DISSERTATION

# CHANGES IN AUTOREACTIVE B CELL LIFESTYLE EARLY IN DEVELOPMENT OF AUTOIMMUNITY

Submitted by

Mia J Smith

Department of Microbiology, Immunology, and Pathology

In partial fulfillment of the requirements

For the Degree for Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2017

Doctoral Committee:

Advisor: Steven Dow
Co-Advisor: John Cambier

Anne Avery
Craig Webb

ABSTRACT

CHANGES IN AUTOREACTIVE B CELL LIFESTYLE EARY IN DEVELOPMENT OF AUTOIMMUNITY

Type 1 diabetes (T1D) is an autoimmune disorder characterized by destruction of the pancreatic beta cells, leading to decreased production of insulin and hyperglycemia. Although environmental factors contribute, genetic factors are likely the primary determinants of risk. With recent advances in GWAS studies, hundreds of risk-conferring alleles have been discovered for T1D. For most cases the exact mechanisms by which these genes and their gene products contribute to development of autoimmunity remains to be elucidated. However, given that T1D requires the activation of autoantigen-specific T and B cells that are normally silenced by immune tolerance, it is likely a combination of HLA and non-HLA alleles act in concert to undermine normal tolerance mechanisms, allowing activation of these autoreactive cells.

Although T cells are the primary effectors of beta cell destruction in T1D, autoreactive B cells are thought to act primarily as antigen presenting cells. In a healthy individual, autoreactive B cells are normally silenced by one of three mechanisms: receptor editing, clonal deletion, or anergy. In this work I determined B cells bearing antigen receptors with high affinity for insulin are found only in the anergic B cell compartment, termed $B_{ND}$, of healthy individuals. Importantly, these cells leave this compartment in a proportion of first-degree relatives (FDRs), and in all autoantibody positive pre-diabetics and new onset diabetics. We posited people at risk for development of T1D carry autoimmune risk alleles that impair proper silencing of autoreactive B cells by anergy, allowing these cells to become activated and contribute to disease. In order to test this, I analyzed the HLA class II alleles and over 50 high risk non-HLA alleles in $B_{ND}$ sufficient and deficient FDRs. I found loss of anergic insulin-binding B cells (IBCs) in FDRs was associated with the high risk T1D HLA alleles and polymorphisms in the high risk non-HLA loci, *INS, PTPN2,*

*PTPN22*, and *IKZF3*. The associations of loss of B cell anergy with these particular risk alleles suggest insulin-reactive T cells and changes in negative regulation of B cell signaling contribute to the unstable anergic phenotype observed in autoimmune patients.

In our T1D studies, we found loss of anergic IBCs was correlated with loss of the entire anergic B cell population, irrespective of their specificity, suggesting loss of B cell anergy could be a common phenomenon in other autoimmune diseases. In addition, many risk alleles for T1D are shared among other autoimmune diseases, including *HLA* and *PTPN22*, suggesting B cell anergy could be compromised in other autoimmune disorders in which similar contributing risk alleles are at play. Hence, I also analyzed the frequency and phenotype of thyroglobulin (Tg) and thyroid peroxidase (TPO) binding B cells, as well as total B cells, in early onset and long standing autoimmune thyroid disease (AITD) patients compared to healthy controls. Similar to studies in T1D, early onset AITD patients had a significant decrease in anergic Tg and TPO-binding B cells that was correlated with a decrease in total anergic B cells. Furthermore, loss of anergic Tg-binding B cells was inversely correlated with Tg autoantibodies and Tg-binding B cells expressed high levels of the activation marker CD86. These findings suggest activation of high affinity thyroid reactive B cells that are normally silenced by anergy, likely leads to production of autoantibodies.

In order to further elucidate the possible contribution a breakdown in anergy of autoreactive B cells has in development of autoimmunity, I studied the phenotype and functional status of IBCs in diabetes susceptible (NOD) and diabetes resistant (C57BL/6) mice transgenic for the 125Tg heavy chain. This transgene increases the frequency of peripheral IBCs to a level that is easily detectable (~0.5-2% of total splenic B cells depending on the strain) [33]. In these mice, I found that high affinity IBCs were phenotypically and functionally anergic in C57BL/6 mice, but the equivalent in NOD appeared activated and functionally responsive, accumulated in the pancreas, and expressed insulin peptides in association with MHC II on their cell surface. Accumulation of these B cells in the pancreas correlated with retention and activation of insulin-reactive CD4 T cells. Hence, these mouse studies nicely summarize what I hypothesize occurs

in autoimmune humans; namely, anergy is impaired in autoreactive B cells, likely due to genetic risk alleles, which allows them to become activated and provide critical antigen presenting function to cognate antigen-reactive T cells. These studies are significant in that they are the first studies to identify a breach in B cell anergy occurs early in development in multiple autoimmune disorders in humans, which is likely driven by a combination of autoimmune risk alleles that alter thresholds for B cell activation, enabling them to become activated and participate in disease through antigen presentation and autoantibody production. Furthermore, these studies highlight the utility of loss of B cell anergy as a possible biomarker for increased risk for development of autoimmune disorders.

ACKNOWLEDGEMENTS

I would like to thank Dr. John Cambier for his excellent guidance and mentorship over the past seven years. It is because of you and your lab that I decided to pursue a Ph.D. and why I have so thoroughly enjoyed myself and my studies over the past years. I am sad to leave my studies and your lab, but am excited for what the future holds. Thank you for encouraging me to learn new things, accept new challenges, and to build the confidence to be an independent thinker and researcher. Your passion for science is evident and contagious and I hope to one day be as great of a scientist and mentor as you have been to me. I would also like to thank Drs. Steven Dow, Anne Avery, Craig Webb, Edward Hoover, and Susan VandeWoude for allowing me to continue my research in Dr. Cambier's lab while pursuing my degree at CSU.

I would also like to thank the many members of the Cambier lab, in particular Drs. Thomas Packard and Andrew Getahun, who over the past years have contributed to the great scientific discussions and heated debates. In addition, I would like to thank the other members of the lab, past and present: Drs. Soojin Kim, Rochelle Hinman, Melissa Walker, Ian Hardy, Janie Akerlund, Shannon O'Neill, Elena Hsieh and Elizabeth Franks, Bergren Crute, and Scott Wemlinger. Also, thanks to members of the Katie Haskins lab, especially Brenda Bradley, who taught me all of my mouse skills, and first introduced me to laboratory research and type 1 diabetes.

I would also like to thank my family and friends who have provided so much support over the many years. My parents, Gary and Celia Smith: To my father for his unending love and support and for always tell me how proud he is of me, and to my mother who I am sure would be proud of my achievements if here today. My sister, Aja, and my extended family, Randy and Sheri McKee, who always supported me. And lastly, but most importantly, to my husband Ryan for your love, support, and understanding during this graduate school journey. One day I promise I will make some money…you just have to wait a few more years.

DEDICATION

*To the two loves of my life, Ryan and Ellis.*

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

# CHAPTER 1

# Introduction

## PATHOGENESIS OF TYPE 1 DIABETES

Type 1 diabetes (T1D) is an autoimmune disorder characterized by self-reactive lymphocyte destruction of insulin producing beta cells in the pancreas. Although genetics is considered the major contributor to risk for development of T1D, the environment is also important given the discordance rate of monozygotic twins is around 50% [1, 2]. In healthy individuals self-reactive lymphocytes are kept in check through various tolerance mechanisms, both central, i.e. occurring in the bone marrow or thymus, and peripheral, i.e. occurring at other sites, that prevent their accumulation, activation and consequent development of autoimmunity [3, 4]. In T1D the combination of genetic risk alleles and environmental factors, allows self-reactive lymphocytes to escape tolerance checkpoints leading to development of autoimmunity [4]. In T1D, self-reactive CD4 T cells, CD8 T cells, and B cells infiltrate the pancreas causing significant beta cell death and destruction. Over time the capacity to secrete insulin in response to a glucose load declines, leading to overt elevation in serum glucose and clinical diagnosis of T1D (Figure 1.1).

## EMERGENCE OF SELF-REACTIVE B CELLS

Studies have shown that as much as 70% of B cells born in the bone marrow bind self-antigens, such as DNA or insulin, via their B cell receptor (BCR) [5]. In order to prevent development of autoimmunity, these self-reactive B cells must be culled, rehabilitated, or suppressed. In the bone marrow, B lymphocytes that bind self-antigen with high avidity undergo a process called receptor editing wherein strong BCR signals induce them to rearrange their antigen receptor light chain genes, silencing one allele and expressing a second, leading to

expression of a BCR with altered specificity [6, 7]. For many cells this process eliminates autoreactivity, but when it does not, continuing strong BCR signals lead to death by apoptosis (Figure 1.2A). These rehabilitation and culling processes are classified as central tolerance mechanisms since they occur in the bone marrow, the birthplace of B cells. On the opposite end of the spectrum, newly produced B cells lacking significant autoreactivity proceed to the periphery and become mature naïve B cells that function in protective immunity. Due to their lack of autoreactivity, these cells are "ignorant" of self-antigen and pose no danger unless they acquire autoreactivity as a consequence of somatic mutation in the germinal center reaction. B cells that bind to self-antigen with moderate avidity, however, can also enter the periphery where they are silenced via a peripheral tolerance mechanism termed anergy (Figure 1.2A). Anergic B cells are characterized by an inability to become activated, proliferate, and produce antibody [8-12] as a consequence of chronic BCR binding to self-antigen. Anergic B cells continue to express BCR, but at lower levels, and the output BCR signals changes. The BCR signals in anergic B cells become biased towards activation of regulatory pathways involving phosphatases such as PTPN22, PTEN and SHIP-1, that prevent anergic B cells from responding to self- and cross-reactive exogenous antigens [13-15]. Anergy is sometimes undermined by genetic polymorphisms that compromise these pathways. Importantly, the anergic status of autoreactive B cells is reversed by antigen dissociation from the BCR, raising the possibility that environment influences may adversely affect anergy [10]. Hence, it is thought that breakdown in the tolerance mechanisms, such as anergy, that are in place to prevent accumulation and activation of self-reactive B cells leads to development of autoimmunity [3].

## EVIDENCE OF A ROLE FOR B CELLS IN T1D

While T cells have been implicated as the major players in destruction of the beta cells in the pancreas leading to T1D, recent studies have highlighted the necessity for B cells. In the non-obese diabetic (NOD) mouse model, which develops spontaneous diabetes, B cells are required

for disease development [16]. In addition, depletion of B cells using anti-CD20 or anti-CD22 prevents diabetes in the NOD model [17, 18]. Moreover, a clinical trial using Rituximab (anti-CD20) to deplete B cells in recent onset T1D subjects showed decreased beta cell loss and a delayed requirement for insulin one year after treatment [19, 20]. Though the effects of Rituximab were minimal two years after treatment, this study demonstrates a role for B cells in this disease. It is likely that by the time Rituximab was administered, many B cells had already committed their crimes, since at the time of T1D diagnosis beta cell mass is sufficiently low to foster hyperglycemia (Figure 1.1).

A study often cited as evidence that B cells are not important in T1D is a report of a person who developed T1D despite being deficient in B cells [21]. However, other studies have taught us that conditions of lymphopenia, be it by genetic defects or pharmacologically induced, can support the accumulation and expansion of self-reactive T cells, without the need for B cell help [22, 23]. Such an event is suspected to have occurred in this individual.

## B CELLS AS ANTIGEN PRESENTERS

So what role do B cells have in T1D? Studies have shown B cells are likely exerting their affect by presenting islet autoantigens to diabetogenic T cells [24, 25], but also by production of cytokines [26, 27] and autoantibodies [28-30]. In the NOD mouse model, inhibition of the ability of B cells to present antigen by either class I or class II prevents diabetes [24, 31], demonstrating the importance of B cells to present antigen to CD4+ T cells and cross-present to CD8+ T cells. In addition, restriction of the B cell repertoire to an irrelevant islet antigen, thus disallowing presentation of islet antigens, prevents diabetes [32]. On the other hand, skewing BCR specificity towards insulin accelerates disease [33]. Hence, these studies demonstrate that islet-reactive B cells are necessary and are likely acting as antigen presenting cells to diabetogenic T cells. Anergic B cells are not competent to present antigen, thus their provision of this function likely depends on compromise of anergy, as occurs in the NOD model, discussed in Chapter 6, and

T1D patients (Chapter 4). T cells activated and expanded as a consequence of antigen presentation act as pancreatic beta cell executioners.

It was recently discovered that post translational modification of islet cell antigens, such as insulin and chromogranin A, leads to the creation of hybrid peptides capable of activating T cells to a high degree [34]. These hybrid peptides have now been identified in both mouse and human [34-36]. B cells reactive with native islet antigens could bind, process and present molecules containing these hybrid peptides. The idea that hybrid peptide-reactive T cells could be the major diabetogenic force is attractive because these T cells would not normally be censored in the thymus due to autoreactivity.

## B CELLS AS AUTOANTIBODY PRODUCERS

To date studies have shown that the presence of islet autoantibodies is one of the best predictors for disease development [28]. Islet autoantibodies that have been identified thus far and are routinely assayed include those against insulin, insulinoma-associated antigen 2 (IA-2), glutamic acid decarboxylase 65 (GAD65), and zinc transporter 8 (ZnT8) [29]. Typically in patients the first antibodies identified are against insulin, which can occur many years before onset of disease [37]. Subjects who have high affinity anti-insulin antibodies or antibodies to more than one islet antigen are almost assured of developing diabetes at some point in life [38-40]. While the presence of autoantibodies suggests a role for B cells in T1D and is a harbinger for disease, the pathogenicity of these antibodies is ambiguous. In mice, production of autoantibodies is dispensable for disease [41] and this may be the case in humans as well.

## PHENOTYPE OF B CELLS IN T1D PATIENTS

Given the evidence for a role of B cells in T1D, recent studies in human have sought to characterize changes in the B cell compartment during disease development. Menard et. al. found that B cells reactive with autoantigens expressed by HEp-2 cells increase in frequency in T1D

patients [42]. As noted above, under normal circumstances autoreactive B cells are silenced at two distinct checkpoints during development: at the immature B cell stage in the bone marrow and prior to entry into the mature naïve stage in the periphery [5] (Figure 1.2A). However, it was discovered that T1D patients had an increased number of autoreactive B cells at both the new emigrant/transitional and mature naïve B cell stages, suggesting impairment of both central and peripheral B cell tolerance [42] (Figure 1.2B). It is also worth noting that the accumulating autoreactive cells are polyreactive, binding to ssDNA, dsDNA, insulin, and LPS. Not surprisingly, a later study by the same group found that treatment with Rituximab failed to reset these impaired tolerance checkpoints [43]. The B cells generated after Rituximab treatment were as auto/polyreactive as the B cells that were present in the periphery prior to treatment.

In order to determine whether B cells in T1D subjects have a decreased propensity for receptor editing, one study analyzed the frequency of recombining sequence (RS) rearrangements in lambda positive B cells in T1D subjects compared to controls. RS sequences accumulate in, and therefore, mark cells that have undergone receptor editing. Results indicated T1D patients had reduced RS rearrangements compared to healthy controls, suggesting T1D patients may have a higher autoreactivity threshold for initiation of receptor editing, which may allow increased numbers of autoreactive B cells to enter into the periphery [44] (Figure 1.2B). Taken together, T1D patients show impaired central and peripheral B cell tolerance, and as discussed in Chapter 3, this includes anergy.

