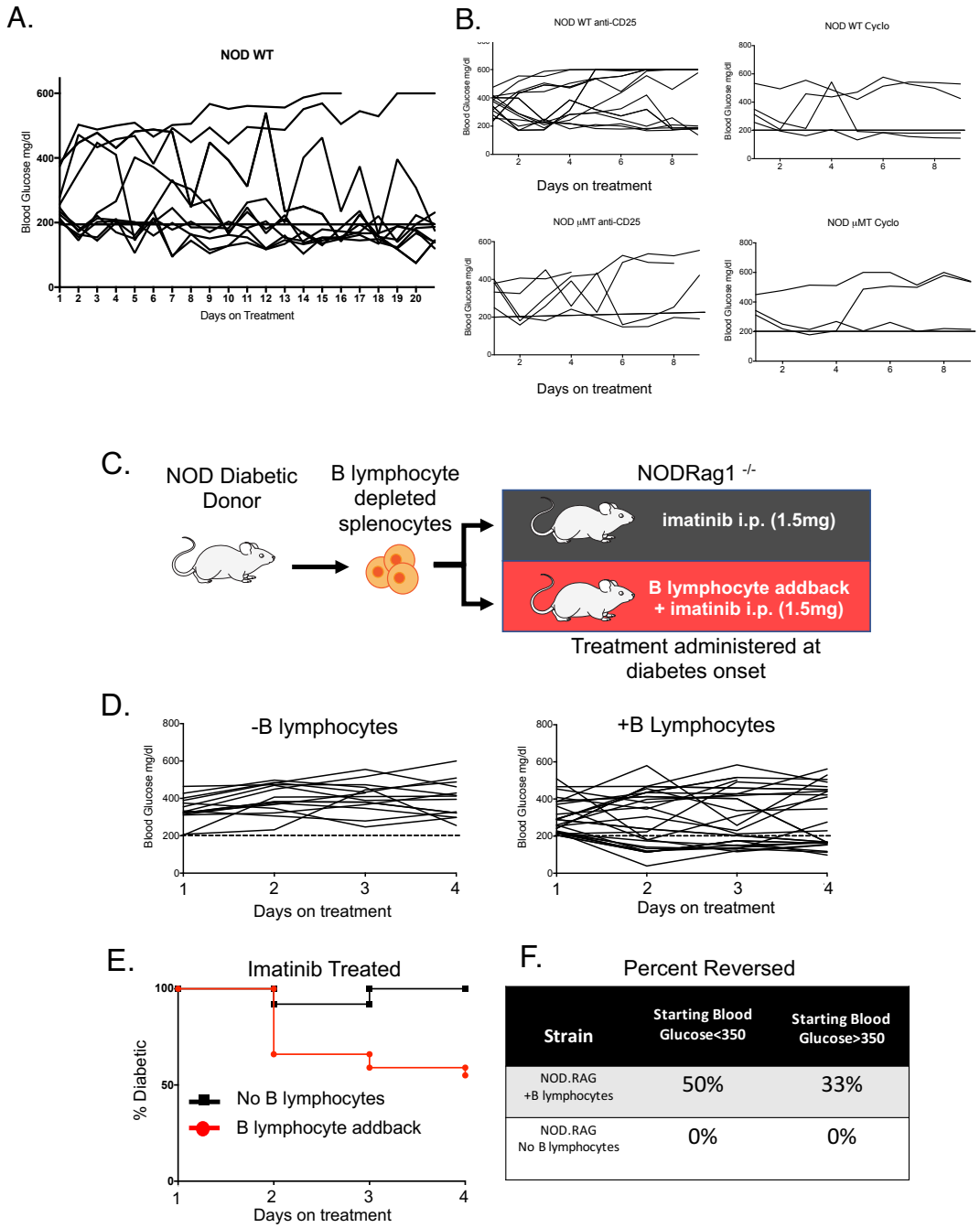


Figure 3.4 Intraperitoneal imatinib mesylate injection reverses type 1 diabetes in NOD B cell sufficient mice. A) To test the efficacy of imatinib mesylate i.p. injection in reversal of diabetes in WT NOD we allowed NOD mice to become diabetic. These mice were then enrolled on therapy for 21 days. At the end of this trial ~67% of mice had normal blood glucose levels (< 200mg/dL). A) The efficacy of imatinib in a model of Treg depletion by anti-CD25 or cyclophosphamide in NOD WT and NOD.uMT (B lymphocyte deficient mice). Once mice became diabetic they were started on imatinib. NOD WT experienced approximately 50% reversal at the end of the study no matter what induction method was utilized. NOD.uMT mice demonstrated reversal of only 1 mouse of a total of 7 enrolled. Due to difficult induction of diabetes in NOD.uMT, we utilized the NODRag1$^{-/-}$ diabetes transfer model for the rest of our studies. C) B220 depleted splenocytes (10x10^6) from a diabetic NOD donor mouse were transferred into immunodeficient NODRag1$^{-/-}$ mice. Mice were allowed to become diabetic and at time of diabetes were given imatinib injections alone or 20x10^6 B lymphocytes and imatinib injections. D) Blood glucose levels of diabetic mice on imatinib therapy revealed that only mice that received B lymphocytes and imatinib together had normalization of blood glucose. E) Approximately 50% of mice with B lymphocytes recovered glycemia (Blood glucose < 200mg/dl) by the end of the study and no B lymphocyte deficient mice were reversed. F) When stratified by blood glucose, mice enrolled in therapy with B lymphocytes were reversed at a rate of 50% when the enrollment blood glucose level was <350mg/dl while mice with blood glucose ≥ 350 mg/dl reversed at a rate of 33%. When no B lymphocytes were present mice did not reverse regardless of starting blood glucose.

Imatinib restores beta cell function only in the presence of B lymphocytes.

To understand how the islet cell population was impacted in imatinib-mediated, B lymphocyte-dependent reversal of diabetes, we collaborated with the Stein lab to anlyze beta cells in imatinib-treated NODRag1$^{-/-}$ mice in the presence or absence of B lymphocytes. We found that 4 days following imatinib treatment, neither the beta cell nor alpha cell area appeared changed (**Figure 3.6A**). Moreover, there was also no obvious change in Pax6+ endocrine cells, proliferating insulin+ Ki67+ cells, or apoptotic insulin+ TUNEL+ cells (**Figure 3.6B**). Collectively, these results suggested that the rapid and robust improvement in glycemia in imatinib-treated mice with B lymphocytes reflected improved islet beta cell function, as further indicated by the relatively elevated serum insulin levels in fasted mice (**Figures 3.6C**).

Increased levels of ROS have been shown to deleteriously impact islet beta cell function, in part, through reductions in beta cell-enriched transcription factors important in maintaining beta cell function[249,250]. To understand the role B lymphocytes play in modulating beta cell function in imatinib therapy, I stained for markers of beta cell identity, function, and maturation in non-diabetic control mice, newly diabetic NOD mice, and NOD mice treated with imatinib with or without B lymphocytes [234,251–255]. Levels of the Pdx1, Nkx6.1, and Nkx2.2 transcription factors (**not shown**) appear unchanged in all conditions, whereas MafA was significantly reduced in newly diabetic and B lymphocyte deficient NODRag1$^{-/-}$ islet beta cells. Strikingly, MafA levels were partially recovered in insulin-positive cells of NODRag1$^{-/-}$ mice when imatinib therapy included B lymphocytes (**Figure 3.6D and E**). MafA, a potent insulin-driving transcription factor important to adult beta cell function, is extremely sensitive to increased levels of oxidative stress [256]. In contrast to MafA, Ucn3 (urocortin 3), a mature beta cell marker and hormone co-packaged within insulin secretory granules, did not recover following imatinib

treatment (**not shown**) [234,255,257–259]. The recovery of MafA and improvement in blood glucose homeostasis suggests that B lymphocytes act to increase antioxidant capacity, as observed in hematologic cells treated with imatinib but previously unrecognized in tissue recovery from autoimmune attack [242].

B lymphocytes acquire enhanced ROS antioxidant capacity after imatinib therapy

Resistance to the effects of imatinib is a common problem encountered in the treatmeof hematologic malignancies and is associated with increased ROS handling proteins, including SODs and the glutathione system [242–244]. I hypothesized that the remaining B lymphocytes enhance ROS handling and beta cell recovery following imatinib treatment. Although I observed no increase in the uptake of cystine, a precursor to glutathione assembly and a primary mechanism of imatinib-induced ROS regulation (**not shown)** [260], I did find a modest increase in SOD2 (i.e. the manganese dependent superoxide dismutase) **(Figure 3.7A)**. Interestingly, SOD2 expression was highest in the marginal zone at baseline, a potential second mechanism for its resistance to imatinib-induced depletion, whereas the transitional and follicular B lymphocytes demonstrated an increase in SOD2 after imatinib therapy **(Figure 3.7B**).

To assess whether a change in ROS handling was induced by imatinib treatment, a cellular dye (H2DCFDA) that fluoresces with increasing levels of intracellular ROS was used. A robust decrease in intracellular ROS was found in NOD mice treated with imatinib (**Figure 3.7C).** Previous studies have demonstrated that lymphocytes can release antioxidant proteins to handle increased oxidative stress [261]. Assaying cell supernatants from B lymphocytes incubated in media alone or in the presence of imatinib in the Hydroxyl Radical Antioxidant Capacity (HORAC) activity assay revealed that imatinib treatment increased ROS-neutralizing capacity

(**Figure 3.7D).** These results explain how islet-invading B lymphocytes could extend tissue protection to nearby islet beta cells following imatinib treatment (**Figure 3.7E)**. While I found robust changes in the splenic B lymphocyte compartment in response to imatinib, it was important to directly assess lymphocytes from the pancreas. Flow cytometry analysis revealed no changes in the distribution of CD4, CD8 or B lymphocytes among intrapancreatic $CD45^+$ cells (**Figure 3.7F**). There was no decrease in B lymphocytes in the pancreas in response to imatinib as had been observed in the spleen (**Figure 3.4F**). Consequently, I hypothesized that this difference could be due to heightened imatinib resistance in the form of antioxidant capacity in pancreatic B lymphocytes. To this end, I found that pancreatic B lymphocytes had a robust increase in SOD2 in comparison to all $CD45^+$ subsets in the pancreas (**Figure 3.7G and H).** This increase in SOD2 expression in imatinib-treated B lymphocytes from the pancreas suggested that these cells have enhanced ROS handling capacity, which can preserve beta cell function in response to imatinib therapy.

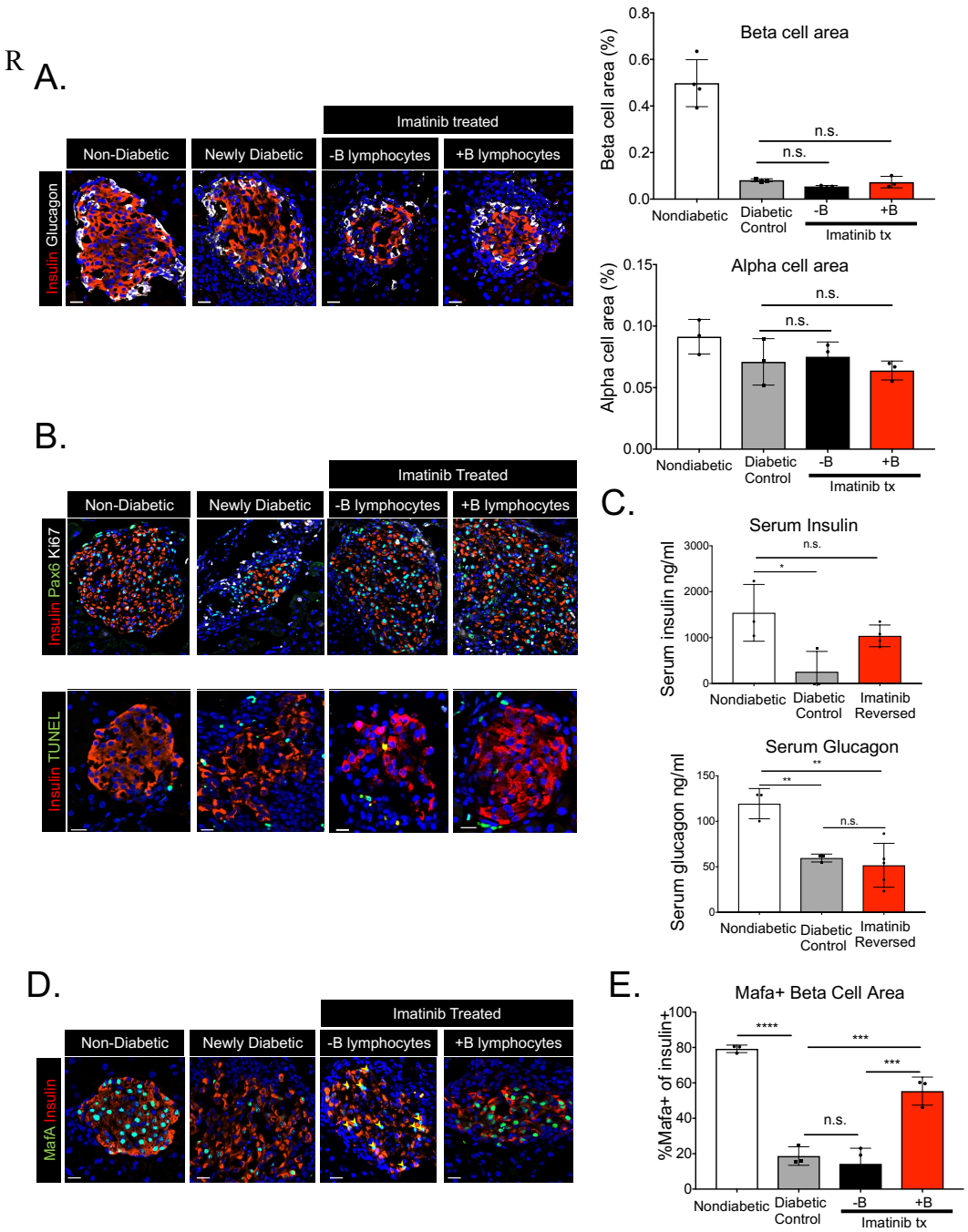


Figure 3.6 Imatinib therapy restores beta cell function but not beta cell mass in mice with B lymphocytes. A) Pancreatic sections from WT, newly diabetic (two consecutive blood glucose readings > 200mg/dL) and imatinib-treated mice with or without B cells (4 days after treatment initiated) were evaluated for beta cell area and alpha cell area by staining with insulin or glucagon specific antibodies. Neither beta cell nor alpha cell area increases following imatinib treatment. B) Pancreatic sections stained with Pax6 (endocrine), insulin, and Ki67 revealed no enhanced islet cell proliferation. Sections subjected to TUNEL assays revealed no enhanced islet apoptosis among any of the groups. C) Serum insulin levels are partially restored in NOD mice following imatinib; in contrast, glucagon levels were not increased following imatinib therapy. D) MafA and insulin staining of pancreatic sections reveals loss of MafA from insulin+ cells in newly diabetic mice. Only imatinib-treated mice with B lymphocytes restored MafA in insulin+ cells. E) Quantification of MafA+insulin+ cells revealed partial recovery in imatinib-treated mice with B lymphocytes as compared to newly diabetic mice or imatinib-treated mice with no B lymphocytes. ****, $p < 0.0001$; ***, $p < 0.0005$; n.s. = not significant. Scale bars = 20μm

Discussion

BCR mediated signaling has been demonstrated to play an important role in driving immune tolerance by preventing emergence of autoreactive B lymphocytes (selection) and preventing activation of autoreactive lymphocytes in the periphery (anergy)[220,229]. Mice and humans with T1D demonstrate abnormal selection processes indicating the potential for abnormal B lymphocyte signaling[130,181,231,262]. My screening assay utilized soluble anti-IgM to crosslink the BCR and initiate signaling. This approach is thought to represent a strong BCR signal that is thought to have a distinct impact on BCR signaling compared to antigens that are not multivalent and fail to fully crosslink the BCR[263,264]. In T1D insulin is a circulating hormone that may elicit a different response than anti-IgM (it is unclear the context in which B lymphocytes first encounter insulin or if insulin is the most important antigen in disease initiation). While this study did not reveal defects in BCR signaling that could explain abnormal selection in autoimmune disease, the reasons may be technical or biological. It is also possible that intrinsic BCR signaling is similar in autoimmunity and healthy individuals but homeostatic factors such as BAFF, TLRs, or circulating Igs subvert normal signaling processes to promote survival of autoreactive specificities (a possibility that will be assessed in Chapter IV). NOD mice and humans with T1D demonstrate defects in B lymphocyte homeostasis that could contribute to the autoreactive B lymphocyte survival.

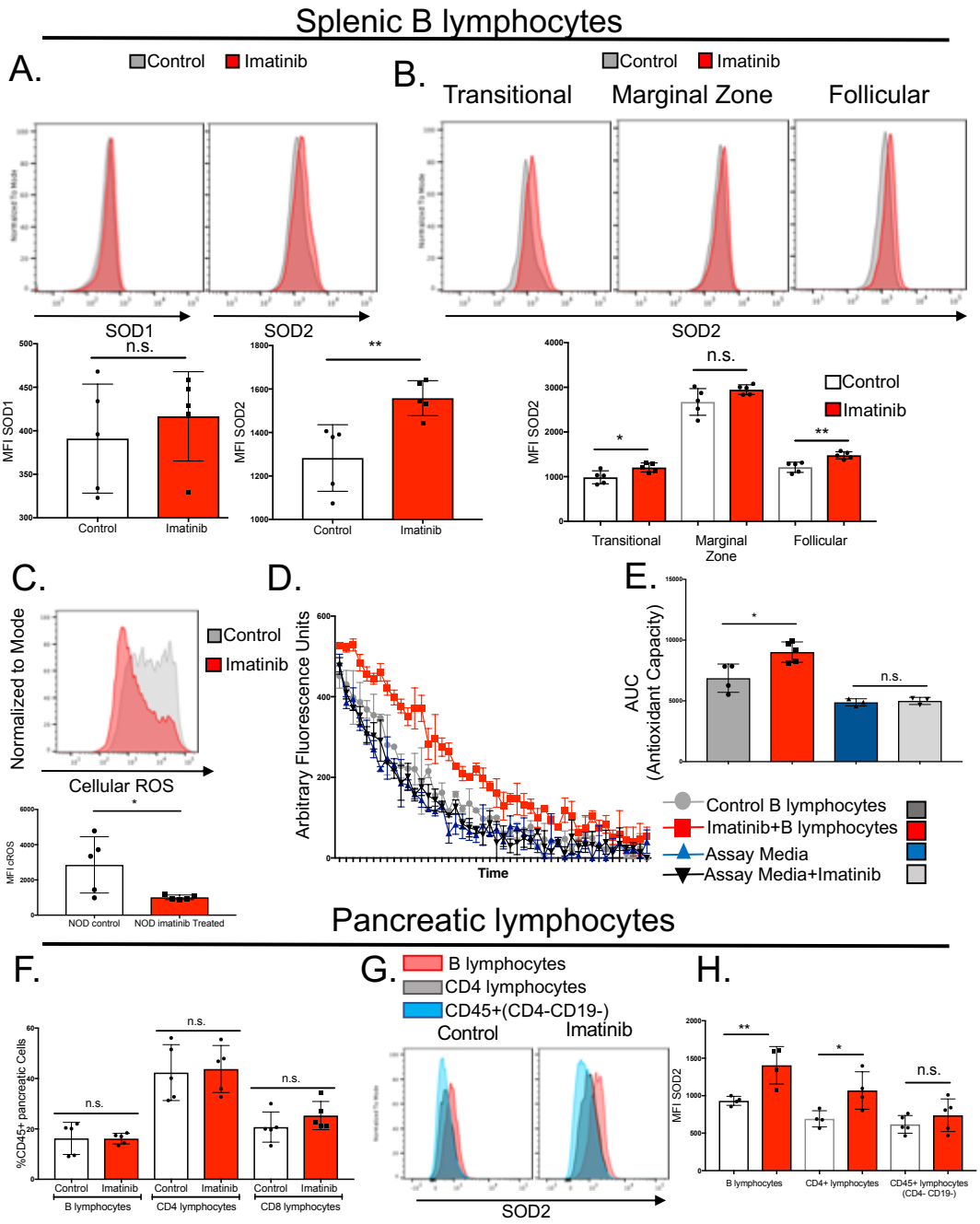


Figure 3.7 B lymphocytes acquire enhanced ROS handling capacity after imatinib therapy. A) Splenic B lymphocytes from imatinib-treated NOD mice and untreated NOD controls were fixed, permeabilized and stained for intracellular SOD1(Cu-Zn) and SOD2 (MnSOD). There was no increase in SOD1 but SOD2 expression did increase as measured by flow cytometry (** p = 0.0026). B) Marginal zone B lymphocytes possessed the highest levels of SOD2 and did not increase SOD2 after imatinib. Transitional and follicular B lymphocytes persisting after imatinib therapy also increased SOD2 expression (* p = 0.024 and ** p = 0.0075 respectively) C) Cellular ROS analysis by flow cytometry of B lymphocytes from imatinib-treated and control NOD mice indicated that B lymphocytes from imatinib-treated mice possessed reduced cellular ROS (p = 0.0330). D) B lymphocytes from NOD mice were purified by FACS and incubated in HBSS+FCS with or without imatinib (10uM). Supernatants were collected and their antioxidant capacity was measured by a fluorescent quenching assay (HORAC). Antioxidant capacity is revealed by prolongation of a ROS-sensitive fluorescent probe. Supernatants from imatinib-treated B lymphocytes prolonged fluorescence (red) as compared to B lymphocytes incubated with HBSS+FCS (gray), HBSS+FCS alone (blue), or HBSS+FCS+10uM imatinib(black). E) The area under the curve was calculated for biologic replicates and plotted demonstrating a significant increase in antioxidant capacity of B lymphocytes (* p = 0.015) F) Flow cytometry analysis revealed no changes in the proportions of B, CD4 or CD8 lymphocytes in the pancreas. G) B lymphocytes were isolated from the pancreas of imatinib-treated and untreated control NOD mice. SOD2 expression is increased in these lymphocytes (* p = 0.013). H) B lymphocytes expressed the highest level of SOD2 of all CD45+ lymphocytes at baseline and in response to imatinib.

It has been unclear what specific signaling pathways drive B lymphocyte mediated regulation. In this study I uncover a surprising role for c-Abl signaling in a novel mode of "tissue specific" B lymphocyte regulation. Whether c-Abl signaling plays a role in tissue protective function in healthy individuals or if this is a peculiarity of the NOD genetic background remains unclear. This study, however, indicates an interesting instance of context specific regulation of beta cell health by B lymphocytes in T1D, again reinforcing the role for B lymphocytes in T1D.

This study demonstrates that B lymphocytes modulate ROS in the islet microenvironment to preserve beta cell function in NOD mice treated with imatinib. Whether B lymphocytes acting as a "ROS-sink" are important in mediating tissue protection in other normal and autoimmune conditions remains to be seen. While ROS is detrimental to some tissues such as beta cells, it can also enhance proliferation of others, like stem cells[263]. So the net effect of ROS modulation is probably dependent on both the tissue and amount of ROS being produced. In NOD mice B lymphocytes robustly invade the islets. It is unclear if the same is true for humans with T1D; studies of cadaveric islets from patients with T1D demonstrate less B lymphocyte infiltration than NOD mice. (Although acquiring a cohort of similarly staged islets is problematic, thankfully)[265,266]. Conversely, the ROS handling activity of B lymphocytes in malignant tumors (especially from patients treated with imatinib) may serve as a negative correlate for tumor regression, as low ROS levels are maintained in tumors to promote optimal tumor growth[267].

The role of ROS at the site of an active immune response is multifaceted. ROS production can have direct effects on immune cells acting as a signal to enhance T cell activation ("Signal 3"), as well as other immune cell subsets. ROS can in part mediate this process by transiently inhibiting phosphatases, which restrict immune signaling, leading to increased and

nonspecific signaling[268]. Additionally, ROS inhibition of phosphatase action may be counterproductive to tolerogenic therapies such as anti-CD45RB that target phosphatase function in lymphocytes. Secondly, ROS can also impact the target tissue itself. Indeed immune cells can produce ROS as a mechanism of cell killing in infection and autoimmunity. Immune cells and cytokines can also influence tissues to undergo stress and produce ROS that further damage the mitochondria and DNA leading to apoptosis or necrosis[249]. In the case of the beta cell, both of these processes occur; additionally glucotoxicity further enhances ROS production in beta cells in T1D[249,250]. While it is to yet established how B lymphocytes modulate intracellular beta cell ROS, some studies have demonstrated the capacity for MnSOD (SOD2) to be secreted in exosomes and acquired by other cells[261].

The beta cell is exquisitely sensitive to oxidative stress and has little of its own internal defense mechanisms to prevent oxidative injury [249,269,270]. The MafA transcription factor is a highly sensitive target of this oxidative stress, with levels being substantially reduced in T1D and T2D islets [234,256,271]. Here I observed a significant reduction in MafA+ insulin+ islet cells of hyperglycemic NOD mice, which was partially reversed in animals under imatinib therapy, but only in the presence of B lymphocytes. This finding adds new insight into the previously established effects of imatinib at the level of the beta cell, which have focused more on the alleviation of ER stress and prevention of apoptosis [233,239]. Prior studies performed *in vitro* have established that imatinib can enhance insulin production through increasing levels of Nkx2.2 [238]. I did not observe an increase in Nkx2.2 levels *in vivo*; however, I did similarly observe improved insulin secretion from the remaining beta cells post recovery. Imatinib has previously been shown to significantly reduce ER stress-mediated beta cell apoptosis in NOD islets prior to diabetes diagnosis [233,239]. Here I did not observe a profound change in beta cell apoptosis, which

could be due to the relatively late time-point at which I did our assessment, after NOD mice were hyperglycemic. I predict, however, that the residual beta cells remaining upon initiation of imatinib therapy recover their functional status due to the enhanced ROS handling capacity of the pancreatic B lymphocytes and improvement in MafA expression.

The translation of Gleevec (imatinib) to human clinical trials represents an exciting opportunity for utilization of an FDA approved drug that could be rapidly approved for use in humans with T1D. Our studies indicate a need to analyze the immune compartment with a focus on B lymphocytes in clinical studies and highlight a novel interaction between immune and beta cells. It is important to point out that our data indicate altered c-Abl signaling in B lymphocytes from NOD mice at baseline that could play a role in the predisposition of these cells to gain ROS regulatory capacity. Our data suggest that NOD B lymphocytes are especially sensitive to imatinib therapy (**Figure 3.4C**), but it is unclear whether the same is true in humans with T1D. However, it is likely to be efficacious since clinical data suggest that the ROS system is a universal target of imatinib in hematologic cells [242–244]. As mounting evidence indicates T1D is a heterogenous disease, the cellular antioxidant response to imatinib may represent an important biomarker for predicting efficacy of imatinib therapy in patients with T1D.

Overall this study illustrates an important role for B lymphocytes in regulating tissue function. Whether this represents an antigen-specific regulation of beta cell function is still unknown. Studies have demonstrated that the islet is enriched for autoreactive specificities, which is where I observed the most robust response to imatinib. Interestingly, B lymphocytes have been demonstrated to play a role in mediating insulin resistance in nonautoimmune mice, indicating crosstalk between the beta cell and B lymphocytes. This study may also help explain why "Bregs" demonstrate counterregulatory capacity in certain circumstances as Bregs may modulate processes that are deleterious to some tissues but beneficial to others. Future studies of Breg function need to account for the target tissue responses when determining the beneficial function of B lymphocytes in tissue protection.

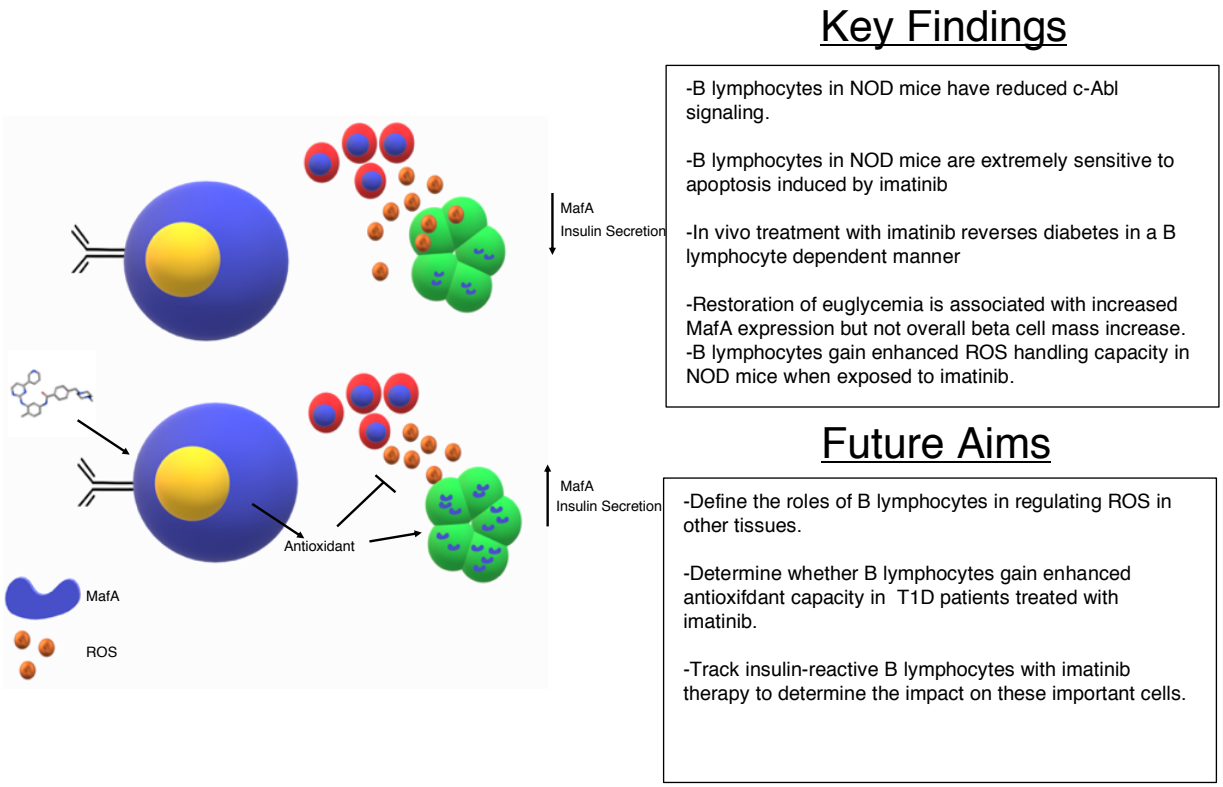


Figure 3.8 Model for B lymphocyte antioxidant role in imatinib mediated beta cell recovery.

CHAPTER IV

NATURAL IGMS REGULATE B LYMPHOCYTE HOMEOSTASIS AND TREG PRODUCTION TO REVERSE T1D

Scientific Goal

My previous chapters highlight novel ways that B lymphocytes regulate tissue autoimmunity in NOD mice. It remains unclear if "immune diversion" or regulation of ROS in the islet microenvironment represent *bona fide* immune tolerance mechanisms. It is clear that B lymphocyte development and homeostasis is abnormal in T1D and other autoimmune conditions leading to B lymphocytes that foster autoreactive T lymphocyte activation. It remains unclear what governs these defects and how to therapeutically correct B lymphocyte abnormalities in T1D. In Chapter IV I elucidate how dysregulated B lymphocyte homeostasis impacts immune tolerance in NOD mice and determine that B lymphocyte derived IgM has the capacity to correct these defects and restore immune tolerance. Findings in this chapter uncover a B lymphocyte mediated tolerance mechanism that is missing in T1D. In addition, it links, for the first time, abnormal B lymphocyte homeostasis and development of Tregs in NOD mice. Overall my studies in this chapter define a surprising mode of B lymphocyte regulation that fulfills the definition of a tolerance-inducing therapy.

Introduction

My previous work uncovered defects in B lymphocyte biology in NOD mice and how to target them to induce B lymphocyte mediated protection of islets. Defective B lymphocyte development and function is a hallmark of many autoimmune diseases (**Illustrated in Fig 4.1**).

In T1D there is a gradual decrease in B lymphocyte output as mice age; in NOD mice this loss occurs at about 4-5 weeks of age[121,124,129]. This event is marked by a loss in transitional B lymphocytes, a subsets important in mediating negative selection of autoreactive specificities. Thereafter, autoreactive B lymphocytes are allowed to pass through this peripheral stage of negative selection and persist to maturity where they can activate effector T lymphocytes. As such several genetic and therapeutic strategies have demonstrated that depletion of B lymphocytes leads to diabetes prevention and reversal in NOD mice.

In light of this knowledge, B lymphocytes have been targeted for depletion in human clinical trials to prevent and reverse T1D[103,119,272–274]. These clinical trials, while demonstrating partial preservation of beta cell functionality, ultimately failed to prevent beta cell loss permanently. This failure was linked to a rapid and prolific reemergence of autoreactive B lymphocytes, indicating depletion failed to "reset" defective homeostasis characteristic of T1D[119]. Due to this perceived failure of B lymphocyte directed therapy in T1D, no further B lymphocyte directed therapeutic interventions have been attempted to date that target this pivotal cell type.

While many researchers believed that depletion of B lymphocytes would activate mechanisms that drive restoration of B lymphocyte selection and homeostasis, this hope was clearly not fullfilled in humans with T1D, in which emergence of autoreactive B lymphocytes may have been accelerated by therapy. While abnormal immune homeostasis is common among many autoimmune diseases, most therapeutic strategies do not directly address this problem. In fact, there is very little understanding of the cellular and molecular mechanisms that govern B lymphocyte homeostasis in autoimmune disease. While B-cell activating factor (BAFF) has been targeted in NOD mice, it also failed to reset immune homeostasis, instead depleting the B

lymphocyte compartment[121]. B lymphocyte homeostasis is controlled by complex interactions between other cell types, metabolic need, intrinsic signaling pathways, secreted molecules, antigen availability, and microbial influences. B lymphocytes begin development in the bone marrow where BCR specificity is determined through recombination of heavy and light chains (kappa or lambda). While some have demonstrated that autoreactive specificities emerge in the bone marrow of NOD mice, it is generally agreed that once they emerge from the bone marrow the transitional zone dictates the ability of these cells to persist[127]. In NOD mice B lymphocytes are lost as they exit the bone marrow and enter the transitional compartment[111]. This loss leads to homeostatic pressure to quickly transit the transitional stage and skip further selection leading to the persistence of autoreactive B lymphocytes (**see Figure 4.1B**).

Studies in non-autoimmune mice determined that developing B lymphocytes exiting the bone marrow are controlled by homeostatic feedback exerted by mature B lymphocytes. While the authors of this study concluded loss of mature B lymphocytes would allow for a homeostatic "reset" of mice in which B lymphocytes had been depleted, they acknowledge this reset was under the control of an undefined homeostatic factor provided by mature B lymphocytes[275]. In humans with T1D it is clear that depletion does not reset defective homeostasis further validating that abnormal homeostatic processes in T1D are not corrected by depletion therapy. One potential reason for failure of depletion therapy to overcome abnormal homeostasis in T1D would be the absence of a pivotal factor that allows return to normal homeostasis (i.e. secreted factors). One such factor secreted from B lymphocytes demonstrated to possess potent capacity to regulate immune homeostasis is natural IgM, a B lymphocyte secreted antibody[115–117,276–282].

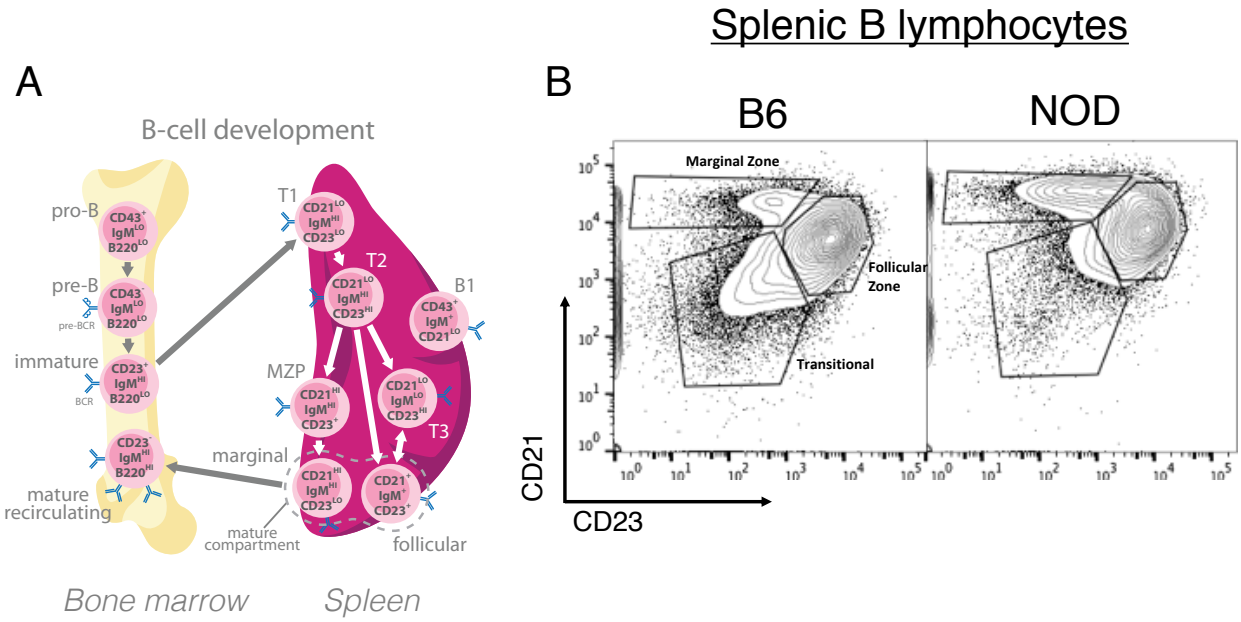


Figure 4.1 B lymphocyte homeostasis is abnormal in T1D. A) B lymphocyte development, illustrated here, is abnormal in NOD mice. Abnormalities in splenic B lymphocyte development included loss of transitional ($CD21^{Lo}CD23^{Lo}$) and expansion of marginal zone ($CD21^{Hi}CD23^{Lo}$). This is illustrated in B). It can clearly be appreciated by the density plot that marginal zone B lymphocytes are expanded (top left gated population) and the transitional compartment is reduced (bottom left population) in NOD mice.