Since T1D is an organ-specific autoimmune disorder, studies should ideally analyze B cells in the target tissues, namely the pancreas and pancreatic lymph node. One of the earliest studies that analyzed infiltrating cell populations in the pancreas using cadaveric tissues found that B cells appear late during insulitis along with CD8+ T cells (Figure 1.1) [45]. Based on this study, one could speculate B cells are acting as antigen presenting cells to the CD8+ T cells. Indeed, B cells have been shown to be essential antigen presenting cells for CD8+ T cells in development of T1D in NOD [31]. With the formation of the Network for Pancreatic Organ Donors

with diabetes (nPOD), more recent studies have been able to extend these findings by analyzing B cells in the pancreas and pancreatic lymph node in more subjects at various stages of disease. Recently, one group found two distinct patters of insulitis, designated CD20Hi (many B cells present) and CD20Lo (few B cells present), distinguish T1D subjects. Subjects who were diagnosed before the age of 7 always display the CD20Hi phenotype, while subjects diagnosed after 13 years of age always show the CD20Lo phenotype [46]. These findings suggest the two forms are differentially aggressive. In line with this, they found that subjects who display the CD20Hi profile show loss of beta cell mass at a more rapid rate than those with the CD20Lo phenotype [46]. This study may help explain why treatment with Rituximab is more efficacious in the younger subjects in the clinical trial [19]. Studies in our lab have been able to track high affinity insulin-binding B cells to the pancreas and pancreatic lymph node where they appear activated in both NOD mice [47] and recent onset T1D patients (unpublished). Hence, it is likely that islet-reactive B cells that escape anergy migrate from the peripheral blood into the pancreas and pancreatic lymph node, where they participate in disease through antigen presentation (Figure 1.2B).

Interestingly, a recent study looking at the frequency of germinal centers in the pancreatic lymph nodes of nPOD donors found that newly diagnosed subjects had a decrease in the number of germinal centers and follicular dendritic cell networks compared to long standing T1D subjects and healthy controls [48]. The authors hypothesize that maturing autoreactive B cells could be pushed into antibody secreting cells at a more rapid rate, therefore causing a disruption in normal follicular development. Since the authors used CD20 as a marker for B cells in their study, they would not have been able to enumerate plasma cells that are CD20lo/-. Future studies using more subjects and more B cell markers could help elucidate the role of B cells in the pancreatic lymph nodes early in disease.

GENETIC RISK ALLELES IN B CELLS AND T1D

Since development of T1D is driven by genetic risk alleles and the environment, it seems likely that the these factors mediate their effects in part by promoting loss of central and peripheral B cell tolerance. The T1D risk allele most affecting likelihood of disease development is HLA class II. DR4-DQ8 followed by DR3-DQ2 alleles confer greatest risk [2, 49]. CD4 T cells recognizing autoantigen. e.g. insulin, peptide-associated DR4-DQ8 could evoke loss of B cell tolerance. The genetic polymorphism conferring second highest risk is in the VNTR region of the insulin (*INS*) gene [2]. This polymorphism is thought to increase the number of insulin-specific T cells in the periphery due to impaired T cell tolerance induction in the thymus [50]. Increased insulin-specific T cells likely promote activation of insulin-reactive B cells, driving them to participate in disease. As discussed in Chapter 4, we found that loss of anergic insulin-binding B cells is associated with risk conferring HLA class II and insulin allotypes, suggesting T cells contribute to loss of B cell anergy.

In addition, our studies have shown that loss of B cell anergy is associated with polymorphisms in the *PTPN22* and *PTPN2* genes (Chapter 4). PTPN2 and PTPN22 are negative regulators of B cell signaling [51]. Recent GWAS studies have shown that the *PTPN22* genotype is the third highest contributor to T1D risk, after HLA and the *INS* genes [2]. *PTPN22* encodes the lymphoid tyrosine phosphatase, Lyp, expressed in T and B cells. Previous studies have shown that PTPN22 functions to dampen B cell signaling and expression of a risk-conferring allele is associated with increased autoreactive B cell frequency in the peripheral blood of T1D patients [42, 52]. Interestingly, one study found that T1D subjects carrying the PTPN22 risk allele had increased anergic ($B_{ND}$) B cells in their periphery [52]. The disparity of their findings with our study could be due to the difference in subjects since they looked at presumably long standing T1D subjects and we analyzed first-degree relatives. It could also be due to a difference in gating strategies to identify anergic B cells. Targeted ectopic expression of the risk allele in B cells *in*

*vivo* leads to autoimmunity [53]. Hence, we hypothesize the *PTPN22* risk allele may promote loss of anergy via compromising anergy, leading to development of autoimmunity.

The *PTPN2* gene encodes another protein tyrosine phosphatase that is expressed in both B and T cells and has been shown to have a range of functions, including negative regulation of JAK/STAT signaling [54] and dephosphorylation of Lck and Fyn after stimulation through the T cell receptor [55]. The *PTPN2* SNP rs1803217 was shown to be associated with 40% reduction in PTPN2 mRNA in primary human Treg and CD4+ memory T cells [56]. Though the study did not examine B cells, one would expect a similar reduction in mRNA in some B cell subsets as well. A study in which *PTPN2* was inducibly deleted in the hematopoietic compartment of adult mice developed autoimmunity characterized by an increase in the number of B cells, including germinal center B cells, and anti-nuclear autoantibody production [57]. Thus, polymorphisms in genes whose products function in negative regulation of BCR may confer T1D risk by impairing peripheral tolerance. Other T1D-associated allelic variants of genes expressed in B cells, such as *BACH2 and SH2B3* may in time also be proven to impair B cell tolerance.

## RATIONALE AND HYPOTHESES

Given the evidence for the importance of islet-reactive B cells, and in particular insulin-reactive B cells, in the pathogenesis of T1D and studies indicating impaired central and peripheral tolerance of autoreactive B cells in T1D subjects, the aim of this study was to analyze the phenotype of insulin-reactive B cells in the peripheral blood of subjects along a continuum of T1D development. We hypothesize that potentially pathogenic insulin-reactive B cells (IBCs) that are normally silenced by anergy become activated and participate in T1D development. Since antigen-reactive B cells occur at a very low frequency (<0.1%) in the peripheral blood, I first developed a method to identify and enrich for antigen-reactive B cells from blood and tissues using magnetic particles, discussed in detail in Chapter 2. Using this method to identify IBCs, I found that anergic, termed $B_{ND}$, insulin-binding B cells were lost in subjects who had

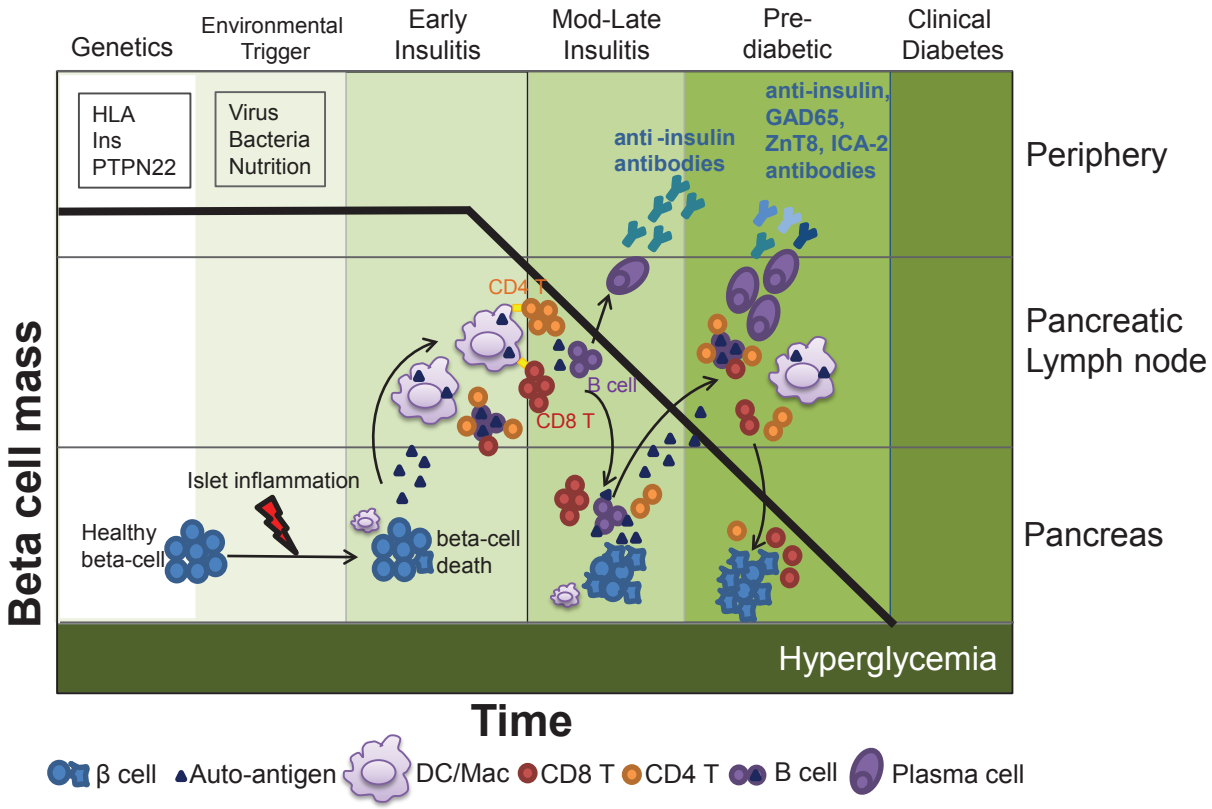
autoantibodies but were not yet diabetic and those who had been diagnosed with T1D within the past year compared to healthy controls (Chapter 3). Subjects who had been diabetic for more than one year had levels of insulin-binding anergic B cells similar to controls. Interestingly, in a portion of first-degree relatives these cells were also decreased in frequency, suggesting that loss of anergy precedes production of autoantibodies. In addition, loss of insulin-binding $B_{ND}$ cells was correlated with loss of total $B_{ND}$ cells, irrespective of their specificity. These results indicate a possible predisposing genetic defect could drive loss of B cell anergy and loss of anergic B cells may be an early event in other autoimmune disorders sharing similar risk alleles.

Hence, we hypothesize people at risk for development of T1D carry autoimmune risk alelles that impair proper silencing of autoreactive B cells, allowing these cells to become activated and contribute to disease. In Chapter 4, I sought to test this by determining whether loss of B cell anergy is associated with high risk HLA and non-HLA T1D risk alleles. I found that loss of B cell anergy was associated with high risk HLA haplotypes and polymorphisms in the non-HLA risk loci, *INS, PTPN2, PTPN22,* and *IKZF3*. These results indicate insulin-reactive T cells and changes in inhibitory signaling of autoreactive B cells likely contribute to impaired B cell anergy in T1D.

Given that many of the genetic risk alleles in genes such as *PTPN22, CTLA4*, and *IL2-RA*, are shared among many autoimmune diseases, such as autoimmune thyroid disease (AITD), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) [42, 58-61], I determined whether loss of anergic B cells was a common phenomenon in other autoimmune disorders by analyzing the phenotype and activation status of thyroid-reactive B cells in autoimmune thyroid patients (Chapter 5). Similar to findings in T1D patients, I found that there was a significant decrease in anergic thyroglobulin (Tg) and thyroid peroxidase (TPO) binding B cells, as well as total anergic B cells, in early onset AITD patients that returned to normal levels in patients diagnosed more than one year prior to analysis. In addition, loss of anergic Tg-binding B cells was inversely correlated with anti-Tg antibody titers and Tg-binding cells, particularly the few

remaining anergic B cells in early onset patients, showed increased CD86 expression. These results suggest loss of anergic Tg-binding cells is an early event in AITD and activation of these cells likely leads to the production of autoantibodies.

In order to further understand the contribution of insulin-reactive B cells in T1D, I studied the phenotype and functional status of IBCs in diabetes susceptible (NOD) and diabetes resistant (C57BL/6) mice transgenic for the 125Tg heavy chain, which increases the frequency of IBCs in the periphery [33] (Chapter 6). C57BL/6 mice also carried the same MHC class II known to be required for T1D in the NOD mouse model ($H2^{g7}$), thus ensuring all B cells were capable of presenting peptides to diabetogenic CD4 T cells. In these mice, I found that high affinity IBCs were phenotypically and functionally anergic in C57BL/6 mice, but the equivalent in NOD appeared activated and functionally responsive, accumulated in the pancreas, and expressed insulin peptides in association with MHC II on their cell surface. Accumulation of these B cells in the pancreas correlated with retention and activation of insulin-reactive CD4 T cells. Hence, by utilizing mouse models, these studies nicely summarize what we hypothesize occurs in autoimmune humans; namely, anergy is impaired in autoreactive B cells, likely due to genetic risk alleles, which allows them to become activated and provide critical antigen presenting function to cognate antigen-reactive T cells.




Adapted from Van Belle et al. 2011. *Physiol Rev.*

**Figure 1.1 Proposed pathogenesis of type 1 diabetes**

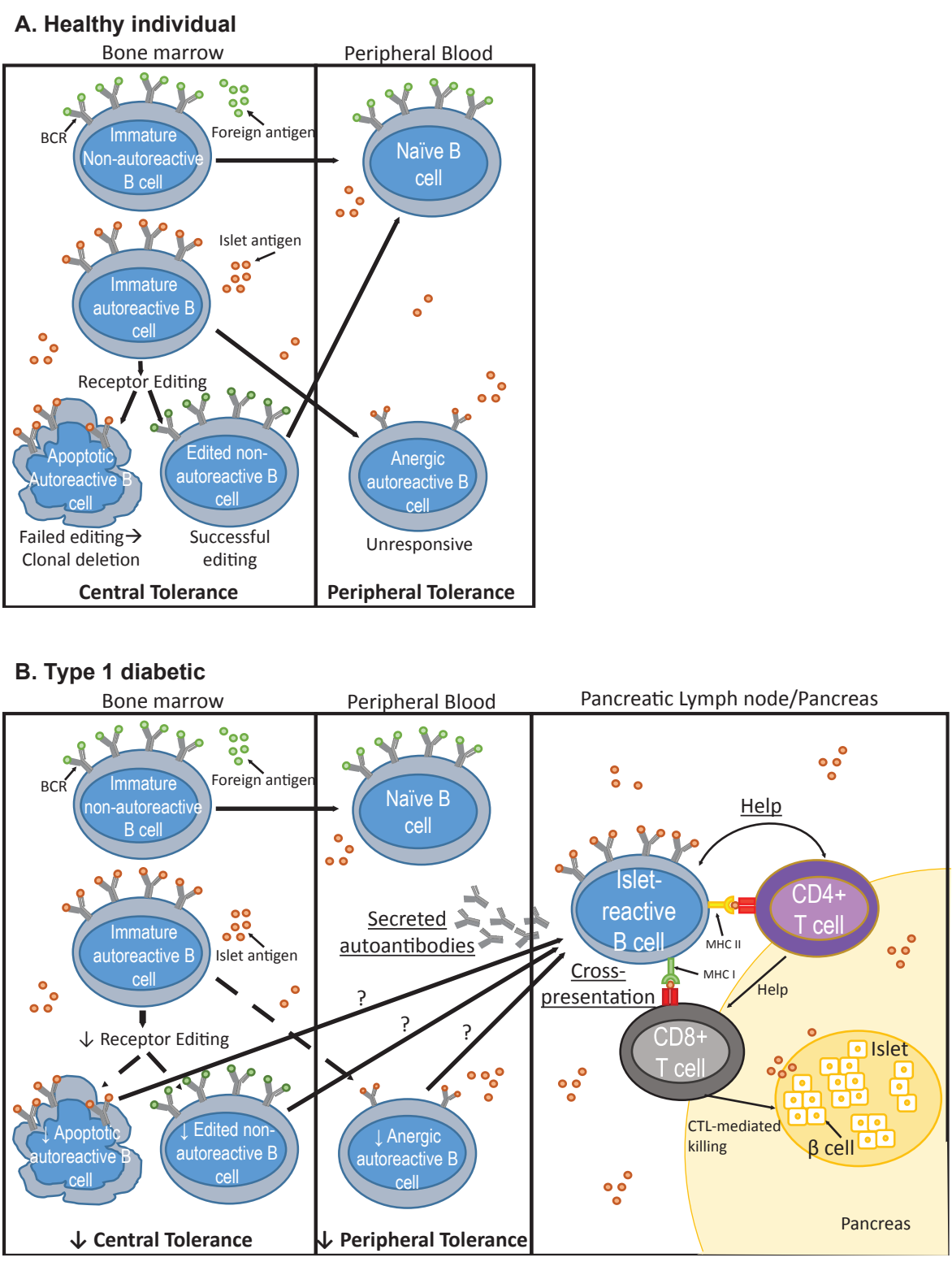



**Figure 1.2 Autoreactive B cell tolerance in healthy individuals and their escape and participation in type 1 diabetics**

**Figure 1.2. Autoreactive B cell tolerance in healthy individuals and their escape and participation in type 1 diabetics. A.** In healthy individuals non-autoreactive B cells migrate into the periphery and become mature naïve B cells. Autoreactive B cells with a high avidity for self-antigen undergo central tolerance in the bone marrow where receptor editing or clonal deletion occurs. Autoreactive B cells with moderate avidity for self can enter the periphery and become tolerized by the process termed anergy. **B.** In type 1 diabetics, it has been shown autoreactive B cells undergo decreased central and peripheral tolerance allowing escape of these autoreactive B cells. These cells participate in disease by entering the pancreas or pancreatic lymph node where they produce autoantibodies and present antigen to islet-reactive CD4+ and CD8+ T cells.