Present at low levels in healthy individuals, IgMs increase during inflammatory disorders and various infections. Studies in animal models have indicated that the natural IgMs are an important part of the normal homeostatic mechanism of the immune system because they limit inflammatory responses[115,282]. Studies in mice have revealed the secreted IgM is an important endogenous regulator of B lymphocyte development. Mice that lack the ability to secret IgM ($sIgM^{-/-}$) or the ability to detect IgM through the TOSO receptor ($FcmR^{-/-}$), demonstrate perturbations in B lymphocyte development that are similar to B-lymphocyte development in NOD mice and that permit the maturation of autoreactive B lymphocytes[117,277]. Furthermore, secreted IgM has the capacity to preserve Treg function and phenotype in chronic inflammatory conditions characteristic of autoimmune disease[117,277]. These findings make treatment with therapeutic IgM an attractive option to restore immune homeostasis and reverse disease in NOD mice.

While NOD mice possess the same amount of circulating IgMs as nonautoimmune B6 mice, there is evidence that the "IgM network" is abnormal[114]. Studies utilizing IgM therapy in inflammatory conditions have linked the therapeutic function of these molecules to their ability to bind self-epitopes on immune cells[283]. Studies that characterized the binding selectivity of the polyclonal IgM repertoire in NOD and B6 mice revealed NOD IgM displayed less autoreactivity than the repertoire of their B6 counterparts[114]. Secretion of polyclonal IgM has been attributed to both marginal zone and peritoneal B lymphocytes[142]. Both of these compartments have been demonstrated to contribute to disease in NOD mice, indicating a potential contribution of abnormal IgM secretion from these subsets to disease pathology[124,284]. The contribution of non-

antigen specific interactions via the newly discovered IgM receptors Fcmu (TOSO) in NOD mice remains undetermined but is an area of future interest.

In this study I utilized nIgM isolated from Swiss Webster mice ($nIgM_{SW}$) to determine the impact of healthy IgM on the NOD immune system. I determined that these IgMs restore B lymphocyte homeostasis, increase stringency of B lymphocyte selection, and reverses disease in NOD mice. This disease reversal was related to expansion of Tregs both centrally and peripherally, which was required for restraint of disease in $nIgM_{SW}$ reversed NOD mice. Surprisingly, expansion of Tregs was dependent on B lymphocytes in the thymus, mediated by a robust increase in circulating BAFF levels. Furthermore, IgM derived from prediabetic NOD mice and administered to diabetic NOD mice was unable to stably reverse disease or mediate a return to immune homeostasis, indicating an undefined defect in IgM mediaed immune regulation in NOD mice. Furthermore, this restoration of B lymphocyte homeostasis rendered NOD mice responsive to tolerance inducing therapy anti-CD45RB, a therapy resisted by NOD mice and thought to be linked to abnormal B lymphocyte function. Lastly, healthy human IgM was able to mediate diabetes protection and expand human Tregs in a humanized mouse model. Overall, this study distinguishes a new regulatory molecule secreted from B lymphocytes with the potential to restore B lymphocyte homeostasis and Treg function in T1D, linking for the first time B lymphocyte homeostasis and Treg development in T1D.

$nIgM_{SW}$ restores B lymphocyte homeostasis and eliminates autoreactivity in NOD mice

To determine whether inappropriate action of the secreted IgM system accounted for abnormal B lymphocyte homeostasis in NOD mice, I treated NOD and B6 mice with $nIgM_{SW}$. B lymphocyte numbers are greatly reduced in NOD mice; this defect was corrected by $nIgM_{SW}$.

B lymphocyte numbers returned to B6 levels (**Figure 4.2A**) Therapy with $nIgM_{SW}$ normalized the abnormal B lymphocyte subset distribution of transitional and marginal zone B lymphocytes in NOD (**Figure 4.2B and C**). I hypothesized that this correction of B lymphocyte developmental defects in NOD mice would foster elimination of autoreactive B lymphocytes and thus eliminate the B lymphocytes important for initiation of disease. To test this hypothesis, I utilized transgenic models with increased frequency of anti-insulin B lymphocytes. The V_H125^{SD}.NOD and V_H125^{SD}.B6 mice have heavy chains specific for human insulin knocked into the endogenous heavy chain locus, which combines with endogenous light chains to produce a functional B cell receptor[193,194]. On the NOD background these mice develop a small but identifiable population of insulin-binding B lymphocytes and have accelerated diabetes onset [193,285]. NOD or B6 mice on the V_H125^{SD} background were treated with $nIgM_{SW}$ and insulin-reactive B lymphocytes were analyzed via flow cytometry using a biotin-conjugated human insulin followed by a streptavidin-conjugated fluorophore. V_H125^{SD}.NOD mice treated with $nIgM_{SW}$ experienced a complete loss of detectable insulin-reactive lymphocytes in the spleen (**Figure 4.2D and D**). Similarly, I identified a complete abrogation of insulin autoantibody production in NOD mice treated with $nIgM_{SW}$ as detected by ELISA (**Figure 4.2F**). Taken together the data demonstrate that $nIgM_{SW}$ therapy corrects defects in B lymphocyte selection characteristic of autoimmune diabetes in NOD mice and which predict disease in humans, thus demonstrating nIgM interferes with the pathologic process in T1D. These data demonstrate the regulatory capacity of secreted IgM in healthy mice and the potent capacity to restore immune homeostasis in NOD mice. As the immune effects were profound, I next assessed whether resetting the immune system halted beta cell destruction and reversed disease in NOD mice.

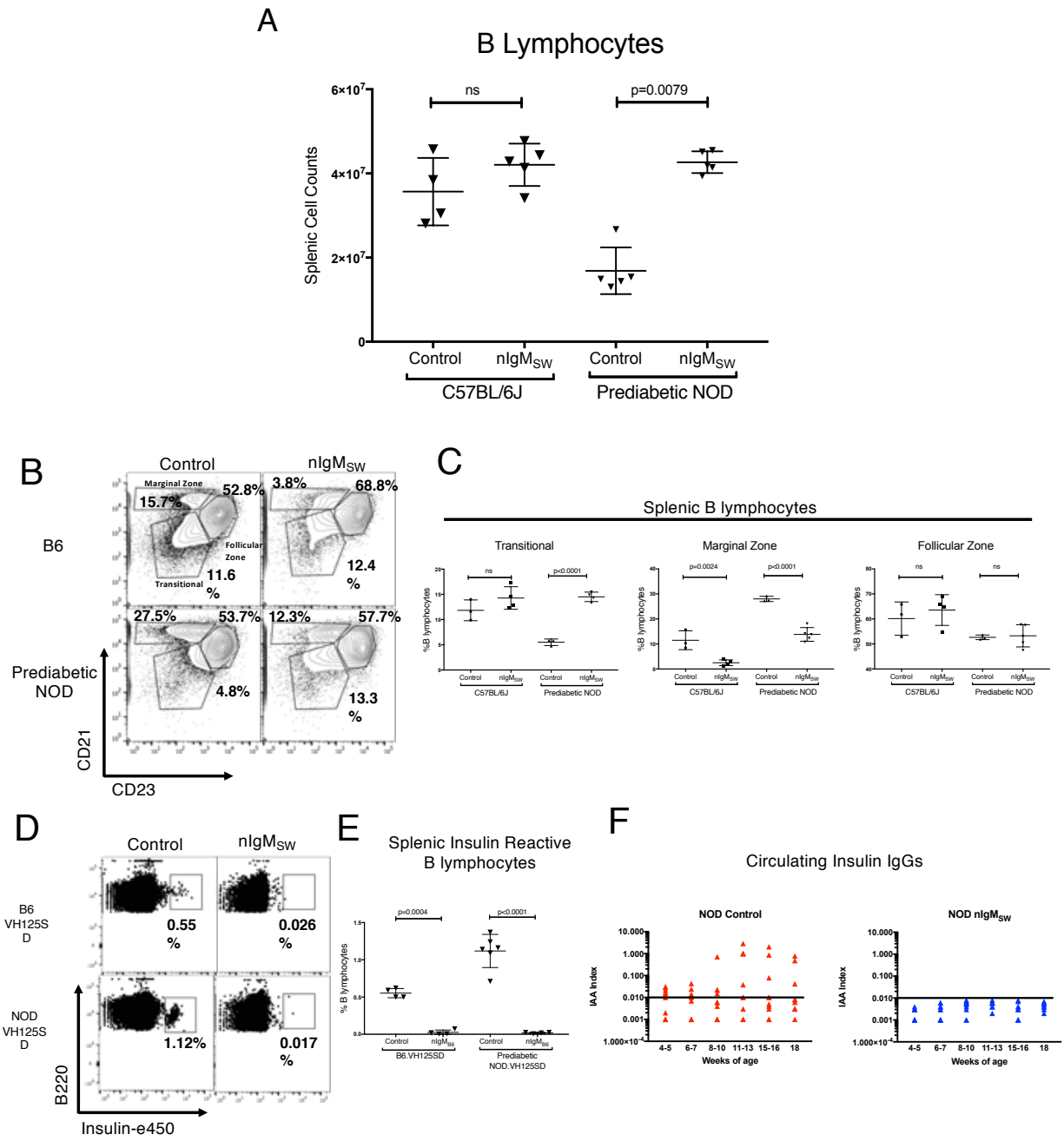


Figure 4.2 B lymphocyte homeostasis in NOD mice is corrected by nIgM from a healthy donor. A) NOD mice demonstrated an increase in B lymphocytes after $nIgM_{sw}$ injection. B) Flow cytometric analysis of B220+ cells revealed normalization of marginal zone and transitional B lymphocyte subsets in NOD mice after $nIgM_{sw}$ treatment. Marginal zone expansion and loss of the transitional compartment are hallmark defects of B cell development in NOD mice that were corrected by $nIgM_{sw}$ therapy. Subsets are quantified in C**.** (representative data of at least 7 experimental repeats) D) B6 and NOD $VH125^{SD}$ possess a heavy chain specific for human insulin knocked into the endogenous IgM locus. This transgenic heavy chain combines with endogenous light chains to produce a population of insulin-reactive B lymphocytes in NOD mice. These mice were treated for 10 days with $nIgM_{sw}$ and insulin-reactive B lymphocytes were identified by staining with biotinylated human insulin followed by strepavidin e450. We were unable to detect insulin-reactive B lymphocytes in NOD mice after treatment, which is quantified in E. F)) A longer course of treatment was undertaken to determine how IgM_{sw} impacted production of anti-insulin Igs. A RIA of circulating IgGs reactive to insulin revealed loss of anti-insulin antibodies in $nIgM_{sw}$ treated mice while control NOD mice possessed insulin-reactive IgGs as expected.

nIgM$_{SW}$ reverses diabetes in NOD mice

As humans are rarely identified before experiencing beta cell loss, it remains important to develop therapies that facilitate beta cell recovery after patients present with hyperglycemia. To this end I tested the ability of IgM derived from Swiss Webster (SW) donor mice (nIgM$_{SW}$) to reverse hyperglycemia in newly diabetic NOD mice (blood glucose 200-300mg/dl on two consecutive measurements). Mice were administered two doses of nIgM$_{SW}$ (100μg) on day 1 and day 4 after onset (n=11). Utilizing this strategy, I determined that nIgM$_{SW}$ normalized hyperglycemia and maintained blood glucose $\leq$ 200mg/dl in 63% of treated mice. (**Figure 4.3 A and B**).

Tregs expand and restrain diabetes in nIgM$_{SW}$ treated NOD mice

The capacity for a short therapy of nIgM$_{SW}$ to reverse diabetes and cull autoreactivity from the B lymphocyte repertoire was a surprising finding. While elimination of B lymphocytes prevented diabetes, in rituxan study, depletion was demonstrated to be less effective in reversal of diabetes, most likely due to the presence of activated beta cell reactive T lymphocytes[273]. IgM therapy has been shown to boost the capacity of protective capacity of Tregs, especially in conditions of chronic immune activation, a cell subset that would have the potential to quell ongoing autoimmunity. I hypothesized that nIgM boosted the Treg function in NOD mice allowing for reversal of diabetes in treated mice. Direct analysis of CD4$^+$CD25$^+$Foxp3$^+$ Tregs in the spleen revealed that nIgM$_{SW}$ expanded Tregs, suggesting the hypothesis that CD4$^+$ Tregs promote reversal of T1D by nIgM$_{SW}$ (**Figure 4.4A and B**).

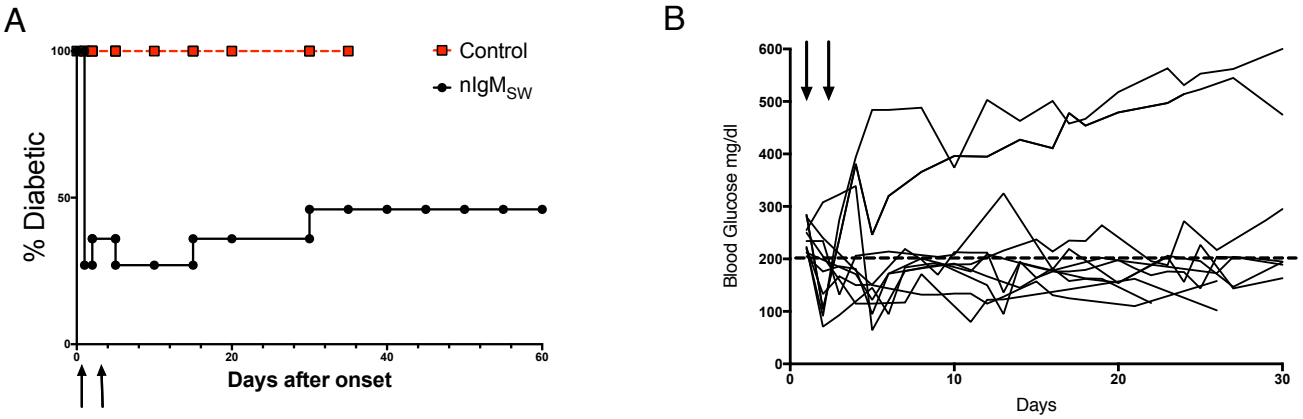


Figure 4.3 nIgMSW reverses T1D and modifies immune subsets in NOD mice. A and B) NOD mice were allowed to become diabetic as defined by two consecutive blood glucose readings between 200-300mg/dl. These mice were then treated with two injections of 100ug nIgM isolated from Swiss Webster mice ($nIgM_{sw}$) on days 1 and 4 post-diagnosis. Blood glucose was monitored serially and approximately 63% of mice reversed and maintained blood glucoses below 200mg/dl for the duration of the time they were monitored. (n=11 mice and control n=15)

To determine whether CD4 Tregs were requisite for durable disease reversal following $nIgM_{SW}$ therapy, I depleted Tregs from NOD mice that had their disease reversed with $nIgM_{SW}$ for more than 30 days by administering two injections of anti-CD25(PC61), an approach that consistently breaks Treg dependent tolerance. These mice developed hyperglycemia in ~14 days after the initial administration of anti-CD25 as compared to control (mice that received no anti-CD25 and remained euglycemic (**Figure 4.4C**). Transfer of Tregs into NOD/SCID mice with diabetic splenocytes also demonstrated the ability of these cells to restrain disease (**data not shown**). Taken together these data demonstrate that Tregs are expanded in response to $nIgM_{SW}$ and restrain disease in reversed mice.

Thymic Tregs expand in a B lymphocyte dependent manner in $nIgM_{SW}$ treated miceI noted an increase in Tregs in $nIgM_{SW}$ treated NOD mice, suggesting that $nIgM_{SW}$ mediated a peripheral expansion of Tregs able to restrain disease. Alternatively, I hypothesized that correction of B lymphocyte homeostasis fostered development of new, potent Tregs in the thymus. To investigate this hypothesis, I assessed the production of $CD4^+$ $Foxp3^+$ Tregs in the thymuses of B6 and NOD $nIgM_{SW}$ treated and untreated mice. Following $nIgM_{SW}$ treatment, NOD mice experienced a two-fold expansion in Tregs in the thymus (Figure 4.5A). Surprisingly, I also noticed a robust expansion of B lymphocytes in thymuses of $nIgM_{SW}$ treated NOD mice (Figure 4.5B and C). While little is known about the role of thymic B lymphocytes in Treg development, some studies suggest that B lymphocytes can boost the output of Tregs from the thymus under certain conditions[286].

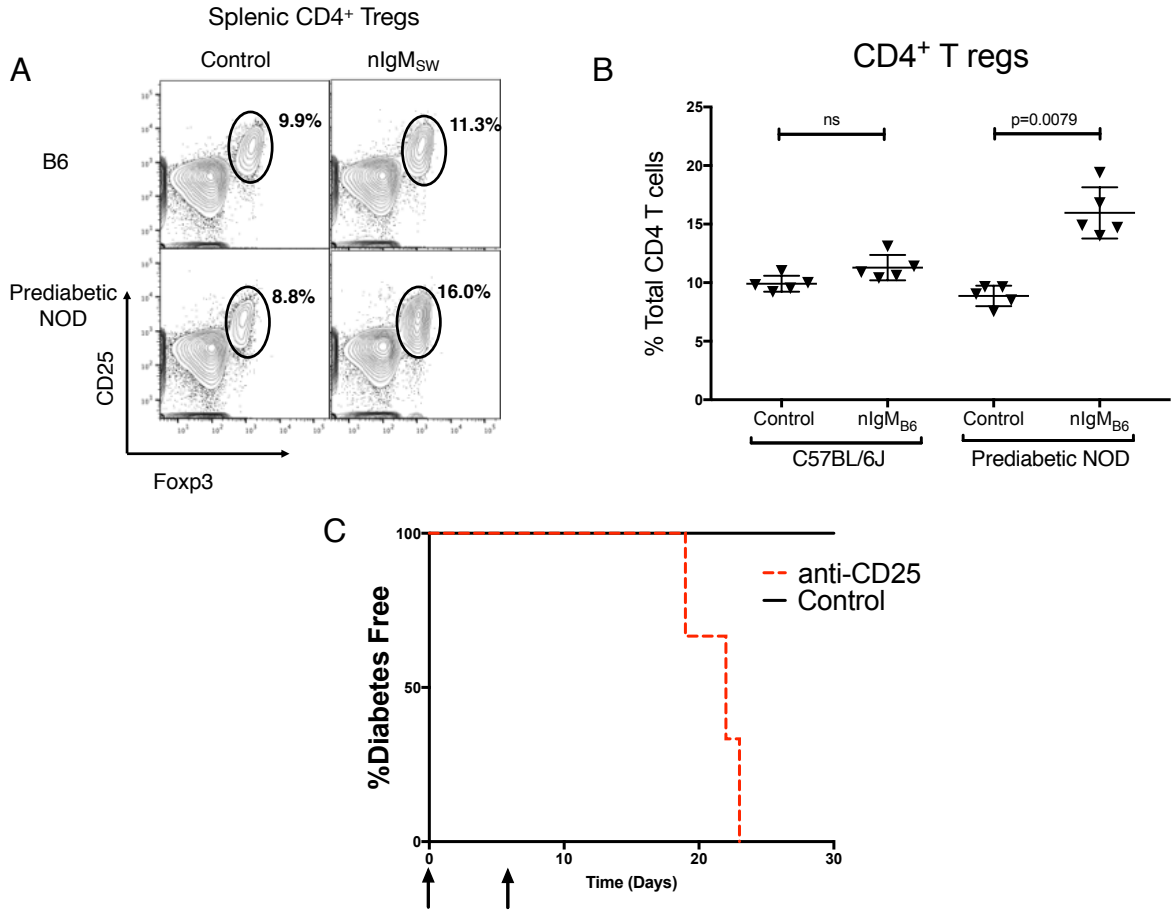


Figure 4.4 nIgMSW expands Tregs and these cells are essential for diabetes reversal. A) B6 and NOD mice were treated with $nIgM_{sw}$ and spleens were analysed for CD25+ FoxP3+ CD4 T cells. B) Treated NOD mice had an almost 3-fold increase in peripheral Tregs after the therapy. B6 mice showed only a modest increase in Tregs after therapy. (Representative data of at least 7 experimental repeats shown) C) To determine whether Tregs were responsible for stably preserving beta cell mass and preventing hyperglycemia, mice that had remained stably reversed with $nIgM_{sw}$ for 30 days were then treated with T cell depleting anti-CD25 antibody (PC61) at a dosage of 2mg/kg on day 1 and day 7 (dashed lines n=3). PC61 treated mice became hyperglycemic approximately 3 weeks after the first injection of anti-CD25 and were sacrificed according to animal protocols. Control mice (Black lines n =3) remained euglycemic even out to 60 days post initial $nIgM_{sw}$ therapy

To test the hypothesis that interactions between thymic B cells and developing T cells were required for Treg generation, I injected $nIgM_{SW}$ into NOD.μMT mice that lack B lymphocytes. With treatment thymic Tregs did not expand in B cell deficient NOD.μMT mice (Figure 4.5D).

Histologic analysis of treated thymuses in B lymphocyte sufficient NOD mice revealed considerable infiltration of B lymphocytes into the medullary spaces of the thymus, an area essential for Treg development [285], in treated NOD mice (Figure 4.5E). Interestingly, this analysis revealed clustering of B lymphocytes (red) and Foxp3+ Tregs (dark blue) in B6 mice at baseline; this same clustering was not present in NOD mice at baseline. Treatment with $nIgM_{SW}$ induced $B220^+$ and $Foxp3^+$ cells to become more localized in the medulla of treated NOD mice than untreated control groups (Figure 4.5F). These data demonstrate a unique role for B lymphocytes in fostering thymic Treg development, which is enhanced by treatment with $nIgM_{SW}$, and may depend on location of B lymphocyte-Treg interactions during thymic development.

BAFF is required for expansion of thymic B lymphocytes and thymic T regs

Others have shown overexpression of B Cell Activating Factor (BAFF) in mice on a B6 background expands thymic B lymphocytes, induces medullary colocalization of B lymphocytes with Tregs, and increases thymic Treg output[286]. Surprisingly, the action of BAFF was dependent on B lymphocytes, as BAFF overexpressing mice did not experience Treg expansion when placed on a B cell deficient background [286]. To date, the role of BAFF in autoimmune disease has been thought to be deleterious especially in the progression of T1D [121]. Surprisingly, $nIgM_{SW}$ therapy increased circulating BAFF almost 8-fold in NOD mice (**Figure 4.6A**).

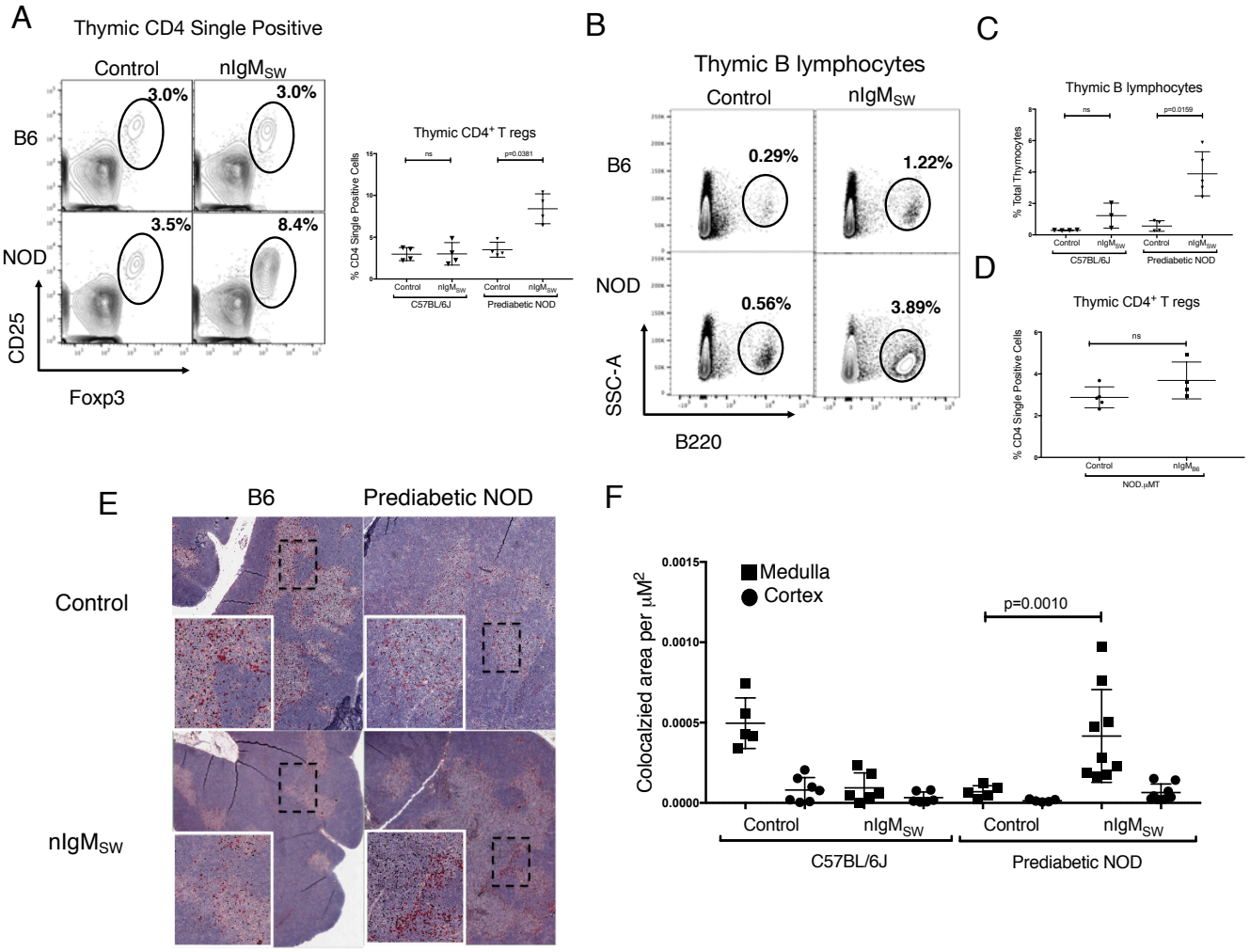


Figure 4.5 nIgMSW expands thymic Tregs in a B lymphocyte dependent manner. A) B6 and NOD mice were treated with nIgM$_{sw}$ and thymuses were analyzed for CD25+ FoxP3+ CD4 T cells. Treated NOD mice had a 2-fold increase in thymic Tregs after the therapy. B6 mice had no increase in Tregs. Represenative flow data. B) Thymic B lymphocytes also increased as shown by measurement of B220$^+$ cells. Further staining demonstrated that these cells are also CD19$^+$ and IgM$^+$. This is quantified in C) D)To determine whether Treg expansion was reliant on B cells, NOD.□MT mice that are genetically deficient of B cells were treated with nIgM$_{sw}$. These mice demonstrated no increase in thymic Tregs indicating a role for B lymphocytes in thymic Treg induction. E) We assessed whether B lymphocytes and Tregs were located in proximity to each other in the thymus by staining B220 (in red) and Foxp3 (in dark blue). We observed clusters of B lymphocytes near the thymic medulla (indicated by the fainter H&E staining) in B6 control and treated groups. In NOD control mice, we observed B lymphocytes in the medulla but at a much lower frequency than B6. After nIgM$_{sw}$ treatment, the number of B lymphocytes near the thymic medulla greatly expanded in NOD thymuses. F) Colocalization analysis revealed that the medullary spaces of the thymus have the most Treg and B lymphocyte interactions in B6. In NOD mice this interaction occurs at a much lower rate. Treatment of NOD mice increased the B-Treg interaction in the thymic medullary spaces.

This increase in BAFF signaling did not lead to an increase in NFkB signaling, it's canonical signaling pathway, but instead normalized levels of NFkB in NOD B lymphocytes were observed (**Figure 4.6B and C**).To determine the role of BAFF in thymic Treg expansion, I blocked circulating BAFF with anti-BAFF (Sandy-2) and treated NOD mice with $nIgM_{SW}$. Blockade of BAFF led to a decrease in both thymic B lymphocytes and thymic Tregs (**Figure 4.6D and E**) indicating an essential role for BAFF in generation of thymic Tregs in NOD mice in response to $nIgM_{SW}$.

NOD nIgM (IgM_{NOD}) does not possess the immunoregulatory properties of $nIgM_{SW}$

Because nIgM isolated from Swiss Webster donors reversed disease and modulated the immune compartment of NOD mice, I hypothesized that NOD-derived nIgM lacks the capacity to reverse disease and restore immune homeostasis. IgMs were prepared from age 8-12 week old prediabetic NOD donors. Diabetic NOD mice were treated with two doses of 100μg of $nIgM_{NOD}$ and blood glucose was monitored. Treated NOD mice experienced an early reprieve from high blood glucose at the beginning of the treatment course but returned to hyperglycemia shortly thereafter (n=4). (**Figure 4.7A**). To determine the immune changes induced by $nIgM_{NOD}$, I treated prediabetic NOD mice with $nIgM_{NOD}$ for 10 days (100ug on day 1 and 50 ugs on days 3,5,7, and 10). I then assessed B lymphocyte numbers and subset distribution via flow cytometry. I did not see an increase in total B lymphocyte numbers (**Figure 4.7B**). I did note a decrease in marginal zone B lymphocytes and a slight increase in transitional B lymphocytes but not to the levels of $nIgM_{SW}$ treatment (**Figure 4.7C and D**). Peripheral Tregs also expanded with $nIgM_{NOD}$ (**Figure 4.7E**) but when I assessed the thymus, there was no significant expansion of thymic B lymphocytes or Tregs in the thymus of treated mice (**Figure 4.7F, G, and H**).

These data demonstrate that IgM isolated from NOD mice is deficient in components important for long term reversal of diabetes.

Natural IgM mediates its effects through mechanisms other than alloreactivity

While I demonstrated robust effects of IgM isolated from Swiss Webster on immune function and diabetes reversal, I was unable to achieve the same results with infusion of IgM isolated from NOD mice. I entertained the hypothesis that this difference in effectiveness might be due to alloreactivity to the infused IgM, inducing immune changes unrelated to homeostatic control. To test this hypothesis, I first assessed the allotype of Swiss Webster mice. NOD and B6 mice both express the IgM(b) allotype, but the Swiss Webster mouse is an outbred mouse so it is unclear the IgM allotype of this mouse. Lineage analysis of the Swiss Webster mouse indicated that it shared a common ancestor with NOD increasing the likelihood that this mouse also utilized IgM(b) (**Figure 4.8A**). Utilizing antibodies against IgM(b) I determined that Swiss Webster mice expressed IgM(b) on roughly half of the B lymphocytes, but also expressed another allotype (**Figure 4.8B**). Further lineage analysis revealed that other progeny of Swiss Webster mice also expressed IgM(a) allotype. I then utilized an antibody against IgM(a) to demonstrate that the B lymphocyte in Swiss Webster mice expressed both the IgM(a) and IgM(b) allotypes (**Figure 4.8B**).

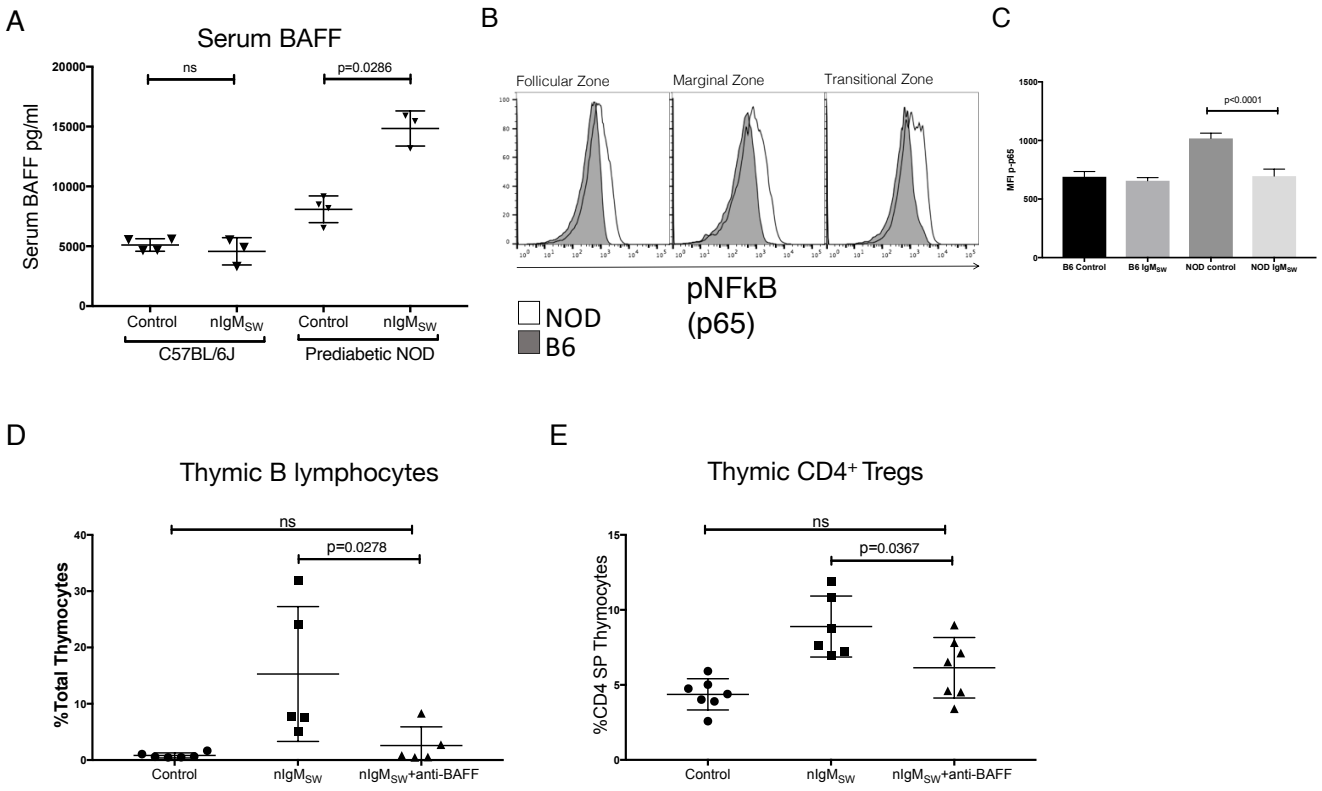


Figure 4.6 BAFF is essential for thymic Treg and B lymphocyte expansion in nIgMSW treated mice. A) Serum BAFF levels were demonstrated to be increased almost 8-fold in NOD mice treated with nIgM$_{sw}$. B) BAFF downstream target NFKB is more active in NOD B lymphocytes in all subsets assessed via phosphorylation measurement by flow cytometry. C) Treatment with nIgM normalized phosphor-NFKB in NOD mice. Blockade of BAFF with anti-BAFF (Sandy-2) led to a reduction in the ability of nIgM$_{sw}$ to expand B cells D) and Tregs C) in the thymus of NOD mice.