# CHAPTER 2

## Detection and enrichment of rare antigen-specific B cells for analysis of phenotype and function


## SUMMARY

B cells reactive with a specific antigen usually occur at a frequency of <0.05% of lymphocytes. For decades researchers have sought methods to isolate and enrich these rare cells for studies of their phenotype and biology. Approaches are inevitably based on the principle that B cells recognize native antigen by virtue of cell surface receptors that are representative in specificity of antibodies that will eventually be secreted by their differentiated daughters. Perhaps the most obvious approach to the problem involves use of fluorochrome-conjugated antigens in conjunction with fluorescence-activated cell sorting (FACS). However, the utility of these methods is limited by cell frequency and the achievable rate of analysis and isolation by electronic sorting. Here we describe a novel method to enrich rare antigen-specific B cells using magnetic nanoparticles that results in high yield enrichment of antigen-reactive B cells from large starting cell populations. This method enables improved monitoring of the phenotype and biology of antigen reactive cells before and following *in vivo* antigen encounter, such as after immunization or during development of autoimmunity.


## INTRODUCTION

Limiting dilution analyses of antibody-secreting cell precursor frequency have suggested that B cells reactive to a particular antigen typically occur at a frequency of 0.05 to 0.005% in the normal repertoire, depending on vaccination status and size/number of epitopes present on the antigen. The low frequency of these cells has made it difficult to study changes in their status during development of immune responses, such as following vaccination or exposure to a foreign antigen, or during development of autoimmunity. Previously, researchers have undertaken

isolation of antigen-reactive B cells using techniques ranging from antigen coated plates or column adsorbents, to antigen-coated red blood cell resetting, to fluorescence-activated cell sorting [62-67]. Though these techniques have been successful in identifying and isolating antigen-reactive B cells, the results have varied in terms of yield, purity and scalability. Recently we developed a novel method to both detect and enrich rare B lymphocyte subpopulations using magnetic nanoparticles. The method enables enrichment with relatively high yield and purity from large starting populations, and is compatible with analysis of responses to antigen. By enriching from populations of cells in suspension, the method eliminates constraints that are associated with the geometry of antigen-coated plates or columns, and limit throughput. Finally, since enriched cells remain associated with antigen and a fluorescent reporter, they can be further purified by FACS sorting. As described herein we have used this approach for study of peripheral blood tetanus toxoid-reactive B cells before and following immunization of human subjects, as well as autoantigen-reactive B cells from subjects with various autoimmune disorders, including type 1 diabetes, Graves' disease, and Hashimoto's disease [68]. The method works equally well in mouse and human, and is compatible with analysis of antigen-reactive B cells from a variety of tissues.

In its basic format, peripheral blood mononuclear cells are first incubated with biotinylated antigen along with antibodies to cell surface antigens required for phenotypic analysis. This labeling step is followed by washing and fixation, and addition of streptavidin coupled to Alexa Fluor 647 for detection of the biotinylated-antigen binding cells (Figure 2.1). Previous studies have identified antigen-specific B cells in a similar manner but using antigens directly conjugated to a fluorochrome [33, 69-71]. Although this is a worthy approach, use of biotinylated antigens in conjunction with streptavidin enables greater signal amplification (hence better differentiation of positive and negative binding cells), particularly when antigens are small [72-74]. An additional consideration is the use of streptavidin instead of avidin because streptavidin is deglycosylated, decreasing non-specific binding. Further, we use Alexa Fluor 647 as our fluorochrome due to its

photostability, quantum yield (brightness), and its small size (~1.3 kD). Protein fluorochromes such as phycoerythrin (~250 kD) and allophycocyanin (~105 kD) [75] are not optimal because they potentially contain many antigenic epitopes. Use of a small organic fluorescent dye composed of a single epitope, such as Alexa Fluor 647, reduces complexity of the isolated cell population.

Once cells are biotinylated-antigen and Alexa 647-streptavidin adsorbed, they are enriched using anti-Alexa 647 conjugated magnetic nanoparticles. Single nanoparticles are not detected by most flow cytometers and therefore need not be removed prior to purification by FACS sorting and downstream assays [76]. Magnetic selection for antigen-specific B cells enriches the population of interest, eliminating the time and cost of sorting rare events using a flow cytometer.

In figure 2.3, we show representative results from enrichment of tetanus-toxoid (Tet Tox)-specific B cells from a subject before and seven days after tetanus toxoid booster immunization. We chose this particular application as an example in order to demonstrate the ability of this method to enrich antigen-specific B cells following acute *in vivo* stimulation. When coupled with flow cytometry, this method is capable of enriching and differentiating antigen-specific naïve, memory, and plasmablast B cells and allows the researcher to follow changes in their frequency over time. In addition, we include another possible downstream assay, an ELISPOT, which demonstrates that cells retain the ability to secrete antibody following enrichment. Another application of this method could involve adoptive transfer of enriched cells into a host. We have previously shown cells maintain the ability to act as antigen presenting cells to antigen-specific T cells following isolation and transfer (data not shown). Hence, there are a number of possible downstream assays that could be coupled to the method, which together informs our understanding of the antigen-specific immune response. We have described the method below, including controls to determine overall yield, purity, cell specificity, and fold-enrichment.

PROTCOL

*1. Isolation of human PBMCs*

1.1) Collect 30-50 mL of blood using heparinized blood collection tubes. The amount of blood used depends on the particular experimental question and frequency in blood of antigen-specific B cells of interest. For our purposes we generally start with ~30 mL of blood. Heparinized blood can be processed immediately or rocked gently overnight at room temperature for processing the following day. The delay in processing has very little effect on the efficiency of enrichment and is associated with minimal loss of viability.

1.2) Mix whole blood 1:1 with sterile, room temperature magnesium and calcium free phosphate-buffered saline (PBS).

1.3) In a 50 mL sterile conical tube, add 10 mL of room temperature Ficoll-Paque PLUS. Opened Ficoll bottles can be stored in the dark at 4°C, but should be warmed to room temperature before use.

1.4) If possible set your pipette gun to the slow setting and/or apply very little, yet consistent, trigger pressure to slowly layer 30-35 mL of the diluted blood on top of the Ficoll, being careful not to mix the two. Placing the tip of the pipette against the edge of the 50 mL conical while adding the diluted blood will help ensure a consistent steady flow.

1.5) Centrifuge at 400 x g for 30 minutes at 20°C with the brake turned off.

1.6) Remove the upper layer, which contains plasma and platelets, being careful not to disturb the mononuclear cell layer below.

1.7) Using a sterile pipette, collect the mononuclear cell layer (buffy coat) and place into a separate sterile 50 mL conical tube.

1.8) Add PBS to the cell suspension to a volume of 50 mL.

1.9) Centrifuge at 400 x g for 10 minutes at 20°C with brake.

1.10) Remove supernatant and resuspend pelleted cells in 10 mL of PBS. Count cells and determine viability using a hemocytometer or automated cell counter.

1.11) Resuspend cells at $10^7$ cells/mL and place on ice until ready for further processing.

*2. Staining of cells*

2.1) Remove 1-2 x $10^6$ cells for each fluorescence compensation/FMO control needed, if applicable.

2.2) Centrifuge remainder of cells at 400 x g for 10 minutes at 20°C with brake.

2.3) Resuspend cells in sterile filtered, cold FACS buffer (PBS + 1% bovine serum albumin (BSA) + 0.01% sodium azide) at 3-6x$10^7$ cells/mL. FACS buffer should be cold and remain on ice throughout the staining procedure. It is a good idea to divide your cell population into fourths (A-D) when optimizing the assay. Fraction A will be used to enrich for antigen-specific B cells of interest, fractions B and C to determine the specificity of enrichment/staining (see 2.6 and 2.7 below), and fraction D to determine the frequency and number of antigen-specific cells in the unenriched population to enable calculation of yield and fold-enrichment.

2.4) Add human FcR blocking reagent to fractions A-D on ice to prevent binding of antibodies to Fc receptors. Follow manufacturer's instructions for appropriate conditions.

2.5) Add fluorochrome-conjugated antibodies to cell surface antigens of interest to fractions A-D for 30 minutes on ice in the dark, being careful not to include fluorochromes that may cross react with the anti-Alexa Fluor 647 antibodies coupled to magnetic nanoparticles. These include antibody conjugates to Alexa680, Cy5, Cy5.5, and Cy7. Any viability stains that are added should be compatible with the use of a fixative. If you do not intend to analyze your cells using FACS, but will determine antibody-secreting frequency, for example, by ELISPOT, surface staining with antibodies is unnecessary.

2.6) To test the specificity of the assay, incubate Fraction B with sufficient amount of unlabeled antigen, such that the majority of the receptors with an affinity of at least $10^{-6}$ M are blocked. Typically, a good starting concentration is a 50-100 fold excess of the amount of antigen deemed sufficient in 2.7 below (e.g. 50-100 μM). Incubate with the excess unlabeled antigen for 30 minutes on ice. This should block any B cells with a high affinity for the unlabeled antigen to bind to the biotinylated antigen, which is added in step 2.7 below, thus allowing confirmation of the specificity of the cells enriched. This step can be completed in conjunction with step 2.5 above.

2.7) Add biotinylated antigen to Fractions A, B, and D. Concentration of biotinylated antigen should be titrated to determine the optimal amount needed to ensure adequate detection and separation of binding cells from non-specific bystander cells. Typically a good starting concentration to test is 1-5 μM. Biotinylated antigen is omitted from Fraction C in order to determine any binding of B cells to other reagents in the protocol, such as streptavidin-Alexa Fluor 647 and the magnetic beads. For Fractions A and D, this step can be done concurrently with step 2.5 above. For Fraction B, this step should be done after step 2.6 (washing in between steps is unnecessary).

2.8) Wash cells twice by suspension of cells in 1 mL of cold FACS buffer per 5 x $10^7$ cells and centrifugation at 400 x g for 5 minutes at 4°C.

2.9) After the final wash, dilute concentrated cell suspension in 1 mL 2% formaldehyde per 5 x $10^7$ cells and let sit in dark on ice for 5 minutes. Fixation of the cells at this point ensures that the biotinylated antigen remains bound to the BCR for the remaining of the procedure. If you need viable cells for downstream analysis, such as in ELISPOTs or adoptive transfers, omit the fixation step.

2.10) Wash cells twice with 1 mL of cold FACS buffer per 5 x $10^7$ cells and centrifuge at 400 x g for 5 minutes at 4°C. Resuspend in 1 mL cold FACS buffer per 5 x $10^7$ cells.

2.11) Add 1-2 µg Streptavidin-Alexa 647/mL and incubate on ice for 20 minutes in the dark. The amount of streptavidin added should be titrated for each application.

2.12) Wash cells twice with 1 mL cold FACS buffer per 5 x $10^7$ cells and centrifuge at 400 x g for 5 minutes at 4°C. Fraction D preparation is completed at this point as it will not undergo enrichment using the magnetic beads. This sample will be used to determine the frequency and number of antigen-specific B cells in your sample. This will be used to determine your yield and fold-enrichment achieved by enrichment.

*3. Magnetic nanoparticle-based enrichment*

3.1) Resuspend cells from samples A-C in 1 mL of cold separation buffer (PBS + 0.5% BSA + 2mM EDTA) per 5 x $10^7$ cells and pass through a 40 µm filter to eliminate any clumps that could clog the column.

3.2) Add Anti-Cy5/Anti-Alexa Fluor 647 nanoparticles. For optimal results, the concentration of nanoparticles should be titrated for each use, but a good starting point is 50 µL suspension per 5 x $10^7$ cells/ mL. Rotate in the dark at 4°C for 10 minutes.

3.3) Wash twice with 1 mL cold separation buffer at 400 x g for 5 minutes at 4°C.

3.4) Resuspend cells in 1 mL of cold separation buffer per 5 x $10^7$ cells.

*4. Magnetic enrichment using LS or LD columns*

4.1) Place three LS or LD columns in the magnetic field of a MACS separator and add 3 mL of separation buffer to wet the columns (see product description to determine which type of column is best suited for your needs). Allow all 3 mL to pass through the column and discard after collection. Although separation of magnetic nanoparticle labeled cells can be accomplished using any one of the magnetic separation devices that are commercially available, our laboratory has had the most success with the use of LS columns, in terms of yield, purity, and efficiency of enrichment.

4.2) Place three 15 mL conical tubes labeled “Fraction A/(B)/(C) negative”, for negatively selected cells, under the column. Add the magnetic particle labeled cells to their respective column and allow the entire volume to pass through.

4.3) Add 3 mL of cold separation buffer on top, and allow this to pass through the column and repeat with 2 mL.

4.4) Collect the negatively selected cells and set aside on ice.

4.5) Remove the column from the magnetic field and place on top of a 15 mL conical tube labeled “Fraction A/(B)/(C) positive”, for positively selected cells.

4.6) Fill the column to the top with approximately 6 mL of separation buffer and immediately plunge this volume through the column to collect cells that had bound to the magnetic field using the provided plunger.

4.7) To increase purity, the positively selected cells can be further enriched using a second clean column, repeating the procedure.

4.8) Spin both the negatively and positively selected fractions at 400 x g for 10 minutes at 4°C and suspend in desired final volume.

4.9) Proceed with downstream analysis, such as FACS, ELISPOT or adoptive transfer.

*5. Analysis of purity, yield, and fold-enrichment using flow cytometry*

5.1) Populations enriched as described above inevitably contain contaminating cells that have not bound streptavidin-Alexa 647 but are trapped in the matrix. These impurities can be removed from enriched populations by FACS sorting. To estimate purity of enriched populations, gate on live cells based on forward and side scatter and/or live/dead stain and analyze Alexa $647^+$ cell frequency. The percentage within this latter gate is indicative of purity. Further analysis of the specificity and antigen affinity of these cells requires FACS sorting of single cells, followed by cloning and sequencing of expressed immunoglobulin genes and recombinant antibody production and analysis.

5.2) Yield refers to the number of isolated antigen-binding B cells recovered in Fraction A relative to the number in the starting population (Fraction D). Since you started with the same number of cells in Fraction D as Fraction A, you can directly determine the yield (# of antigen-binding B cells in Fraction A ÷ # of antigen-binding B cells in Fraction D).

5.3) Fold-enrichment (E) is reflected by the percentage of Alexa 647+ B cells in the "positively" enriched Fraction A relative to that in Fraction D. This value indicates the proportional increase in antigen-specific B cells achieved by nanoparticle enrichment. The value can be found using the following equation: $\mathrm{E} = \frac{\%\ \text{antigen-specific in enriched}}{\%\ \text{antigen-specific in unenriched}}$

RESULTS

To demonstrate this technique, we show representative results obtained using tetanus-toxoid (Tet-Tox) as a model antigen to detect and enrich for Tet-Tox-reactive B cells in the peripheral blood of subjects who have been vaccinated against tetanus at varying time points. Figure 2.1 is a schematic of the method and the antigen adsorbent. Figure 2.2a shows the frequency of Tet-Tox-reactive B cells from an individual who was last vaccinated against tetanus more than a decade ago. Shown are the enriched ("positive") and depleted ("negative") populations from Fraction A and the baseline frequency of Tet-Tox-specific B cells from Fraction D. Hence, in this example we are able to enrich binding cells by around 7-fold and achieved a purity of 4% in the enriched fraction.

Figure 2.2b shows the specificity of the reaction using blood from a subject who had been vaccinated ~3 years earlier. When we added a 50-fold excess (50 μM) of unlabeled tetanus-toxoid to the cells before addition of the biotinylated antigen (Fraction B) we are able to block binding by 83%, demonstrating the specificity of antigen binding. When we omitted biotinylated antigen from the procedure (Fraction C), a small number (~ 0.2%) of B cells still bound; this small fraction of B cells presumably reflects binding to some non-antigen in the adsorbant, such as the streptavidin-Alexa Fluor 647, the anti-Alexa 647 antibody or the magnetic beads.

To demonstrate use of the method to analyze changes in antigen-binding cell phenotype following immunization we show in Figure 2.3 the frequency of Tet-Tox-specific B cells in the naïve, memory, and plasmablast populations in blood drawn before and 7 days after booster vaccination of a healthy subject. While the percentage of naïve B cells among total Tet-Tox-

binding B cells decreased from 84% to 64% after boost, the percentage of memory and plasmablast populations among total Tet Tox-reactive cells increased from 16% to 36% and 0% to 40%, respectively. Figure 2.3b shows representative ELISPOT data from peripheral blood from the 7 days post-boost healthy subject. Isolation of the Tet-Tox-binding B cells led to 4-fold enrichment in tetanus toxoid antibody secreting-cell frequency.

## DISCUSSION

Here we describe a novel method to accomplish isolation and enrichment of antigen-binding B cells from human peripheral blood. The method is readily applicable to mice and to other tissues, such as the spleen and lymph nodes, and is compatible with post-enrichment analysis of cell phenotype and function.

The user should be cognizant of a number of variables that can affect success of this procedure. In our experience dead cells tend to stick to the magnetic beads and “take up” fluorescent dyes, such as the Streptavidin-Alexa Fluor 647, leading to non-specific increased background. It is critical to remove as many dead cells from samples as possible before beginning this procedure. Use of stains that enable live/dead discrimination to gate out dead cells from the analysis may be helpful, but presence of dead cells may compromise interpretation of assays of antibody secreting cell frequency and function following adoptive transfer.

In addition, we have found that careful titration of the biotinylated antigen added is essential to distinguishing antigen-specific and non-specific binding. Use of too little antigen will lead to underestimation of the frequency of antigen-binding cells, while too much antigen can cause false positive detection/enrichment. Use of antigen concentrations that are too high may can be indicated by apparent binding to non-B cells in the samples. Further, apparent detection of an excessively high frequency (>1%) of antigen reactive cells may be an indication of binding specificity issue. The ultimate goal when determining the optimal amount of antigen to use is to add a little bit more antigen than is present at physiological concentrations, such that the majority

of the B cell receptors with an affinity of $<10^{-6}$ M, for example, for the antigen are saturated. This ensures you are detecting antigen-specific B cells with an affinity that has pathogenic potential. Addition of too little antigen, i.e. at or below physiological concentrations, may lead to detection of very few antigen-specific B cells due to decreased antigen occupation of the BCRs. On the other hand, addition of too much antigen, far exceeding physiological concentrations, can lead to increased non-specific binding which will include B cells with a low affinity for the antigen ($>10^{-6}$ M) and are actually ignorant of the antigen *in vivo*. Hence, it is vital to first titrate the biotinylated antigen across a few logs in order to be confident that only true antigen-reactive B cells are being detected. Similarly, we have found that use of too much streptavidin-Alexa647 can increase background.

One of the limitations to this technique is its reduced effectiveness for enrichment of antigen-binding B cells from cryopreserved compared to fresh cells. This could relate to greater cell death and associated "stickiness" in frozen samples. Nonetheless, the method can be applied to frozen samples but it is probably important not to directly compare results from enrichment and analysis of fresh and frozen cells. Another limitation is the purity of antigen-specific B cells in your enriched sample. In our experience, there are always some cells that "tag along", which includes both non-specific B cells and T cells. This is to be expected given the rarity of the cell population you are trying to enrich for. However, you can increase the purity of your cells by ensuring you have a single cell suspension before adding your cells to the column, enriching a second time on a new column, or by FACS sorting your antigen-specific B cells. Depending on the type of study you are doing, the researcher can decide which option is best and whether it is necessary.

As we optimized this technique we envisioned that it would be amenable to discrimination of high affinity versus low affinity antigen-reactive cells based on antigen titration or staining intensity. However, in a previous study, we showed antigen-reactive B cells with up to a 1000-fold difference in affinity to their antigen were equally enriched using this method[68]. Hence, formal measurement of the affinity of enriched cells using surface plasmon resonance analysis of

expressed recombinant antigen receptors is required if knowledge of the affinity is pertinent to your study.

Inherent in this technique is the limitation that addition of the biotinylated antigen and streptavidin can activate the cells by cross-linking the BCR. This could pose a problem for particular downstream functional assays, such as those that seek to compare stimulated cells to a resting control. This problem may be mitigated by keeping control samples cold to prevent transduction of signals. It is also possible that BCR signals could affect an ongoing biological response. In our experience antigen-enriched cells maintain their ability to secrete antibody, as indicated by ELISPOT analysis (Figure 2.3b) and function as antigen presenting cells to T cells *in vitro* and after adoptive transfer into mice (data not shown). A final limitation to this technique is the use of anti-fluorochome antibodies to accomplish nanoparticle binding to antigen adsorbed cells. Anti-Alexa 647 cross-reacts with certain structurally related fluorochromes, including Cy7 and Cy5.5. This can limit the options of antibody conjugates available for measurement of phenotypic markers. However, with increased availability of new fluorochromes, this is becoming less of an issue.

Using this detection and enrichment method, it is possible to easily identify antigen-binding B cells in *ex vivo* peripheral blood, spleen, or other tissues. This method is particularly useful in studying the immune response of antigen reactive cells following immunization or exposure to foreign antigens and in the setting of autoimmune disease. Although no method to detect antigen-reactive B cells has proven to be without its faults, this method is fast, simple, and produces a relatively high purity, yield, and enrichment of antigen-reactive cells that can be coupled to a variety of downstream assays. In addition, due to the basic principles of the method, this procedure could easily be tailored to a variety of experimental needs.

**Table 2.1 Materials needed**

| Name of material/Equipment | Company | Catalog number | Comments/Description |
|---|---|---|---|
| Antigen of interest | variable | variable | At least 100 µg to biotinylate easily; if using protein try to use protein that has been validated by ELISA. It must be carrier protein free. |
| Biotin for labeling; e.g. EZ link Sulfo-NHS-LC-Biotin | e.g. Thermo Scientific | e.g. 21335 | Biotin is available in different formulations, such as those containing various length spacers, so the type used should be determined by the researcher |
| Streptavidin-Alexa Fluor 647 | Invitrogen | S21374 | Can obtain from other suppliers. |
| Anti-Cy5/Anti-Alexa Fluor 647 Microbeads | Miltenyi | 130-091-395 | |
| LS Columns | Miltenyi | 130-042-401 | |
| MACS manual separators | Miltenyi | variable | |
| Formaldehyde | | | Dilute to 2% with PBS; optional if downstream assay requires live cells |
| PBS without calcium and magnesium | | | |
| Ficoll-Paque PLUS | GE Healthcare | 17-1440-02 | |
| Whole blood in heparinized collection tubes | | | |
| FACS buffer (PBS + 1% BSA + 0.01% sodium azide) | | | |
| Separation buffer (PBS + 0.5% BSA + 2mM EDTA) | | | |
| 50 mL conical tubes | | | |
| 15 mL conical tubes | | | |
| 1.5 mL Eppendorf tubes | | | |
| Surface marker reactive antibodies, Fc Block, live/dead discriminating stain, if needed | | | |
| ELISPOT supplies, if needed | | | |

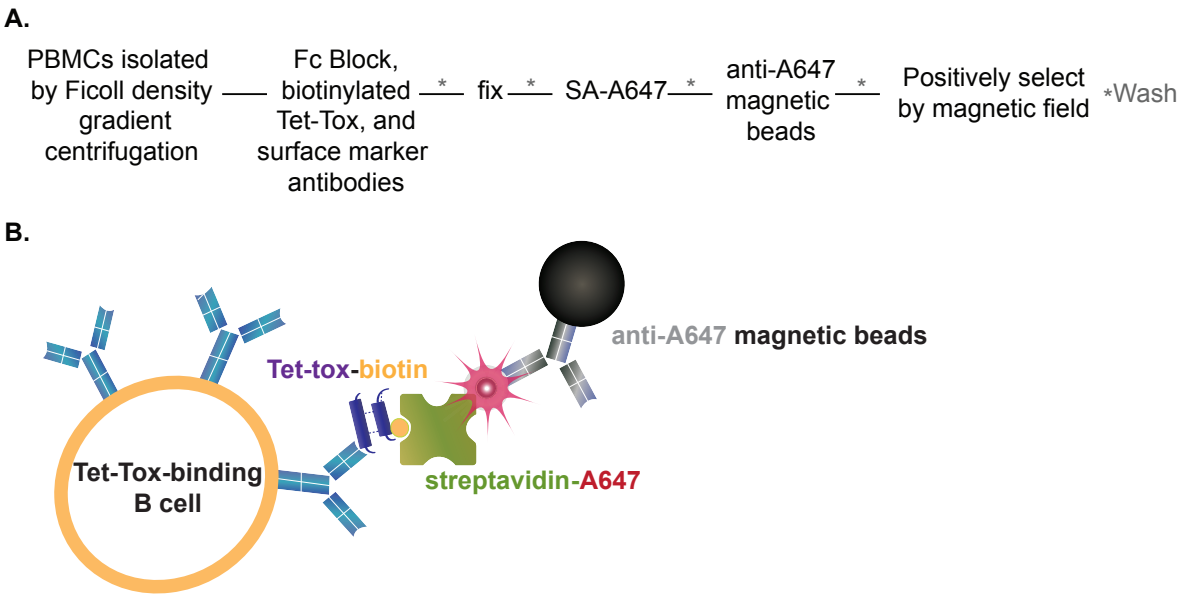



**Figure 2.1 Method for detection and enrichment of tetanus toxoid (Tet Tox)-binding B cells**. **A.** Protocol for enrichment and isolation of Tet-Tox-binding B cells. **B.** Diagram of adsorbent employed to identify and enrich Tet-Tox-binding B cells.

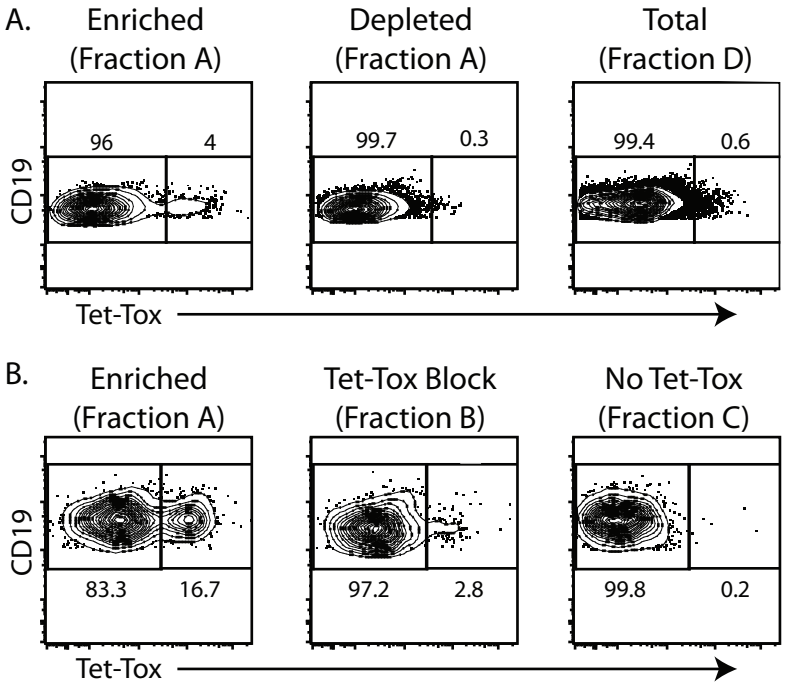



**Figure 2.2 Representative cytograms demonstrating enrichment of Tet Tox-binding B cells from human peripheral blood. A.** Cytograms of enriched and depleted Tet Tox-reactive B cells from Fraction A and total Tet-Tox-reactive B cells from Fraction D from a normal subject last vaccinated >10 years previously. **B.** Cytograms of PBMCs from the "positive" populations from a subject vaccinated ~3 years previously and enriched as in A (Fraction A), after pre-incubation with excess of 50 uM unlabeled Tet-tox (Fraction B), or enriched with Tet-Tox-biotin omitted during the procedure (Fraction C). All analyses were gated on CD19+ lymphocytes. Percentages represent the percent of Tet-Tox-binding cells of all B cells (CD19+) in the final fraction.