To determine whether alloreactvitiy against IgM(a) accounted for the immune changes in NOD mice, I utilized a monoclonal antibody of IgM(a) allotype raised against Keyhole-limpet hemocyanin (KLH), an irrelevant antigen. I treated NOD mice with the same dosage and treatment schedule of this monoclonal IgM(a) as the $nIgM_{SW}$ therapy. After analyzing the immune compartment of these treated mice, I determined that it did not induce an expansion of splenic B lymphocytes (**Figure 4.8C**). It moderately decreased marginal zone B lymphocytes but had no effect on transitional, follicular or splenic Treg subsets **(Figure 4.8D, E, F, and G)**. Analysis of the thymus revealed no change in B lymphocyte or Treg subsets in NOD mice treated with monoclonal IgM(a) (**Figure 4.8H and I**). This indicated that a majority of the immune changes could not be accounted for by alloreactivity.

nIgM restores responsiveness of Tregs to toleranc- inducing therapy anti-CD45RB

The therapeutic potential of nIgM in treating T1D is high, as it is the first non-immunodepleting therapy to demonstrate potent and permanent reversal of T1D in mice. While NOD mice are amenable to diabetes reversal by several immune therapies, these therapies have consistently failed in humans. Interestingly, NOD mice are stringently resistant to transplant tolerance mechanisms of all kinds[287–289]. Recent work in our lab has demonstrated that NOD mice resist expansion of Tregs mediated by anti-CD45RB, and in kind resist this Treg mediated tolerance induction therapy (**Figure 4.9A**). Attempts to tolerize NOD.μMT, mice that are genetically deficient in B lymphocytes but maintain all the genetic loci that contribute to disease, resulted in high-rates of graft acceptance; while untreated NOD.μMT mouse still maintained the capacity to reject these islets (**Figure 4.9A**). Furthermore, NOD.μMT were able to expand Tregs in response to anti-CD45RB similar to B6 mice (**Figure 4.9B and C and not shown**).

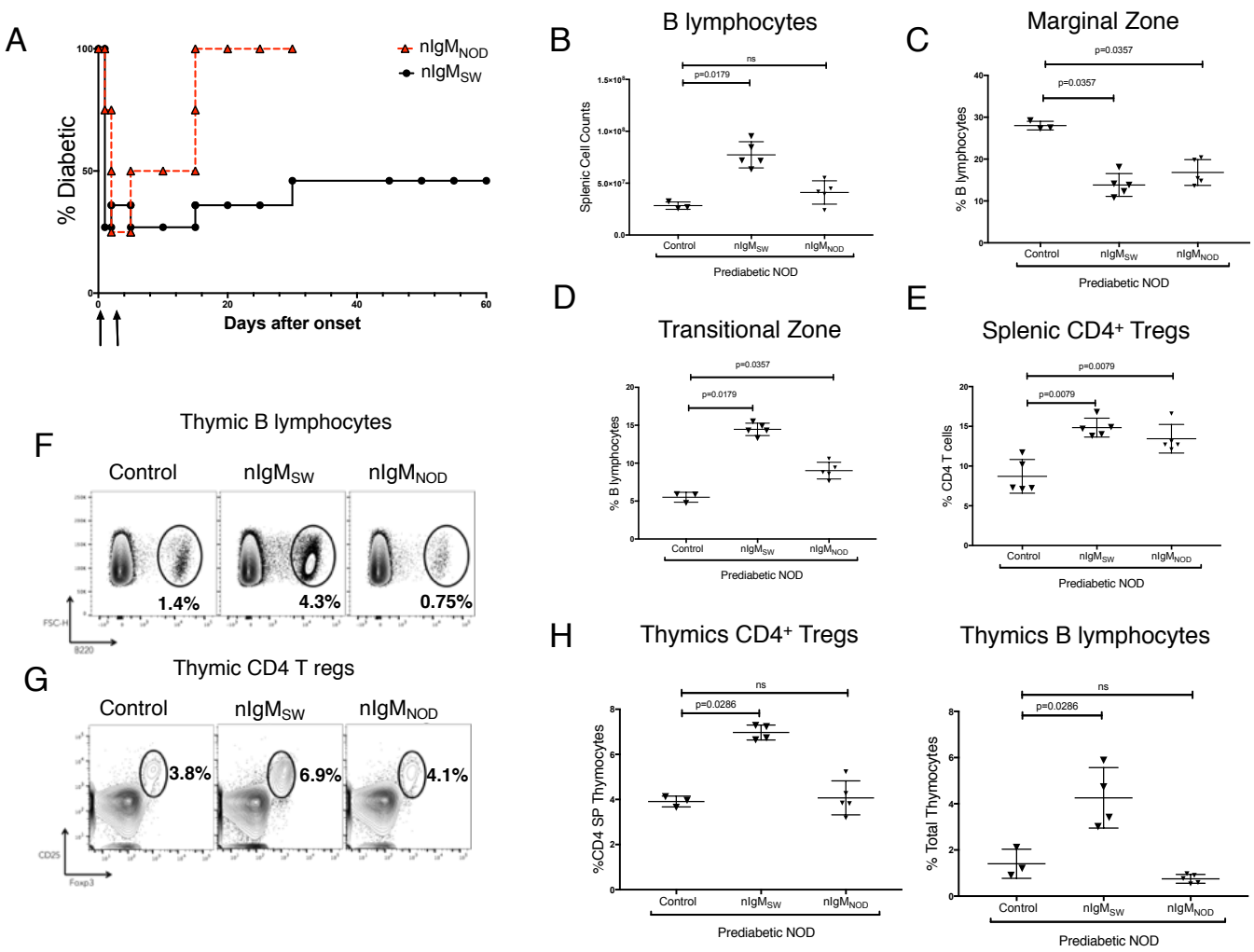


Figure 4.7. nIgMNOD does not reverse diabetes and lacks some of the immunomodulatory capacity of nIgMSW. A) Injection of IgM derived from pre-diabetic NOD donors (IgM_{NOD}) did not reverse diabetes in NOD mice. Mice were administered two doses of 100μg of IgM_{NOD}. While all treated mice experienced a brief reprieve in high blood glucose, all mice had recurrent and permanent hyperglycemic shortly after thereafter. Comparison shown to IgM_{SW}-treated mice from Figure 1. B) It did not increase total B lymphocyte numbers. It modestly decreased marginal zone B cells C) and modestly increased transitional B lymphocytes D) but not to the level of treatment with IgM_{SW} E) IgM_{NOD} expanded splenic Tregs in NOD mice. F) IgM_{NOD} did not expand B lymphocytes in the thymus of NOD mice. G) IgM_{NOD} also failed to expand Tregs in the thymus of NOD mice. This is quantified in H.

I hypothesized that abnormal development of Tregs in the thymus prevented them from becoming activated by tolerizing therapy anti-CD45RB. To test this hypothesis I utilized a transfer model of peripheral Tregs from NOD WT and NOD.μMT mice. Transfer of NOD.μMT CD4+ T cells into a B lymphocyte replete NOD environment and transfer of NOD CD4+ T cells in to a B lymphocyte deficient recipient did not change the capacity for anti-CD45RB to mediate Treg expansion (**Figure 4.9D and E**). These findings indicate that B lymphocytes in NOD mice foster abnormal T lymphocyte development, bolstering our findings about abnormal localization of thymic B lymphocytes in NOD mice (**See figure 4.5F**). I hypothesized that restoration of B lymphocyte homeostasis via treatment with nIgM before anti-CD45RB would allow for the production of Tregs capable of responding to anti-CD45RB in NOD mice. Mice were treated with two doses of anti-IgM_{SW} over 4 days and rested 1 week before being given anti-CD45RD. Pre-treatment with nIgM allowed for expansion of Tregs after anti-CD45RB therapy, indicating restoration of B lymphocyte homeostasis may be a key step in returning the NOD mouse to a state in which it is amenable to immune tolerance (**Figure 4.9F**), a hypothesis that will be investigated in the future.

Human IgM expands human Tregs and prevents diabetes

To assess the translational potential of this therapy, I obtained human IgM from a healthy donor, which I injected i.p. at the same dosage as $nIgM_{SW}$ into NOD mice. I noted moderate normalization of B lymphocyte subsets (**not shown**) and the expansion of Tregs in these mice (**Figure 4.10A**). I determined that human IgM was strikingly effective at preventing diabetes, with complete protection of treated NOD mice lasting for >12 weeks after therapy was discontinued (**Figure 4.10B**). Having determined that IgM immunotherapy expands thymic Tregs to promote long term diabetes prevention and reversal, I modeled this effect in the humanized BLT mouse to assess the response of human Tregs. In this model, human T-cell development originates in the bone marrow (B) of immunodeficient NSG mice from human hematopoietic, liver derived (L) CD34+ stem cells passing through human thymic (T) development (**Figure 4.10C**)[290,291]. Treatment of a cohort of BLT mice with human IgM resulted in a doubling of the Treg proportion within the CD4 T-cell compartment (**Figure 4.10D**); these expanded Tregs had a Foxp3+ Helios+ phenotype that is indicative of thymus-derived Tregs.

Discussion

This study introduces two unrecognized but linked pathologic processes in T1D, loss of B lymphocyte homeostasis due to abnormal function of secreted IgM and inappropriate B lymphocyte-Treg interactions in the thymus. Considerable amounts of work are still needed to completely understand the roles of these processes in impeding tolerance and inducing autoimmunity. However, this study highlights potential problems with current clinical strategies that rely on depletion to restore defective homeostasis in T1D. Namely, that B lymphocyte depletion may be ineffective due to persistence of pathologic IgM (or absence of protective IgM) that fosters a return to abnormal development and selection. Furthermore, my study suggests thymic B lymphocyte and Treg interactions play a role in establishing disease reversal and potentially disease progression, a function that has not been evaluated clinically.

My findings illustrate that naturally occurring polyclonal IgM from healthy donor animals and humans was highly effective not only in preventing diabetes occurrence but also reversing new onset disease. Although IVIg therapy has been previously evaluated in patients with T1D, these infusions were not successful [104]. These products contain relatively low amounts of IgM, which our investigation suggests is the key immunomodulatory component for the treatment of T1D. Interestingly some new immunoglobulin preparations such as Pentaglobin are enriched in IgM although they are still not purified IgM preparations and contain relatively low amounts of IgM [292]. These preparations have not been evaluated in T1D or other autoimmune disorders but have been effectively applied in sepsis, suggesting that this approach would not cause deleterious immune suppression in patients with T1D[142].

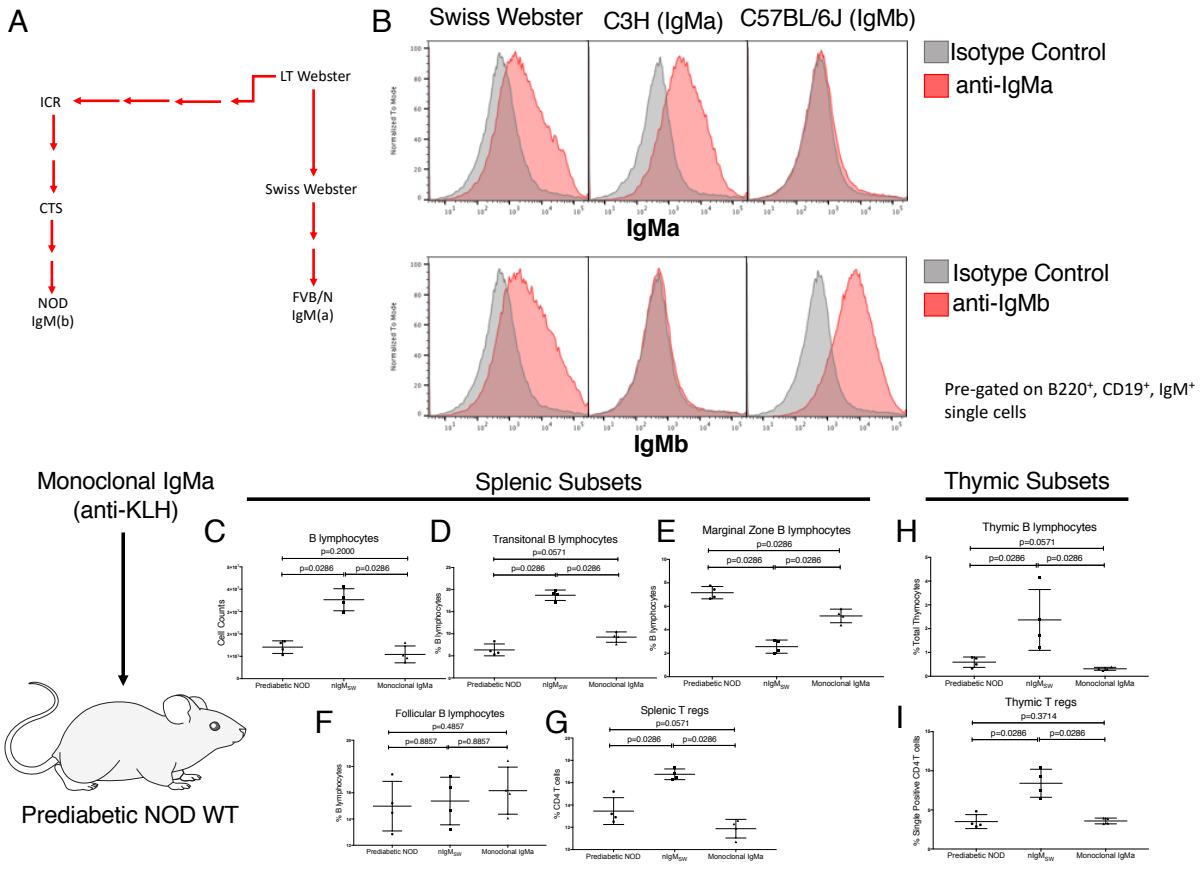


Figure 4.8 Swiss Webster mice express dual IgM isotypes but exert a majority of immunomodulatory effects independent of alloreactive immunity. A) Lineage analysis reveleas that Swiss Webster gave rise to mice that possess both IgM(a) and IgM(b) alloytpes. B) Antibodies against IgMa and IgMb were utilized to determine the allotype of IgM expressed on B lymphocytes. C57BL/6J mice (IgMb), C3H (IgMa) were compared to Swiss Webster to determine efficacy on antibody staining. As characteristic of an outbred strain, the IgM loci was heterozygous for both IgMa (top row) and IgMb (bottom row). C) To determine if alloreactivity played a role in mobilizing immune changes in the immune compartment in NOD mice we treated mice with IgMa raised against KLH at the same dosage as $nIgM_{sw}$. We observed no changes in total B lymphocyte numbers. D) There were no changes transitional B lymphocyte subsets and only a moderate decrease in marginal zone B lymphocytes seen in E. F) Similar to $nIgM_{sw}$ there were no changes in Follicular B lymphocytes. G) Splenic T regs were not expanded by IgMa as compared to $nIgM_{sw}$. H) Thymic B lymphocytes were also not impacted by IgMa and as a consequences neither were thymic T regs I).

While not directly related to diabetes reversal, deep immunophenotyping using mass cytometry (CyTOF) and Spanning-Tree Progression Analysis of Density-Normalized Events (SPADE) revealed multiple immune modulating effects of IgM (**Figure 4.11A**)[293]. These changes include alteration of the CD8 T lymphocyte compartment and expansion of Myeloid-Derived Suppressor like cells, which were unable to restrain disease in our hands, but have been demonstrated to have potent immunomodulatory properties in other models (**Figure 4.11B and C**)[294]. Together these data indicate far-reaching immunomodulatory capacity of IgM that warrants further investigation.

My work illustrated that the IgM mediated regulation of homeostasis is abnormal in NOD mice, a completely unrecognized pathologic process in T1D. This surprising finding indicates that failed B lymphocyte mediated regulation may be an important contributor to the fundamental pathogenesis of disease. While the immunomodulatory capacity of IgM is known, it is rarely mentioned in discussions with traditional "B regs", possibly due to the ubiquitous nature by which IgM is secreted from a majority of naive B lymphocytes. Nonetheless my data indicates that only a portion of IgMs contain immunomodulatory function. At this point I do not know whether NOD mice are deficient in this capacity from birth, whether it develops later, or whether a pathologic IgM over-rides any protective IgM the NOD mouse possesses. A striking change in B cell development with loss of the transitional zone occurs around 4-8 weeks in NOD mice, which could lead to shifts in antibody production [111]. However, many of these IgMs are expected to arise from the innate like B1 B cell compartment; this compartment has been associated with diabetes pathogenesis rather than protection suggesting it may be deficient in producing this regulatory component[284].

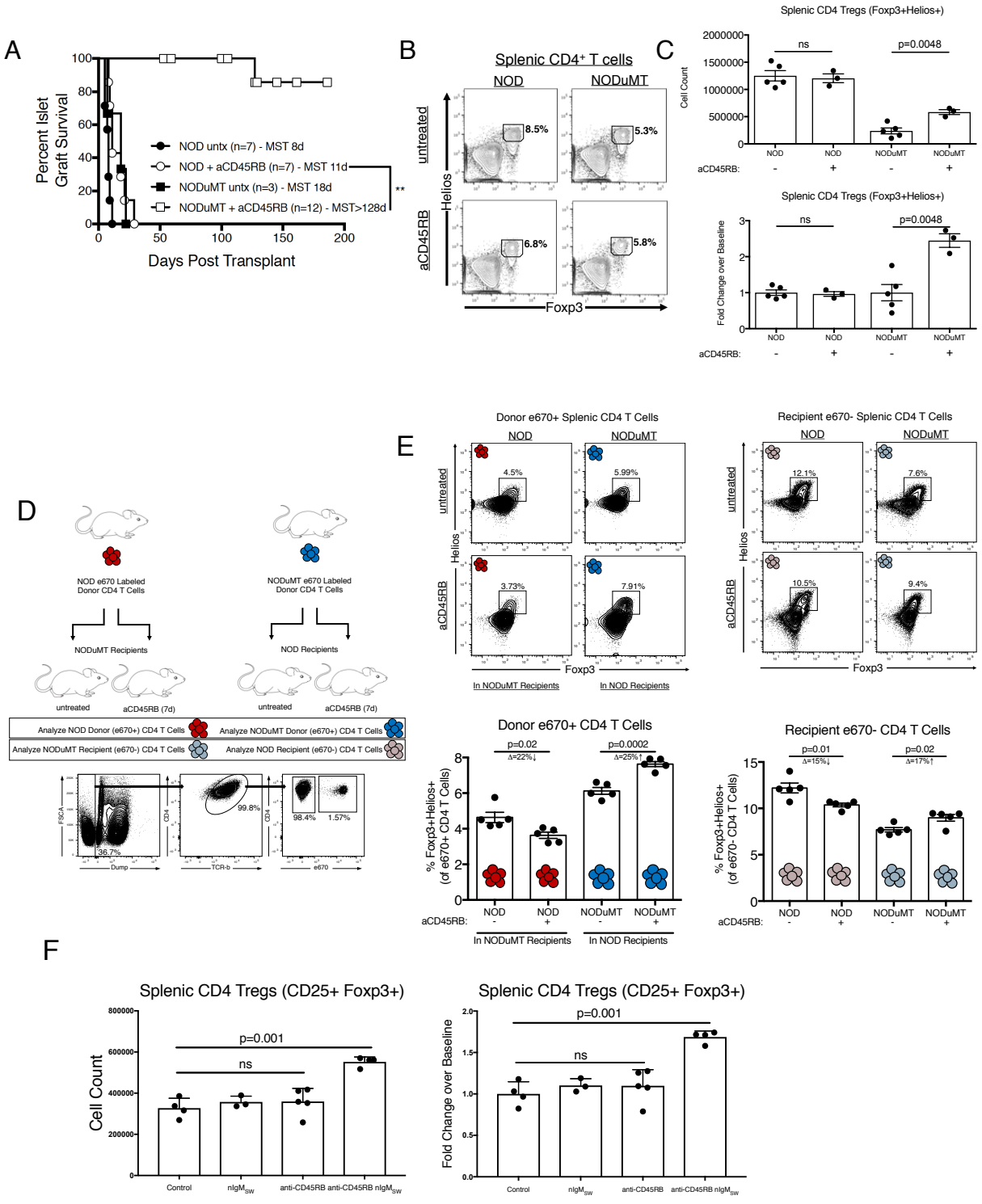


Figure 4.9 Abnormal B lymphocyte homeostasis precludes tolerance in NOD mice by inhibiting Treg expansion, that can be corrected by nIgM. A) Transplant of allografted islets in NOD and NODuMT mice reveals that NOD mice are resistant to tolerance induction by anti-CD45RB, but B lymphocyte deficient are completely permissive to tolerance induction. Important these mice retain the capacity to reject islets when no tolerance therapy is administered (**=??ns are indicated in figure). B) Treatment with anti-CD45RB expanded thymically derived Tregs (Helios^{+}Foxp3^{+}) in NODuMT mice but this was resisted in NOD WT mice. D) Transfer of purified CD4 T cells from NOD and into NODuMT or vice versa revealed that even when reintroduced new environments Tregs from either NOD or NODuMT reacted the same way to anti-CD45RB, indicating a developmental programming event prevented Treg from expanding in NODWT mice. F) Pretreatment of NOD mice with nIgM restored B lymphocyte homeostasis and allowed for expansion of Tregs in NODWT mice.

To identify cellular targets of IgM, I have proposed co-incubating $nIgM_{SW}$ with different immune cell subsets then extracting the unbound IgM. I can then utilize the remainder in reversal and immunophenotyping studies in NOD mice to determine which cell subset depletes the therapeutic capacity of IgM. A receptor for the Fc portion of IgM (Fc□R or TOSO) is known and I found it to be expressed on B lymphocytes but not Tregs (**not shown**). It was however shown to be robustly expressed on transitional B lymphocytes, a subset known to be lost in NOD mice, and demonstrated to be altered by $nIgM_{SW}$ therapy. (**Figure 4.12A**). Animals deficient in TOSO or missing secreted IgM also demonstrate abnormal B cell development with loss of the transitional zone and accumulation of autoreactive specificities [277,279,281]. Backcrossing these mice onto NOD background is currently underway by our collaborators. Whether there are other biophysical alterations in the NOD IgM such as changes in glycosylation, folding, or other modification is not known but is an important area for future investigation.

Antigenic targets of IgMs remain unclear at this point but will be important in establishing the IgMs important for immune modulation. As mentioned previously the IgM repertoire of NOD has a reduction in enrichment for autoreactive specificities, while the B6 has relatively enriched numbers of autoreactive IgMs. Previous reports suggest that polyclonal IgM can neutralize several inflammatory cytokines including IL-17, but none that have been reported or that I observed in our studies (**not shown**) would clearly account for changes in B cell development or thymic Treg selection [183]. The therapeutic potential of IgM will most likely be a combination of antigen specific and Fc mediated interactions.

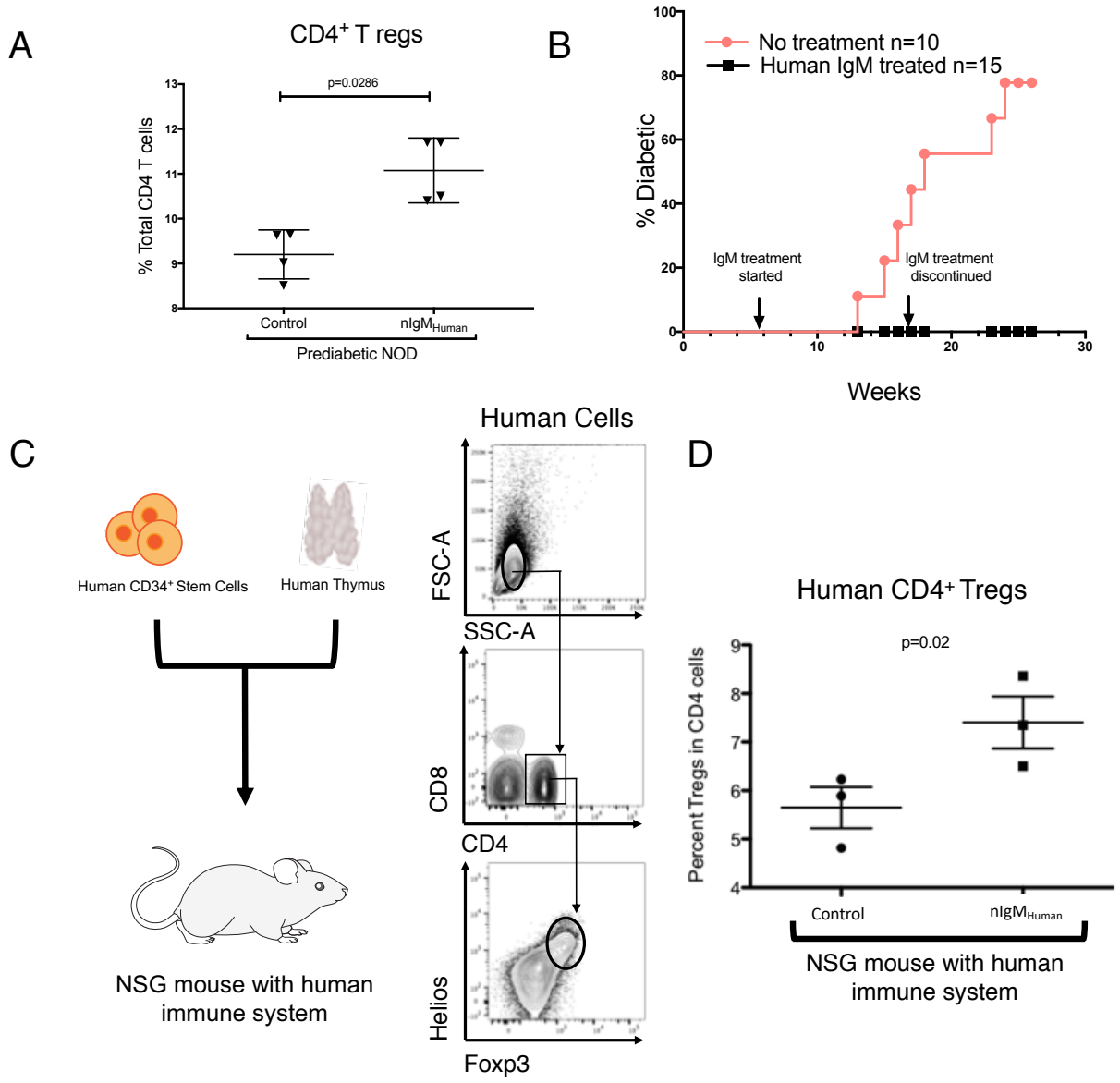


Figure 4.10 Human IgM expands Tregs to prevents diabetes in NOD mice and expands human Tregs. A) Prediabetic NOD mice were treated with human nIgM and Tregs were measured. Mice treated with human IgM experienced an increase in Helios+Foxp3+ Tregs as compared to untreated controls demonstrating the therapeutic potential of human IgM. (n=3 in each group) B) NOD mice were given human IgM from healthy human donors starting at week 5 and ending at week 15. These mice did not develop overt diabetes at up to 25 weeks of age (black line) while 80% of untreated mice (red line) developed diabetes by week 25 C) NOD/SCID mice were transplanted with fetal thymus and liver CD34+ hematopoietic stem cells and allowed for the immune system to reconstitute. Illustrated is the flow cytometry gating scheme to identify human T regulatory cells. D) Human IgM was capable of expanding human Tregs in the NSG humanized mouse system.

My results suggest that IgM therapy in the NOD mouse in part enhances immune function as is evidenced by the substantial increase in B lymphocyte numbers **(Figure 4.2A)**. This effect positions therapy with IgM as an important alternative to immune depletion, which has been ineffective in T1D treatment to date but has remained the paradigm for most clinical approaches to autoimmunity [120,274,295]. Indeed, enrichment of immune development may be a critical mechanism to address the defective B lymphocyte selection that allows the emergence of islet-reactive B lymphocytes that drive disease. This interpretation is supported by studies in animal models in which animals that have lower B cell numbers allow more autoreactive cells to escape to maturity [296,297]. This escape typically occurs at the stage of development known as the transitional stage, which is the stage at which B cells emerge from the bone marrow to the periphery and sample circulating antigens and is similar to patients with B cell immunodeficiency in whom B cell autoreactivity is also increased [298,299]. NOD mice have a loss of the transitional B cell compartment as they age; restoration of B cell numbers genetically has been demonstrated to improve B cell selection and reduce autoreactive lymphocyte numbers [111]. Similarly, IgM treatment increased transitional B cell numbers while eliminating insulin-reactive B lymphocytes and insulin autoantibody production **(Figure 4.2B, C, D, E, and F)**. Individuals with T1D also have a decrease in circulating transitional B cell numbers in the blood [130]. NOD mice have additional B cell developmental abnormalities including an accumulation of marginal zone B lymphocytes, which have been suspected to contribute to pathogenesis [124,300]. Treatment with IgM similarly targeted this differentiation step to produce normal B cell frequencies.

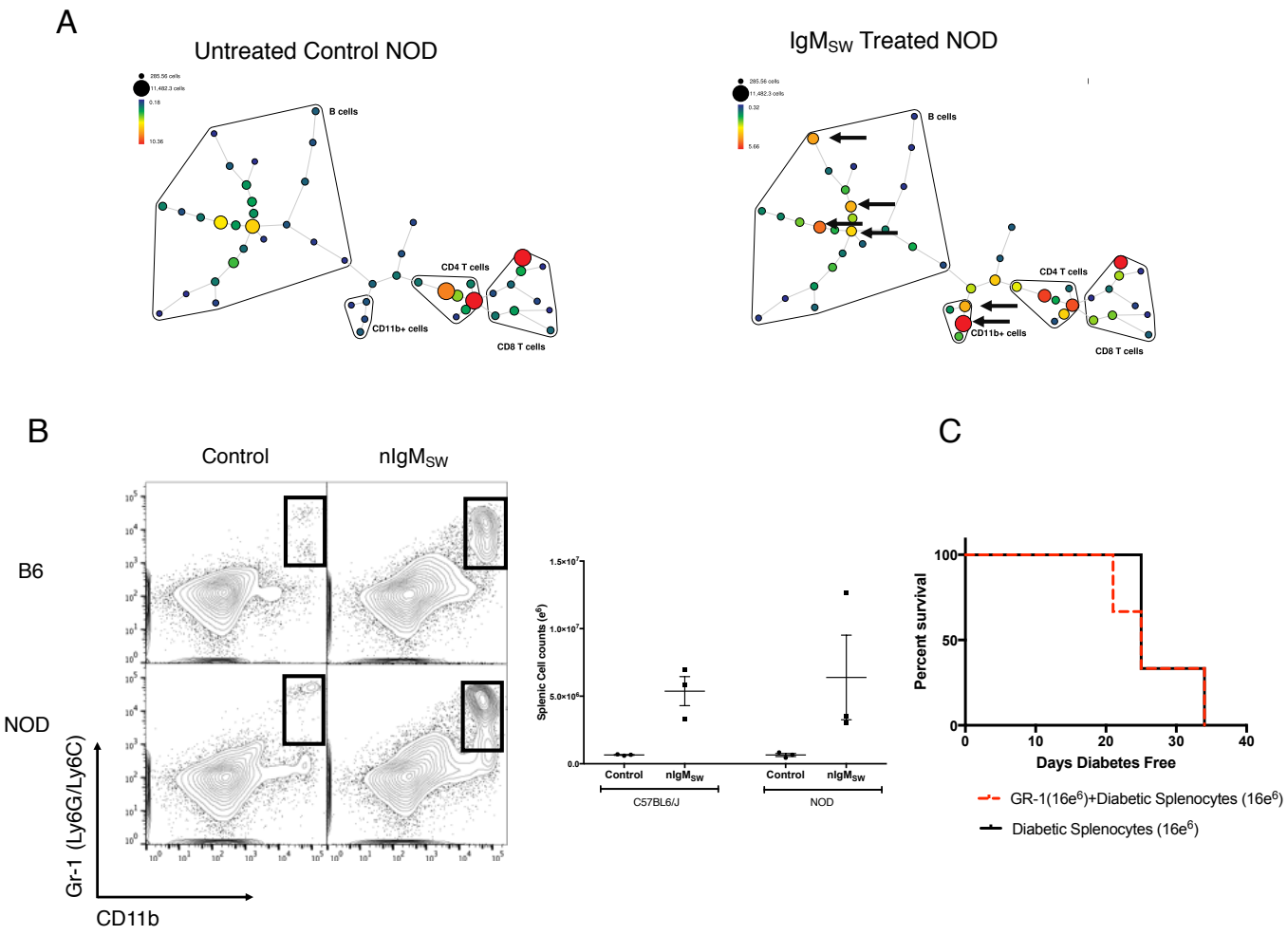


Figure 4.11 CYTOF reveals an nIgMSW mediated expansion of MDSC-like cells that provide no diabetes protection with. A) nIgM$_{sw}$ mice was injected into NOD mice on days 1,3,5,7, and 10. The mice were sacrificed on day 13 and the spleen was harvested. Time of flight mass cytometry (CyTOF) was carried out utilizing 24 markers of immune cell subsets. Analysis was carried out in Cytobank using Spanning-tree Progression Analysis of Density-normalized Events (SPADE). This tree diagram depicts cells classified based on all immune markers assessed. A bubble is drawn around subsets. The size of the circle denotes the number of cell events in that circle while the color scale indicates the percentage of the total events these cells represent. This analysis revealed changes in B cells (top black arrows) and in GR-1+, CD11b+ cells of a Myeloid Derived Suppressor Cell (MDSC) phenotype (bottom black arrows). (n=3 mice per group) B) Treatment with IgM$_{sw}$ led to expansion of GR-1+, CD11b+ double-positive cells in the spleen of both B6 and NOD mice, as demonstrated in the flow diagrams and graph. C) Transfer of these MDSC-like cells with splenocytes from a diabetic donor mouse at a ratio of 1:1 into NOD.RAG mice offered no protection from diabetes onset in these mice. (n=4 per group)

Furthermore, I have established BAFF has a complex role in development of autoimmune disease as many have found its effects accelerate disease[142]. I demonstrate in the context of nIgM it is essential for the expansion of T regs in the thymus. While blockade of BAFF has been shown to prevent disease in NOD mice, it does so by exacerbating B lymphopenia[121]. Whether this resets defective homeostasis remains unknown. BAFF signaling is largely perpetuated through the NFkB pathway. Studies in NOD mice indicate enhanced NFkB signaling in B lymphocytes as compared to B6[199,200]. My work illustrated that in spite of an almost 8-fold increase in BAFF there was a reduction in the level of activated NFkB (phospho-p65) in NOD mice treated with $nIgM_{SW}$ (**see Figure 4.6B and C**) The reason for this normalization of signaling could not be accounted for by changes in BAFF receptor expression (TACI, BR3, or BAFFR) (**not shown**). While an increase in BAFF may represent an increase in production of BAFF others have speculated that circulating BAFF levels are modulated via utilization by target cell types. While BAFF is a potent survival factor for B lymphocytes, nIgM has been shown to also promote robust B lymphocyte development and survival. One hypothesis for increases circulating BAFF would be that B lymphocytes in NOD mice are no longer utilizing BAFF to the same extent, as the proper nIgM repertoire now supplements their survival and persistence[142]. Indeed the IgM receptor has been illustrated to have antiapoptotic properties related to inhibition of Fas mediated apoptosis.[142] Interestingly, blockade of Fas-L in NOD leads to diabetes prevention related to an alteration of the B lymphocyte repertoire[301]. Nonetheless, further studies will need to determine the interplay between BAFF signaling and nIgM that contribute to B lymphocyte defects in NOD mice.

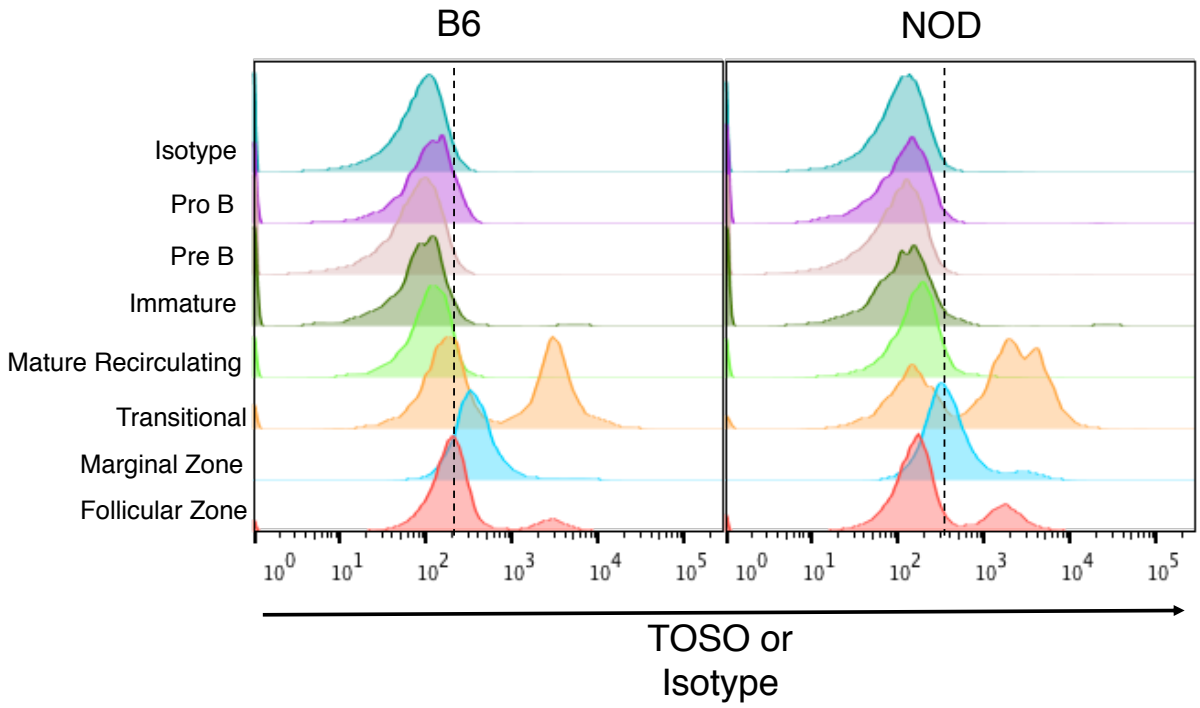


Figure 4.12 Transitional B lymphocytes in both NOD and B6 mice have robust expression of nIgM receptor TOSO. A) Flow cytometry analysis revealed that transitional B lymphocyte (orange histogram) express the highest level of TOS receptor in both NOD and B6. This would be a possible explanation for the robust recovery of transitional B lymphocyte in NOD.