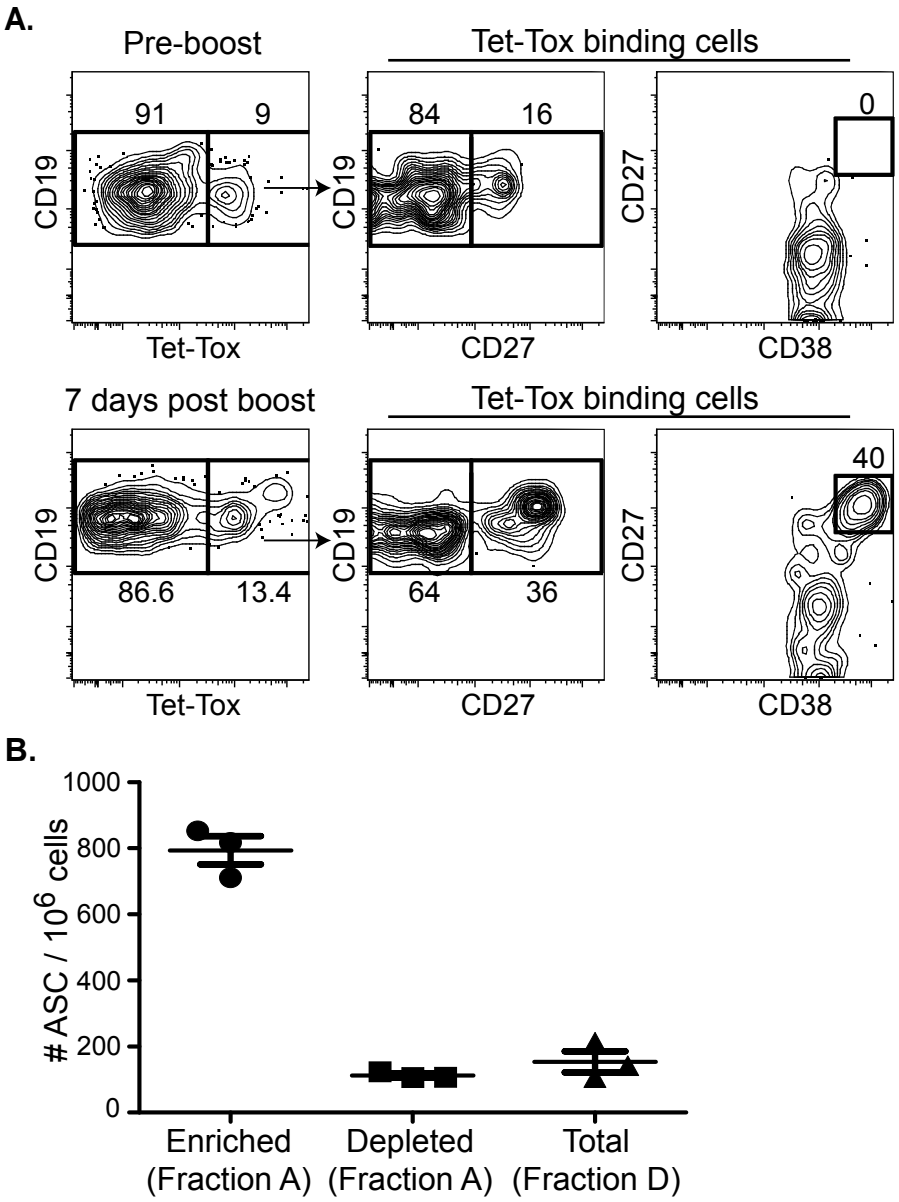



**Figure 2.3 Examples of post-enrichment analysis of antigen-binding cells.**
**A**. Representative flow cytometric analysis of cell phenotype following enrichment of Tet-Tox-binding B cells. Cytograms depict identification and analysis of Tet-Tox-reactive naïve (CD27-), memory (CD27+), and plasmablasts (CD27++ CD38++) in B cell subpopulations of a subject before and 7 days after booster vaccination against tetanus. All cells were gated on CD19+ lymphocytes from the enriched “positive” populations. **B.** Representative ELISPOT analysis of antibody secreting cells in enriched, depleted, and total unenriched B cell fractions from peripheral blood of a healthy subject 7 days after boost.

# CHAPTER 3

## Loss of anergic B cells in pre-diabetic and new onset type 1 diabetic patients


### SUMMARY

Although dogma predicts that under normal circumstances potentially offensive autoreactive cells are silenced by mechanisms of immune tolerance, islet antigen-reactive B lymphocytes are known to play a crucial role in the development of autoimmunity in Type 1 Diabetes (T1D). Thus participation of these cells in T1D may reflect escape from silencing mechanisms. Consistent with this concept we found that in healthy subjects, high affinity insulin-binding B cells (IBCs) occur exclusively in the anergic $B_{ND}$ compartment. Antigen receptors expressed by these cells are polyreactive and have N-region additions, Vh usage, and charged CDR3 regions consistent with autoreactivity. Consistent with a potential early role in autoimmunity, these high affinity insulin-binding B cells are absent from the anergic compartment of some first degree relatives, and all pre-diabetic and new-onset (<1yr) T1D patients tested, but return to normal levels in individuals diabetic for >1 year. Interestingly, these changes were correlated by transient loss of the entire $B_{ND}$ compartment. These findings suggest that environmental events such as infection or injury may, by disrupting B cell anergy, dispose individuals toward autoimmunity, the precise nature of which is specified by genetic risk factors, such as HLA alleles.


### INTRODUCTION

Although effector T cells mediate islet destruction in type 1 diabetes (T1D), it has become clear that B cells also play an important role in disease development. Rituximab (anti-CD20), a B cell depleting therapy, has shown efficacy in clinical trials in which newly diagnosed patients had

preserved beta-cell function one year after treatment [77]. In non-obese diabetic (NOD) mice, disease development requires B cells specific for islet antigens such as insulin [33].

While autoreactive B lymphocytes play a critical role as producers of pathogenic autoantibodies in diseases such as Lupus and Rheumatoid Arthritis, they appear to function differently in T1D. Although production of high affinity islet antigen reactive autoantibodies indicates increased risk, such antibodies appear dispensable for disease, indicating that B cells may instead contribute by antigen presentation and/or cytokine production [25, 41].

We hypothesized that insulin-binding B cells (IBCs) that function in T1D are normally silenced by anergy, a mode of B cell tolerance wherein autoantigen reactive cells populate peripheral lymphoid organs but are antigen unresponsive [9, 11, 78, 79]. Recent description of the surface phenotype of a cohort of anergic human B cells, termed $B_{ND}$ cells, allowed testing of this hypothesis [9]. $B_{ND}$ refers to "naïve $IgD^{+}$, $IgM^{-}$ B cells" that normally represent ~2.5% of peripheral blood B cells. More than 75% of cells in the $B_{ND}$ compartment bear autoreactive antigen receptors and are refractory to antigen receptor stimulation *in vitro*, and thus appear anergic. More recently, Quach et al. [79] extended these findings, showing that the anergic population includes cells that express low membrane IgM but are otherwise $B_{ND}$ in phenotype. This $IgM^{low/-}$ $IgD^{+}$ phenotype is typical of anergic B cells in the mouse [11, 78].

To explore the relationship between development of autoimmunity and integrity of the anergic B cell compartment we undertook studies of the affinity, frequency, and surface phenotype of IBCs in the peripheral blood of subjects along the continuum of T1D development. We report that IBCs are present in the anergic $B_{ND}$ B cell compartment, and that antigen receptors expressed by these cells are of high affinity and polyreactive. Importantly, IBCs are present in the anergic B cell compartment of healthy subjects, but absent from this compartment in some first degree relatives (FDR), all pre-diabetic subjects, and all new onset patients. Interestingly, individuals who are diabetic for >1 year have anergic IBC levels similar to healthy controls. These findings indicate loss of BND cells in FDRs and pre-diabetic individuals may reflect breach of

anergy predisposing subjects to development of anti-islet antibodies and participation in development of T1D.

## MATERIALS AND METHODS

### *Peripheral Blood Processing*

Samples were obtained with informed consent at the Barbara Davis Center for Childhood Diabetes using protocols approved by the University of Colorado Institutional Review Board. Eligible subjects were male or female, who met the American Diabetes Association criteria for classification of disease. GAD antibody, islet cell antibody, insulin autoantibody, and zinc transporter 8 antibody titer tests were used to confirm diagnosis of pre-diabetes and type 1 diabetes. PBMCs from autoantibody negative first degree relatives, autoantibody positive pre-diabetics (identified in the Type 1 Diabetes TrialNet Natural History study), new onset T1D patients, long standing T1D patients, and healthy age/sex matched controls were isolated from heparinized blood by Ficoll-Hypaque fractionation.

### *Flow cytometry analysis and enrichment of insulin-binding B cells*

In order to maintain consistency of gating, each day a patient sample was analyzed alongside an age/sex matched healthy control and the healthy control cells were used to draw gates, which were then copied to the patient cells. PBMCs were stained in PBS/1%BSA/0.02%NaAzide with human FcR Blocking Reagent (Miltenyi Biotec), 0.1 µg/$10^6$ cells insulin-biotin, and mouse monoclonal anti-human antibodies against CD19-V450 (BD Bioscience), CD27-PerCP (BioLegend), IgM-PE (Southern Biotech), and IgD-FITC (BD) for 20 minutes at 4ºC. For the insulin receptor studies, cells were stained with anti-CD19, CD27, as well as PE-anti-CD220 (BD) to detect insulin receptors, and FITC-anti-CD14 (BD) to detect monocytes. After washing, cells were then fixed with 2% formaldehyde at 4ºC, followed by incubation with streptavidin-AlexaFlour647 for 20 minutes at 4ºC. Cells were washed, suspended

in MACS buffer (PBS/0.5%BSA/2mMEDTA), and incubated with Anti-Cy5/Anti-Alexa647 microbeads (Miltenyi) for 15 minutes at 4ºC. Samples were then passed over magnetized LS columns (Miltenyi), washed 3 times with 2 mL of MACS buffer, and bound cells were eluted with 6 mL of MACS buffer. Flow cytometry was performed on an LSR II (BD) and data analyzed with FlowJo software ver. 8.8.4.

*B cell Calcium Flux Analysis*

Using a strategy similar to previous reports [9, 79], PBMCs freshly isolated from buffy coats were enriched for B cells using a no-touch method (Miltenyi Naïve B cell Isolation Kit II). Cells were suspended in warmed 37° RPMI, 2% BSA, and 1 μM Indo1-AM (Molecular Probes). Cells were stained with antibodies, as previously described [9] for 30 minutes and washed two times with warmed RPMI containing 2% BSA. Cells were then placed on a LSRII flow cytometer and after 20 seconds of baseline readings were stimulated with 20 μg/mL of $F(ab')_2$ goat anti-human IgD (Southern Biotech) or 1 μL of 1mg/mL ionomycin as a control. The mature naïve fractions were gated as $CD19^+CD27^-IgG^-$, while the $B_{ND}$ fractions were gated as $CD19^+CD27^-IgM$. Calcium mobilization analysis was conducted using FlowJo software.

*PhosFlow Analysis*

B cells were enriched using the no-touch B cell enrichment kit, as described above, and serum starved by incubation in serum free RPMI medium for 1 hour at 37°C. Cells were stimulated with 20 ug/mL rabbit $F(ab')_2$ anti-human IgG (H&L) (Jackson Labs) for 5 minutes at 37°C, or left unstimulated. Cells were immediately fixed with 2% PFA at 37°C for 10 minutes, spun down, and then resuspended with ice cold 100% methanol and placed on ice for 30 minutes. Cells were washed and stained with mouse monoclonal anti-human antibodies against CD19, CD27, IgM, and IgD for 15 minutes. Cells were also stained with mouse anti-pZAP70/Syk, anti-

pPLCγ2, anti-pAKT, or PTEN antibodies (BD Phosflow) or isotype control. Flow cytometry was performed as described above.

*Immunoglobulin Gene Cloning*

Cells were stained and enriched for insulin-binding B cells, as indicated in the flow cytometry section above, before $B_{ND}$ or mature naïve cells were single cell sorted into 96 well plates. V region amplification, sequence analysis, and recombinant monoclonal antibody production was completed as previously described [9, 80]. Briefly, variable region genes were amplified by RT-PCR to identify and clone the Ig heavy and light chain genes. The variable region genes were then cloned into expression vectors and expressed with human IgG constant regions in the 293A human cell line. A total of 11 antibodies from $B_{ND}$ cells and 16 antibodies from mature naïve B cells were compared.

*Assay of antibody by ELISA*

To screen expressed antibodies for antigen reactivity, ELISA microtiter plates (Costar) were coated with 10 ug/mL of recombinant human insulin (Sigma-Aldrich), LPS (Sigma-Aldrich), or calf chromatin (kindly provided by L. Wysocki, National Jewish Health) in PBS overnight at 4°C, followed by incubation with blocking buffer solution (PBS with 3% BSA) for 1.5 hours at room temperature. Recombinant antibodies or human sera were serially diluted in blocking buffer and incubated in the 96 well plates for 2 hours at room temperature. Between all steps the plates were washed 4 times with PBS containing 0.05% Tween-20. HRP Goat-anti-human IgG (H&L) (Bio-Rad Laboratories), followed by development with TMB single solution (Invitrogen) were used to detect antibody binding. Reactions were stopped using 1N $H_2PO_4$ (Sigma). Antibody was measured based on optical density at 450nm using a VERSAMax plate reader (Molecular Devices) and the data were analyzed with Softmax software. For recombinant antibody ELISA figures, each curve represents results from an individual recombinant antibody. For serum ELISA

figures, the curves from each subject are combined into one new onset or one healthy control curve using replicate values.

*Statistics*

Data were analyzed using Prism software (GraphPad Software, Inc.). One-way ANOVA followed by a Bonferroni's Multiple Comparison post-test was used to determine significance of differences among the five patient groups. One-way ANOVA with repeated measures tests were used to compare differences in ELISA curves. Chi-squared tests were used to compare differences in variable region sequences. Spearman's Correlation tests were used to determine correlation between two data sets.

RESULTS

Anticipating that insulin-binding cells (IBC) likely occur in very low frequency in the normal B cell repertoire, we first developed the magnetic microparticle adsorbent method described schematically in Figure 3.1A and B to stain and enrich IBCs for subsequent analysis [75]. In a typical PBMC sample, ~0.37% of total B cells bound the adsorbent (Fig 3.1C), and selection resulted in ~20-fold enrichment of IBCs (Fig 3.1C). Omission of insulin led to a ~90% reduction in IBC yield, indicating that most cells are enriched by virtue of insulin recognition (Fig 3.1D). We also verified insulin specificity by saturating anti-A647 magnetic beads with free biotin-SA-A647 and saw near complete blocking of cell binding (data not shown). In order to address the possibility that cells are enriched by virtue of insulin receptor (CD220) binding, which seemed unlikely given the 100 fold lower expression of insulin receptors than antigen receptors on B cells [81], we stained our enriched cells using antibodies against the insulin receptor. As shown in Figure 3.1E and F, enriched IBCs and non-IBCs express equivalent levels of insulin receptors indicating that IBCs are not selected based on insulin receptor level. Hence, cells isolated by this procedure bind the adsorbent by virtue of antigen receptor recognition of insulin. At ~0.37% of peripheral

blood B cells, IBCs occur at higher frequency than those that bind the large exogenous antigen phycoerythrin [75]. This might be explained in part by findings shown in figure 3.3 that some insulin-binding antibodies are polyreactive, binding multiple structurally unrelated antigens [5].

In order to analyze the distribution of IBCs in B cell subpopulations, we used a gating strategy employed by Duty and Quach, which allows recognition of $B_{ND}$ cells [9, 79] (Fig 3.2A). As previously described, cells are $CD19^{+}CD27^{-}IgM^{low/-}IgD^{+}$, while mature naïve B cells are $CD19^{+}CD27^{-}IgM^{+}IgD^{+}$. In healthy individuals ~50% of these $B_{ND}$ cells expressed little or no CD21, a phenotype also previously associated with anergy (data not shown) [82]. To confirm that $B_{ND}$ cells recognized using these markers are anergic, we assessed their response to BCR stimulation based $Ca^{2+}$ mobilization and phosphorylation of B cell signaling molecules (Fig 3.2B/C). Analysis of these parameters using IBC $B_{ND}$s was not possible due to the low frequency of these cells, and thus we analyzed total $B_{ND}$s. As expected based on previous literature [9, 79], $B_{ND}$ cells exhibited greatly reduced BCR-mediated $Ca^{2+}$ mobilization and significantly reduced Syk, Akt, and PLCγ2 phosphorylation relative to naïve B cells. We also analyzed the relative expression of PTEN, a negative regulator of the PI3-kinase pathway, in $B_{ND}$ and naïve cells (Fig 3.2B/C). $B_{ND}$ cells showed significantly increased expression of PTEN compared to naïve cells, a finding that has been shown in mouse models of B cell anergy [14, 83], but has not been previously demonstrated in human anergic B cells.