B lymphocytes are thought to contribute to T1D pathogenesis primarily through activation of islet-reactive T lymphocytes. The presence of autoreactive B lymphocytes is an absolute requirement for disease pathogenesis in the NOD mouse model [246]. Similarly patients at risk for Type 1 diabetes can be stratified by the presence of autoantibodies in their serum [302]. Increasing numbers of serum autoantibodies confer substantial increases in T1D risk with the presence of two or more autoantibodies now conferring a diagnosis of Stage 1 T1D [303]. Additional studies in the animal model have demonstrated that B lymphocytes primarily interact with CD4 T cells via MHC class II interactions and drive epitope spreading leading to diversification of the immune response against the pancreas [182,304]. I now establish that treatment with nIgM interferes with this pathologic process by eliminating instigating B lymphocytes in the periphery (**Fig 4.2D, E, and F**). In addition, our data also suggest a previously unrecognized mechanism by which B lymphocytes may control T1D pathogenesis. In this study I demonstrate that treatment with IgM leads to an expansion of thymic B cells that yields diabetespreventingTregs (**Fig 4.5**).

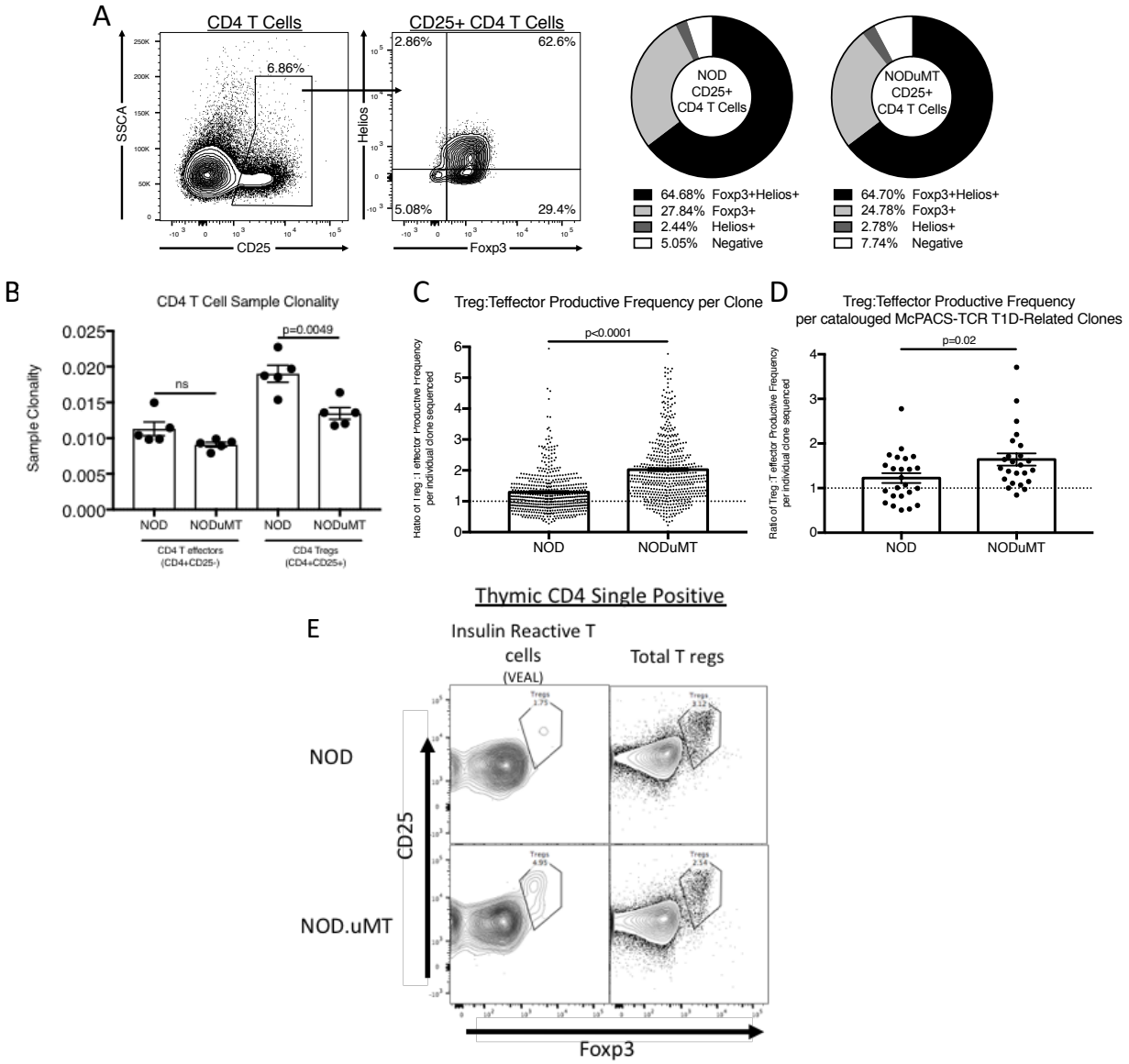
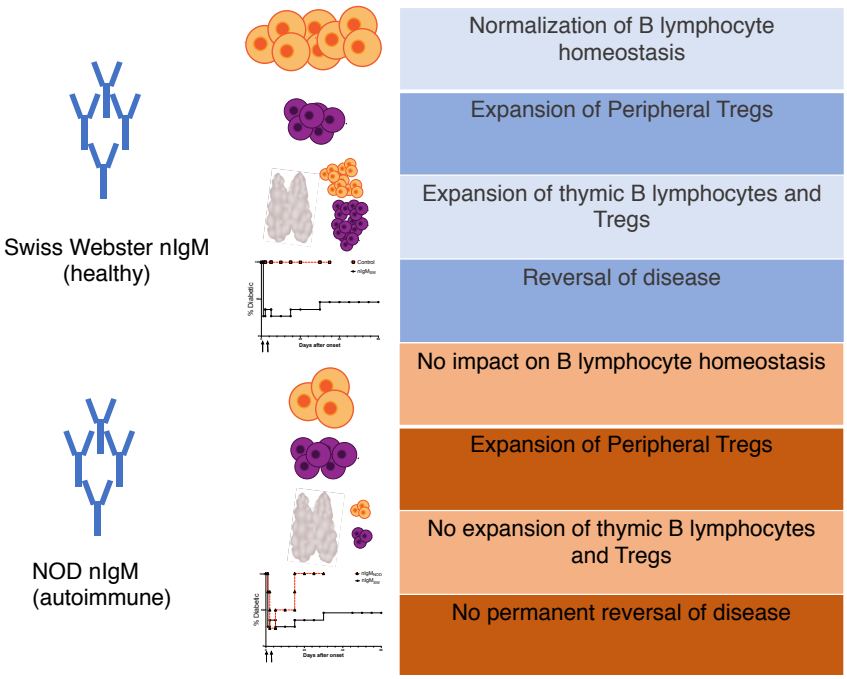


Figure 4.13 B lymphocytes restrict the overall clonality of the Treg repertoire and insulin reactive Tregs in NOD mice. A) Flow cytometry analysis revealed that the CD25$^+$compartment was composed of similar cell subsets based on Foxp3 and Helios expression. This allowed for a direct comparison of the TCR repertoire of CD25$^+$, MACS purified, CD4$^+$ T lymphocytes. B) Sequencing of Tcra in both effectors and Tregs from NOD and NODuMT revealed increased clonality (indicated reduced diversity) in NOD B lymphocyte sufficient mice as compared to NODuMT mice. C) A ratio of the productive frequency of Tregss to Teffectors revealed that on a per clone basis there was a skewing toward a T regulatory phenotype in NODuMT mice. D) Analysis of T1D-related clones curated by the MCPACS-TCR database revealed that the ratio of Tregs to T effectors was again increased in NODuMT indicating that islet invading TCR-specificities were more likely to become Tregs in the absence of B lymphocytes. E) Flow cytometry analysis of T cells that bind insulin in an Ia-g7 tetramer reveal that NODuMT contain a greater percentage of insulin binding Tregs than NOD WT mice (left two panels). They contain approximately the same amount of total Tregs, indicating B lymphocytes cull-autoreactive Tregs on the NOD background.

Relatedly, I also demonstrated that NOD mice resist Treg mediated tolerance induction by anti-CD45RB in a B lymphocyte dependent manner. Further analysis revealed that Treg resistance to tolerance was developmentally imprinted in NOD mice by B lymphocytes, as reintroduction of Tregs to B lymphocytes did not inhibit mature Treg expansion (**see Figure 4.9D and E**). I demonstrated that correction of B lymphocyte homeostasis by nIgM led to a restoration of responsiveness to this therapy in the form of Treg expansion, indicating B lymphocytes restrict Treg tolerance mechanisms. The impact of inappropriate B lymphocyte and Treg interactions remain incompletely understood but I propose two hypotheses supported by preliminary data. First, I have sequenced the TCRB chain from Tregs from NOD and B lymphocyte deficient NOD.μMT. Preliminary results indicate that B lymphocytes in NOD mice restrict the clonality of developing Tregs and prevent the emergence of Tregs that target endogenous antigens (**Figure 4.13A-D**). Utilizing Ia-g7 tetramers loaded with insulin peptide, I was able to further demonstrate a reduction in insulin-reactive Tregs in NOD mice as compared to NOD.μMT mice (**figure 4.13E**). In this model autoreactive B lymphocytes could deplete Tregs of autoreactive specificity due to enrichment of autoreactive epitopes in the thymus coupled with improper thymic localization. I have also determined that B lymphocytes may impede Treg functionality by regulating cell surface expression of GARP, a TGFB receptor important for Treg function[306]. Analysis in our lab reveals a reduction in GARP+ Tregs in NOD mice compared to NOD.μMT. As GARP is expressed on activated Tregs, this reduction in the GARP expression in NOD mice could reflect reduction in autoprotective Tregs that are actively restricting autoimmunity at a baseline (**not shown**).

Previous studies have focused on the positive effects of IgM on immune function. These studies largely focus on IgM derived from C57BL6/J administered at supraphysiologic doses in an autologous manner or, as in our own research, into NOD mice[283,307,308]. While striking, these results neglect the clinical reality that IgM for therapeutic intervention will most likely be administered from diverse and possibly multiple donors. In this chapter, I addressed this clinical caveat by using IgM from the Swiss Webster mouse. By utilizing this model, I was able to provide an important control by administering this drug to both NOD and C57BL6/J mice. In this way, I can identify key mechanistic biomarkers that could provide clues to therapeutic responsiveness in patients (increase in circulating BAFF) while defining other immunologic reactions that, while true, may not be wholly important in the therapeutic activity of nIgM (expansion of MDSCs). Furthermore, I was able to use autologous transfer of IgM from NOD mice back into NOD recipients to further define the immunologic responses necessary to drive permanent reversal in mice (expansion of thymic Tregs). These mechanistic findings provided candidate biomarkers for translation including, Tregs in circulation, B lymphocyte subsets, serum BAFF levels, and thymic output via thymic excision circles, and insulin reactive B lymphocytes via recently described methods, now exists in humans [181]. In conclusion, loss of protective nIgM represents a new pathologic mechanism of T1D and could be utilized as a safe and effective T1D reversal therapy.



Key Findings

-nIgM from healthy mice normalizes immune homeostasis and reverses diabetes in NOD mice.

-nIgM isolated from NOD mice does not possess the same immuneregulatory properties and cannot reverse disease.

-Human nIgM possesses similar immunoregulatory properties and can prevent disease in NOD mice.

Future Aims

-Define the subset of therapeutic nIgMs.

-Determine whether nIgM makes NOD mice permissive to tolerance induction by anti-CD45RB.

-Determine how abnormal B lymphocyte homeostasis culls autoreactive Tregs in NOD mice.

Figure 4.14 Model for nIgMs role in immunoregulation and reversal of diabetes.

CHAPTER V

ENHANCED CD4 T LYMPHOCYTE METABOLISM IS A KEY BARRIER TO TOLERANCE INDUCTION IN SLE123 MICE

Scientific Goal

As I have demonstrated in previous chapters, the immune system contains powerful regulatory mechanisms that can be harnessed to prevent autoimmunity. This immune regulation requires that effector T lymphocytes be amenable to the regulatory mechanisms utilized by regulatory B and T lymphocytes. B6.SLE123 mice (a preclinical model for Systemic Lupus Erythematosus) possess enhanced effector function that prevents proper regulation by multiple tolerance mechanisms. We recently demonstrated that SLE123 fail tolerance induction by anti-CD45RB[153]. In Chapter V I investigate the mechanistic underpinnings of effector resistance to immune tolerance in SLE and link it back to abnormal metabolic function in $CD4^+$ T lymphocytes, which when targeted therapeutically improves immune tolerance. This chapter will highlight the metabolic and signaling abnormalities in B6.SLE123 $CD4^+$ T lymphocytes that contribute to resistance to immune tolerance. Furthermore, it will demonstrate that a short metabolic intervention combined with tolerance-inducing anti-CD45RB therapy leads to improved acceptance of grafted islets and prevention of SLE in mice.

Introduction

Systemic Lupus Erythematosus (SLE) is an autoimmune disease that, much like T1D, is characterized by inappropriate B and T lymphocyte collaboration, leading to T lymphocyte activation and autoantibody production. While the pathogenic potential of autoantibodies appears

to be low in T1D, in SLE these autoantibodies (often directed against nuclear antigens) collect in the kidney, occlude nephrons, and activate complement causing nephritis[309,310]. Patients experience severe kidney damage and oftentimes require kidney transplants that are subject to both remitting and relapsing autoimmunity as well as allorejection[311].

To interrogate transplantation in this system we previously attempted to tolerize B6.SLE123 mice (a congenic mouse model of SLE) to C3H mismatched islets,with anti-CD45RB[153]. Even though this mouse possessed no detectable islet autoreactivity and was transplanted at a time before fulminant lupus disease, these islets were still rejected (**Figure 5.1A and B**). We hypothesized that this outcome might be due to resistance to regulation by effector T lymphocytes in B6.SLE123 mice. We demonstrated that B6.SLE123 derived $CD4^+$ $Foxp3^+$ Tregs could not suppress B6.SLE123 derived effector T lymphocytes but robustly inhibited B6 effector T lymphocytes (**Figure 5.1C)**. Furthermore, Tregs isolated from B6 mice could not inhibit B6.SLE123 effector cells (**Figure 5.1C**). This observation led us to conclude that effector resistance, but not absence of regulation, was to blame for failed tolerance in B6.SLE123 mice.

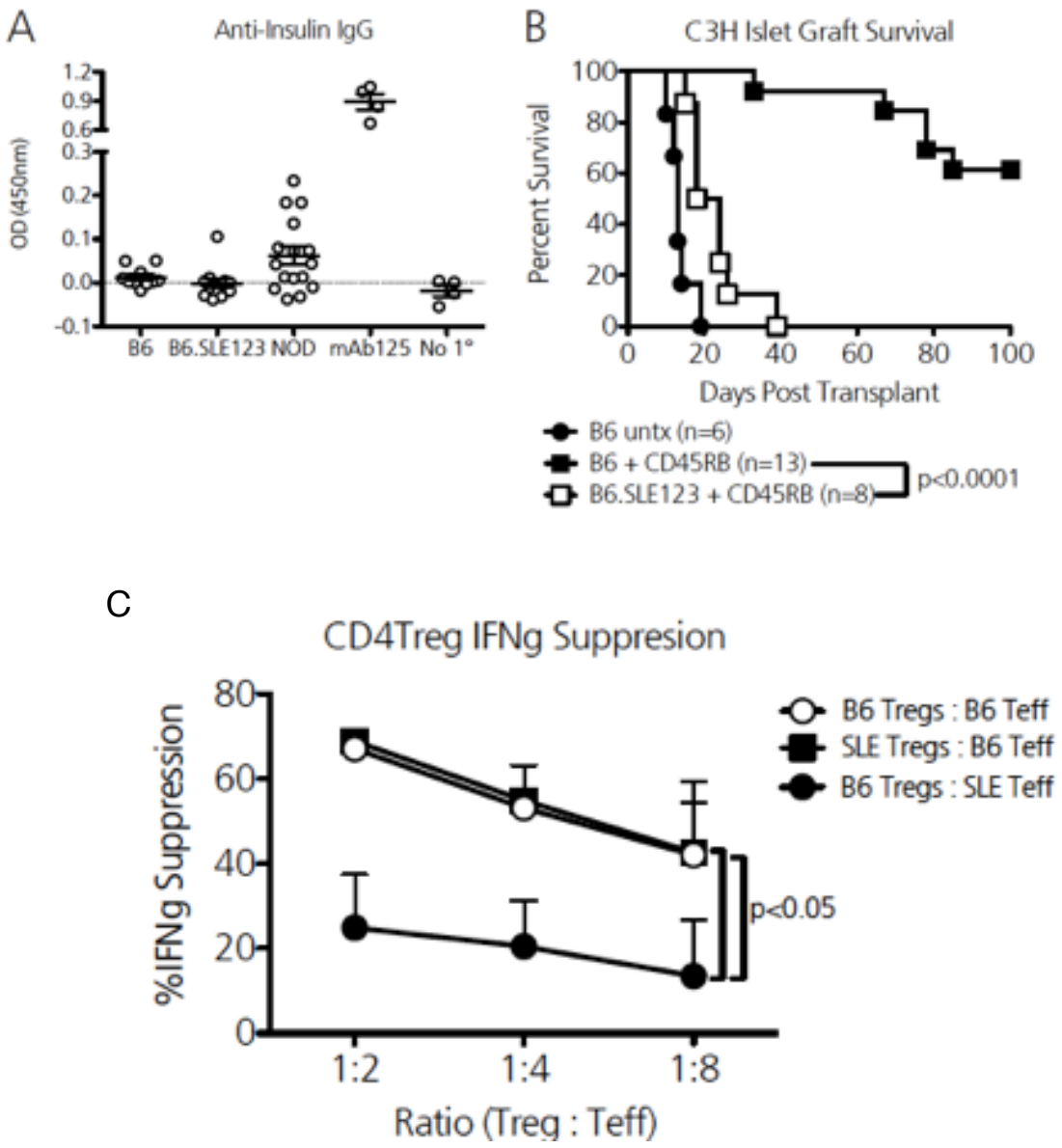


Figure 5.1 SLE resist transplantation tolerance due to effector resistance. A) Measurement of anti-insulin IgGs revealed no baseline islet autoreactivity in SLE123 mice. This is compared to B6 and NOD mice. Insulin binding antibody mAb125 was used as a control of assay sensitivity. B) Transplant of allo-mismatched C3H islets into in SLE123 mice revealed that SLE123 mice resisted transplant tolerance induction by anti-CD45RB, while B6 mice became tolerant to grafted islets as previously demonstrated. C) Interferon gamma suppression assays revealed that B6 Tregs were capable of inhibiting B6 effector IFNg production. SLE Tregs were likewise competent to inhibit IFNg production in B6 Teff cells. B6Tregs were unable to mediate IFNg suppression in SLE Teff indicating effector resistance to tolerance induction in SLE Teff cells.

The B6.SLE123 mouse is a triple congenic mouse derived from the NZM4120 mouse and placed on the B6 background[312]. This mouse was created in the lab of Edward Wakeland by the lupus investigator Laurence Morel (a postdoc at the time whose dissertation work related to ant behavior). It was determined that 3 genetic regions-SLE1 risk locus from Chromosome 1 (154-197 Mb), the SLE2 risk locus from Chromosome 4 (50-129 Mb), and the SLE3 risk locus from Chromosome 7 (50.7-124 Mb)-were sufficient to induce fatal nephritis in 100% of B6 congenic mice. These mice develop anti-chromatin and dsDNA autoantibodies that deposit in the nephrons of the kidney causing complement activation and damage, similar to humans. The relative contribution of each genetic locus has been dissected and is outlined in **Table 5.1**[312–331]. As can be appreciated from the table, there is a large contribution of each genetic locus to the interactions of B and T lymphocytes in disease.

Interestingly, anti-CD45RB has been demonstrated to also rely on B and T lymphocyte interactions during tolerance induction[88]. Moore et al. demonstrated, in a model of mouse cardiac transplant, that B lymphocytes are required for graft acceptance. These studies demonstrated that anti-CD45RB impacted B lymphocyte antigen presenting function through modulation of ICAM, and depended on CD40, CD80 and CD86, but not antibody secretion. At the time, failed tolerance was hypothesized to relate to an inability to produce graft-protective Tregs in the absence of B lymphocytes. We, as well as others, have demonstrated that B lymphocytes are not required for Treg expansion by anti-CD45RB, and expansion is actually hindered by B lymphocytes in states of autoimmunity[79,332]. These data do not eliminate the role of B lymphocytes in induction of Tregs but point to additional roles for B-T lymphocyte interactions during tolerance induction.

One potential mechanism would be the ability of anti-CD45RB treated B lymphocytes to acquire and concentrate antigen and present it in a tolerogenic context to effector T lymphocytes. B lymphocytes have been shown to present antigen and induce T lymphocyte anergy in some contexts[60]. Additionally, others have demonstrated that anti-CD45RB induces anergy in effector T lymphocytes. Regardless of the mechanistic underpinnings, success relies on the ability of T lymphocytes to be regulated by a tolerizing signaling.

In this chapter I demonstrate that CD4 T lymphocytes from B6.SLE123 mice (henceforth referred to as SLE123 mice) resist anti-CD45RB induced anergy as scored by proliferation. Additionally, anti-CD45RB drives expansion of T follicular helper (Tfh) and Germinal Center B lymphocytes in SLE123 mice, suggesting inappropriate signaling responses to anti-CD45RB. RNA analysis of anti-CD45RB treated B6 CD4 T lymphocytes revealed that metabolism is a major target of anti-CD45RB. Metabolic analysis of anti-CD45RB treated CD4 T lymphocytes revealed a reduction in glucose uptake and mitochondrial function in B6 mice, which were resisted by SLE123 CD4 T lymphocytes. Signaling analysis reveals that abnormal CREB/ATF-1 signaling and not enhanced AKT/mTOR signaling may be responsible for metabolic abnormalities in

SLE123 CD4 T lymphocytes. Unable to directly target this pathway, I targeted downstream metabolic processes of glucose uptake and mitochondrial respiration with 2-deoxy glucose and metformin, combined with anti-CD45RB. A short 2 week course of this triple therapy improved tolerance to grafted islets and prevented SLE in ~50% of SLE123 mice 6 months after the initial intervention. Overall these studies suggest abnormal metabolism mediated by the CREB/ATF-1 pathway is an immunologic barrier to tolerance induction in SLE123 mice that can be overcome by metabolic conditioning.

Congenic Strain	Subcongenic Strains	Chr	Congenic Interval	Immunologic and Cellular Phenotype
B6.SLE1		1	154-197 Mb	anti-H2A/B DNA + anti-Histone IgG CD4Teff resistance to CD4Treg suppression ↑ CD69+/CD40L+ CD4 cell number ↓CD4Treg number with ↓Foxp3 expression ↑DC derived IL-6 ↑CD4 proliferation ↑CD4 IL-2, Il-4, and IFNg production Failed FcgRIIb upregulation in GC B cells ↑Ly-6A/E lymphocyte expression Autoimmune phenotype dependent on Estrogen Receptor alpha
	B6.SLE1a.1 B6.SLE1a.2	1	170 Mb 171-173 Mb	Same phenotype as B6.SLE1 mice ↑CD4 ICOS expression from SLE1a.1 locus ↑CD4 expression of PBX-1d splice isoform -> ↓T cell apoptosis
	B6.SLE1b	1	173-174 Mb	Restored CD4 Treg and Teff # and function to B6 mice Varied SLAM/CD2 structure and expression in T/B cells Ly108.1 allele impairs B cell anergy, receptor editing, deletion Increased spontaneous GC formation and Tfh cell number
	B6.SLE1c.1 B6.SLE1c.2	1	194-197 Mb 190-193 Mb	Same phenotype as B6.SLE1 mice + Novel CR2 glycosylation-> altered C3d binding altered GC formation and ↓humoral response B6.SLE1c.2: ↓Esrrg expression in T cells -> ↓mitochondrial mass/function and altered metabolic pathway utilization
	B6.SLE1d	1	175-188 Mb	Contribution to lupus pathology currently unknown
B6.SLE2		4	50-129 Mb	↓mature B Cells in Bone Marrow early expansion of splenic CD23lo B cells ↑polyclonal/polyreactive IgM ↑B1a cells with ↑CD80/86, CD24, ICAM-1
	B6.SLE2a	4	50-80 Mb	strongest contribution to ↑IgM^{lo}CD5+ B1a cell #
	B6.SLE2b	4	91-100 Mb	↓IFNa production
	B6.SLE2c	4	104-117 Mb	strongest contribution to total ↑B1a cell # decreased lymphocyte response to G-CSF
B6.SLE3.1		7	50.7-124 Mb	↑CD4:CD8 cell ratio ↑activated CD69+CD4 cells ↑I-A2 and CD44 B cell expression ↑T-dependent antibody response ↓CD4 cell proliferation with ↓apoptosis mild penetrant glomerulonephritis When combined with SLE1 -> severe glomerulonephritis IRAK1 dependent dendritic cell hyperactivity Failed renal ↑of Kallikrein during anti-GBM ab ligation
	B6.SLE3.2	7	53.5-124 Mb	Same as B6.SLE3.1 mice + When combined with SLE1 -> severe glomerulonephritis
B6.SLE5		7	4-20 Mb	No observed autoimmune phenotype When combined with SLE1 -> severe glomerulonephritis

Table 5.1 Phenotypes associated with each genetic portion introduced on the B6 background to make the B6.SLE123 mouse.

SLE123 mice resist anti-CD45RB anergy induction and demonstrate expanded effector subsets after treatment

Anti-CD45RB has been demonstrated to drive signaling that promotes Treg function while simultaneously inhibiting the effector function of T lymphocytes[333]. Studies have demonstrated that anti-CD45RB induces a nonresponsive or anergic state in effector T lymphocytes from treated mice[333]. Based on previous data of effector resistance in SLE123 mice, I hypothesized CD4 T cells in SLE123 mice would resist anergy induction by anti-CD45RB. To test this hypothesis I treated B6 and SLE123 with anti-CD45RB for a standard 7 day course. I utilized mice between 6-12 weeks of age, long before kidney pathology and T lymphocyte abnormalities emerge. At the end of this treatment regime, cells were isolated from the spleens of treated and untreated mice and stained with a proliferation dye, which is diluted upon each subsequent division. Total splenocytes were then plated and stimulated with soluble anti-CD3 and anti-CD28 (1ug/ml of each). In agreement with published data, anti-CD45RB B6 CD4 T lymphocytes demonstrated refractory responses to TCR stimulation as measured by proliferation (**Figure 5.2A left two panels and B**). CD4 T lymphocytes from SLE123 mice proliferated regardless of anti-CD45RB treatment (**Figure 5.2A right two panels and B)**.

Further analysis revealed that anti-CD45RB treated SLE123 demonstrated expansion of Tregs($CD4^+$, $CD25^+$, $Foxp3^+$), as previously reported (**Figure 5.2C and F).** Additionally, post anti-CD45RB SLE123 mice experienced expansion of Tfh ($BCL\text{-}6^+$, $PD\text{-}1^+$), GC B lymphocytes (IgMlo, BCL-6+) (**Figure5.2D, E, G, an H**) and enhanced $CD44^+$ T lymphocytes indicating an abnormal response to anti-CD45RB (**not shown**). B6 and SLE123 both responded to anti-CD45RB by downregulating CD62L, a phenomenon well documented in anti-CD45RB

treatment, making it difficult to identify effector and central memory subsets by standard subsetting strategies (**Figure 5.2I and J)**[334].

Metabolism is a major target of anti-CD45RB in CD4 T lymphocytes from B6 mice

To understand how SLE123 CD4 T lymphocytes resist anergy induction by anti-CD45RB, I determined the gene pathways targeted by this therapy. I utilized RNA-sequencing from purified $CD4^+$ T lymphocytes from B6 and SLE123 untreated and anti-CD45RB treated mice. B6 mice demonstrated a robust regulation of gene transcripts in response to anti-CD45RB while SLE123 mice did not (**Figure 5.3A**). Genes from B6 mice were then classified into downregulated and upregulated gene categories. (methods indicate p-value and fold change cutoffs for regulated genes). These gene-lists were then separately subjected to Kyoto Encyclopedia of Gene and Genomes (KEGG) enrichment analysis to identify pathways and processes enriched among the genes listed (**Figure 5.3B**). Surprisingly, metabolic pathways made up a large portion of the genes downregulated by anti-CD45RB (**Figure 5.3B)**.

Further Analysis of the genes in this Analysis of T lymphocyte and B lymphocytes in B6 and SLE123 mice pathway revealed modulation of pathways associated with glucose metabolism, including the pentose phosphate pathway and synthesis of purine and pyrimidine bases, a process important for T lymphocyte function and proliferation (**Figure 5.3C upper panel**). I also noted a downregulation of N-glycan biosynthesis. The meaning of this regulation is not immediately apparent. One possibility is it may be in response to the direct action of anti-CD45RB binding a neuraminidase sensitive glycosylated motif on CD45RB, potentially as a compensatory mechanism to reduce glycosylation of newly synthesized CD45RB (**Figure 5.3C upper panel**).

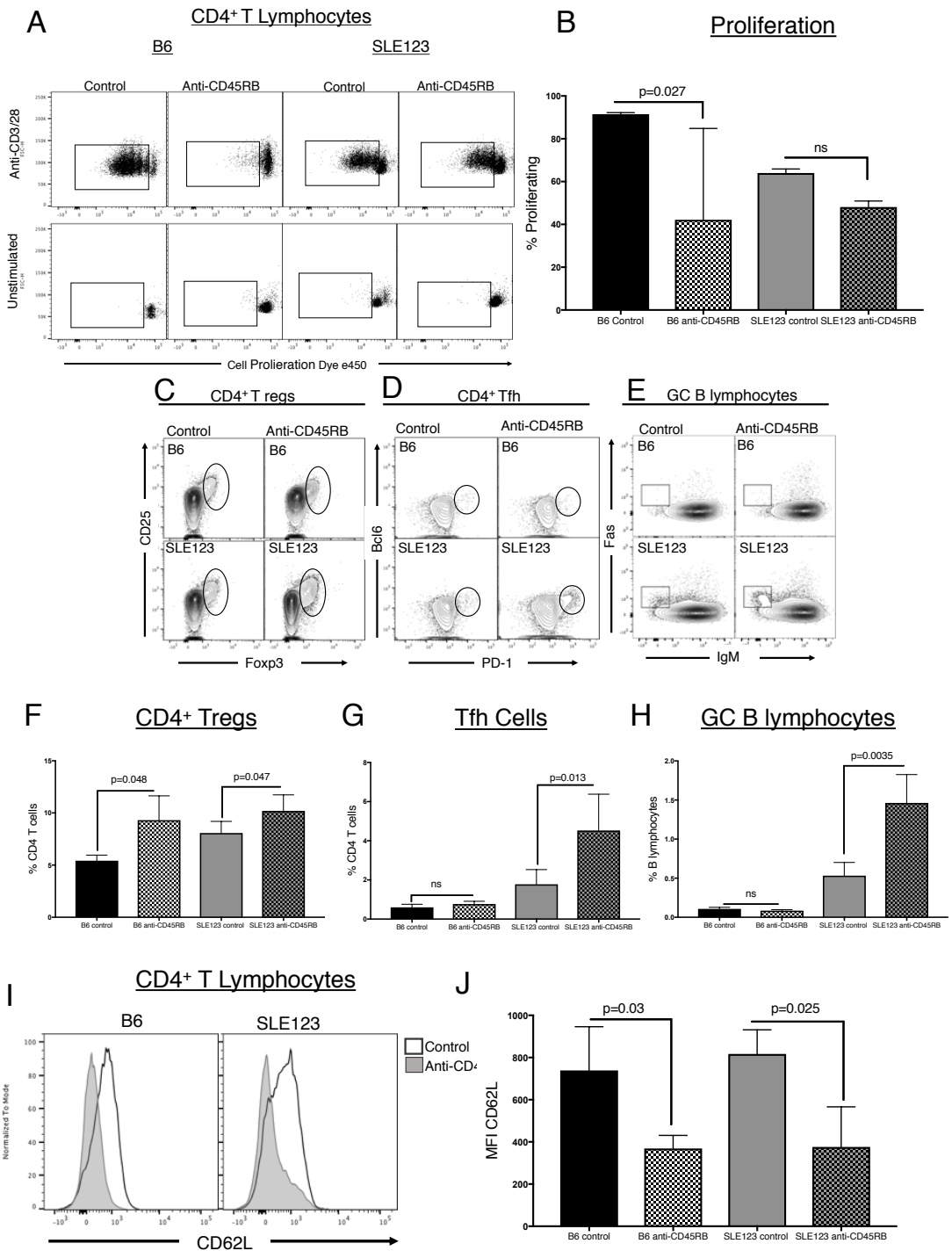
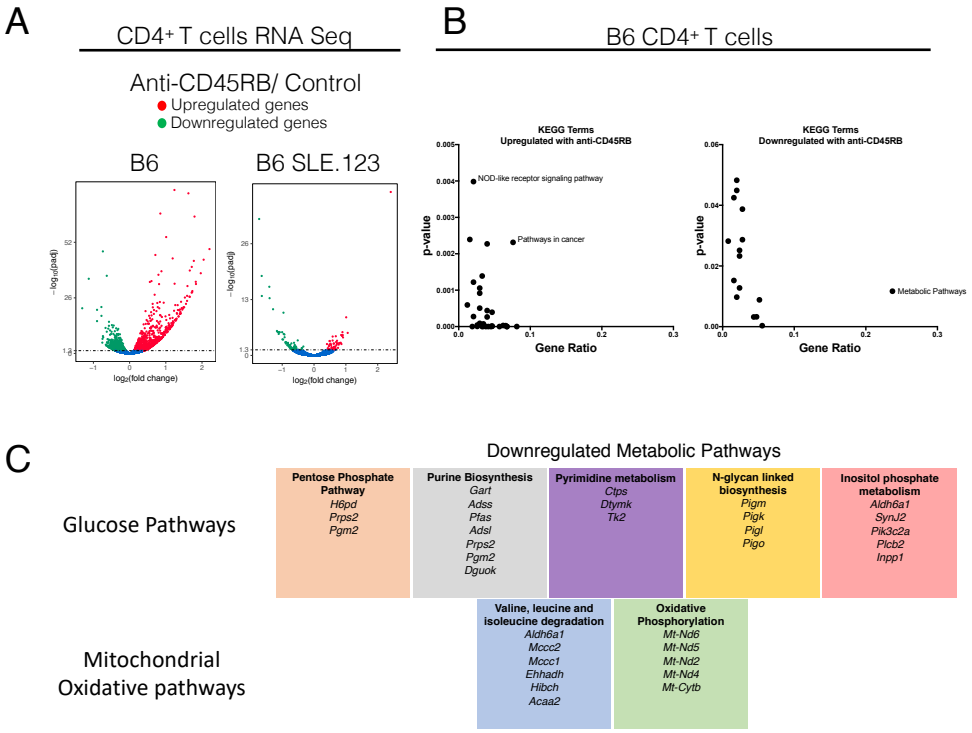


Figure 5.2 Anti-CD45RB induces CD4 T lymphocyte unresponsiveness in B6 but activates SLE123 CD4 T lymphocytes. A) Proliferation analysis of anti-CD3/CD28 stimulated CD4 T lymphocytes isolated from anti-CD45RB treated and untreated B6 and SLE123 mice revealed that anti-CD45RB inhibits proliferation in B6 mice, but SLE123 mice resist this inhibition. This is quantified in B). C) Treg ($CD25^{+}Foxp3^{+}$) expansion was similar between B6 and SLE123 anti-CD45RB treated mice. D) Anti-CD45RB enhanced expansion of Tfh ($Bcl6^{Hi}$, $PD\text{-}1^{Hi}$) and D) GC B lymphocytes ($B220^{+}IgM^{-}Fas^{+}$) in SLE123. F-H) Demonstrates the quantification of CD4 Tregs, Tfh cells, and GC B lymphocytes. I) Anti-CD45RB mediates similar downregulation of CD62L in CD4 T lymphocytes from B6 and SLE123 mice after anti-CD45RB. This is quantified in J.

I also noted a downregulation of inositol phosphate metabolism, a pathway important in modulating AKT and mTOR signaling (**Figure 5.3C upper panel**). Mitochondrial localized metabolic processes were also impacted, including degradation of valine, leucine and isoleucine (**Figure 5.3C lower panel**). Leucine has been demonstrated to contribute to mTOR signaling and its breakdown leads to energy production via the Krebs cycle and ketone bodies. I also identified downregulation of components of the electron transport chain, including Complex I and III (**Figure 5.3C lower panel**). Metabolomic analysis of anti-CD45RB treated CD4 T lymphocytes revealed a loss of precursor nucleosides, guanosine and inosine in treated cells and an accumulation of 6,8-Dihydroxypurine (a marker of DNA damage due to lack of purine and pyrimidine bases needed for DNA repair)[334]. This finding was in agreement with the RNA-seq data that suggest downregulation of purine and pyrimidine biosynthesis (**Figure 5.3D**). Overall these data suggested that anti-CD45RB targets multiple metabolic processes in T lymphocytes that involve glucose metabolism and the mitochondrion.



D

Untargeted Mass Spec Metabolites

B6 CD4+ T lymphocytes

Upregulated metabolites

Metabolite	P-value	FC
4-Methylhistamine	0	16.00
6,8-Dihydroxypurine	0	16.00
6,8-Dihydroxypurine	0	16.00
docusate	0	16.00
Glyodin	0	16.00
Nitrilacarb	0	16.00
GalCer(d18:0/16:0)	3.81E-04	2.60
GlcCer(d18:2/22:0)	0.00429552	2.54
Galbeta-Cer(d18:1/22:0)	0.03171751	2.38
PGH2-EA	2.73E-04	2.33
PS(P-20:0/19:1(9Z))	7.77E-05	2.30
PE(24:0/18:2(9Z,12Z))	1.85E-05	2.26
CerP(d18:1/24:0) Esi-1.3639991	0.00750449	2.23
Galbeta-Cer(d18:1/16:0)	3.81E-04	2.22
PS(20:3(8Z,11Z,14Z)/18:0)	5.72E-04	2.21
O6-Methyl-2'-deoxyguanosine	2.73E-04	2.19
O6-Methyl-2'-deoxyguanosine	0.02108133	2.17
PE(O-20:0/22:0)	4.50E-05	2.13
Galbeta-Cer(d18:1/16:0)	1.07E-04	2.11
PS(O-16:0/20:0)	1.85E-05	2.04
PS(O-20:0/19:1(9Z))	2.30E-05	2.03
PE(24:0/18:1(9Z))	0.00864221	2.01
PS(O-18:0/21:0) Esi-1.3450006	0.03045165	2.00

Downregulated metabolites

Metabolite	P-value	FC
Inosine	0	-16.00
Guanosine	0	-16.00
1-Naphthalenesulfonic acid	1.85E-05	-2.89
Chryso-obtusin glucoside	3.85E-04	-2.73
Cimicifugoside	0.02636899	-2.36
4-Hydrocinnamoyl-2,2,5-trimethyl-4-cyclopentene-1,3-dione	0.01719775	-2.15
Guanosine	0	-2.12
Fexofenadine	0.00633477	-2.06
Fexofenadine	0.00633	-2.04

Figure 5.3 Bioinformatic analysis reveals anti-CD45RB targets CD4 T lymphocyte metabolism. A) RNA was isolated and sequenced from anti-CD45RB treated B6 and SLE123 purified CD4 T lymphocytes. Genes that were downregulated with anti-CD45RB are indicated by green dots while those upregulated are indicated by red dots on the volcano plots. As can be appreciated B6 CD4 T lymphocytes experienced robust transcriptional regulation while SLE123 did not. B) KEGG pathway analysis of upregulated and downregulated genes from the RNA-seq data revealed that metabolism was the most robustly downregulated process in CD4 T lymphocytes in B6 mice. C) Analysis of genes derived from the KEGG-derived metabolic gene list revealed genes involving glucose metabolism and mitochondrial metabolism. Of not purine and pyrimidine biosynthesis pathways were downregulation D) Untargeted mass spec from purified CD4 T lymphocytes revealed upregulation of 6,8-Dihydroxypurine, in agreement with a loss of nucleotides for DNA repair by downregulation of purine and pyrimidine biosynthesis.

Anti-CD45RB decreases glucose uptake and alters mitochondrial function in B6 but not SLE123 mice

Having identified that anti-CD45RB targeted CD4 T cell metabolic pathways in B6 mice, I determined whether this downregulated glucose uptake and impaired mitochondrial function. I first utilized Seahorse Metabolic Analyzer to interrogate the glycolytic and respiratory rate of purified $CD4^+$ T lymphocytes from treated and untreated B6 and SLE123 mice. Briefly, this instrument measures the glycolytic rate of isolated cells by monitoring changes in pH of the media in response to glucose (extracellular acidification rate or ECAR, a surrogate readout for lactic acid production, a byproduct of glycolysis). The analyzer also measures the partial pressure of oxygen in the media to gauge the oxygen consumption rate or OCR of the cell (a more detailed explanation can be found in the Methods). Analysis of CD4 T lymphocytes from B6 and SLE123 mice left untreated or treated with anti-CD45RB revealed little differences in ECAR or OCR between these groups (**Figure 5.4A**). This can be partially explained by the relatively low metabolic activity of naïve T lymphocytes, which a majority of these assayed cells were[334].

Even though I measured no change in glycolysis or respiration, due to the low metabolic demand of assayed cells, I hypothesized that anti-CD45RB may still alter glucose uptake and mitochondrial activity. I utilized 2-NBDG (a fluorescent but metabolically inert analog of glucose) to measure CD4 T lymphocyte uptake in response to anti-CD45RB in B6 and SLE123 mice by flow cytometry. Analysis revealed that anti-CD45RB treated CD4 T lymphocytes from B6 mice had reduced glucose uptake in comparison to controls. SLE123 CD4 T lymphocytes experienced no reduction in glucose uptake in response to therapy (**Figure 5.4B and C**). Analysis of Glut-1 expression via flow cytomtery revealed no downregulation of Glut-1, the

primary glucose transporter in T lymphocytes (the antibody utilized detects an intracellular epitope of Glut-1 and thus does not reflect the extracellular expression of this protein) (**Figure 5.4D**). The apparent discrepancy between glycolytic rate and glucose uptake can be partially explained by glucose utilization in naïve lymphocytes being shunted towards pentose phosphate pathway and oxidative phosphorylation in the mitochondria[334]. These two pathways are negatively impacted by anti-CD45RB thus reducing the need for glucose.

The RNA-seq data suggested that components of the electron transport chain were also impacted by anti-CD45RB, including Complex I (Mt-Nd5,Mt-Nd6, Mt-Nd2, and Mt-Nd4) and Complex III (Mt-Cytb) (**See figure 5.3C**). I was unable to directly assess the protein expression levels of Complex I via flow or western blot, as the available antibodies detect nuclear encoded subunits of Complex I that assemble even in the absence of the mitochondrially-encoded genes. I, therefore, chose to assess other parameters of mitochondrial function to determine the impact of anti-CD45RB on T lymphocytes.

Studies that utilized metformin to inhibit Complex I demonstrate that these mitochondria favor respiration uncoupled from ATP production (a potential explanation for maintained OCR), and demonstrate inefficient utilization of glucose via t[334]he citric acid cycle (offering an explanation for reduced glucose uptake). This change leads to alterations in the mitochondrial membrane potential ($\Delta\psi$), a measure of the negative charges in the inner membrane modulated by active shuttling of H^+ ions out of the mitochondrion. During uncoupled respiration the ETC continues pumping H^+ out of the mitochondrion in the absence of H^+ influx and ATP production via ATP-synthase, leading to a hyperpolarized inner membrane[334]. I utilized dye MitoTrackerCMXRos, that accumulates in the inner-membrane of the mitochondria in response to increased membrane potential to assess the membrane potential of CD4 T lymphocytes from

B6 and SLE123 treated and control mice. I noted that B6 anti-CD45RB treated CD4 T lymphocytes possessed heightened mitochondrial membrane potential (hyperpolarized) compared to controls (**Figure 5.4E and F)**. SLE123 T lymphocytes actually presented with decreased mito-membrane potential in response to anti-CD45RB indicating an abnormal response (**Figure 5.4E and F)**. Mitochondrial membrane hyperpolarization in T lymphocytes has been linked to enhanced apoptosis but was not directly assessed here[334].

In times of nutrient deficiency and mitochondrial dysfunction, mitochondrial maintenance through mitophagy is reduced leading to large fused mitochondria[334]. To assess this with anti-CD45RB treatment, I analyzed mitochondrial mass via MitoTracker Green, which labels mitochondria regardless of the membrane potential, via flow cytometry. Utilizing this dye I uncovered an increase in mitochondrial size in B6 CD4 T lymphocytes treated with anti-CD45RB, but no such change in SLE123 treated mice (**Figure 5.4G and H)**. Further studies to determine whether this increase in mitochondrial mass represents large, fused mitochondria or multiple hypofunctional mitochondria will need to be carried out. Overall these data demonstrate anti-CD45RB targets both glucose and mitochondrial metabolism in B6 mice, a process that is resisted by SLE123 CD4 T lymphocytes.

Anti-CD45RB does not depend on the purinergic signaling network to modulate CD4$^+$ T lymphocyte metabolism

In light of the surprising finding that anti-CD45RB robustly targeted CD4 T lymphocyte metabolism in B6 but not SLE123 mice, I attempted to dissect the responsible signaling pathways. My bioinformatic analysis revealed a strong modulation of the purinergic pathway in CD4 T lymphocytes from B6 anti-CD45RB treated mice, including the modulation of purine,

pyrimidine, and ATP metabolism (**See figure 5.3C and D**). These pathways all involve the nucleoside

adenosine. I, however, did not detect increased adenosine, as endonucleases and ectonucleases can quickly degrade these molecules. Nevertheless adenosine, has been demonstrated to possess potent immunosuppressive capacity in CD4 T lymphocytes. In lymphocytes, catabolism of ATP by CD39 and CD73 (lymphocyte expressed ectonucleases) leads to creation of adenosine. This adenosine binds to G-protein coupled receptor A2AR and mediates inhibition of T lymphocytes (**illustrated in Figure 5.5A**)[335,336]. Furthermore, this system has been demonstrated to be abnormal in SLE123, implicating its function in anti-CD45RB mediated mechanisms[337]. To determine whether this system played a role in anti-CD45RB modulation of metabolism, I treated B6 mice with anti-CD73 (TY/23 a monoclonal antibody that inhibits the catabolism of AMP to adenosine) and an A2AR antagonist (SCH 58261) with doses demonstrated to provide robust inhibition, in combination with anti-CD45RB (**Figure 5.5B**)[338,339]. After a standard treatment course of anti-CD45RB +/- anti-CD73 and A2AR inhibitor, I measured metabolism in $CD4^+$ T lymphocytes. I determined that inhibition of this pathway did not alter the ability of anti-CD45RB to modulate metabolism in $CD4^+$ T lymphocytes through any parameter assessed (**Figure 5.5C, D, and E**). I did note that anti-CD45RB increased the expression of A2AR in multiple lymphocyte subsets, implicating it in other non-metabolic regulatory mechanisms not investigated here (**not shown**). Overall the purinergic modulation observed in anti-CD45RB may not necessarily contribute to the metabolic abnormalities by a signaling process, but may be a result of inadequate purine biosynthesis due to abnormal glucose and mitochondrial metabolic processes.

SLE123 mice do not possess enhanced AKT/mTOR signaling at the time of anti-CD45RB treatment nor is this pathway inhibited by anti-CD45RB

Anti-CD45RB targets the RB isoform of CD45, a membrane phosphatase whose role in immune signaling has been extensively studied by Arthur Weiss and others[228,340]. CD45 is a ubiquitously-expressed tyrosine phosphatase whose genetic manipulation leads to robust changes in the tyrosine signaling network in immune cells. Signaling analysis of B6 $CD4^+$ T lymphocytes after treatment with anti-CD45RB did not demonstrate overt defects in their ability to signal as assessed by phosphorylation of key signaling molecules or calcium flux nor did anti-CD45RB independently induce phosphorylation of any signaling molecules assessed (**not shown**). Similarly, other studies with anti-CD45RB have failed to define robust signaling events induced by the therapy, instead focusing on the cellular and intercellular mechanistic underpinnings of this therapy. One study, which utilized the human form of this antibody, indicated a slight inhibition of the AKT/mTOR pathway[340]. In agreement, our RNA-seq analysis revealed downregulation of inositol phosphate metabolism, which is important for AKT signaling. An important study in SLE123 carried out by the Morel Lab indicated that CD4 T lymphocytes possessed an upregulated mTOR signaling axis that could contribute to enhanced CD4 metabolism as the mice age, and possibly contribute to resistance to anti-CD45RB[341].

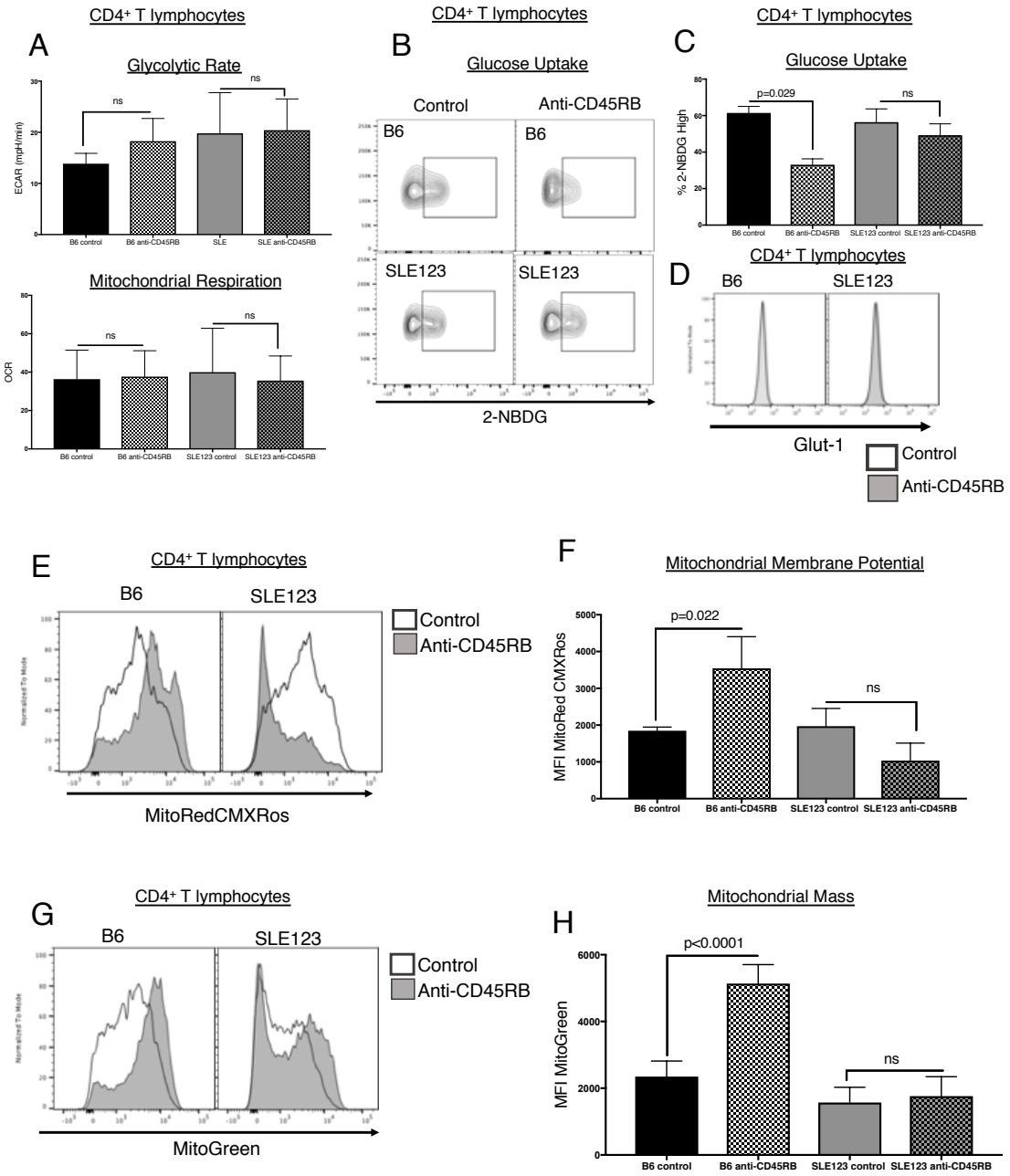


Figure 5.4 Cellular analysis revealed downregulation of glucose uptake and altered mitochondrial function only in B6 CD4 T lymphocytes from anti-CD45RB treated mice. A) Seahorse metabolic analysis of purified CD4 T lymphocytes revealed no major changes in either glycolytic rate or mitochondrial function as scored by oxygen consumption after anti-CD45RB. B) Measurement of glucose uptake, by 2-NBDG, before and after anti-CD45RB in B6 and SLE123 mice demonstrated a reduction in glucose uptake only in B6 CD4 T lymphocytes after anti-CD45RB. SLE123 took up the same amount of glucose regardless of treatment. Quantified in C. D) Analysis of glucose receptor Glut-1 revealed no changes in Glut-1 expression that could explain reduced glucose uptake in B6 mice or enhanced glucose uptake in SLE123 mice. E) Measurement of mitochondrial membrane potential (by MitoRedCMXROS) after anti-CD45RB in CD4 T lymphocytes revealed that B6 mitochondria became hyperpolarized, while SLE123 mice demonstrated reduced polarity. Quantified in F. G) Additionally, CD4 T lymphocytes in B6 anti-CD45RB treated mice demonstrated enhanced mitochondrial mass. This was again resisted by SLE123 CD4 T lymphocytes. Quantified in H.

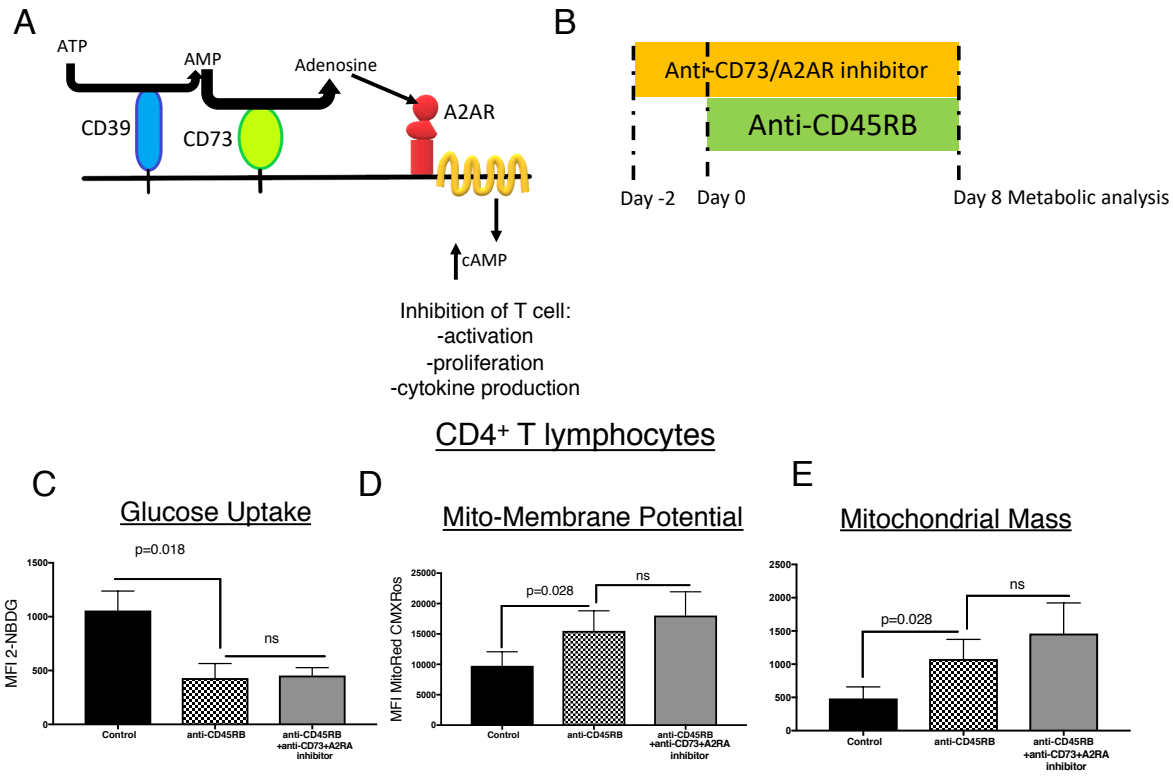


Figure 5.5 Purinergic signaling does not account for the metabolic phenotype of anti-CD45RB treated CD4 T lymphocytes. A) Cartoon illustrating the capacity for CD39 to catabolize ATP to AMP, which is converted to adenosine by CD73, and finally recognized by G-couple protein receptor A2AR. This binding leads to T lymphocyte inhibition. B) To test the role of adenosine signaling in anti-CD45RB modulation of metabolism I treated mice with CD73 blocking antibody and A2AR inhibitor in conjunction with anti-CD45RB according to the outline dosing schedule. C-E) Inhibition of the CD39/CD73/A2AR system did not impact any metabolic parameter that was modulated by anti-CD45RB.

I assessed the phosphorylation patterns of AKT and mTOR in SLE123 and B6 mice post TCR stimulation via phosphoflow cytometry (full methods described in Methods section). Analysis of the signaling kinetics in SLE123 and B6 CD4 T lymphocytes revealed no difference in mean fluorescence intensity (MFI) or fold-change over baseline between these strains in AKT or mTORC1 signaling (**Figure 5.6A, C, and D**). Furthermore, I noted no reduction in phosphorylation of mTOR or AKT at baseline or in responseto TCR stimulation after anti-CD45RB treatment in B6 or SLE123 mice (**Figure 5.6 C and D**). These data indicate that AKT and mTOR signaling are not major targets of anti-CD45RB therapy.

Anti-CD45RB inhibits CD4 T lymphocyte metabolism via a Cyclosporine A dependent but NFAT independent pathway

While neither the purinergic signaling nor the AKT/mTOR signaling system seemed to be targeted to impact the metabolism of CD4 T lymphocytes, I next investigated established pathways that induce T lymphocyte anergy. Studies with anti-CD45RB demonstrate that treatment of mice with Cyclosporine A during anti-CD45RB treatment prevents tolerance to cardiac allografts (a problematic finding as cyclosporine has been used clinically to inhibit organ rejection in transplant and autoimmunity)[342]. Complete mechanistic understanding of this phenomenon was never completed in these original studies. Cyclosporine A targets phosphatase Calcineurin A whose most noteworthy function in T lymphocytes is to mediate the dephosphorylation and nuclear localization of Nuclear Factor of Activated T cells (NFAT1c)[342] . In T lymphocyte anergy, import of NFAT1c, in the absence of other signals, is required[342]. I first tested whether anti-CD45RB induced translocation of NFAT1c to the nucleus in B6 mice. I stimulated purified CD4 T lymphocytes with anti-CD45RB (20ug/ml) or isotype control over a

time course. I then utilized a protocol described in detail in the methods to isolate whole, fixed nuclei from purified CD4 T cells. I was then able to assess the nuclear content of NFAT1c in the absence of cytoplasmic NFAT1c via flow cytometry. Utilizing this technique I noted a modest entry of NFAT1c into the nucleus of treated cells (**Figure 5.7 A and B**). Due to nuclear localization of NFAT1c, I hypothesized that treatment of B6 mice with anti-CD45RB in conjunction with Cyclosporine A would prevent the metabolic inhibition induced by anti-CD45RB. As in the original study, mice were treated with 20mg/kg of Cyclosporine A via i.p. injection on day-2 before starting anti-CD45RB and then treated every day during the standard 7-day course (**Figure 5.7A**). I assessed both glucose uptake and mitochondrial membrane potential.

Anti-CD45RB targets ATF-1/CREB signaling via modulation of CREB regulated transcriptional coactivators (CRTCs)

Having determined that cyclosporine inhibits the metabolic action of anti-CD45RB, in a manner independent of NFAT, I sought to define a regulator of these changes by revisiting the metabolic genes defined by the RNA-seq analysis. I took this metabolic gene list and subjected it to TRANSFAC analysis (via the Duke GATHER database), comparison against a curated database of transcription factors and their experimentally defined genomic binding sites and DNA-binding profiles[343]. This tool allowed for the analysis of this 60 gene list to determine whether regulation of this genes set was enriched for any transcription factor. This analysis gives both a p-value and a Bayes Factor. The Bayes Factor is a ratio of the relatedness of identified transcription factor

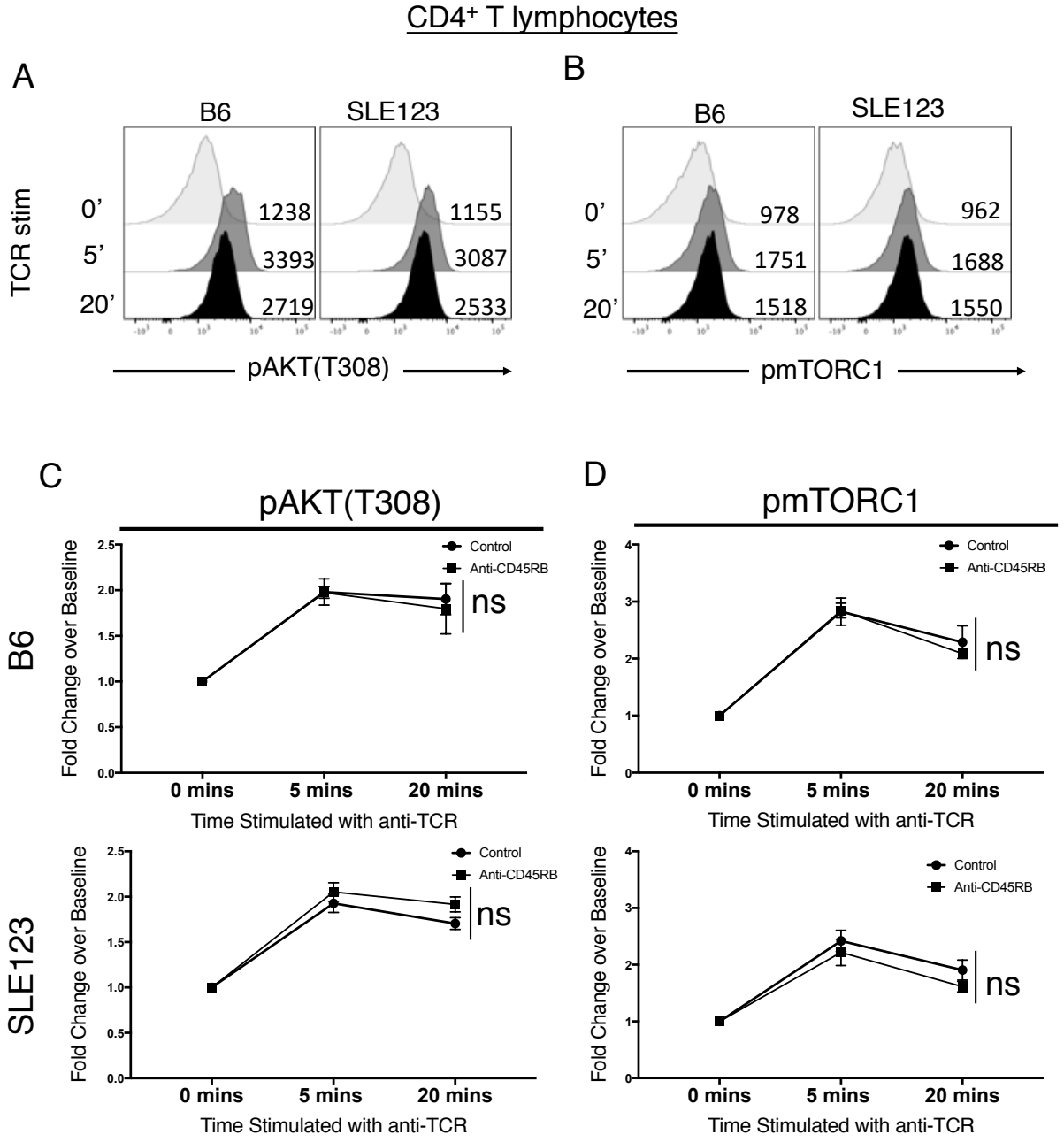


Figure 5.6 AKT/mTOR signaling is not enhanced in SLE123 mice at the age assessed nor is it targeted by anti-CD45RB. A) CD4 T lymphocytes from B6 and SLE123 mice were stimulated with anti-TCR stim (anti-CD3/CD28/CD4) over time. These cells were then analyzed for AKT phosphorylation by phosphoflow cytometry. As indicated by the MFIs there was no difference in AKT signaling in response to TCR stimulation between B6 and SLE123 mice. B) There was also no difference in downstream signaling through mTORC1. C) The capacity for CD4 T lymphocytes to signal through AKT after anti-CD45RB was measured after TCR stim by phosphoflow. There was no difference detected in TCR mediated phosphorylation of AKT after anti-CD45RB in B6 or SLE123 mice. D) There was also no difference in phosphorylation of mTORC1 after anti-CD45RB in B6 or SLE123 mice.

relative to your gene set to the relatedness of the identified transcription factor to the rest of the genome. Thus, a higher Bayes Factor indicates the identified transcription factor is more related to your gene list than other genes in the genome. This analysis revealed that CREB Binding protein and ATF-1 were the most highly associated transcription factors within the list of metabolic genesanalyzed (**Figure 5.8A**). The CREB/ATF-1 family are highly associated with metabolic regulation in many cell types[344]. CREB and ATF-1 share much homology in both structure, regulation, and genomic motifs they bind; thus I chose to assess the regulation of both proteins with anti-CD45RB.

ATF-1 and CREB are regulated via phosphorylation of a serine residue that mediates binding to coregulators (CBP-1 and p300) leading to translocation to the nucleus and promotion of transcription[344]. While neither calcineurin nor CD45 have been demonstrated to directly regulate CREB, I determined whether anti-CD45RB may modulate phosphorylation to modify CREB activity. To assess this possibility, I stimulated CD4 T lymphocytes with anti-CD45RB from B6 and SLE123 mice and assessed CREB/ATF-1 phosphorylation (Ser133) via phosphoflow cytometry, using an antibody reactive to both phosphorylated CREB and ATF-1. I was unable to demonstrate an increase or reduction in CREB/ATF-1 phosphorylation in response to anti-CD45RB (**not shown**). Nevertheless, I sought to determine whether anti-CD45RB reduced the nuclear localization of CREB or ATF-1 to modulate transcription of metabolic genes. I first utilized imaging cytometry to determine the nuclear localization of ATF-1 and CREB before and after anti-CD45RB in CD4 T lymphocytes. To do this I utilized SyTOX Green to stain the nucleus and observe the overlap between nuclear stain and ATF-1 or CREB. I was unable to detect a change in nuclear localization after anti-CD45RB treatment (**Figure 5.8B**). I

then isolated nuclei as described before and determined that nuclear content of ATF-1 and CREB was not altered by anti-CD45RB (**Figure 5.8C**). Mice treated with Cyclosporine A demonstrated resistance to anti-CD45RB induced changes in glucose uptake and mitochondria function (**Figure 5.7 D and E**).

To determine whether NFAT played a direct role in the metabolic modulation by anti-CD45RB, I utilized NFAT inhibitor VIVIT[345]. This peptide inhibitor displays specificity for NFAT by blocking the interaction of NFAT with Calcineurin specifically while not inhibiting the phosphatase activity of Calcineurin A. Mice were treated with 10mg/kg, a concentration consistently demonstrated to provide in vivo inhibition of NFAT, -2 days before anti-CD45RB then every day during the standard treatment course (**Figure 5.7 F**)[344]. This VIVIT peptide did not inhibit the metabolic changes induced by anti-CD45RB in CD4 T lymphocytes from B6 mice (**Figure 5.7G and H)**. These data suggest that calcineurin targets another protein important for metabolic modulation or some off target effect of Cyclosporine A was inhibiting the metabolic modulation by anti-CD45RB.

I was unable to demonstrate that CREB or ATF-1 was modulated en masse, but I still entertained the hypothesis that other factors may impact the genomic interactions of CREB/ATF-measure by the CREB regulated transcriptional coactivators (CRTCs) have been demonstrated to alter the genomic association of both CREB and ATF-1. Interestingly, these CRTCs are regulated by the phosphatase activity of Calcineurin A, making them a prime target for anti-CD45RB. When not active, these CRTCs are sequestered in the cytoplasm in a phosphorylated state, in association with homeostatic factor 14-3-3 sigma, calcineurin then dephosphorylates CRTC and leads to their nuclear localization (**Figure 5.8D**)[344]. I first assessed whether anti-CD45RB led to

the dephosphorylation of these proteins in CD4 T lymphocytes after anti-CD45RB.There are 3 known isoforms of the CRTC with denotations of 1, 2, and 3. Due to reagent availability, I assessed the phosphorylation status of CRTC2 after anti-CD45RB. I noted a decrease in the phosphorylation of CRTC2 after anti-CD45RB in B6 mice, as phospho-CRTC2 low population. Dephosphorylation was inhibited with pretreatment with Cyclosporine A (**Figure 5.8F**).As the result of this dephosphorylation is nuclear localization, I assessed nuclear localization in response to anti-CD45RB utilizing nuclei isolation and flow cytometry. I chose to assess nuclear localization of CRTC2 at the 15 min timepoint (as dephosphorylation peaked at 10 mins and was reduced at 30 mins). I then assessed nuclear localization after anti-CD45RB in B6 and SLE123 CD4 T lymphocytes utilizing isolated nuclei, as described before. I noted that B6 mice experienced robust nuclear localization after anti-CD45RB while SLE123 mice did not. (**Figure 5.8G and H**) I additionally determined that this nuclear localization was inhibited by Cyclosporine A in B6 mice. This suggests CRTCs may be the target of anti-CD45RB leading to alteration of the CREB/ATF-1 transcriptional program.

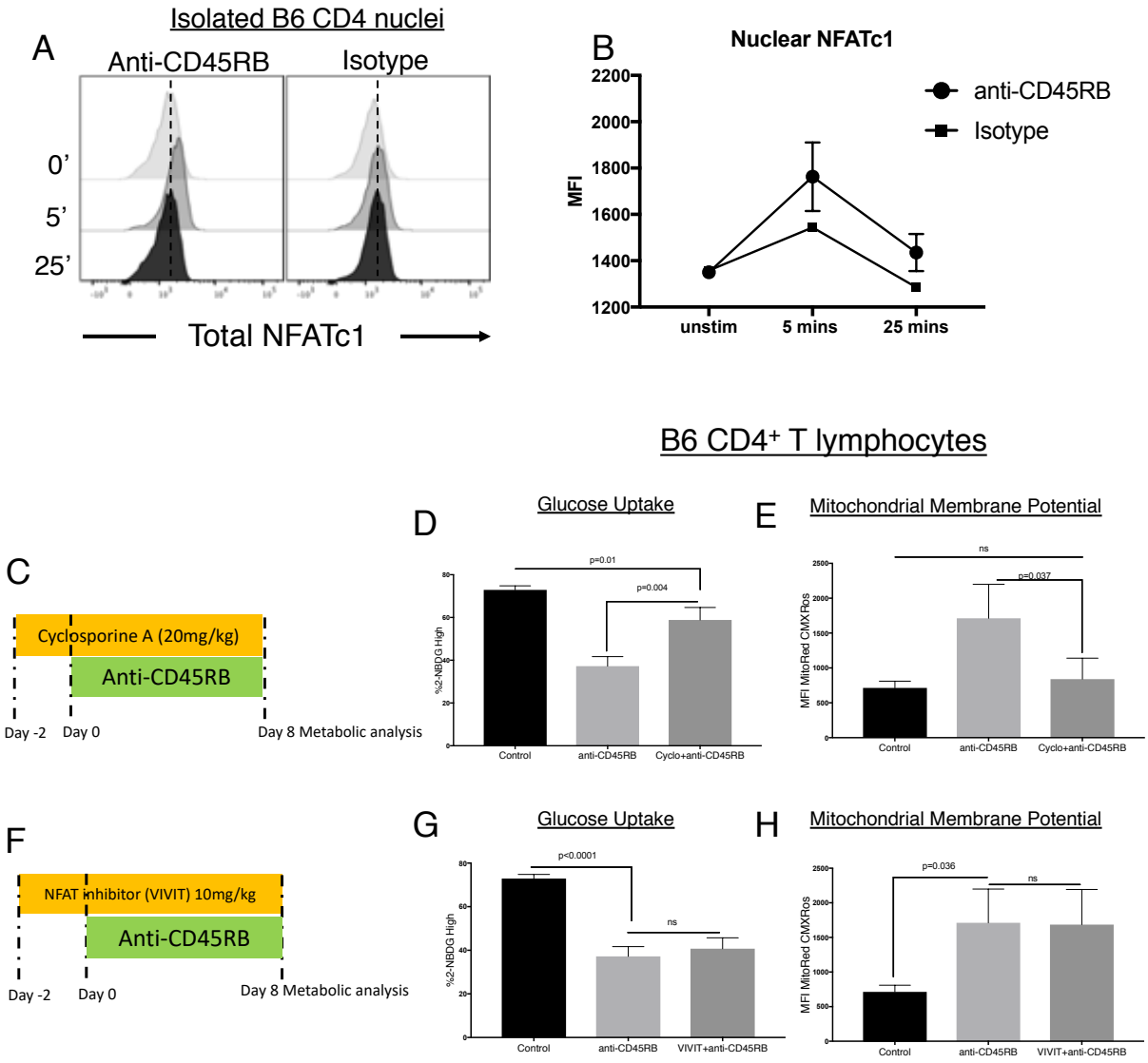


Figure 5.7 Metabolic modulation by anti-CD45RB in CD4 T lymphocytes depends on Calcineurin A but not NFAT. A) Flow cytometry measurement of the NFAT content of CD4 T lymphocytes after anti-CD45RB revealed a modest increase in nuclear NFATc1 in B6 mice. This is quantified in B. C) Treatment strategy to target Calcineurin A via cyclosporine A to inhibit NFATc1 nuclear import by anti-CD45RB. D-E) Pretreatment with cyclosporine A inhibited the metabolic changes induced by anti-CD45RB in CD4 T lymphocytes in B6 mice. F) Treatment strategy to inhibit NFATc1 nuclear import specifically in B6 mice. G-H) NFATc1 inhibitor VIVIT did not prevent metabolic modulation by anti-CD45RB, indicating NFATc1 was not important for metabolic modulation by anti-CD45RB.