Interestingly, we found IBCs in almost all B cell subpopulations (Fig 3.2A). We considered the possibility that many of the cells captured by the adsorbent employed may have such low affinity for insulin that they are effectively ignorant of ambient autoantigen. Studies in other systems have shown that while B cells bearing receptors with moderate to high affinity for self-antigen can be rendered anergic, those bearing a low affinity for the same self-antigen can be effectively ignorant of antigen in their environment [84, 85]. To test this possibility as well as to confirm the specificity of isolated $B_{ND}$ IBC for insulin, we cloned immunoglobulin genes from single naïve and BND IBCs, expressed their variable regions in the context of IgG constant regions, and

analyzed the specificity and approximate affinity of these recombinant immunoglobulins by ELISA [80]. As shown in Figure 3.3A, while $B_{ND}$ IBCs expressed high affinity anti-insulin BCR, naïve IBC BCR affinity for insulin appears >100-1000 fold lower. Based on the titration curves, the $B_{ND}$ IBCs have an approximate affinity for insulin of ~6.6 x $10^{-10}$ M, which is sufficient to maintain anergy in the face of normal blood levels of insulin (6.0 x $10^{-11}$ – 6.0 x $10^{-10}$ M) when at least 30-40% of B cell receptors are occupied, which has been reported to sustain anergy [86]. However, the mature naïve IBCs appear to have an insulin affinity of only ~1.67 x $10^{-7}$ M, which is too low to maintain anergy, thus these cells are likely ignorant, i.e. unresponsive to insulin, and therefore, fail to be tolerized. Moreover, given that earlier studies have shown that self-reactive B lymphocytes often have polyreactive BCRs [5], we determined the reactivity of our recombinant antibodies to LPS and chromatin. All high affinity $B_{ND}$-derived antibodies displayed significant reactivity to LPS and chromatin, indicating polyreactivity (Fig 3.3A).

We then analyzed heavy chain variable region gene usage by $B_{ND}$ IBCs and found that their sequences showed no signs of somatic mutation indicating their auto/polyreactivity is not generated by participation in prior immune responses (data not shown). Auto/polyreactivity has been previously associated with long IgH complementarity-determining regions (CDR3) regions with increased numbers of positive amino acid residues [5, 87, 88]. Comparison of the number of positive charged amino acid residues in CDR3s of BCR of $B_{ND}$s and naïve IBCs revealed that the $B_{ND}$ cells have more positive charged amino acids than mature naïve cells (mean of 3 versus 1.5, respectively; $p<0.001$) (Fig 3.3B). Further, both $B_{ND}$ and mature naïve IBCs had significantly more positive charged amino acids in their CDR3 regions than seen in the normal human mature B cell repertoire [5] ($p<0.01$ for mature naïve IBCs and $p<0.001$ $B_{ND}$ IBCs) (data not shown). In addition, the frequency of IBC $B_{ND}$s with long CDR3 regions (>17 amino acids) was significantly higher ($p<0.03$) than the mature naïve cells (Fig 3.3C) consistent with auto/polyreactivity. Lastly, we analyzed variable region gene usage and found the $B_{ND}$ cells use predominantly $J_H6$ gene segments (Fig 3.3D), which is indicative of increased receptor editing and autoreactivity ($p<0.04$)

[5, 9, 89]. Together, these data not only confirm that B cells isolated using this adsorbent do indeed bear insulin-binding BCR, but also that $B_{ND}$ IBCs have high insulin affinity consistent with anergy, are polyreactive, and their BCR have structural features consistent with autoreactivity.

If insulin-reactive B cells that in healthy individuals occur in the anergic $B_{ND}$ fraction participate in autoimmune responses in Type 1 Diabetics, one would predict that insulin reactive antibodies produced by diabetic patients would be polyreactive. If this is the case, sera from new onset patients should contain antibodies reactive with both insulin and chromatin. We analyzed insulin and chromatin reactivity of serum antibodies from new onset diabetic, insulin autoantibody positive (IAA+) patients who had not yet received exogenous insulin treatment. Interestingly, new onset diabetic patients have increased reactivity to both insulin and chromatin relative to healthy controls serum (Fig 3.4). LPS was also tested but reactivity was not statistically different (data not shown). Taken together these data are consistent with T1D associated loss of anergy by $B_{ND}$ cells bearing polyreactive antigen receptors with high affinity for insulin.

In order to test further this possibility, we compared the frequency of IBCs captured by our adsorbent, including anergic IBCs, in blood B cell subpopulations from various subject groups (Fig 3.2A). Consistent with the hypothesis, in autoantibody positive pre-diabetic and new onset patients, significantly fewer IBCs were $B_{ND}$ ($CD27^{-}IgM^{lo/-}IgD^{+}$) and $CD27^{-}$ $IgM^{lo/-}IgD^{-}$ cells relative to healthy controls (Fig 3.5A and B). Most significant were differences in frequency of IBC that are $B_{ND}$, which showed reduction from 2.4% in healthy controls to 0.57% and 0.41% in pre-diabetic and new onset diabetics, respectively ($p<0.001$ for both). Parenthetically, this decrease in $B_{ND}$ IBCs was not simply a function of subject age and in follow-up studies appeared stable over >6-12 months (data not shown). Interestingly, some first-degree relatives (FDRs) showed a BND frequency among IBCs similar to unrelated healthy controls while others were similar to pre-diabetic and new onset patients (Fig 3.5A). These findings suggest that departure of IBC from the $B_{ND}$ compartment precedes, and may therefore predispose individuals to development of insulin autoantibodies and, eventually, T1D.

Loss of the anergic $B_{ND}$ IBC population may reflect entry of these cells into a non-anergic state competent to present antigen to diabetogenetic CD4+ T cells. However the size of the $B_{ND}$ pool is so small that such a shift would not significantly affect frequency of much larger mature populations. Nonetheless we examined the relative frequency of IBCs in mature compartments and observed a trend toward increased frequency of IBCs among mature naïve ($IgM^{+}IgD^{+}$) B cell populations in pre-diabetic and new onset patients compared to the long standing T1D and healthy controls (Fig 3.5A). Thus loss of IBCs from the $B_{ND}$ compartment may reflect upregulation of mIgM, consistent with loss of anergy [78]. This change may also reflect relocalization of these cells to other anatomical sites, particularly the pancreas and pancreatic lymph nodes that are rich in autoantigen. This possibility would be consistent with reported B cell accumulation in the pancreases of diabetic individuals [45] and the recent study suggesting anergic insulin-specific B cells are capable of entering the pancreases of NOD mice [90].

As noted above we also observed a statistically significant decrease in $CD27^{-}IgM^{low/-}IgD^{-}$ IBCs in pre-diabetic subjects and new onset patients relative to healthy controls (Fig 3.5A). In healthy individuals ~50% of these cells expressed little or no CD21, suggestive of anergy (data not shown) [82]. Thus, we suggest that these CD27, IgM, IgD triple negative IBCs may represent a second anergic population that is lost in pre and early T1D. Additionally, these cells may be members of a recently described population that lacks expression of CD27, IgD, and IgM, but bear somatically mutated mIgG, and are thought to have class-switched outside a germinal center [91, 92].

Perhaps most importantly, some autoantibody negative first-degree relatives of diabetic patients showed an IBC $B_{ND}$ frequency similar to pre-diabetic and new onset patients while others were similar to unrelated healthy controls (Fig 3.5A). These findings suggest that loss of $B_{ND}$ IBC cells precedes, and therefore may predispose individuals to develop insulin autoantibodies and, eventually, T1D.

Although the majority (~70%) of the IBCs were found in the $CD27^-$ compartment, some occurred among $CD27^+$ memory B cells. As shown in Figure 3.5B, pre-diabetic and new onset patients exhibit a decrease, though not statistically significant, in the frequency of IBCs among $CD19^+$ $CD27^+$ $IgM^-$ $IgD^+$. Cells in this population were previously shown to be IgD heavy chain class switched and enriched in autoreactive cells (C$\delta$–CS) [93]. Thus anergic IBCs that normally occur in this population may also relocalize during onset of diabetes.

Studies in a variety of systems suggest that environmental factors such as infection and/or injury, as well as colonization by certain commensal microbes, may promote development of autoimmunity [1, 94, 95]. If such triggers act by causing loss of B cell anergy in diseases such as T1D, they may do so nonspecifically, i.e. irrespective of B cell autoantigen specificity. If such factors are in play, loss of $B_{ND}$ IBCs may be accompanied by loss of all $B_{ND}$ cells. To test this possibility we analyzed total $B_{ND}$ frequency, and found a correlated reduction in $B_{ND}$ in the IBC and total B cell population of pre-diabetic and new onset patients compared to long-standing T1D and healthy controls (Fig 3.6). This finding suggests that a generalized loss of anergic B cells may precede development of autoimmunity.

## DISCUSSION

We have characterized insulin autoantigen-binding B cells in the previously defined anergic $B_{ND}$ population in peripheral blood of healthy humans. These cells were found to express high affinity insulin-binding antigen receptors that are cross-reactive with chromatin and LPS. They possess germline-encoded hypervariable regions consistent with origination in the primary repertoire. In addition, they exhibit longer N-region additions and biased usage of Jh6, features previously associated with autoantibodies. Finally their CDR3 regions contained increased positively charged amino acid residues typical of DNA-binding autoantibodies. These potentially pathogenic anergic B cells were lost or exhibited altered phenotype in some first-degree relative,

and all pre-diabetic, and new onset diabetic patients consistent with an early role in development of T1D.

This is the first reported analysis of alterations in autoantigen-reactive B cells in the context of development of type 1 diabetes in humans. An initial surprising finding was that IBCs occur at higher frequency in the normal repertoire than cells reactive with the large exogenous antigen phycoerythrin [75]. However, given our findings that our IBCs were also reactive to chromatin and LPS, the higher frequency we observed may be explained in part by the polyreactivity of these cells.

Also surprising, was the finding that IBCs occur in all major B cell compartments, suggesting that, in addition to potentially harmful high affinity receptor-bearing cells, the isolation procedure captures cells that bear antigen receptors of such low affinity that they are ignorant of low concentration antigen in their surroundings.

Consistent with this, the population containing the most IBCs, the mature naïve population, is composed of low affinity B cell clones, whereas the anergic IBC population in the healthy individual is almost entirely composed of high affinity, polyreactive B cell clones. The chromatin and LPS crossreactivity of anergic high affinity IBCs is surprising given previous evidence that polyreactive autoreactive B cells appear to be preferentially purged from the repertoire very early in development [5, 96]. However, polyreactivity could have functional importance since these cells may be activated by virtue of cross-reactivity with chromatin released by tissue damage, and subsequently present insulin also bound by their antigen receptors.

Importantly, this polyreactive anergic population is lost in a subset of FDRs, all pre-diabetic, and all new onset patients, suggesting that a breach in peripheral B cell tolerance has occurred. Consistent with this, new onset insulin autoantibody positive patients have elevated serum antibodies reactive with chromatin relative to healthy controls, which is consistent with activation of anergic, polyreactive B cells found in healthy individuals. Although previous studies have found increased anti-DNA antibodies in the serum on T1D patients, as well as some of their

first-degree relatives [97, 98], this is the first study to correlate this finding with loss of the anergic polyreactive B cell population. Moreover, given that T1D patients are prone to development of other autoimmunities [99, 100], including autoimmune thyroiditis and Addison's disease, we suspect these polyreactive B cells are also reactive with self-antigens, such as thyroglobulin, as has been observed in the NOD mouse [101]. Studies in our laboratory are currently underway to examine this possibility.

In addition to the loss of anergic IBCs, we found a correlated decrease in total anergic B cells, irrespective of their specificity, in pre-diabetic and new onset patients, as well as some FDRs. These results suggest that loss of anergy may predispose individuals to multiple types of autoimmunity, and that loss of B cell anergy may be a phenomenon that also precedes other non-T1D related autoimmune disorders. Consistent with this, Kinnunen et al. recently found that MS patients, as well as RA and long standing T1D patients, have increased frequency of auto/polyreactive B cells in their mature naïve B cell pool [102], suggesting loss of peripheral B cell tolerance. Given that recent genome-wide association studies (GWAS) have revealed common polymorphisms among multiple autoimmune disorders [59], we suspect the loss of B cell anergy we observe in T1D patients, and perhaps other autoimmune patients, is associated with combinations of susceptibility alleles that compromise anergy globally. *PTPN22* polymorphisms have been shown to dampen B cell signaling and are associated with increased autoreactive B cells in the peripheral blood of T1D patients [42, 52]. In addition, susceptibility alleles encoding BLK, Lyn, PTPN2, BANK1, CSK and FcγRIIb, are associated with B cell signaling and may promote loss of anergy via alterations in thresholds for activation [58]. Hence, we speculate that risk alleles that compromise regulation of BCR signaling increase the risk of development of autoimmunity by undermining immune tolerance.

The apparent transience of loss of anergic B cells observed seems inconsistent with a genetic basis, but rather suggests that loss of anergy may be due to an environmental insult, such as infection, injury, or a diet/microbiota change. This seems plausible given that multiple systems

have shown environmental factors can promote development of autoimmunity [1, 94, 95] and that it is well established that both genetics and the environment must be playing a role in development of T1D [103].

In conclusion, we have defined changes in the surface phenotype of peripheral blood B cells of patients along the continuum of T1D. Our findings reveal a potential biomarker for increased risk of T1D: a significant decrease in the $B_{ND}$ population of IBCs in peripheral blood. Indeed, we have uncovered a possible biomarker for risk of development of B cell-dependent autoimmunity in general, namely a decrease in total $B_{ND}$. Longitudinal studies in progress should help establish the utility of these changes as prognostic and diagnostic biomarkers. Finally, findings are consistent with a role for perturbation of B cell anergy in development of T1D and perhaps other autoimmune diseases.

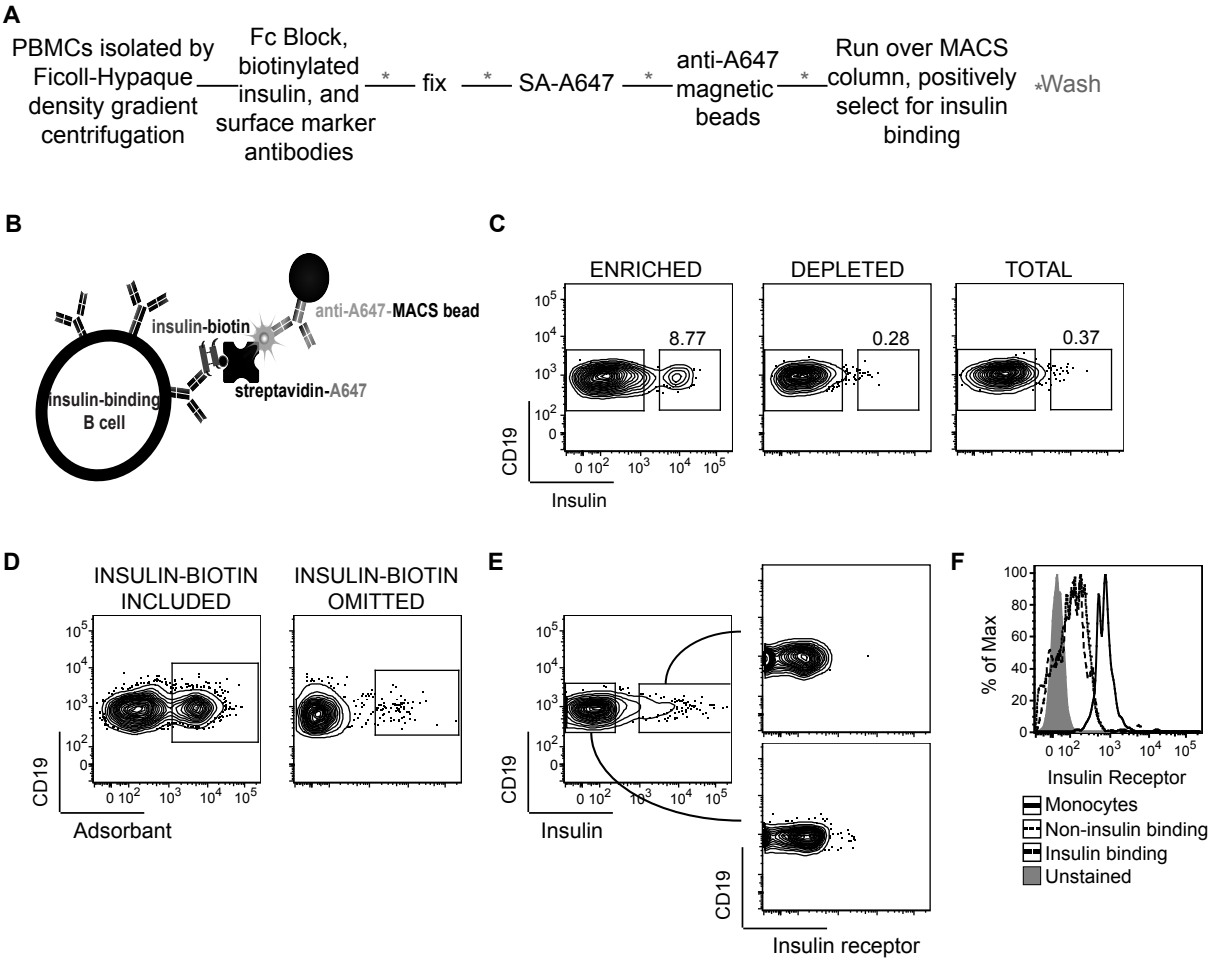



**Figure 3.1 Detection and enrichment of IBCs is dependent on insulin binding to the BCR. A.** Protocol for enrichment and isolation of IBCs. **B.** Diagram of adsorbent used to identify and isolate IBCs. **C.**Representative cytograms of IBCs enriched from blood of a new onset patient. Cells that bound to the adsorbent and eluted from magnetic column are termed enriched, non-binders termed depleted, and un-enriched termed total. **D.** Cytograms of PBMCs enriched as in (A) or enriched with insulin-biotin omitted during the procedure. **E.F.** Representative cytograms of insulin receptor antibody staining of enriched IBC populations. Monocyte staining is shown as a positive control. All cells were gated on CD19+ lymphocytes.