It was unclear whether anti-CD45RB induced an altered transcriptional profile in CREB signaling or inhibited its interaction of CREB with certain genes through activation of CRTCs to alter metabolism. I hypothesized that perhaps inhibiting CREB transcription directly may impact the metabolic changes in CD4 T lymphocytes treated with anti-CD45RB. To test this I treated mice with 10mg/kg of CREB inhibitor 666-15 at day -2 and every day during a 7-day course of anti-CD45RB. I noted no changes in the metabolic response of CREB inhibited/anti-CD45RB treated CD4 T lymphocytes (**not shown**). The explanation could be many-fold for this finding, including the inhibitor not restraining ATF-1 transcriptional activity, CREB/ATF-1 transcriptional activity not being required for metabolic suppression by anti-CD45RB, or the inability of 666-15 to inhibit interactions between CRTCs and CREB/ATF-1. Inhibitor 666-15 mediates its inhibitory effect by blocking binding of CBP-1 to CREB and preventing it's translocation to the nucleus. Studies have demonstrated that CRTCs can compensate for the function of CBP-1 in transcriptional activation of CREB leaving open the possibility the inhibitor was unable to inhibit CRTC-CREB/ATF-1 interactions[344].

Short course inhibition of CD4 T lymphocyte metabolism in combination with anti-CD45RB restores deranged metabolism and eliminates deleterious cell subsets

Having defined abnormal CRTC activity in SLE123 mice, it was unclear how to target this pathway to restore tolerance, as no *in vivo* therapeutic modulators of CRTCs exist. I instead opted to target the deranged glucose and mitochondria metabolic processes utilizing 2-deoxyglucose (a non-hydrolyzable glucose analog) and metformin (an inhibitor of Complex I of the ETC), respectively. This strategy has been utilized as a chronic therapy in SLE123 mice to prevent disease. I hypothesized that inhibiting metabolism during anti-CD45RB therapy would

make CD4 T lymphocytes amenable to B and Treg tolerance mechanisms, thus resetting autoimmunity long term with a short course of therapy. I treated mice with 2-DG and metformin in their drinking water for 1 week before beginning anti-CD45Rb for 7-days while continuing 2-DG/met therapy (**Figure 5.9A**) SLE123 mice treated with the triple therapy demonstrated inhibited metabolism similar to B6 mice (**Figure 5.9B and C**). Furthermore, I noticed elimination of Tfh and GC B lymphocytes with the triple therapy as compared to anti-CD45RB alone (**Figure 5.9D**).

Metabolic inhibition combined with anti-CD45RB improves allograft tolerance and prevents disease

I hypothesized that this triple therapy would restore faulty tolerance mechanisms in SLE123 both transplant and autoimmunity. Mice transplanted with C3H islets and treated with the triple therapy experienced improved islet survival compared to anti-CD45RB alone(**Figure 5.10A**). I then utilized the same 2 week therapy in 9 week old SLE123 mice (**Figure 5.10B**). After 9 months I demonstrated that these mice experienced a reduction in anti-dsDNA titers and an overall reduction in IgG deposition in the kidneys as scored by fluorescence staining **(Figure 5.10C, D, and E)**. Overall this demonstrates that metabolic inhibition coupled with signaling driven by anti-CD45RB improves tolerance and disease outcome in SLE123.

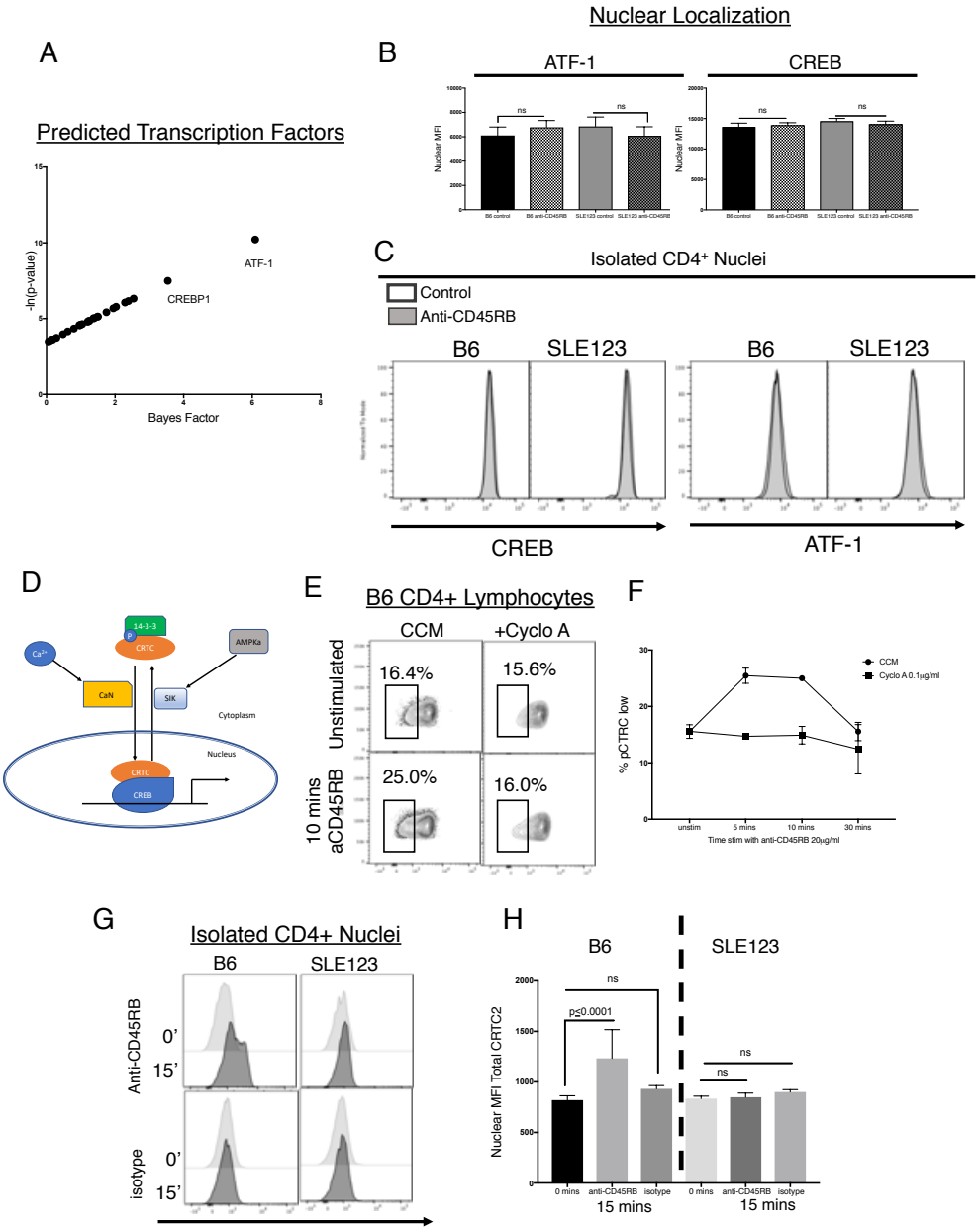


Figure 5.8 CREB/ATF-1 transcription is a likely target of anti-CD45RB for metabolic modulation of CD4 T lymphocytes. A) Transfac analysis of metabolic genes modulated by anti-CD45RB indicated that CREBP1 and ATF-1 binding sites were enriched in this gene list. B) Measurement of ATF-1 or CREB by imaging flow cytometry or flow cytometry of isolated nuclei C) revealed no change in ATF-1 or CREB nuclear content. D) Cartoon illustrating regulation of CRTCs, that regulate CREB and ATF-1 transcriptional activity. CRTCs nuclear localization is induced by Calcineurin A dephosphorylation and opposed by SIK phosphorylation. E) Analysis of anti-CD45RB induced dephosphorylation of CRTC2, revealed anti-CD45RB induced dephosphorylation of CRTC2 that was inhibited by Cyclosporine A. Quantified in F. G) Flow analysis of total CRTC2 content in isolated nuclei revealed import of CRTC2 after anti-CD45RB treatment in B6 CD4 T lymphocytes only. There was no quantifiable import of CRTC2 in SLE123 CD4 T lymphocytes. Additionally, there was no import with an isotype control antibody. This is quantified in H.

Discussion

This study illustrates an important role for metabolism as a modifiable barrier to T lymphocyte tolerance induction in SLE123 mice. The importance of the glucose and mitochondrial metabolism in T lymphocyte function has been borne out repeatedly in murine and human studies. During T lymphocyte activation, oxidative phosphorylation increases and is required for efficient and complete activation[344]. Similarly, during activation and effector function glucose is shunted to the glycolytic pathway, a phenomenon termed the Warburg Effect[344]. This metabolic switch allows for byproducts and intermediates of glycolysis to be utilized for the cell division and production of molecules for proper effector function. CD4 T regulatory cells are thought to utilize lipids and mitochondrial oxidative phosphorylation to mediate their function, although studies are emerging that question that paradigm[346–348]. Studies in other immune cell subsets illustrate that metabolism is also important in mediating activation and function of these cells, but in the interest of conciseness will not be reviewed here.

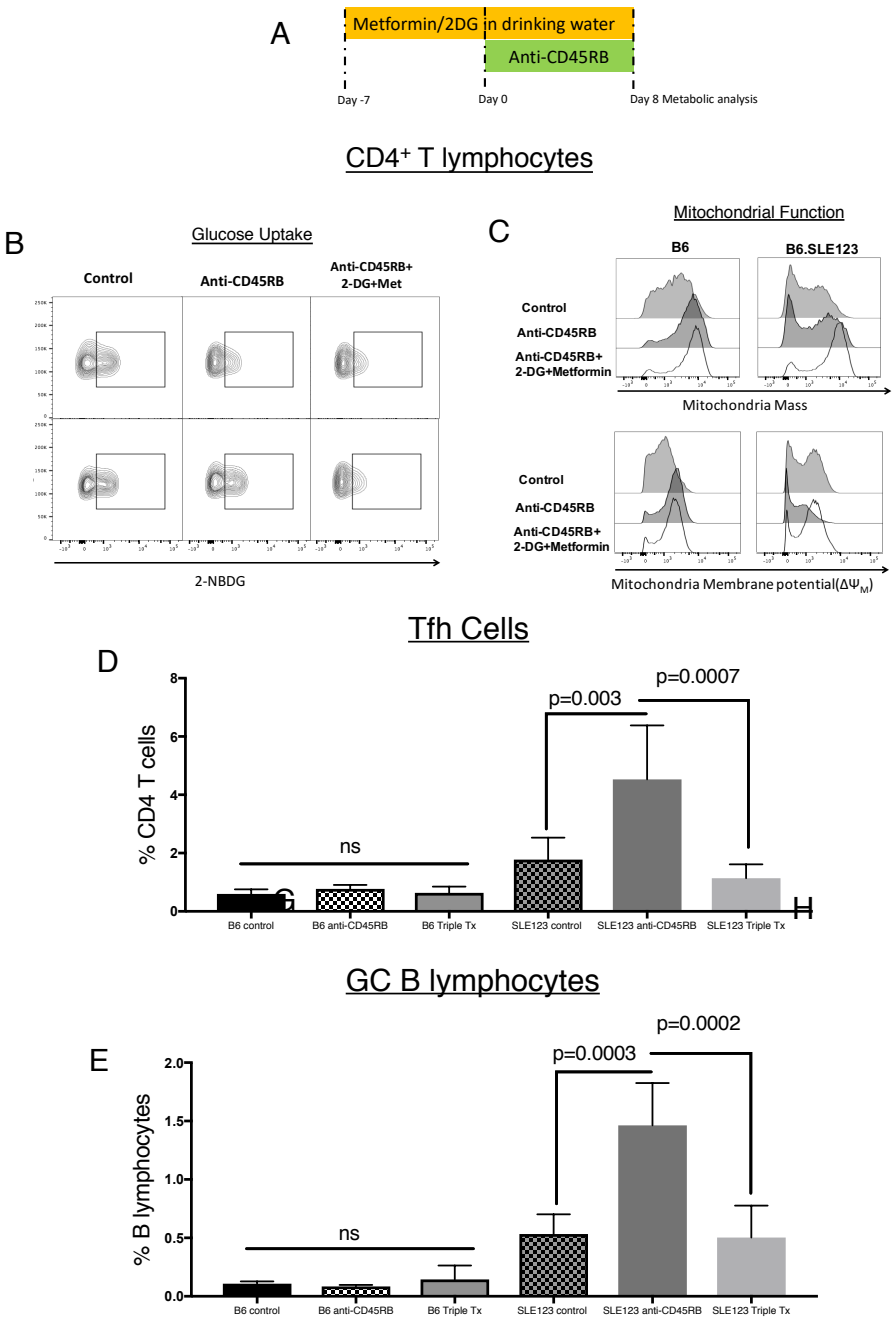


Figure 5.9 Metabolic therapy restores responsiveness to anti-CD45RB in SLE123 mice. A) Treatment strategy for metabolic inhibition in SLE123 mice. B and C) Treatment with metformin and 2-DG in combination with anti-CD45RB restored metabolic modulation in SLE123 mice. D and E) This triple therapy also reduced both Tfh and GC B lymphocytes that were increased anti-CD45RB alone. This indicated that metabolic modulation restored the suppressive capacity of anti-CD45RB.

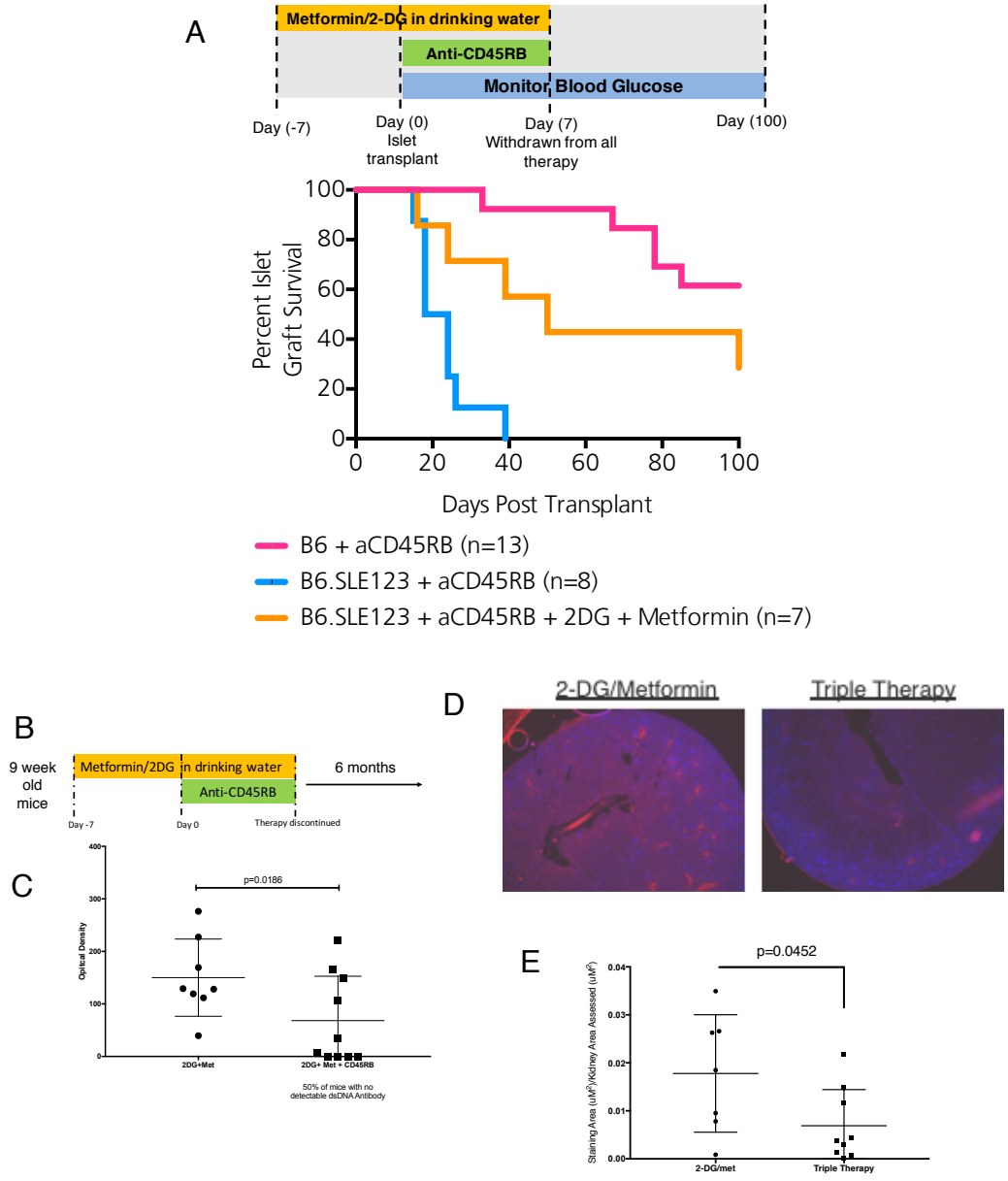


Figure 5.10 Metabolic therapy combined with anti-CD45RB improves transplant outcomes and improves disease in SLE123 mice. A) Treatment strategy for metabolic inhibition and anti-CD45 in SLE123 mice before and during islet transplant. Islet transplant survival was compared between B6 anti-CD45RB treated (pink line), SLE123 anti-CD45RB (blue line) or SLE123 anti-CD45RB+2DG/Met (orange line). As can be appreciated, SLE123 mice treated with metabolic therapy and anti-CD45RB had improved islet transplant survival. B) Illustration of a short course metabolic inhibition+ anti-CD45RB for prevention of SLE in SLE123 mice. C) Measurement of anti-dsDNA titers by revealed mice treated with triple therapy (n=10) experience a reduction in titers as compared to mice only receiving 2-DG/Metformin (n=8). D) Histologic analysis of kidneys from each treatment group for Ig deposition revealed a reduction in antibody deposition in kidneys from ice treated with 2-DG/Met and anti-CD45RB as compared to 2-DG/Met alone. This is quantified by fluorescence intensity in E.

My study in B6 mice revealed that both glucose uptake and mitochondrial function were inhibited by anti-CD45RB. As can be appreciated from **Figure 5.2**, this inhibition limits the ability of T lymphocytes to proliferate in response to a TCR stim. As the bulk of cells analyzed were resting naïve cells, glycolytic rate was unaffected before activation. It is of interest but outside of the scope of this study to determine whether anti-CD45RB would have the same effect on T lymphocytes of a memory or effector phenotype, or if these antigenically primed cells resist inhibition. RNA-seq and metabolic analysis revealed a decrease in pathways important for nucleotide synthesis (pentose phosphate, purine biosynthesis, and pyrimidine biosynthesis). These pathways are extremely important in T lymphocytes as they utilize them exclusively to maintain a pool of nucleotides for division and DNA repair[349]. Although not directly tested, this lack of free nucleotides may have contributed to the inability of anti-CD45RB treated T lymphocytes to proliferate, as my data revealed T lymphocyte activation signaling was intact after therapy. Interestingly, methotrexate, a chemotherapeutic drug that targets the first step of purine biosynthesis, has been utilized in lupus, rheumatoid arthritis, graft versus host disease, and in some instances of transplant[350–353]. The moderate level of success of this drug is possibly due to the nonspecific way methotrexate targets effectors and regulatory cells who both rely on purinergic signaling (although not shown, our metabolomic analysis reveals Tregs did not demonstrate the same alteration of purine biosynthesis).

My RNA-seq analysis also revealed alterations in branched-chain amino acid metabolism (valine, leucine and isoleucine). These amino acids are utilized by the mitochondria for ATP production, via the Krebs Cycle, and to stimulate protein production. Leucine, isoleucine and valine makeup ~40% of the free amino acids in plasma, as such they could be an important fuel source for T lymphocytes[354]. In many cells types leucine is metabolized and used as a fuel source

or nitrogen donor for synthesis of alanine and glutamine[354]. A growing interest in the role glutamine in T lymphocyte differentiation and function has emerged[355]. Glutamine has been targeted in transplant studies with 6-Diazo-5-oxo-L-norleucine (DON), a glutamine antagonist that inhibits enzymes in the glutamine metabolic pathway[355]. It will be of interest to see whether combining DON with anti-CD45RB, 2-DG, and metformin will improve disease and transplant outcomes. As branched chain amino acid metabolism requires mitochondrial activity and I achieved mitochondrial inhibition with my triple therapy strategy, I did not attempt to include DON to improve outcomes in these studies.

As stated previously recent studies in the SLE123 mouse model illustrate that CD4 T lymphocytes from aged and diseased mice possess enhanced mitochondrial function and enhanced glycolysis. This enhancement was linked back to AKT/mTOR signaling. The mice in my study did not demonstrate enhanced signaling via this pathway at the time assessed. The senior author of these studies (whom I have had the privilege of discussing the interpretation of their findings) did not determine whether enhanced mTOR signaling is a primary defect or the result of inappropriate T lymphocyte activation accumulating over time. Similarly, T lymphocytes isolated from patients with SLE also demonstrate enhanced metabolic signaling, which may also emerge secondarily to continued T lymphocyte activation[356,357].

In the previous studies in SLE123 mice, mice were treated chronically with 2-DG and metformin and demonstrated a reduction in lupus pathology[341]. This metabolic inhibition presumably led to inhibition of effector T lymphocyte activation, essentially equivalent to chronic immune suppression. We demonstrated that a short-course metabolic inhibition coupled with a tolerizing therapy, anti-CD45RB, led to improved disease outcomes in SLE123 mice without chronic intervention for over 6 months. In SLE123 mice anti-CD45RB did not alter the

metabolic programming of T lymphocytes. Instead these mice resisted these changes and deleterious subsets of T lymphocyte appeared to be enhanced by anti-CD45RB prompting me to investigate the pathways responsible for the effect anti-CD45RB had on metabolism.

It must be stated that the signaling pathways I defined in this study are most likely not the only signaling pathways modulated by anti-CD45RB, as a role for other intercellular an intracellular pathways have been described in literature[86,289,332]. In this study I was specifically interested in the signaling pathways that contributed to metabolic modulation by anti-CD45RB. My data suggested that targeting of CREB/ATF-1 influenced by CRTCs was responsible for the metabolic modulation by anti-CD45RB. The role of CREB in T lymphocytes is widely studied and impacts cytokine production, NF-KB activation, antiapoptotic signaling, and differentiation of Th1, Th2 and Th17 response[358,359]. While CREB has been demonstrated to modulate metabolic processes in other cell types, it has not been demonstrated to have a direct role in T lymphocyte metabolism. Additionally, CREB/ATF-1 is thought to be important for expression of Foxp3 and thus maintenance of Tregs[360]. Additionally, cyclic-amp (cAMP) is a powerful immunosuppressive molecule that is dependent on CREB to carry out its suppressive activity[360]. Not shown here, but in additional studies I carried out with anti-CD45RB, I demonstrated that cAMP was not produced as a result of therapy.

CREB abnormalities have been extensively documented in SLE123 mice, including reduced activity and abnormal function of CREB and cAMP responsive element binding modulator (CREM), an alternative splicing isoform of CREB with capacity to inhibit CREB transcriptional activity[361]. Additionally, abnormal nuclear activity of CamKIV in T lymphocytes from lupus patients lead to binding of CREM to the IL-2 promoter, inhibiting transcription of this gene[362]. This interaction leads to abnormal T lymphocyte function and putative Treg

abnormalities. Others have determined that IgGs secreted by B lymphocytes bind T lymphocytes and contribute to abnormal CREM signaling[361]. Finally, it cannot be ruled out that abnormal response to anti-CD4RB is due to changes in epigenetic gene regulation described in SLE mice, due to reduced function of DNA methyltransferase 1 (DMNT1) responsible for maintaining proper epigenetic patterning[363].

The role of CRTCs is poorly understood in the immune system. The CRTC protein family is composed of 3 isoforms CRTC1, found mostly in the brain and in lymphocytes, and ubiquitously expressed CRTC2 and CRTC3. Loss of CRTC2 in an EAE model of autoimmune disease results in protection[363]. This protection is thought to be related to the loss of Th17 cells that are regulated by CRTC2. While these results run counter to my findings, they illustrates an important point about the function of proteins being specific to cell subset, context, and co-activated pathways. (i.e. NFAT driving anergy in the case of a TCR signal with no costimulation and full activation when activated in the presence of costimulation). In the case of CRTC/CREB/ATF-1 interactions the net response may be dependent on the phosphorylation status of both CREB/ATF-1 and the CRTCs. Thus, CRTCs may be important for establishing responsiveness to tolerogenic signaling in resting, naïve T lymphocytes but may have other roles in activated lymphocytes.

The signaling regulation of CRTC activity is a complex process. As can be seen in the **Figure 5.8D**, phosphorylated CRTCs are sequestered in the cytoplasm due to the kinase activity of Salt-inducible Kinases (SIKs). There are 3 isoforms of SIK; interestingly SIK2 whole-body knockouts exhibit overt metabolic defects[363]. Either due to activation of Calcineurin A, the cAMP/PKA dependent inhibition of SIK , or both, the CRTCs become dephosphorylated and enter the nucleus, where they can regulate CREB activity. Of interest to my studies, SIK is

activated downstream of AMPKa and LKB1 signaling, a target of metformin. Metformin is thought to activate AMPKa thus enhancing SIK inhibition of CRTCs. So, while metformin may have improved metabolism in the SLE123 T lymphocytes, it may interfere with the already inhibited CRTC signaling system. (although my data demonstrate that relatively no CRTCs made it into the nucleus in SLE123 T lymphocytes when stimulated with anti-CD45RB).

While nuclear localization of CRTCs and CREB/ATF-1 was assessed in this study, it is important to note that the mitochondrial genes downregulated by anti-CD45RB were encoded from the mitochondrial genome. CREB and CRTCs have been of interest in studies of oxidative stress and aging. In these studies translocation of CRTC and CREB to the nucleus lead to inhibition of respiration, reducing oxidative stress and increasing longevity[363]. Modulation of the mitochondrial genome by anti-CD45RB and whether CRTCs are localized to the mitochondria after anti-CD45RB is an important area of future study.

CD4 T lymphocytes in SLE demonstrate defects in proteins important for activation and inhibition of CRTCs. Downstream of the TCR, SLE T lymphocytes experience enhanced calcium flux that could lead to aberrant CRTC activity or an increase in counter-regulation of CRTCs[364]. Importantly, the activity of PKA signaling has been consistently demonstrated to be reduced in SLE T lymphocytes due to under-expression of PKA[365]. As PKA serves as a negative regulator of SIK activity, a protein whose kinase activity opposes translocation of CRTCs to the nucleus, this might partially explain why, even in the presence of phosphatase activity, CRTCs did not translocate to the nucleus. A pan-SIK inhibitor (HG-9-91-01) has been successfully utilized *in vitro* but has not been optimized for *in vivo* use, and thus was not utilized in these studies[365]. It must be stated that the phospho-residue I assessed in this study is not important for nuclear translocation of CRTC2. It was assessed to demonstrate robust phosphatase activity

induced by anti-CD45RB. No reliable reagents exist for the 14-3-3 sigma binding residues; thus the activity at these sites is not directly measured in this study. The nuclear translocation data would suggest this site was not dephosphorylated or was rapidly rephosphorylated in SLE123 T lymphocytes.

In focusing on the derangement of CD4 T lymphocyte metabolism, it can be easy to forget that a systemic response to anti-CD45RB is necessary for proper tolerance induction. Studies have borne out the important role of B-T lymphocyte interactions in autoimmunity and tolerance. It is of great interest whether B lymphocytes play a role in the metabolic derangement of T lymphocytes. While it has not been demonstrated that anti-CD45RB induces T lymphocyte anergy in a B lymphocyte dependent fashion, it is likely that therapeutic success depends on direct anergic effects mediated by anti-CD45RB binding T lymphocytes, and the action of anti-CD45RB on B lymphocytes to induce nonresponsiveness in T effectors recognizing graft relevant antigens presented by B lymphocytes. Indeed, improper B and T lymphocyte interactions are characteristic of SLE, and interactions such as ICOS-ICOSL and CD40-CD40L have been demonstrated to play roles in the disease process[366,367].

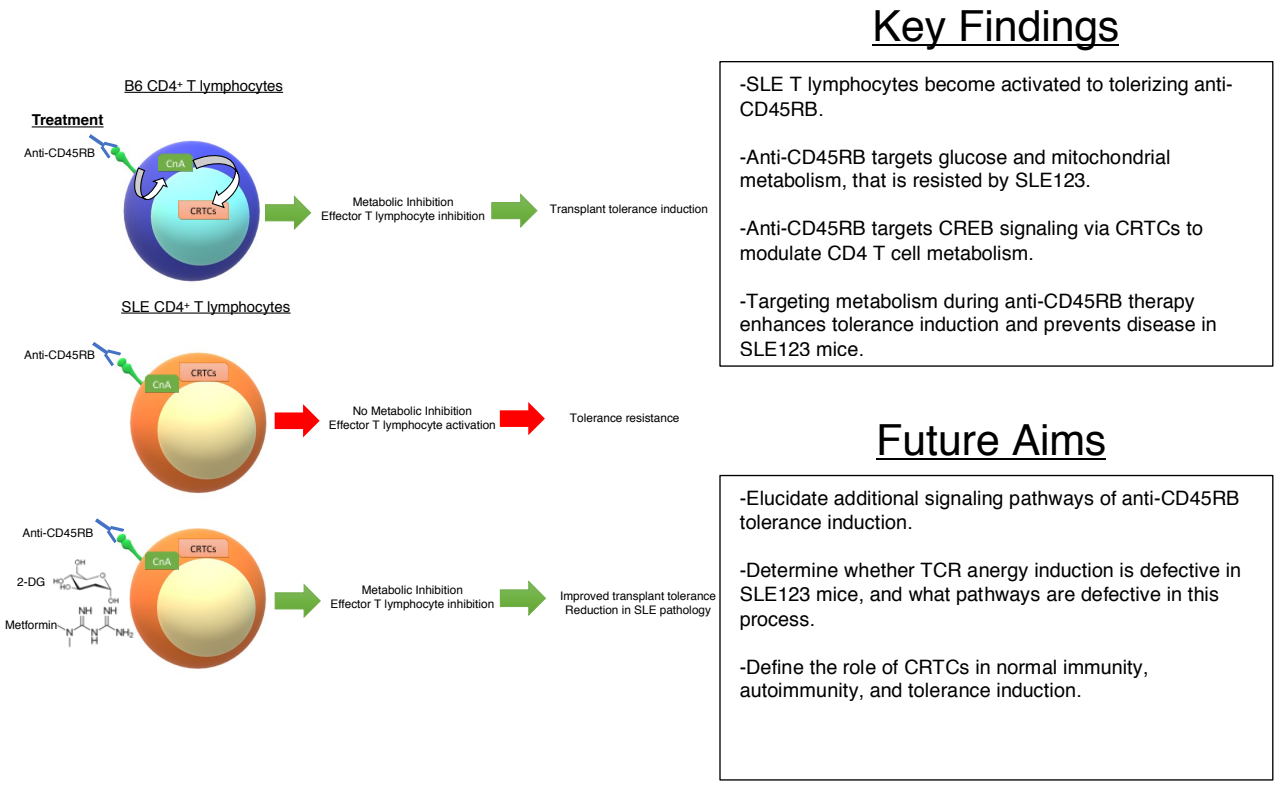


Figure 5.11 Model of the impact of anti-CD45RB on B6 and SLE123 CD4+ T lymphocytes.

Anti-CD45RB has been demonstrated to alter the distribution and expression of ICAM on B Lymphocytes, leading to the likelihood of enhanced interactions with T lymphocytes[368]. In the case of a non-autoimmune setting this interaction may lead to a tolerogenic effect, while on the SLE123 background this may lead to exacerbation and activation of T lymphocytes as seen in our studies (changes in mitochondrial function and increase in Tfh and GC subsets). Future studies in SLE123 should determine whether B lymphocytes drive abnormal T lymphocyte activation, and determine the molecules that govern this interaction. It would be of interest to see whether a therapy that normalizes B lymphocyte homeostasis (such as nIgM) may allow the restoration of normal T lymphocyte metabolism. Taken together, these studies highlight the importance of metabolism in immune tolerance and define new signaling pathways important for tolerance induction.

CHAPTER VI

DISCUSSION AND FUTURE DIRECTIONS

Preventing or curing autoimmune disease is an important and noble goal. With the incidence of autoimmune disease such as T1D on the rise, it is important that we find better and more benign immune interventions to permanently reverse disease. B lymphocytes have consistently been demonstrated to play important roles in autoimmune disease in both initiation and perpetuation, but current clinical interventions fail to induce long-term tolerance and also fail to deal with B lymphocyte biology in a meaningful and directed way. My thesis illustrates new ways B lymphocytes can prevent and reverse autoimmunity. In Chapter VI, I will highlight the unexpected modes of B lymphocyte regulation I have uncovered in my studies, future questions and studies that should be carried out in light of these results, My findings from each chapter are summarized in **Fig 6.1**. Hopefully, my findings will provide a framework for understanding the diverse ways that B lymphocytes can be harnessed to treat autoimmunity.

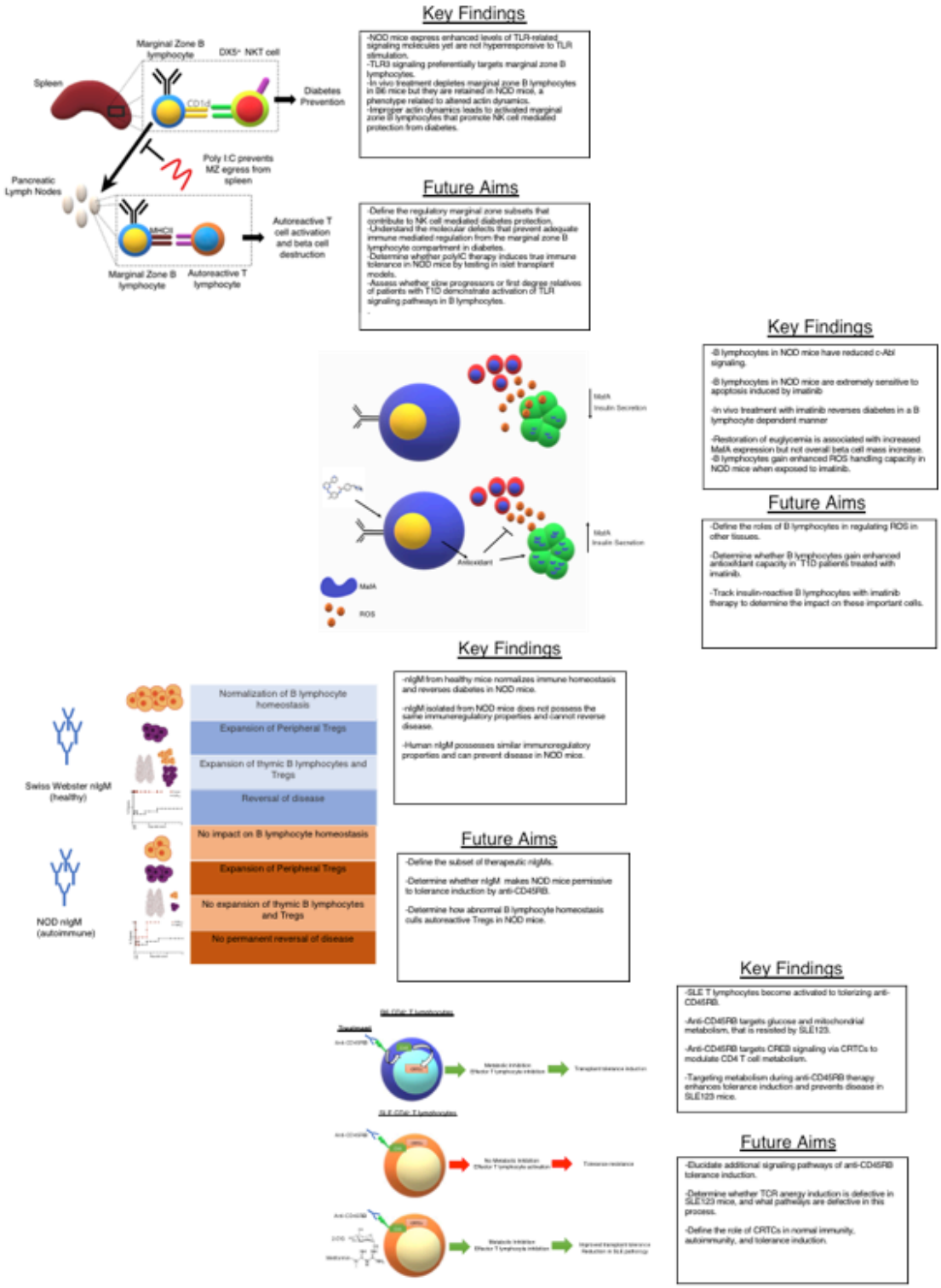


Figure 6.1 Model, key findings and future directions from each chapter.