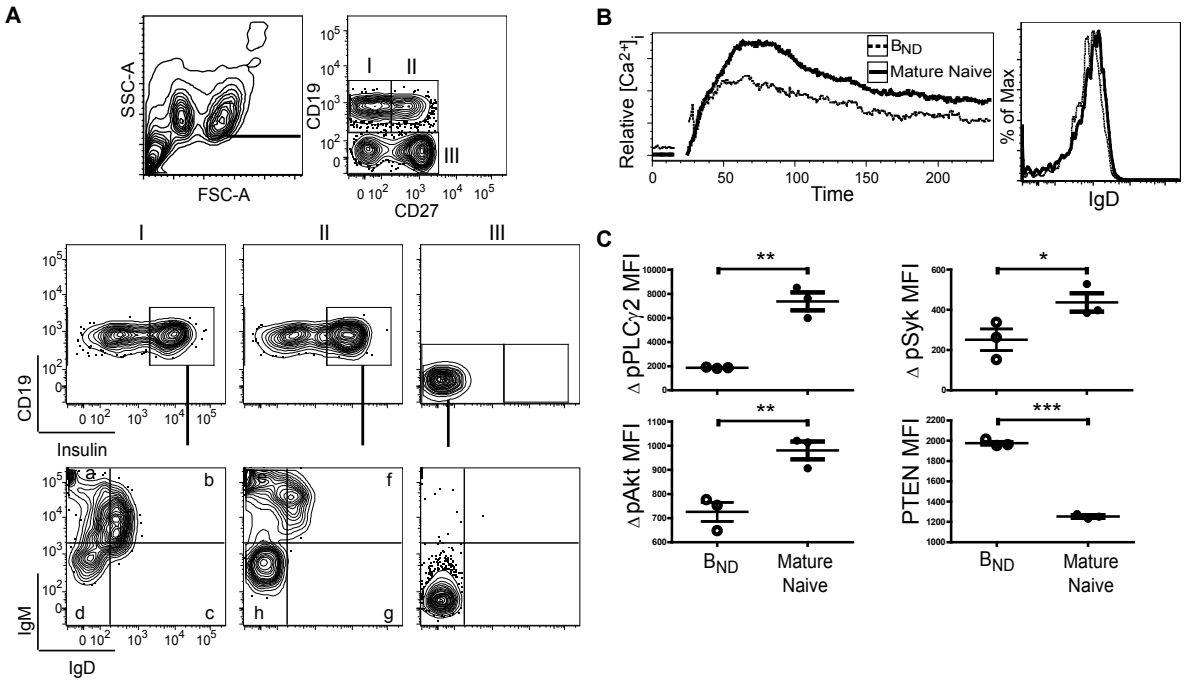



**Figure 3.2 Total BND cells show reduced response upon anti-BCR stimulation compared to their mature naïve counterparts, suggesting they are anergic. A.** Gating strategy for identification and analysis of insulin-binding naïve and memory B cell subpopulations illustrated using cells from a healthy control. IgM vs IgD gates of CD19+CD27- (I) IBCs produce the following subpopulations: (a) IgM+ IgDlo/-, (b) Mature Naïve, (c) BND, and (d) IgMlo IgD-. IgM vs IgD gates of CD19+ CD27+ (II) IBCs produce the following subpopulations: (e) IgMhiIgDlo/-, (f) Pre-switch, (g) Cδ-CS, and (h) class-switch (CS). Staining of T (CD19-) cells (III) are shown as verification of gating strategy. **B.** Representative Ca2+ mobilization plot over time for BND and mature naïve B cells upon stimulation with 20 ug/mL of goat anti-human IgD F(ab')2. Representative histogram showing the relative expression of IgD on BND and mature naïve B cells is similar. **C.** Difference of pPLCy2, pSyk, pAkt, and PTEN median florescence intensity (MFI) between unstimulated and stimulated naïve and BND cells in three different experiments (**p<0.002, Student's t test).

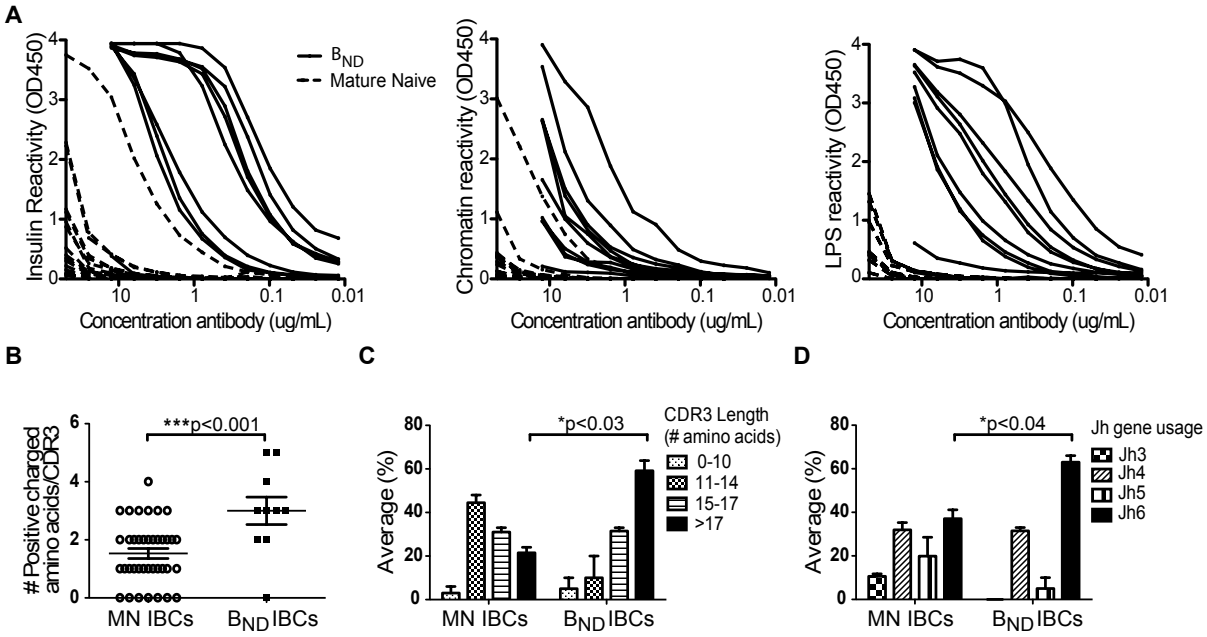



**Figure 3.3 IBCs found in the BND fraction bear high affinity, polyreactive antigen receptors and have VH regions consistent with auto/polyreactivity.**
**A.** ELISA data showing the affinity and specificity of recombinant antibodies made from cloned immunoglobulins from single cell BND (n = 11) and mature naïve IBCs (n = 16). Anergic BND IBCs have higher affinity for insulin and are polyreactive relative to IBCs in the mature naïve compartment. **B-D.** Sequencing of the heavy chain variable region genes revealed BND cells have more positive charged amino acids in their CDR3 regions (***p<0.001, Student's t test), have longer CDR3 regions (*p<0.03 for >17 aa and 11-14 aa, Student's t test), and have increased use of JH6 (*p<0.04, Student's t test), which is consistent with auto/polyreactivity.

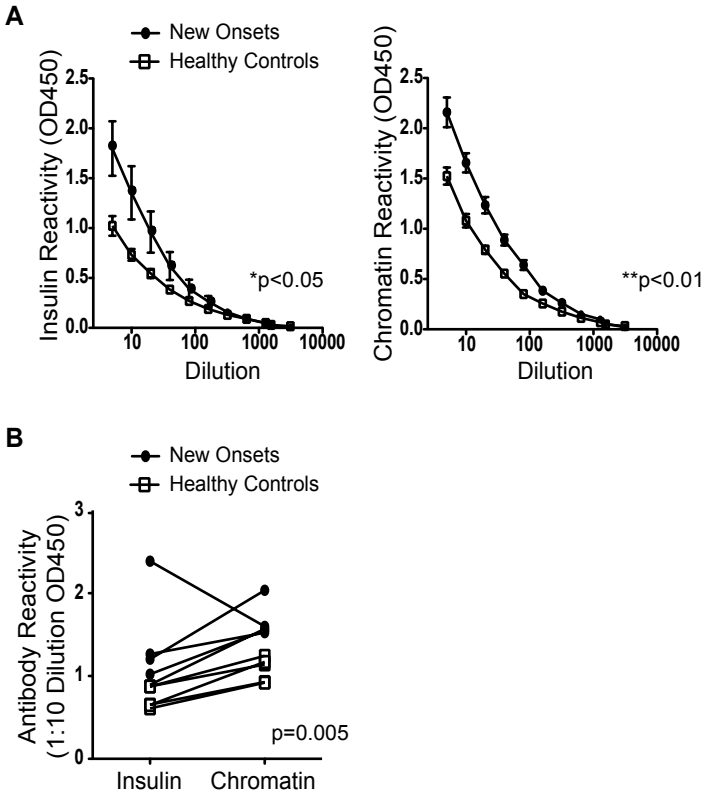



**Figure 3.4 Sera from insulin autoantibody positive new onsets show increased reactivity to both insulin and chromatin. A.** ELISA data showing sera from insulin autoantibody (IAA) positive new onset patients (n=5) have increased reactivity to not only insulin (*p<0.05), but also chromatin (**p<0.01), compared to healthy control IAA- samples (n=5) (significance determined by One-way ANOVA with repeated measures). Curves depict mean +/- SEM of OD values from 5 individual subjects.
**B.** Correlation between insulin and chromatin reactivity for each human sample (each bar represents data from one individual) at the 1:10 dilution (p = 0.005, Spearman's correlation)

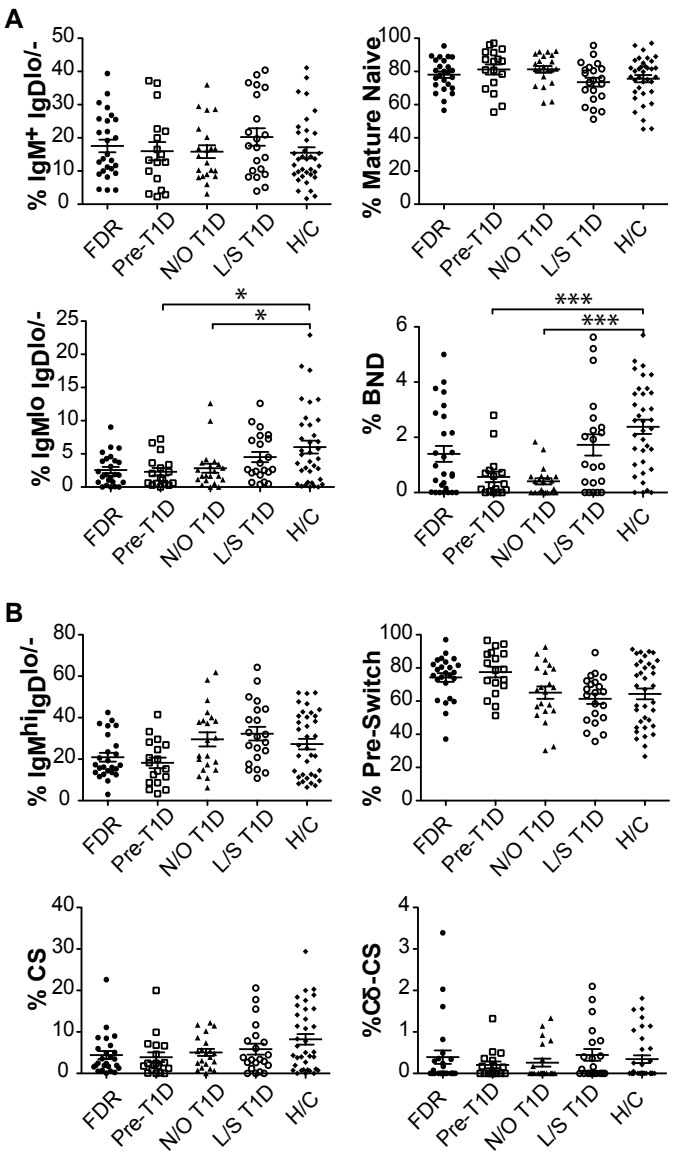



**Figure 3.5 Surface phenotype of naïve and memory IBCs shows significant differences between some "at risk" first-degree relatives, pre-diabetic, and new-onset patients compared to long-standing T1D patients and healthy controls.** **A.** Percentage of CD19+CD27-IBCs captured by the adsorbent that fall in the IgM vs IgD gates. Percentages reported as percent of total CD27- IBCs ±SD. **B.** Percentage of CD19+CD27+IBC "memory" subpopulations from subjects reported as percent of total CD27+ IBCs ±SD. (Statistical significance determined by One-way ANOVA followed by Bonferroni's multiple comparison post test; *p<0.05, ***p<0.001). Samples include autoantibody negative first-degree relatives (FDR, n=25), autoantibody positive pre-diabetics (Pre-T1D, n=17), new onset diabetics (<1 year from diagnosis, N/O T1D, n=21), long standing diabetics (>1yr, L/S T1D, n=21) and healthy age matched controls (H/C, n=36).