B lymphocyte regulation is perpetuated by numerous cell subsets and mechanisms

Current literature on B lymphocyte-mediated regulation indicates that their immunosuppressive capacity can be traced to multiple, phenotypically distinct subsets. In **Table 6.1** I illustrate the phenotypic definitions of B lymphocytes with regulatory capacity described in the literature[369–371]. Interestingly, most of these subsets share IL-10 as a primary effector of regulation. It is surprising that these cells utilize identical suppressive strategies even though they are distinct both functionally and anatomically. My studies suggest that these same subsets possess other regulatory properties, suggesting that the importance of IL-10 may have been overestimated in previous studes. As studies in T lymphocytes demonstrate an ever widening array of specialized Tregs with distinct functions and modes of regulation, it is appropriate to consider new modes of B lymphocyte regulation.

B lymphocytes Integrate Innate Signals to Influence Adaptive Immune Responses

As is often the case, returning to a discovery can provide fresh insight in the context of new findings. The existence of an immunoglobulin-producing cell population was first defined in chickens[2,3]. The development of these cells was linked to the Bursa of Fabricius, giving birth to the B lymphocyte moniker. The Bursa of Fabricius is located in the hindgut of chickens, a place in which B lymphocytes develop in close contact with the gut microbiota. Studies in several animal models have demonstrated that, early in life, B lymphocytes traffic to the gut (in mice the lamina propria) to interact with the nascent microbiome[372]. This interaction influences the establishment of the immunoglobulin repertoire and provides a potential mechanism for early life microbial events to set the stage for the lifelong capacity of B lymphocytes to regulate immunity.

Murine Breg Phenotypes

Breg Subtype	Phenotype	Mechanism of Suppression
T2-MZP	$CD19^{+}CD21^{Hi}CD23^{Hi}CD24^{Hi}IgM^{Hi}IgD^{Hi}CD1d^{Hi}$	IL-10
MZB10	$CD19^{+}CD21^{Hi}CD23^{-}CD24^{Hi}IgM^{Hi}IgD^{Lo}CD1d^{Hi}$	IL-10
B-10	$CD19^{+}CD1d^{Hi}CD5^{+}$	IL-10
B-1a	$CD5^{+}$	IL-10
Killer B	$CD5^{+}CD178^{+}$	FasL, IL-10
GIFT-15	$B220^{+}CD21^{+}CD22^{+}CD23^{+}CD24^{+}CD1d^{+}$ $CD138^{+}IgD^{+}IgM^{+}$	IL-10
Plasma	$CD138^{Hi}IgM^{+}TACI^{+}CXCR4^{+}CD1d^{Hi}Tim\text{-}1^{int}$	IL-10, IL-35
Plasmablasts	$CD138^{+}CD44^{+}$	IL-10
Tim-1^{+}	$CD19^{+}Tim\text{-}1^{+}$	IL-10
PD-$L1^{Hi}$	$CD19^{+}PD\text{-}L1^{Hi}$	PD-L1
Adenosine	$B220^{+}CD39^{+}CD73^{+}$	Adenosine

Human Breg Phenotypes

Breg Subtype	Phenotype	Mechanism of Suppression
Immature	$CD19^{+}CD24^{Hi}CD38^{Hi}$	IL-10, PD-L1
B10	$CD19^{+}CD24^{Hi}CD27^{+}$	IL-10
GrB^{+}	$CD19^{+}CD38^{+}CD1d^{+}IgM^{+}CD147^{+}$	GrB, IL-10, IDO
Br1	$CD5^{+}$	IL-10, IgG4
Plasmablasts	$CD5^{+}CD178^{+}$	IL-10
Adenosine	$CD39^{+}CD73^{+}$	Adenosine
iBregs	$CD138^{Hi}IgM^{+}TACI^{+}CXCR4^{+}CD1d^{Hi}Tim\text{-}1^{int}$	TGF-β, IDO

Table 6.1 Current understanding of defined Breg phenotypes in mice and humans.

Indeed, modulation of the gut microbiome in early life of NOD mice has an impact on disease outcomes with germ free conditions, accelerating disease in some instances[171,173,373]. Several studies in humans have attempted to dissect the contributions of the microbiome and other pathogens to T1D incidence and onset, but so far no consensus has been reached on any common microbial mediator. Of interest to my studies, it would be important to determine whether B lymphocytes from NOD mice raised in germ-free or specific-pathogen free conditions possess IgM with altered regulatory capacity.

Even before B lymphocytes emerge into the periphery, they begin to interact with microbial products. Studies have demonstrated even hematopoietic stem cells (HSCs) have the capacity to recognize TLR agonists[374]. Repeated exposure to TLR4 agonist LPS leads to injury and failure of the HSC compartment. In studies from our lab, we have demonstrated that hematopoietic stem cell mobilization is defective in NOD mice[375]. It would be of interest to determine whether modulation of TLR4 signaling could improve HSC mobilization and thus lymphopoiesis and B lymphocyte homeostasis in NOD mice. In addition Meffre and colleagues demonstrated that deficient responsiveness to TLR signaling led to abnormal negative selection and autoreactive B lymphocyte survival in bone marrow B lymphocytes[375]. Finally, B lymphocytes in the immature Transitional 1 stage, a stage abnormal in NOD mice, can survive negative selection and become activated when provided the appropriate TLR stimulation[52].

Once B lymphocytes become activated, TLRs can influence their response to antigens, leading to immune activation or regulation. Studies from the Thomas lab illustrate that immunization or stimulation of B6.VH125 mice that possess B lymphocytes with a heavy chain specific for insulin with insulin and TLR4 stimulation lead to a robust T-independent response to insulin (an antigen that when administered alone does not elicit this response)[194]. A similar study

in MRL/lupus-prone mice demonstrated that TLRs mediate extrafollicular activation of autoreactive antibody-producing B lymphocytes, independent of T lymphocyte help[375]. While antibodies are pathogenic in SLE, research in T1D indicates they play no role in islet damage; thus, the role of TLRs in shaping autoimmunity is probably specific to the disease assessed.

My studies illustrate that B lymphocytes respond to TLR3 stimulation and activate regulatory NK cells that can mediate modest disease prevention. This finding does not represent an antigen specific immune regulation, but it would be of future interest to determine whether TLR3 stimulation mediates inhibition or conversion of islet-specific effector cells to Tregs through a B lymphocyte specific interaction. Nevertheless, we have demonstrated that viral and bacterial components have the capacity to therapeutically target B lymphocytes to promote disease prevention in NOD mice. B lymphocytes are unique cells in the immune system as they have the capacity to acquire and concentrate antigen via their BCR. In my study I noted that regulation was seemingly mediated by marginal zone B lymphocytes activating NK cells. NK cells interact with cognate MHC CD1d, which is expressed in high levels on marginal zone B lymphocytes[376]. Bregs, described in literature, often have phenotypes that overlap with marginal zone B lymphocytes. It was clear in my studies that B lymphocytes were required for induction of NK-mediated cell protection from diabetes. These cells have the capacity to then drive a Th2 immune response thought to divert Th1 responses[210]. It is still unclear whether Th1 responses are important for T1D pathology[47]. While I was able to demonstrate TLR3 stimulation mediated protection in the NOD mouse, it is unclear whether the same mechanism would occur in humans.

Regardless of the mechanism of TLR influence on B lymphocyte regulation, microbes do influence onset of autoimmunity, including T1D. There is ongoing support for the "Hygiene Hypothesis", as rates of T1D continue to grow in industrialized countries that widely utilize

vaccines and antibiotics. When considering this hypothesis, it is very important to take into consideration several factors that could change our understanding of it or mitigate its influence. People with the most aggressive forms of T1D often did not survive to reproductive age until the 1920s, upon the advent of exogenous insulin therapy use[377]. We have little historical understanding about the genetic penetrance of disease associated alleles in terms of magnitude or contribution to the fierceness of T1D. The lack of T1D diagnoses in non-industrialized countries may not represent a true decrease in disease, but a reduction in the diagnosis of disease among these impoverished and underserved countries, facing much more immediate and dire health crises. Secondly, the genetic predisposition to T1D of these populations in non-industrialized countries has not been as exhaustively characterized as those in industrialized countries. This lack allows for the possibility that non-industrialized populations may possess protective alleles, patterned during an early migrational event. Although it is not out of the question that these protective alleles could be selected for by the divergent microbes these populations interact with, it is more likely that microbes act as a rheostat over an individual's genetic predisposition to modulate disease onset.

In human studies it may be useful to phenotype B lymphocytes from patients from low-incidence regions to determine whether they have altered B lymphocyte subsets with potential regulatory capacity. Subsequently, we could determine whether TLR stimulation in populations from developed countries could drive similar B lymphocyte subset expansion. In conclusion, further research into the interaction between microbes and B lymphocytes could provide a therapeutic strategy to prevent T1D in patients that are at-risk to develop disease, but may not represent a cure for T1D.

Tissue-specific regulation of ROS by B lymphocytes

The surprising finding that B lymphocytes treated with imatinib upregulated antioxidant capacity and were required for disease reversal demands investigation into the role of ROS in autoimmune tissue destruction and in autoreactive lymphocyte biology. B and T lymphocytes have been demonstrated to produce ROS downstream of the antigen receptor[268]. This production serves to enhance activation, but can also lead to apoptosis in some circumstances, including negative selection. This demonstrates that the role of ROS in B lymphocytes may be stage specific. ROS has been demonstrated to regulate the actin cytoskeleton as well, as was demonstrated in my investigation into the role of TLR signaling in B lymphocytes (**See fig 2.6**). I demonstrated that NOD mice had a reduced capacity to mobilize the actin cytoskeleton in response to ROS generating H_2O_2[192]. Whether this resistance was due to enhanced ROS handling capacity or actin cytoskeletal resistance to the impact of ROS was not determined.

I demonstrated imatinib treatment led to some B lymphocyte loss in NOD mice, but a majority of B lymphocytes persisted, exhibiting enhanced ROS handling capacity. This outcome facilitated recovery of beta cell transcription factor MafA and return to euglycemia in mice that possessed B lymphocytes and were treated with imatinib. Beta cells are exquisitely sensitive to ROS, due to low expression of antioxidant enzymes[270]. Several studies have illustrated that therapeutic treatment with antioxidant proteins and compounds can prevent beta cell loss and diabetes in NOD mice[378,379]. It is still unclear whether this regulation of ROS in the islet microenvironment preserved beta cells by protecting them from the effects of ROS, by dampening immune cell activation, or both. What is clear is that ROS plays an important role in T1D in both beta cell survival and immune activation, an interplay that needs to be thoroughly investigated to effectively preserve beta cell function.

Importantly, we utilized a clinically relevant drug to reverse diabetes, thus increasing the direct impact of these studies on human health. Imatinib (Gleevec) is currently in clinical trials in humans with T1D. Once these studies are completed, it will become clear whether Gleevec worked in preserving beta cell mass in patient with T1D. Regardless of the outcomes it will be important to determine whether Gleevec induces ROS handling capacity in B lymphocytes in patients or a subset of patients with T1D. In this way dosage can be adjusted or therapy can be utilized only in those patients demonstrating robust antioxidant responses to Gleevec.

It is important to note that Gleevec is also utilized in hematologic malignancies. While Gleevec has been widely successful in treating cancers originating from the BCR-ABL fusion protein, resistance to imatinib has been documented and is related to upregulation of antioxidant proteins[243]. Solid tumor cancer biology has focused on T lymphocyte and Treg mediated forms of therapeutic intervention, demonstrating PD-1 and CTLA-4 targeted therapies (as of this writing the current recipients of the Nobel Prize) can improve cancer outcomes when combined with chemotherapeutics. It would be important to determine whether upregulation of antioxidant capacity in B lymphocytes may correlate with worse outcomes during Gleevec or other therapies. This model could lead to the potential for temporary B lymphocyte depletion (via anti-CD20) during Gleevec therapy to improve outcomes.

In kind, B lymphocytes have been targeted in humans with T1D with anti-CD20 therapy (Rituximab). Rituximab has been demonstrated to partially rely on the induction of ROS to mediate its depleting effect on B lymphocytes[380]. As previously stated, rituximab has been used clinically in humans with T1D, with mixed results. This therapy demonstrated a mild reprieve from beta cell loss but beta cell loss resumed after discontinuation of therapy. It would be interesting to investigate whether enhanced ROS handling by B lymphocytes that persisted in the

islets after anti-CD20 (as demonstrated by David Serreze) did so by upregulating antioxidants thus impacting ROS in the islet, facilitating partial beta cell recovery[272]. Conversely, it would be important to determine whether enhanced antioxidant capacity in humans leads to resistance to therapy and rapid reemergence of autoreactive clones after withdrawal form therapy. It is becoming increasingly clear that ROS plays an important role in T1D.

IgM mediated regulation of autoimmunity

Although B lymphocyte antioxidant capacity may allow B lymphocytes to regulate the local immune environment, the IgM network presents an interesting way for B lymphocytes to regulate immunity at a distance. IgMs have the capacity to modulate immune homeostasis[152,279]. Studies have linked this back to binding of the conserved Fc portion of IgM to its cognate receptor and was discussed in Chapter IV. Of interest to many is the autoreactive specificity of IgM against lymphocyte specific antigens. Several studies indicate that these IgMs bind and target immune cell receptors on T lymphocytes that regulate immune activation[283]. This mechanism does not necessarily provide an explanation for the depletion of autoreactive B lymphocytes I demonstrated in my studies. In providing an explanation for this phenomenon, I offer three possibilities (**See Figure 6.2**). One, IgM of the same or similar autoreactive specifcity is secreted and binds autoreactive B lymphocytes via Fc mediated interactions. These Igs then co-localize ITIM associated receptors via extracellular glycosylation patterns with the BCR that prevent signaling and survival of these cells. This phenomenon has been described with other isotypes of antibodies[100]. Second, IgM antibodies specific against autoreactive BCRs alone or bound to antigen foster elimination of autoreactive B lymphocytes. Indeed, this phenomenon occurs as well and the Igs responsible for this are part of the “idiotypic network”, a network of

antigen receptors targeted against motifs of other antigen receptors[380]. It is, however, unlikely that the polyclonal preparations of IgM contained enough of a particular antigen-specific IgM to impact insulin-reactive B lymphocytes via inhibition or depletion. Lastly, it could be that nIgMs impact immune homeostasis by modulating homeostatic factors such as BAFF, leading to improved selection and increased immune regulation through multiple mechanism. There is also the possibility that IgM may have impact directly on the beta cells as expression of TOSO has been found on the beta cell itself and mediates a protective anti-apoptotic effect[380]. Identifying the B lymphocytes that secrete IgM is an important next step to determine whether NOD mice are lacking this important compartment.

My studies with nIgM demonstrated that NOD mice were deficient in their regulatory capacity, a surprising new mode of defective NOD immune biology. It will remain important to determine the characteristics of the IgMs with therapeutic capacity in mice. In applying IgM to humans, longitudinal studies should also be carried out on humans with T1D, first-degree relatives, or those with autoantibodies but no dysglycemia, to measure the effectiveness of isolated IgMs to mediate immune modulation of human immune systems in humanized mice (akin to those used in Chapter IV). Finally, it will be important to determine whether the regulatory mechanisms of IgM are conserved between mice and humans (i.e. antigen specificity, Fc interactions, glycosylation patterns, etc.).

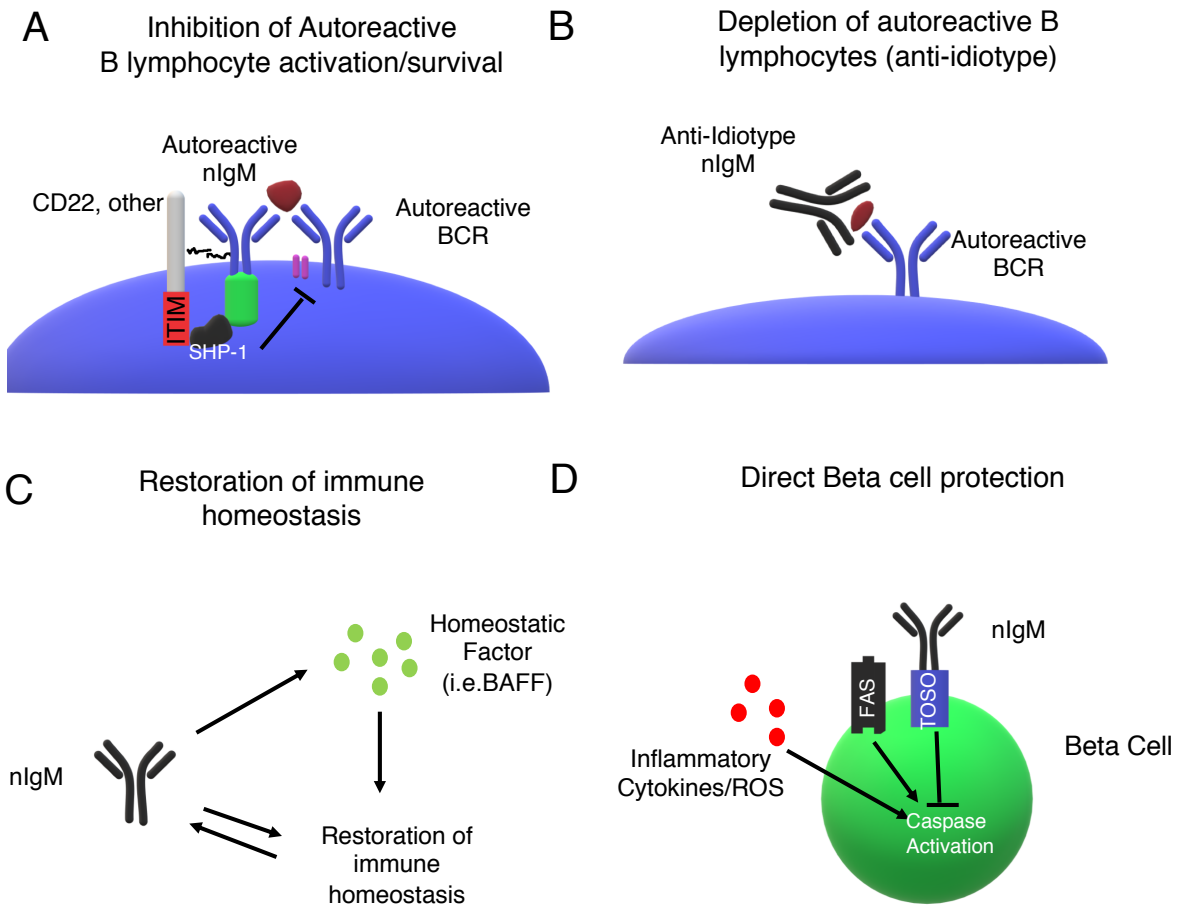


Figure 6.2 Proposed mechanisms of nIgM's regulation of immune homeostasis and diabetes protection. A) IgMs of an autoreactive origin bind B lymphocytes of the same antigenic specificity via Fc mediate interactions. These interactions lead to B lymphocyte inhibition directly or through recruitment of ITIM containing receptors such as CD22. This could occur via extracellular interactions such as differential glycosylation (illustrated by the dark lines). B) IgMs that bind certain autoreactive BCR sequences or autoreactive BCRs when bound to cognate autoantigen mediate depletion through mechanisms such as phagocytosis, complement activation (potentially in humans as NOD lack functional complement) or activation induced cell death (AICD). C) Polyclonal nIgM may also regulate autoimmunity by regulation homeostatic factors such as BAFF, improving selection and dampening autoreactivity. Alternatively nIgM may directly modulated homeostasis through TOSO interactions on developing B lymphocytes. This restoration of homeostasis may enhance the production of protective nIgMs. (indicated by the reciprocal arrows in C). D) Polyclonal IgMs may also mediate beta cell protection directly by binding beta cell expressed TOSO. This interaction has shown to inhibited FAS induced apoptosis but may also inhibit other modes of apoptosis.

It is important to consider also that my studies seemed to illustrate an important role for the thymus in nIgMs capacity for long-term diabetes reversal. In humans the thymus begins to shrink at a rate of ~3% per year beginning after the first year of life[380]. This reduction in thymic epithelial space (TES) results in decreased thymic output. Surprisingly studies in patients receiving bone marrow transplants after 40 failed to fully restore a peripheral T lymphocyte population, indicating reduced thymic function[380]. Optimal thymic output may be essential for IgM or any immune directed therapy to effectively work. Thus, careful monitoring of thymic output in patients treated with IgM (via thymic excision circles and Treg expansion) will be essential to determine the effectiveness of this therapy. If it is found that nIgM is ineffective in expansion of Tregs in aged-humans (the majority of Phase I participants in T1D clinical trials), it may be important to test this in younger individuals. Alternatively it may require IgM be combined with a thymic reinvigoration therapy. As both testosterone and estrogen have been shown to drive thymic involution inhibiting the production of these sex hormones can establish rejuvenation of the thmus. Lupron (Luteinizing Hormone-Releasing Hormone analog) has been demonstrated to prevent release of sex hormones and rejuvenates the thymus[380]. Utilizing Lupron may be undesirable to many, due to the side effects of this therapy.

Central and peripheral B-T lymphocyte collaboration for tolerance induction

My studies with nIgM revealed a surprising interaction between B lymphocytes and T lymphocytes. Indeed, multiple studies have revealed that T-B collaboration can lead to mutual activation unique to the interaction of T lymphocytes with B lymphocytes, as compared to other APCs. When dissecting the types of B-T lymphocyte interactions that may lead to T lymphocyte tolerance vs activation, it may be best to take an anatomical approach to discuss each interaction.

In this section I will discuss central interactions (thymic and bone marrow) and peripheral antigens (pplenic and lymph node interactions including gut-related tissues, circulating, and site of antigen encounter).

B and T lymphocyte interactions in the bone marrow are not completely understood. It is clear that T lymphocytes traffic to the bone marrow and that these cells are mostly of a memory phenotype where they are maintained by the rich stromal environment and IL-7[380]. Additionally, in many patients with autoimmune disease, there seems to be an enrichment of autoreactive T lymphocytes in the bone marrow[380]. It is unclear how B lymphocytes play any role in the maintenance or priming of these autoreactive cells. The bone marrow is composed of developing B lymphocytes (Pre, Pro, and Immature) and mature recirculating B lymphocytes, with the capacity to present antigen. However, there is little evidence of specific T-B lymphocyte interactions that could drive tolerance in the bone marrow.

The thymus is the primary site of T lymphocyte development. As has been discussed previously, there are multiple interactions that have been defined for B lymphocytes in my studies and others. Interestingly, described functions of B lymphocytes in the thymus, negative selection and enrichment of Treg development, would most likely occur in the medulla of the thymus. In fact, my studies with nIgM demonstrated that after treatment B lymphocytes infiltrated the medulla of the thymus *en masse*, leading to increased Treg output that was absent in B lymphocyte deficient (uMT) mice. When comparing NOD mice to B6 mice, it was clear NOD mice possessed reduced numbers of medullary-invading lymphocytes even though they possessed more thymic B lymphocytes overall. While I was unable to define the differences in B lymphocyte subsets pre and post nIgM treatment or in B6 and NOD mice, this may be due to the use of splenic subsetting markers to dissect the thymic B lymphocyte subsets. It may be true that

thymic B lymphocytes express their own unique markers that provide them unique thymic functionality. Techniques such as laser-microdissection, coupled with flow sorting and RNA-seq may provide additional information about the B lymphocytes localized in the cortical and medullary space in the thymus of B6 and NOD mice.

Future studies of thymic B lymphocytes could incorporate markers known to be important for function and localization of other thymic subsets. Dendritic cells that localize to the medulla or cortico-medullary junction utilize chemokine receptors to do so. Recently two populations of medullary localized DCs have been demonstrated based on differential chemokine receptor usage. One class of DCs (defined as pDCs) are defined by their capacity to migrate from the peripheral spaces to the cortico-medullary junction, bringing new antigens to thymus to mediate tolerance[380]. The other class of dendritic cells (resident cDCs) are localized to the medulla and seem to express AIRE and foster Treg development[381,382]. Similarly, B lymphocytes have been thought to both traffic to the thymus or nascently develop in the thymus. It would be of interest to utilize the unique markers of these DCs to determine whether B lymphocytes were similarly composed of two subsets. Utilizing B lymphocyte antigen specific models in NOD and B6 mice, such as the VH125 insulin binding mouse would also allow for tracking of antigen specific cells in the thymus. Thus, one could elucidate whether abnormal trafficking of antigen-specific cells in the thymus plays a role in culling Tregs or mediating inappropriate T lymphocyte selection.

Restoration of B lymphocyte homeostasis seemed to restore normal distribution of B lymphocytes within the thymus in NOD mice. It is completely unknown what homeostatic factors contribute to normal B lymphocyte development and distribution in the thymus and would be an important area of future study. My studies, as well as others, indicated BAFF is an

important modulator of thymic B lymphocyte function as blockade of BAFF led to a reduction in thymic B lymphocytes and Treg production in NOD mice treated with nIgM[96,152]. Overall thymic B lymphocytes may represent the best opportunity for identification of antigen-specific interactions that mediate lasting tolerance in autoimmune disease.

Peripheral interactions of B and T lymphocytes represents a more diverse and complicated process. As illustrated previously the antigen, activation status, the local cytokine milieu, and environmental and metabolic conditions contribute to the type of immune response elicited by B lymphocyte interactions. Future studies in peripheral B lymphocyte tolerance should also focus on antigen specific interactions that seem to foster Treg expansion or effector inhibition. In my studies with nIgM therapy, I illustrated a complete loss of insulin-reactive B lymphocytes from the spleen but did not look for them in the bone marrow, thymus, gut, or pancreas and associated lymph nodes. Additionally, it was unclear whether nIgM may have downregulated the BCR of insulin-binding B lymphocytes, decreasing their ability to be detected with biotinylated insulin. Future studies should utilize more extensive and sophisticated tracking techniques to follow insulin reactive B lymphocytes during and immediately after therapy and any insulin-reactive B lymphocyte that emerges after therapy, to understand the phenotypic changes. Additionally, insulin-reactive Tregs should be tracked in response to IgM in the presence or absence of B lymphocytes. Therapy with anti-CD45RB therapy could be applied to alloreactive B and T lymphocytes during transplant to track their phenotype post therapy. Isolation of islet graft or organ specific lymphocytes may also illustrate the cytokines, cell interactions, and metabolic subtleties of different tissues. This investigation would help explain the differential roles of B lymphocytes in mediating tolerance in various tissues and transplants.

Overcoming effector resistance to promote immune regulation in autoimmunity

While targeting central and peripheral B lymphocytes to drive regulation, T lymphocytes in autoimmunity may possess qualities that make them resistant to tolerance induction. My studies in the SLE123 mouse model reinforced the notion that metabolic activity is a mode of effector resistance. As has been demonstrated previously, metabolism is altered in the SLE123 $CD4^+$ T lymphocytes[341]. I demonstrated that this alteration conferred tolerance resistance. My studies did not demonstrate that overt metabolic defects were present before the time of SLE pathology. This makes it likely that enhanced metabolism observed later in disease is due to inappropriately activated CD4 T cells. Indeed, treatment with anti-CD45RB, which inhibited B6 metabolism, actually increased mitochondrial activity in SLE.123 mice. This increase could possibly be the reason for increased Tfh and GC cell subsets after anti-CD45RB treatment in SLE123 mice. It will be of future interest to determine whether T lymphocyte signals that normally induce T lymphocyte non-responsiveness, or anergy, induce T lymphocyte activation and metabolic abnormalities in SLE123 mice (i.e. signaling in the absence of co-stim) and whether this applies in other forms of autoimmunity and complicates immune interventions for these conditions.

Enhanced T lymphocyte metabolism and activation could be perpetuated by cell-cell interactions or cell intrinsic signaling pathways. My data seemed to suggest that intrinsic signaling defects in the CREB/ATF-1 pathway led to altered responses to anti-CD45RB. Cytokines such as IL-2, IL-6, IL-10 and TNF-a all contain CRE responsive elements, making CREB/ATF-1 an important modulator of immunity[359]. Additionally, these cytokines have been associated with effector resistance in SLE mice, making it possible that a CREB pathway effect may exacerbate metabolism transcriptionally by altering metabolic genes and may drive

improper cytokine production that alters T lymphocytes in other ways[383–385]. Even though my studies did not demonstrate enhanced AKT/mTOR signaling in SLE T lymphocytes, the contribution of this pathway to abnormal metabolism through non-TCR induced signaling pathways cannot be ruled out as contributory to metabolic abnormalities in SLE.123 mice, and would be a barrier in older or more severely affected mice.

Anti-CD45RB induces changes in B lymphocyte adhesion molecules that facilitate T-B lymphocyte interactions[368]. In non-autoimmune mice this change could lead to T lymphocyte inhibition, but in SLE123 mice it could lead to abnormal activation and metabolic resistance. It would be an area of future interest to determine whether genetic or therapeutic depletion of B lymphocytes alters T lymphocyte metabolism in SLE123 mice. In NOD mice T lymphocytes have also been demonstrated to resist immune regulation, but there is still no consensus on the importance of this phenotype[386]. Nevertheless, we demonstrated that NOD mice resist immune regulation and that loss of B lymphocytes restores tolerance induction in NOD mice. In data, not shown here, I determined that NOD T lymphocytes do not resist metabolic modulation by anti-CD45RB, indicating B lymphocytes may mediate effector resistance through multiple mechanisms.

As tetramer and antigen specific technology improves, it will be important to determine the antigen-specific cells that govern effector resistance. Studies carried out by our lab and others have focused on the large effects of T and B lymphocyte mediated regulation via suppression of proliferation or cytokine production of antigenically diverse effector cell populations. While data from our lab indicated Treg development may be altered by B lymphocytes in NOD mice, it would be of interest to determine whether erosion of antigenically-competent Tregs may underlie failed regulation in SLE123 mice. In order to assess this possibility, TCR-sequencing of Tregs

and effectors and antigen-specific tetramer tracking should be carried out in SLE123 mice. Additionally, it would be interesting to determine whether metabolic modulation in the thymus may facilitate the development of competent or antigen-specific Tregs. Data from my work demonstrated thymic NOD B lymphocytes possess increased levels of ICOS-L, which can modulate AKT signaling in thymic T lymphocytes, which express higher levels of ICOS than peripheral T lymphocytes (**not shown**). These data indicate B lymphocytes may also participate in metabolic direction of developing T lymphocytes. The contribution of metabolic competition between Tregs and effectors for survival and emergence of these cells into the periphery is poorly understood and represents a new avenue of exploration

On defining a single-lineage of B regulatory cells

This study illustrates B lymphocytes have the capacity to regulate the immune system in multiple ways. This begs the questions "What is the most important immunoregulatory mechanism or what immune regulatory mechanism defines "Bregs"? At this moment the conventional answer seems to be IL-10, although many studies demonstrate that IL-10 does not absolutely define Bregs nor does it provide immune regulation in all contexts assessed[48,370]. When comparing "Bregs" to traditional $CD4^+$ $Foxp3^+$ Tregs, it is evident that phenotypic definition and conserved mechanistic function of these cell types lags far behind, but maybe that is appropriate.

Tregs are specialized cells that can recognize one peptide sequence in the context of MHCII and its decision is binary--affect immune regulation or ignore the stimulus. B lymphocytes are antigen-specific cells as well, but they can recognize antigens of any origin (protein, lipid, synthetic, etc.), internalize that antigen, process it and present it to T lymphocytes

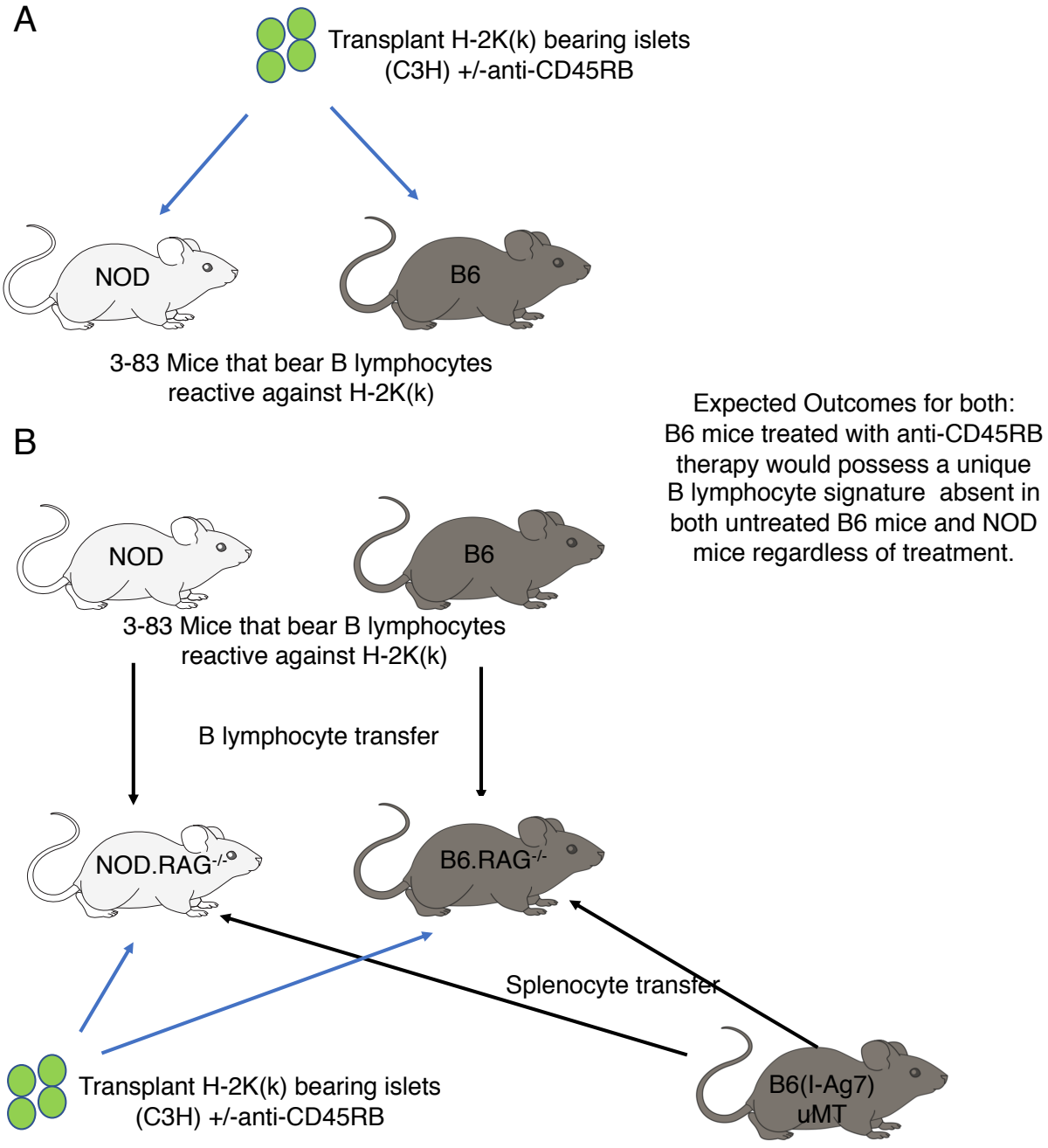
in multiple contexts to drive differential activation. In contrast to other antigen presenting cells, B lymphocytes can concentrate their cognate antigen and related antigens to provide an enriched-antigen surface for T lymphocytes to bind. Additionally, B lymphocytes can facilitate this action at a distance, especially in the case of circulating antigens like insulin. This capacity leads to the possibility that B lymphocytes could orchestrate and mobilize immune regulation at a distance in response to changes in circulating antigen. This potential also means B lymphocytes can concentrate and present antigen absent of the local cytokine milieu, which may influence the context in which they present antigen. Indeed, my work demonstrated that nIgM may be one such mode of B lymphocyte regulation that occurs at a distance. B lymphocytes that most robustly secrete IgM, marginal zone and B1 B cells, overlap with phenotypically with described IL-10 Bregs[183]. This indicates the possibility that the therapeutic contribution of IL-10 may have been overestimated in these studies.

My studies also demonstrated that B lymphocytes have the capacity to modulate tissue health in novel ways. It is still, however, important to emphasize that it is unclear whether any of these B lymphocyte-mediated mechanisms of immune regulation lead to robust and specific immune tolerance. nIgM appeared to have the greatest antigen-specific impact on modulation of autoreactive B lymphocytes and mobilization of disease-reversing Tregs. Still, its ability to allow restoration of immune tolerance needs to be assessed in an antigen-specific way in transplant models. This being said, utilizing the modulatory properties of B lymphocytes I defined in this dissertation may be clinically applicable among certain autoinflammatory conditions in humans. It will be important to carefully determine whether certain pathologic processes occur in humans before applying B lymphocyte mediated regulation (i.e. is ROS important in humans with T1D who are currently enrolled in clinical trials?).

It still remains unclear whether researchers can define a specific lineage of Bregs. I believe the best chance of discovering a Breg lineage will be through the utilization of B lymphocyte antigen-specific models on the NOD and B6 background. Studies from our lab indicate that B lymphocyte modes of regulation are dysfunctional in NOD mice, as is clearly indicated by islet-transplant studies, data from IgM therapy, and historical evidence of B lymphocyte autoantibody secretion and homeostatic defects in humans and mice with T1D. I would first address the role of alloreactive B lymphocytes in transplant tolerance induction utilizing anti-CD45RB. I would utilize the 3-83 mouse that contains Ig specificities against MHC-I H-2K(k) and to a much lesser degree H-2K(b)[386]. These mice exist on both B6 and NOD backgrounds. As can be illustrated in **Fig 6.3**, I would transplant both B6 and NOD 3-83 mice with islets from C3H (H2-K(k) bearing mice) and treat with tolerizing therapy anti-CD45RB. I would then track rejection of islets in both strains. I would expect the B6 would become tolerant in the presence of anti-CD45RB while NOD mice would not. I could then use CyTOF to analyze the phenotype of these antigen specific B lymphocytes in the presence of anti-CD45RB and transplanted islets. I would expect the B6 mouse to contain a unique B lymphocyte signature in tolerance that would be absent in NOD mice. As my work illustrated, B lymphocytes in the spleen, thymus, bone marrow and at the site of the graft could all play a role in mediating immune tolerance. As such, I would make sure to analyze all these compartments. To eliminate the contribution of abnormal NOD T lymphocytes to this interaction, I would also utilize B lymphocytes from B6 or NOD 3-83 mice total from B6.g7 mice on a B lymphocyte deficient background, which bear the same MHCII as NOD mice, in an NOD.RAG model (**Illustrated in Figure 6.3**). It may also be that there are no differences in the capacity for B lymphocytes to regulate alloantigen in B6 and NOD mice or the mild reactivity to H-2K(b) possessed by the B6

might confound the results. If this is the case. these experiments would then be repeated utilizing the insulin-reactive B6 and NOD.VH125SD B lymphocytes utilized in Chapter IV to determine importance of autoreactive B lymphocytes. The advent of antigen specific MHC tetramer technology would allow the tracking of the phenotypes of antigen-specific T lymphocytes in these various models to determine the role of B lymphocytes in directing antigen-specific responses in T lymphocytes.

As demonstrated by my study, the regulatory capacity of B lymphocytes is robust and varied, with multiple mechanisms that could positively or negatively impact tissue function. In moving forward in Breg immunology it will be important to determine how a particular B lymphocyte regulates tissue damage. It is not sufficient to merely attribute regulatory capacity to IL-10 as IL-10 is counterregulatory to immune tolerance in multiple systems. The holy-grail of immune regulation will be the identification of antigen-specific B lymphocytes with the capacity to impact systemic immunity in a durable and antigen-specific.


A
Transplant H-2K(k) bearing islets
(C3H) +/-anti-CD45RB
NOD
B6
3-83 Mice that bear B lymphocytes
reactive against H-2K(k)
B
Expected Outcomes for both:
B6 mice treated with anti-CD45RB
therapy would possess a unique
B lymphocyte signature absent in
both untreated B6 mice and NOD
mice regardless of treatment.
NOD
B6
3-83 Mice that bear B lymphocytes
reactive against H-2K(k)
B lymphocyte transfer
NOD.RAG-/-
B6.RAG-/-
Splenocyte transfer
Transplant H-2K(k) bearing islets
(C3H) +/-anti-CD45RB
B6(I-Ag7)
uMT

Defining B lymphocyte regulation in T1D clinical trials

To effectively determine whether B lymphocyte regulation plays a role in clinical therapy, the clinical application of therapies needs to change in T1D, and quickly. The overwhelming failure of NOD-derived therapies in humans with T1D is a perplexing problem. While it may be

informative to apply other animals models of autoimmunity (like SLE) to gain additional insight into failure of immunologic tolerance, many researchers want to abandon mouse models in favor of utilizing samples from humans with T1D. Unfortunately, humans with T1D do not provide the capacity to comprehensively analyze the immune interactions that may govern disease. The NOD mouse represents our best preclinical model of T1D. So the question is, how do we as researchers improve the clinical efficacy of T1D trials?

Illustrative of this point is the clinical use of anti-CD20 (Rituximab) in humans with T1D. Initial studies in NOD mice (harboring a transgenic human CD20) demonstrated that robust depletion of B lymphocytes with anti-CD20 prevented and even modestly reversed diabetes[387]. This observation was linked to a partial restoration of B lymphocyte homeostasis in repopulated B lymphocytes post-therapy, reduction in inflammatory cytokine production, and Treg expansion[388]. While it was unclear whether these changes were mechanistically important, theywere indicative of a "successful" response to therapy. Clinical trials in humans largely ignored these results and proceeded directly to reversal trials[120]. Once these trials ultimately failed, enthusiasm for anti-CD20 and consequently B lymphocyte therapies waned significantly. Follow-up studies in patients with T1D, treated with anti-CD20, revealed that their repopulated B lymphocyte was not restored to normal homeostasis, indicating the therapeutic dose did not achieve the same immunologic effect in humans as it did in NOD mice[119]. This caveat suggests

possibility that the dosage must be altered or combined with other therapeutics to achieve a similar immunologic result between murine models and patients. Additionally, the therapy may only work on a subset of patients with T1D, or it may be the immunologic mechanisms between mice and humans are distinct in regards to anti-CD20's impact on T1D. Nonetheless, as interest in anti-CD20 in diabetes wanes, it remains unknown what the true answer is.

Improve Clinical Benchmarks of Therapeutic Effectiveness

Genome-wide association studies reveal T1D is a diverse disease[389]. While the overwhelming contribution of MHC polymorphisms reveals the immunologic underpinnings of this disease, the pathologic process is different individual-to-individual, as evidenced by variable time of onset and the diversity of autoantibodies in individuals with T1D. Current clinical trials in T1D have a miserable track record in impacting disease prevention or onset with only a handful of studies demonstrating modest preservation of C-peptide levels at the end of the study, with those levels declining at a similar rate shortly thereafter. C-peptide is traditionally the endpoint measurement for success of type 1 diabetes trials, as it indicates survival of beta cells. While preservation of beta cell function is the end goal of T1D trials, it is a glaring oversight that immunologic readouts have not been primary outcome measures for most clinical trials.

We, as researchers and clinicians, have the responsibility to carefully and methodically proceed with clinical trials. While many drug therapies proceed to market with little understanding of mechanism, their robust activity lends them to FDA approval. Unfortunately, the history of clinical trials in T1D demonstrates that immunologic therapies will require more mechanistic dissection. If T1D human clinical trials are to be successful, it will require understanding of immune responses to therapies in humans, utilizing murine models as guidelines.

Mounting evidence indicates it is likely T1D will not have a "one-size-fits-all" therapeutic option. Careful clinical design in T1D should involve vigorous genetic and immunologic screening of patients before enrollment followed by immunologic outcomes in Phase I trials. These studies can then be compared directly to murine studies to determine whether the dosage reached the immunologic benchmarks achieved in mice. There can then be careful evaluation of responders and non-responders to aid enrollment of the proper candidates for subsequent trials. While the "find a cure now" mentality, the driving mantra of T1D foundations and researchers, drives rapid translation of T1D therapies to clinic, without caution and careful consideration the "cure T1D now" mentality may lead to "cure T1D never" reality.

Test therapies in stringent models of transplant tolerance induction

The immunologic role of beta cell destruction in NOD mice and humans with T1D is clear. In NOD mice many therapies can prevent further beta cell destruction, while a select few are capable of reversing disease. Unfortunately, NO immunologic intervention has ever successfully induced transplant tolerance in NOD mice. This dissertation indicates a failure of generalized immune tolerance mechanisms associated with both T and B lymphocyte mediated regulation. Only a handful of therapies that reverse or prevent disease in NOD mice have been tested in transplant tolerance, and all have failed to provide durable and specific tolerance, even though they have the capacity to do so in non-autoimmune strains[288]. Utilization of a transplant model in NOD mice will allow researchers to determine what therapies have the capacity to restore specific and durable endogenous tolerance mechanisms in states of autoimmunity.

In testing these therapies, preference should be given to those that induce tolerance in NOD mice or those that, when combined with established tolerance mechanisms, make NOD mice amenable to tolerance induction. I hypothesize that the most successful therapies in humans will be those that restore endogenous tolerance mechanisms as opposed to those that deplete or immunosuppress. In regards to my findings, I would utilize nIgM in NOD mice in a transplant model to determine whether it had the capacity to induce tolerance to transplanted islets. As my data indicates it increases the Treg response to anti-CD45RB, I would most likely used it as a preconditioning therapy in NOD mice before anti-CD45RB. Additionally, combination of nIgM with anti-CD45RB in antigen specific B lymphocytes (3-83 or VH125 SD) on NOD or B6 background may reveal emergence of antigen-specific B lymphocytes with regulatory capacity.

Cross scientific lines to gain a complete picture of T1D pathology

While the majority of this discussion has focused on the role of the immune system, it is clear that risk factors related to islet cell health are also associated with T1D[389]. In fact MRI studies in T1D are underway to determine whether defects in pancreatic mass appear even before overt diabetes[389]. Additionally, polymorphisms in insulin, associated with T1D, may contribute to beta cell dysfunction independent of the immune system, making beta cells more sensitive to stressors[390]. My studies with imatinib illustrated the interplay between beta cell health and the immune systems in ways that were unexpected. This discovery would not be possible without collaboration with beta cell biologists Roland Stein and Jason Spaeth. While my original studies with imatinib demonstrated a B lymphocyte mediated effect on diabetes reversal, without observing the increase in ROS sensitive Mafa, the antioxidant capacity of B lymphocytes would have remained uninterrogated. Collaboration with researchers who study islet biology and whole body metabolism will be essential to understand the holistic response of patients to T1D therapeutics.

Conclusion

Reversing autoimmunity is a daunting task. When I first began to consider whether B lymphocytes could play an important role in prevention or reversal of autoimmune disease, I was drawn by their diverse function coupled with their antigen specificity. It is disheartening that the Breg scientific community has boxed the "Breg" into an IL-10 producing cell. This is especially frustrating when IL-10 demonstrates counter-regulation in many immune models, including T1D. Additionally the B lymphocyte possesses so many more potent and specific ways to influence immune regulation; as illustrated by my thesis work, the capacity of B lymphocytes to modulate autoimmune mediated damage is impressive. These B lymphocyte mediated mechanisms may provide profound clinical relief to those who suffer from autoimmune disease, especially T1D. The caveat being, my studies DO NOT illustrate a mechanism of specific and durable tolerance mediated by B lymphocytes (future studies with nIgM may reveal potent antigen-specific effects). Additional studies, utilizing antigen-specific B lymphocytes in states of autoimmunity and health will facilitate the definition or lack thereof of a potent, antigen-specific Breg. Due to a preponderance of evidence, I do believe such a cell exists but will be challenging to find. Once functionally defined, its role in immunoregulation can be elucidated and utilized. Even if this cell demonstrates durable and specific tolerance induction, it must be remembered that the immune system is a complex beast, with every arm contributing some function to both destruction and regulation. Thus, the temptation to practice reductionist science in relation to achieving immune tolerance must be avoided in order to effectively understand how to achieve long-lasting immune homeostasis and health in autoimmune disease.

CHAPTER VII

MATERIALS AND METHODS

Animals

All mice were housed and maintained according to the guidelines for use and care of laboratory animals as set forth by Vanderbilt University. All NOD mice were monitored for the development of diabetes by blood glucose measurement with Accu-chek test strips (Roche Diagnostics, Indianapolis, IN). Two consecutive glucose measurements >220mg/dL constituted a diagnosis of diabetes. Animals in our colony have a spontaneous diabetes incidence of 80-90% in females by 30 weeks of age. Before terminal analysis mice were euthanized with isoflourane followed by cervical dislocation. Mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or Charles Rivers (Wilmington, MA) or kindly donated by the investigators listed in **Table 7.1**. Prior to use, all mouse genotypes were confirmed by PCR using primers published by the strain's donating investigator. Humanized BLT mice were created as described before

Strain name (Abbreviated Strain Name)	Phenotype/Purpose for Study	Source
C3H/HeJ (C3H)	*MHC Mismatched islet donor*	*JAX - 000651*
C57BL6/J (B6)	*Non-autoimmune (control)*	*JAX - 000664*
Sle1NZM2410/Aeg Sle2NZM2410/Aeg Sle3NZM2410/Aeg/LmoJ (B6.SLE123)	*Fully Penetrant SLE model*	*JAX - 007228*
NOD/ShiLtJ (NOD	*T1D prone moues*	*JAX - 001976*
NOD.129S7(B6)-Rag1tm1Mom/J (NOD.RAG)	*T and B lymphocyte deficient T1D prone mouse*	*JAX - 003729*
NOD.129S2-Ighmtm1Cgn/Dvs (NODuMT)	*B lymphocyte deficient T1D prone mouse*	*JAX - 003903*
Swiss Webster (CFW)	*Non-autoimmune source of nIgM*	*Charles Rivers 024*
(VH125^{SD}.B6)	*Insulin specific BCR in small number B cells due to insulin specific heavy chain*	*Donated by James W. Thomas*
NOD.129P2(Cg)-Ightm1.1Jwt/J (VH125^{SD}.NOD)	*Insulin specific BCR in small number B cells due to insulin specific heavy chain*	*Donated by James W. Thomas*

Table 7.1 List of mouse models, phenotypes and sources used in these studies.

Magnetic-activated cell sorting (MACS) purification

B lymphocytes were purified form splenocytes following positive selection via murine CD45R microbeads. (B220 Miltenyi Biotec) Briefly, total splenocytes were incubated in PBS with 1% HI-FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml), with 10uls of CD45R microbeads per 10 million total cells for 10 mins at 4 degrees Celsius. Cells were then passed over an LS columns (Mitlenyi Biotec) in a magnetic field, trapping B lymphocytes in the column. B lymphocytes were eluted with 5mls of the previously described buffer. Untouched $CD4^+$ T lymphocytes were purified by negative selection utilizing $CD4^+$ T lymphocyte sorting kit (Miltenyi Biotec). This kit contains biotinylated-antibodies against CD8a, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, Anti-MHC-class II, Ter-119 and TCRγ/δ. These are incubated with total splenocytes, washed and then followed by incubation with magnetic anti-biotin microbeads. These are also passed over and LS column in a magnetic field. All non $CD4^+$ T lymphocytes are trapped in the column. Unlabled CD^+ T lymphocytes are allowed to pass through, yielding a purity of ~97%.

Standard and intracellular flow cytometry

Lymphocytes were plated at a concentration of 1 million cells per well in 96 well V-bottom plates. Plates were then centrifuged at 550g for 3 minutes, supernatants discarded, and cells were stained in a 50ul volume of FACS Buffer (PBS + 0.1% sodium azide + 3% Fetal Bovine Serum) composed of various panels of fluorophore-conjugated antibodies purchased from either BD Biosciences, San Jose, CA or eBioscience, San Diego, CA. Extracellular fluorophore-conjugated antibodies used are listed **in Table 7.2**. Intracellular flow cytometry involved fixing with 4% PFA and permabilizing on ice with 0.1% Triton or Fixing and permeabilizing simultaneously

with the Foxp3/Transcription Factory Staining Buffer Set (ThermoFisher). All intracellular antibodies utilized are listed in **Table 7.2 and Figure 3.3A**.

Isolation of lymphocytes

Lymphoid organs (Spleens or thymi) were prepared by dispersion of the organ and passage through a 70-μm cell strainer followed by red cell lysis. Cell sieves were then washed three times with ice-cold PBS into the same 50mL conical tube to ensure maximal cell recovery. Isolated cells were then spun at 550g for 3 minutes and the resulting cell pellet was lysed of contaminating red blood cells via ACK Lysis Buffer for 5 minutes. The lysis buffer was then quenched via dilution with PBS. Isolated cells were then centrifuged, resuspended in 10 mL of PBS, and counted via an Automated Cell Counter (BioRad, Hercules, CA). For isolation of lymphocytes from pancreatic tissue cells were digested via collagenase disruption. Briefly, pancreas were isolated and chopped with scissors into 1-2mm pieces. The pancreas was then placed into 15 mls prewarmed digestion buffer. This digestion buffer was made in 50ml aliquots by combining 0.5mL of 0.5M $CaCl_2$, 0.5ml of Collagenase D in Hank's Balance Salt Solution with 5% fetal bovine serum. Chopped pancreas and digestion buffer were incubated at 37 degrees Celsius in a water bath for 15 minutes, with shaking every 5 minutes. Digestion was centrifuged once and then washed HBSS containing 10% FBS. The digest was then passed through a 100uM cell strainer and washed additional times with HBSS with 10% FBS until supernatant was clear. Cells were stored on ice until further analysis.

Antibody	Clone or Species origin
B220	RA3-6B2
IgM	II/41
CD21	7G6
CD23	B3B4
IgD	11-26c.2a
CD43	S7
CD19	6D5
CD4	RM4-5
CD36	No.72-1
CD9	KMC8
CD25	PC61
CD8	53-6.7
CD11b	M1/70
CD62L	MEL-14
CD44	IM7
CD69	H1.2F3
CD40	HM40-3
CD80	16-10A1
CD180	RP/14
MD-1	MD14
CD274	MIH5
CD284	UT41
CD1d	1B1
IgM(a)	MA-69
IgM(b)	AF6-78
TOSO	4B5
Helios	22F6
SOD1	Rabbit Polyclonal
SOD2	Rabbit Polyclonal
pNFkB (p65)	Rabbit Polyclonal
NFATc1	Rabbit Polyclonal
pCreb	Rabbit Polyclonal
ATF-1	Rabbit Polyclonal
Creb	Rabbit Polyclonal
pCRTC2	Rabbit Polyclonal
CRTC2	Rabbit Polyclonal

Table 7.2 List of antibodies used in flow cytometry studies

Phosphoflow cytometry and B and T lymphocyte stimulation

To activate signaling in B lymphocytes total splenocytes were stimulated with 10ug/ml anti-IgM (Jackson) in a 96-well plate. To activate signaling in CD4 T lymphocytes were stimulated with Biotin anti-mouse CD4, Biotin anti-mouse CD3, and anti-mouse CD28 (all from Biolegend), this was then crosslinked with Streptavidin (Sigma). Cells were then fixed and permeabilizing according to the methods described in the intracellular flow cytometry section.

CyTOF mass cytometry and SPADE analysis

CyTOF was carried out by the Mass Cytometry Center for Excellence at Vanderbilt. A list of antibodies and metals utilized are listed in **Table 7.4**. SPADE analysis was carried out on the Cytobank online platform (https://www.cytobank.org/).

***Ex vivo* stimulation with TLR ligands**

Cells were plated in 96-well plates at a density of 1×10^6 total cells/ml in DMEM containing 10% HI-FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), beta-mercaptoethanol (55μM), and varying amounts of the identified stimulus. Stimuli included LPS, Imiquimod, and PolyIC, according to manufacturer's instructions. (Invivogen, San Diego, CA). All cells were incubated for the indicated times at 37°C in 5% CO2.

In vivo treatment with polyIC

9-10 week old female NOD mice were injected with 100ug PolyIC resuspended in 100uls of saline(Invivogen, San Diego, CA) once daily for 9 days.

Adoptive transfer and diabetes protection by polyIC

Female NOD/scid mice were given 20×10ˆ6 cells from the spleen of a diabetic female donor. All donors had diabetes for at least one week. For diabetes protection, DX5+ cells were obtained from polyIC treated NOD or NOD.μMT mice after a 9 day course of polyIC delivered intraperitoneally at 100ug/day. Cells were obtained via MACS (Miltenyi), using two columns for enrichment and reaching final cell purity >95%.

Analysis of Actin Depolymerization

Splenocytes were isolated and plated in 100μl of culture media. Kinase inhibitors Dasatinib, Src-Inhibitor 1, or 1uM Latrunculin were added for 1 hour before stimulation (Sigma) where indicated. Splenocytes were stimulated with 100μl 10mM peroxide or left unstimulated for the times indicated. Cells were fixed with 4% Paraformaldehyde for 20 mins and permeabilized on ice with 0.1% Triton x-100 in PBS. They were stained with Rhodamine Phalloidin and B cell markers (B220, CD21, CD23) in PBS containing 0.1% Triton X-100, 0.1% azide and 3% FCS and analyzed by flow cytometry.

Antibody	Metal Conjugate
CD48	(sm154)di
CD45	(sm147)di
CD5	(gd160)di
CD43	(nd146)di
CXCR5	(nd142)di
CD9	(gd158)di
CD23	(tb159)di
CD1d	(dy162)di
CD44	(yb171)di
CD86	(yb172)di
B220	(yb176)di
CD11b	(sm148)di
CD40	(dy161)di
CD54	(dy163)di
CD21	(yb168)di
CD3e	(sm152)di
IgM	(eu151)di
CD8a	(eu153)di
IAIE	(yb174)di
CD4	(nd145)di
CD19	(sm149)di
IgD	(nd150)di
CD38	(lu175)di
Cisplatin	(pt195)di

Table 7.3 List of antibodies used in CyTOF studies

Imatinib Treatment and Diabetes Reversal

Imatinib Mesylate (Eton Biosciences) was dissolved at a concentration of 1.5mgs/100ul of saline. As the original studies in NOD mice utilized gavage to deliver Gleevec (imatinib) suspended in peanut oil, we first determined whether imatinib delivered via i.p. was equally effective at reversing T1D (1). In initial studies 100uls of Imatinib was injected into diabetic NOD mice for up to 3 weeks. For studies involving cellular analysis Imatinib was injected for 5 days at a concentration of 1.5mg/100uls. Imatinib treated mice were given 100 ul injections (1.5mg) of Imatinib once diabetic (blood glucose >200mg/dl on two consecutive measurements). NODRag1-/- mice were given 10x106 splenocytes depleted of B220+ cells by MACS. Once diabetic these mice were given 1.5mg of Imatinib in 100uls of saline alone or concurrently with B lymphocytes (20x106).

Treg depletion with anti-CD25 or cyclophosphamide

For studies carried out in NOD.uMT T reg depletion was carried out by two injections of anti-CD25 4 days apart (2mgs per mouse) (PC61 from BioXcell) or two injections of cyclophosphamide. For depletion of Tregs in nIgM treated reversed NOD mice, they were injected with anti-CD25 (2mgs per mouse) on day 1 and the again on day 7. (PC61 from BioXcell)

Analysis of imatinib induced apoptosis *ex vivo*

Cells were plated in 96-well plates at a density of 1×10^6 total cells/ml in DMEM containing 10% HI-FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), for 4 hours with the indicated stimulus. To demonstrate imatinib mediate B lymphocyte death via apoptosis some wells were co-incubated with Caspase-9 inhibitor III (CAS 403848-57-7 Santa Cruz). Cell death was measured via intracellular staining with antibodies against Cleaved PARP (BD Biosciences Asp175) and Cleaved Caspase-3 (Cell Signaling Asp175).

Pancreas Preparation and Immunostaining

Pancreata were fixed in 4% (vol./vol.) paraformaldehyde, embedded in OCT, and cut to 6μm. Sections were blocked with 5% (vol./vol.) normal donkey serum in 0.5% BSA/PBS (wt/vol.) and incubated with primary antibodies overnight at 4°C. Cyanine dye (Cy)2-, Cy3-, or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, 1:2,000) were used for fluorescent detection. DAPI (diamidino-2-phenylindole) dye (SouthernBiotech) was used to detect nuclei in immunofluorescent images. The following primary antibodies were used: insulin (DAKO, A056401-2, 1:1,000); glucagon (Cell Signaling, #2760, 1:2,000); Ki67-mouse (BD Pharmingen, 550609, 1:1,000); MafA (Novus Biologicals, NBP1-00121, 1:500); Nkx6.1 (Novus Biologicals, NBP1-49672, 1:500); Urocortin 3 (Phoenix Pharmaceuticals, H-019-29, 1:1000); Pax6 (Covance, PRB-278P, 1:1000); Nkx2.2 (Santa Cruz, sc-15015,1:2000) and Pdx1 (gift from C. Wright, Vanderbilt University, 1:20,000). Images were collected on a Zeiss Axioimager M2 (Jena, Germany) fluorescent microscope.

For beta and alpha cell area measurements, six sections (~240 μm apart) were analyzed for insulin or glucagon positive area with HRP-secondary staining using the DAB substrate kit

(Vector Labs, Burlingame, CA) and counterstained with eosin. Images were collected on an Aperio ScanScope (Leica, Buffalo Grove, IL, USA) whole slide scanner and the percentage of beta and alpha cell area relative to whole tissue area (eosin) was calculated. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed on sections using the Click-iT TUNEL Assay kit (ThermoFisher, C10617).

Measurement of serum insulin

Serum from fasted NODRag1$^{-/-}$ mice was collected via retroorbital bleed. Serum was separated from platelets by centrifugation of whole blood. Insulin concentration was determined by Luminex assay by the Vanderbilt Hormone and Analytical Core.

HORAC Activity Assay

Analysis of the antioxidant capacity of supernatants from Imatinib treated B lymphocytes was carried out according to manufacturer's recommendation (Eagle Biosciences). Briefly, 10x10^6 B lymphocytes were incubated in 60 uls HBSS+1% fetal calf serum for 5 hours at 37C in the presence of 10uM Imatinib or with media only. As a control wells with only 60uls of HBSS+1% FCS with or without Imatinib were incubated on the same plate for the same time period. At the end of the assay cells were pelleted via centrifugation and 20uls of supernatant was acquired for each well, all samples were assayed in duplicate. Supernatant was transferred to a black-walled optical plate to which was added 120uls of assay media, 20uls of Fenton reagent and 20 uls of hydroxyl radical solution. Wells were thoroughly mixed and fluorescence was measured every minute for 45 minutes on a fluorescent plate reader. As an assay control gallic acid with a known antioxidant capacity was run on the same plate.

Measurement of insulin reactive B lymphocytes

Insulin-binding BCRs were detected using biotinylated human insulin (Sigma-Aldrich) followed by staining with streptavidin conjugated to Alexa Fluor 647 (Life Technologies). This staining was coupled with staining of extracellular B lymphocyte markers and analyzed by flow cytometry.

Purification of IgM

IgM was purified by size-exclusion column chromatography (Sephacryl S-300 HR; GE Healthcare, Piscataway, NJ) from irradiated, heat-inactivated (56°C ×1 hour) WT Swiss-Webster murine sera as detailed here. nIgM was not isolated by dialyzing sera in water or by ammonium chloride precipitation because these techniques yield IgM with impaired functional activity. Column-purified nIgM was repassaged through Sephacryl S-300 to remove contaminating IgG and other proteins. With this approach, more than 92% of the protein fraction contained nIgM with less than 1% IgG, less than 3% albumin, and less than 1% other protein contaminants as determined by protein electrophoresis and ELISA. We did not affinity purify nIgM antibodies, as such procedures (binding of nIgM to mannan-binding protein or binding of nIgM to agarose coupled with goat anti-IgM antibodies) yield 10% to 15% of the starting IgM and have the potential to deplete certain IgM fractions. Purified nIgM was concentrated to 1.3 to 1.5 mg/mL (higher concentration led to nIgM aggregation and precipitation), dialyzed against RPMI 1640, and micro filtered using a 0.45-μm Millipore filter, before use in cultures or in vivo. Purified IgM were stored at 4°C to prevent precipitation that occurs when frozen. All preparations had undetectable endotoxin activity.

Dosage of nIgM, hIgM, and anti-BAFF

For diabetes reversal, NOD mice were treated by i.p. injection with two doses (100µg) of NOD or Swiss Webster nIgM on Day 1 and Day 4 after diabetes onset. For cellular analysis NOD and B6 mice were treated by i.p. injection with an initial dose of 100µg of SW nIgM on day 1, followed by 50ug doses on days 3, 5, 7, and 10 and sacrificed on day 13. For human IgM testing, humanized BLT mice were treated by i.p. injection with 100µg of human IgM on Day 1, followed by 70µg on days 5 and 10 with analysis on day 13. In experiments where anti-BAFF (Sandy-2 from AdipoGen) was used 100µg was injected into NOD mice on day -2, concomitantly with 100µg of SW nIgM on day 1 and another dose of anti-BAFF and nIgM on day 3 before being sacrificed and analyzed on day 4.

Measurement of insulin autoantibodies

Longitudinal evaluation of IAA levels was conducted using plasma samples obtained from female NOD/ShiLtJ mice injected with saline or IgM. Plasma insulin autoantibody (IAA) was measured by micro-IAA radio immunoassay at the Barbara Davis Center for Diabetes, Colorado.

Thymus and Kidney Histology and Analysis

Thymuses were harvested into 10% Formalin. Slides were then embedded in paraffin and sectioned in 5-µm sections. The slides were stained for hematoxylin and eosin (H&E) and double-stained for B220 and Foxp3. Thymus sections were then imaged on a brightfield Aperio ScanScope and acquired at 20x using Aperio ImageScope. Sections were analyzed for colocalization using Halo software (Indica Labs). Medullary spaces were defined as areas of light H&E staining.

Kidneys were harvested into 10% Formalin. Slides were then embedded in paraffin and sectioned in 5-µm sections. Rehydration was carried out by sequential exchanges of xylene and ethanol baths. An additional antigen retrieval step was utilized with citric acid based antigen unmasking solution (Vector Labs) and microwaving. Slides were then stained overnight with anti-mouse IgG followed by anti-species antibody conjugated to PE. Images were taken via microscope at 20x and total fluorescence was quantified by Halo software (Indica Labs).

Measurement of T lymphocyte proliferation

Total splenocytes were stained with eBioscience Cell Proliferation Dye eFluor 450 (ThermoFisher 65-0842-85) following manufacturer's recommendations. Total splenocytes were plated in 96-well plates at a density of 1×10^6 total cells/ml in DMEM containing 10% HI-FCS, penicillin (100 U/ml), streptomycin (100 µg/ml), beta-mecaptoethanol (55µM), and incubated with anti-CD3e LE/AF (145-2C11 BD Biosciences) or anti-CD28 LE/AF (37.51 BD Biosciences). As the differences in treatment groups was so stark, proliferation was measured a percent dividing via flow cytometry.

RNA sequencing and Bioinformatic Analysis

MACS purified $CD4^+$ T Lymphocytes were submitted to Novogene (Beijing, China) for HiSeq platforms with paired-end 150 bp (PE 150) sequencing strategy. Upregulated and downregulated genes were entered into KEGG analysis (http://www.kegg.jp/). KEGG analysis was used as a screening tool for regulated pathways. When identifying the metabolic pathway as a pathway of interest both p-value and gene ratio were taken into account. The metabolic gene list was then subjected to cluster analysis via String Database (https://string-db.org/), to determine the individual processes each gene belonged to. To determine transcription factors important for the metabolic gene modulation were analyzed by Transfac (https://changlab.uth.tmc.edu/gather/gather.py). P-value and Bayes factor were taken into account to determine the relevantly enriched transcription fators for that gene set.

Untargeted Metabolomic analysis

MACS purified $CD4^+$ T Lymphocytes were submitted to the Mayo Clinic. They were then analyzed by untargeted Mass spectrometry, aligned, and identified at Mayo Clinic.

Seahorse metabolic analysis

MACS purified $CD4^+$ T Lymphocytes were submitted were plated on a Seahorse 96 well plate at a concentration of 500,000 cells per well. These cells were then subjected to automated Seahorse analysis as outlined in the text.

Measurement of Glucose uptake

Whole splenocytes from B6 and SLE123 mice were plated at a concentration of 1 million cells per well in 96-well V-bottom plates in glucose-free CCM and cultured for 1 hour at 37°C. Cells were then supplemented with 30μM of the fluorescent glucose analogue 2-NBDG (Thermo-Fisher Scientific, Waltham, MA) and cultured for a further 30 minutes at 37°C. Splenocytes were then stained for antibodies directed against CD4 and analyzed by the methods described in the Conventional Flow Cytometry section.

Measurement of mitochondrial function

Whole splenocytes from B6 and SLE123 mice were plated at a concentration of 1 million cells per well in 96-well V-bottom plates in CCM and cultured for 1 hour at 37°C. Cells were then incubated with 100nM MitoTracker Red CMXRos (M7512 ThermoFisher) or 60nM MitoTracker Green FM (M7514 ThermoFisher). Splenocytes were then stained for antibodies directed against CD4 and analyzed by the methods described in the Conventional Flow Cytometry section.

Treatment of mice to inhibit the metabolic effect of anti-CD45RB

Mice were dosed with the anti-CD73 (BioXcell), A2AR inhibitor (SCH 58261 Fisher), Cyclosporine A (Fisher), CREB inhibitor (666-15 EMD Millipore), or VIVIT (480401 Calbiochem), as indicated in the text.

Nuclear isolation protocol

Following Stimulation of MACS purified $CD4^+$ T lymphocytes with anti-CD45RB or Isotype control (20ug/ml). Cells were resuspended on ice for 15 mins in a buffer containing 320mM Sucrose, 10mM HEPES, 8mM $MgCl_2$, 1x Roche EDTA-free cOmplete Protease Inhibitor, and 0.1% (v/v) Triton X-100. Cells were then incubated in the same buffer with 4% Paraformaldehyde, without Triton X-100. The isolated nuclei were then washed and permeabilized with 0.3% Triton X-1000. Cells were then stained with the antibodies indicated in the text.

Analysis of nuclear localization by Flowsight imaging cytometry

Total splenocytes were fixed and permeabilized according to the protocols described in the intracellular flow staining section. These cells were then stained with SYTOX Green Nucleic Acid Stain- 50nM solution in DMSO. They were then stained with the indicated antibodies followed by an detecting antibody. Flowsight analysis was utilized to determine the mean fluorescence intensity of the particular antibody only within the nucleus.

Streptozotocin induced diabetes

Fasting mice were injected with at a single dose of 180-225mg/kg Streptozotocin (Sigma Aldrich, St. Louis, MO) dissolved in ice-cold Sodium Citrate Buffer (pH 4.5), as previously described[344]. Two days after injection, blood glucose levels were checked via an AccuChek Blood Glucose Monitor (Roche Diagnostics, Basel Switzerland). Only those mice with blood glucose levels > 300mg/dL were included in islet transplantation studies.

Islet transplantation and anti-CD45RB mediated tolerance induction

Sub-capsular renal islet transplantation was carried out as previously described[133]. Briefly, chemically diabetic mice (on either the B6[H2-b] or NOD[H2-g7] backgrounds) were transplanted with at minimum 400 female MHC-mismatched C3H(H2-k) or Balb/c(H2-d) islets under their left kidney capsule. Cultured islets suspended in 37°C Islet Culture Media were delivered to anesthetized mice using a 1cc tuberculin syringe fitted with a beveled PE50 tube. Treated mice were injected with 100μg of anti-CD45RB antibody (BioXCell, West Lebanon, NH) on days 0, 1, 3, 5, and 7 after transplantation. IN SLE123 drinking water supplemented with the anti metabolic agents 2-deoxy-D-gluocse (5mg/mL, Sigma Aldrich, St. Lious, MO) and Metformin (3mg/mL, Enzo Life Sciences, Farmingdale, NY) given from days -7 to 14. Successful islet engraftment was confirmed by a blood glucose reading of <140 mg/dL the day after transplantation and graft rejection was recorded when recipient mice demonstrated glucose readings above 250 mg/dL on 2 consecutive days. Recipient blood glucose readings were recorded every 3 days.

Statistics

Statistical analysis was performed with GraphPad Prism V5 (La Jolla, CA), using the Student's t-test for comparison of two normally distributed conditions. One- or two-way analysis of variance followed by Bonferroni post-test was used to compare multiple groups. Statistical comparisons with $p<0.05$ values were deemed significant. In cases of non-normally distributed data, data was compared by a Mann-Whitney Test.

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