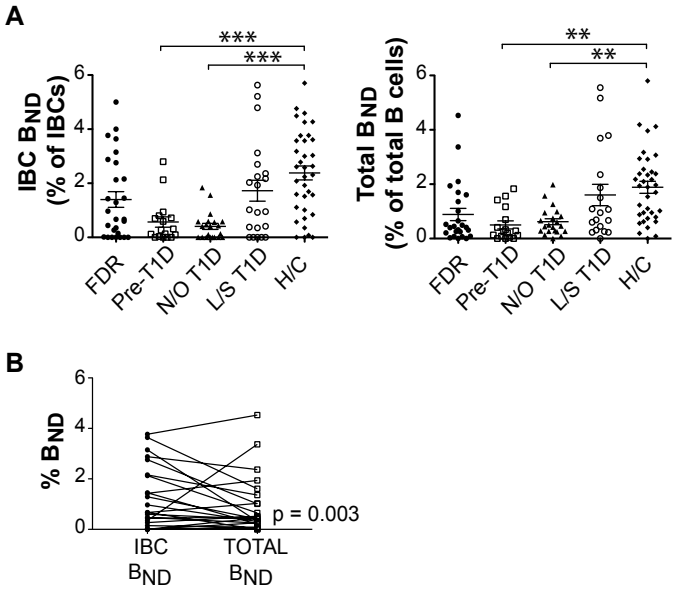



**Figure 3.6 Subjects with low BND frequency among IBCs also exhibit low frequency of BND among total B cells. A.** Percentage ±SD of IBCs that are BND compared to the percentage of total (non-insulin binding) CD27- B cells that are BND in subjects from various groups. (Statistical significance determined by One-way ANOVA followed by Bonferroni's multiple comparison post-test, **p<0.01, ***p<0.001). **B.** Correlation between frequency of total BND and IBC BND in individual FDRs (p=0.003, Spearman's correlation test). Each bar represents data from an individual FDR.

CHAPTER 4

# Loss of anergic insulin-binding B cells in type 1 diabetes is associated with high risk HLA and non-HLA susceptibility alleles


## SUMMARY

While autoreactive B cells are normally silenced in healthy individuals, these cells become activated and contribute to development of type 1 diabetes (T1D). Little is known regarding mechanisms operative in subversion of B cell tolerance leading to participation of these cells in autoimmunity. We have previously shown that high affinity insulin-binding B cells (IBCs) occur exclusively in the anergic ($B_{ND}$) compartment in peripheral blood of healthy subjects. Consistent with their activation very early in disease, these high affinity IBCs are absent from the $B_{ND}$ compartment of some first degree relatives (FDRs), and all autoantibody positive pre-diabetic and new-onset T1D patients, at a time when they appear in pancreatic islets. Loss of $B_{ND}$ IBCs is associated with loss of the entire $B_{ND}$ B cell compartment, consistent with provocation by an environmental trigger or predisposing genetic factor. To explore this possibility we analyzed the association between HLA and non-HLA T1D-associated risk allele genotypes and loss of $B_{ND}$s in FDRs. We found that high risk HLA alleles, as well as a subset of non-HLA risk alleles whose products function in B and T cell development, are associated with a low $B_{ND}$ phenotype. Hence, our results suggest a role for these alleles in perturbation of B cell anergy in development of T1D, which may be dictated by high risk genotypes.


## INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease in which self-reactive lymphocytes destroy the insulin-producing pancreatic beta cells. While genetic variations are thought to be the major contributor to risk for developing T1D, the environment also plays a role. These factors may

impart their effects by compromising maintenance of immune tolerance in T cells and/or B cells, both of which are known to be essential in the pathogenesis of T1D. Studies have shown these B cells are likely acting as antigen presenting cells and autoantibody producers in T1D [28, 31, 33]. How these self-reactive B cells, which are normally silenced in healthy individuals, become activated to participate in disease is not known. Previous studies have shown that as much as 70% of all B cells generated in the bone marrow are autoreactive [5]. Autoreactive B cells are silenced by multiple mechanisms. Those reactive with high avidity self-antigens undergo receptor editing, in which they rearrange their antigen receptor light chains modifying specificity. If this process fails and high avidity persists, cells can be induced to undergo apoptosis, a mechanism referred to clonal deletion [6]. Cells reactive with low avidity autoantigens, even if these have high affinity, do not receive sufficiently strong signals to induce receptor editing. These cells mature and inhabit the periphery where they are maintained in a state of unresponsiveness, called anergy. Anergic B cells show evidence of previous antigen exposure including downregulation of surface IgM and elevated basal calcium, but are refractory to further stimulation [10, 11]. Importantly, studies in mice have demonstrated anergy is rapidly reversed if autoantigen dissociates from the BCR, suggesting that this unresponsive state is maintained by a non-durable, and presumably fragile, biochemical mechanism rather than genetic reprograming. Consistent with this, the activity of inhibitory signaling pathways is upregulated in anergic cells by phosphorylation (SHIP1 and SHP-1) and miRNA regulation of expression levels (PTEN) [10, 14]. B cell intrinsic expression of these regulatory phosphatases is required to maintain anergy [13, 14]. It is likely that additional genetic factors also play an important role in tuning B cell responsiveness to antigen and maintenance of anergy. Obvious candidates lie among the products of genes alleles of which have been shown to confer risk of developing autoimmunity.

Previously we examined the status of insulin-reactive B cell (IBC) in peripheral blood of healthy individuals. We found these cells have a high affinity for insulin and are polyreactive,

binding to LPS and chromatin. Interestingly, these cells disappear from the peripheral blood in islet autoantibody positive and recent onset T1D subjects, as well as a portion of first degree relatives (FDRs) [68]. In order to better understand what might drive loss of anergic B cells early in T1D, in this study we genotyped both $B_{ND}$ sufficient and deficient FDRs for high risk HLA and non-HLA T1D alleles. We found loss of anergic IBC $B_{ND}$ cells is associated with the high risk HLA class II DR4-DQ8 haplotype and polymorphisms in the risk conferring *INS*, *PTPN2*, and *IKZF3* genes. Results suggest insulin-specific T cells and changes in B cell signaling are involved in loss of B cell anergy.

## MATERIALS AND METHODS

### *Peripheral Blood Processing*

Samples were obtained with informed consent at the Barbara Davis Center for Childhood Diabetes using protocols approved by the University of Colorado Institutional Review Board. Eligible T1D subjects were male or female, who met the American Diabetes Association criteria for classification of disease. GAD antibody, islet cell antibody, insulin autoantibody, and zinc transporter 8 antibody titer tests were used to confirm diagnosis of pre-diabetes and type 1 diabetes. PBMCs from autoantibody negative first degree relatives, autoantibody positive pre-diabetics (identified in the Type 1 Diabetes TrialNet Natural History study), new onset T1D patients, long standing T1D patients, and healthy age/sex matched controls were isolated from heparinized blood by Ficoll-Hypaque fractionation. DNA was extracted from the granulocyte layer using the DNA midi kit (Qiagen).

### *Flow cytometry analysis and enrichment of insulin-binding B cells*

In order to maintain consistency of gating, each day a patient sample was analyzed alongside an age/sex matched healthy control and the healthy control cells were used to draw gates, which were then copied to the patient cells. PBMCs were stained in

PBS/1%BSA/0.02%NaAzide with human FcR Blocking Reagent (Miltenyi Biotec), 0.1 µg/$10^6$ cells insulin-biotin, and mouse monoclonal anti-human antibodies against CD19-BV510 (BioLegend), CD27-PerCP or CD27-BUV395 (BioLegend), IgM-PE (Southern Biotech), and IgD-FITC (BD Biosciences) or IgD-BV421 (BioLegend) for 20 minutes at 4ºC. After washing, cells were then fixed with 2% formaldehyde at 4ºC, followed by incubation with streptavidin-AlexaFlour647 for 20 minutes at 4ºC. Cells were washed, suspended in MACS buffer (PBS/0.5%BSA/2mMEDTA), and incubated with Anti-Cy5/Anti-Alexa647 microbeads (Miltenyi) for 15 minutes at 4ºC. Samples were then passed over magnetized LS columns (Miltenyi), washed 3 times with 2 mL of MACS buffer, and bound cells were eluted with 6 mL of MACS buffer. Flow cytometry was performed on an LSR II (BD) and data analyzed with FlowJo software ver. 8.8.4.

*HLA and non-HLA genotyping*

DNA from FDRs and healthy controls was HLA genotyped at the Autoantibody/HLA Service Center at the Barbara Davis Center for Childhood Diabetes using established protocols. DNA for non-HLA genotyping was sent to the University of Florida Diabetes Institute for interrogation of 58 unique SNP loci found to be associated with development of T1D according to ImmunoBase (https://www.immunobase.org/). Genotyping was conducted using a custom made chip which utilizes TaqMan probe and primer sets (Thermo Fisher).

*Statistics*

Data were analyzed using Prism software (GraphPad Software, Inc.). One-way ANOVA followed by a Bonferroni's Multiple Comparison post-test was used to determine significance of differences among the five patient groups. Mann-Whitney non-parametric *t* tests were used to determine differences in genetic non-HLA risk alleles.

RESULTS

As previously described and depicted in figure 4.1A, we used magnetic particles to enrich for insulin-binding B cells (IBCs) in the peripheral blood of subjects along a continuum of diabetes development [68, 104], including FDRs (n=94), autoantibody positive (n=18), recent onset (< 6 months) (n=21), long standing (>1 year) (n=21), and healthy controls (n=46). We further phenotyped these IBCs to determine what proportion fell into the recently identified anergic B cell population in humans, termed $B_{ND}$ (Figure 4.1B). $B_{ND}$ cells are mature naïve B cells that have downregulated surface IgM but retain IgD. We have previously found the IBCs in the $B_{ND}$ population have a very high affinity (~6.6 x $10^{-10}$ mol/L) for insulin, but are refractory to stimulation through BCR based on decreased calcium flux and phosphorylation of Syk, PLCγ2, and Akt [68].

While the absolute number of IBCs did not vary between subject groups (data not shown), we found anergic IBCs were lost in all autoantibody positive and recent onset T1D subjects compared to long standing and healthy controls (Figure 4.1C). Interestingly, a portion of FDRs also displayed the low $B_{ND}$ phenotype. We hypothesized these individuals may be at increased risk for development of autoantibodies and T1D. In order to rule out the possibility loss of $B_{ND}$ was correlated with a younger age, we analyzed the frequency of $B_{ND}$ cells in FDRs in various age groups. As seen in figure 4.1D, there was no significant difference between levels of $B_{ND}$ in all age groups, suggesting loss of anergy in IBCs is independent of age.

Next, in order to further understand what may drive loss of IBC $B_{ND}$ cells and determine whether our FDRs with the low IBC $B_{ND}$ phenotype showed other evidence of increased risk for development of T1D, we HLA genotyped our $B_{ND}$ sufficient and deficient FDRs. Interestingly, the FDRs who carried the very high risk DR3/4-DQ2/8 and DR4/4-DQ8/8 haplotypes had a significant decrease in IBC $B_{ND}$ cells, while subjects heterozygous for the moderate risk DR4-DQ8 and DR3-DQ2 genotypes showed similar levels to subjects carrying non-T1D risk related HLA haplotypes (Figure 4.2A). Surprisingly, the FDRs who carried the T1D protective DQB1*0602 (DQ6) allele [105] had a significant increase in the frequency of IBC $B_{ND}$ cells, suggesting enhanced tolerance

of these autoreactive cells. If the high risk T1D HLA genotypes regulate IBC levels in our FDRs, then the same should be true in our healthy control group. Though none of the controls we genotyped carried the high risk DR3/4-DQ2/8 genotype, we saw a similar significant decrease in IBC $B_{ND}$ frequency with the high risk DR4/4-DQ8/8 T1D genotype and significant increase with the protective DQ6 allele (Figure 4.2B).

Next, we wanted to explore whether any single nucleotide polymorphisms (SNPs) in non-HLA T1D susceptibility genes also help drive loss of B cell anergy, using a gene chip that assays 58 of the high risk non-HLA T1D associated SNPs. Since almost all of our FDRs who carried the DR4-DQ8 haplotype had a decrease in IBC $B_{ND}$, we only genotyped $B_{ND}$ sufficient and deficient FDRs heterozygous for the DR3-DQ2 or non-T1D associated HLA alleles. Results indicate loss of IBC $B_{ND}$ cells is associated with polymorphisms in the *INS* (rs689), *PTNP2* (rs1893217), and *IKZF3* (rs2872507) genes. In addition, loss of IBC $B_{ND}$ cells was lower in carriers of the high risk non-synonymous SNP *PTPN22* (rs2476601), though significance was not reached due to the low presence of carriers in our FDR group (Figure 4.3).

## DISCUSSION

Studies have shown HLA class II is the number one determinant for T1D risk, accounting for up to one-half of the genetic risk [106]. While both the HLA DR4-DQ8 and DR3-DQ2 haplotypes confer the most risk [49], development of insulin autoantibodies (IAAs) are more frequent among patients with DR4-DQ8 [107, 108]. Studies have shown that the HLA-DQ8 molecule has an amino acid substitution at position 57 in the P9 pocket of the binding groove, which creates a wider peptide-binding groove [109, 110]. Some argue this substitution allows promiscuous binding and presentation of peptides, including those of insulin [110, 111]. Hence, it is interesting loss of IBC $B_{ND}$ cells is closely associated with FDRs carrying the DR4-DQ8 haplotype. It is tempting to speculate that CD4 T cells recognizing insulin peptides presented by IBCs may promote loss of anergy.

In line with this, we also found loss of IBC $B_{ND}$ is associated with the SNP (rs689) in the highest risk conferring non-HLA gene, *INS*. It is thought carriers of the risk allele have decreased expression of the insulin protein in the thymus, leading to decreased negative selection of insulin-specific T cells [112]. In addition, a recent study found the rs689 polymorphism was significantly associated with development of IAAs, but not other autoantibodies in T1D patients [113]. Hence, it seems plausible carriers of this polymorphism have increased numbers of peripheral insulin-reactive T cells that escaped central tolerance that could provide the necessary T cell help to an IBC, thus driving them out of a state of anergy or preventing them from ever entering it. These IBCs could then go on and differentiate into insulin autoantibody producing plasma cells.

Loss of IBC $B_{ND}$ was also associated with the *PTPN2* SNP rs1893217. PTPN2 is ubiquitously expressed, including in both B and T cells, as well as beta cells in the pancreas. Studies have shown PTPN2 has a range of functions, including negative regulation of JAK/STAT signaling [54] and dephosphorylation of Lck and Fyn after stimulation through the T cell receptor [55]. In addition, the T1D susceptibility *PTPN2* SNP rs1893217 was correlated with decreased IL-2 signaling in CD4+ T cells in control subjects [56]. In T1D subjects, decreased IL-2 signaling has been shown to affect maintenance of FOXP3 expression in Treg cells [114]. Hence, it is plausible the *PTPN2* SNP rs1893217 could alter B cell signaling, as it has been shown to do in T cells, or impair development of Treg cells. The absence of Treg cells in the periphery has been shown to lead to the accumulation of autoreactive B cells [115] and loss of B cell anergy [116]. Studies in our lab have also shown loss of anergic IBCs is correlated with a decrease in the frequency of CD4+ $CD25^{hi}$ $CD127^{lo}$ Treg cells in the periphery (data not shown).

IKZF3, also known as AIOLOS, is a member of the Ikaros family of zinc-finger proteins that regulates B cell proliferation and differentiation. Though not well studied in the context of T1D, B cells in IKZF3-deficient mice have an activated cell surface phenotype [117] and spontaneously develop human SLE-like phenotypes, including anti-nuclear antibodies and

glomerulonephritis [118]. Hence, loss of IBC $B_{ND}$ cells could be due to changes in the threshold for activation due in part to polymorphisms in Ikaros.

Taken together our results indicate loss of anergy in insulin-binding B cells is associated with high risk HLA and non-HLA T1D genotypes. These risk alleles could impact B cell anergy, and therefore, development of autoantibodies and/or T1D, through an increase in the number of insulin-specific T cells in the periphery, their ability to provide T cell help, loss of Treg cells, or alterations in the signaling thresholds needed to maintain anergy. Future studies are underway to validate our findings, including a longitudinal study to follow our FDRs for development of autoantibodies, in order to better understand the contribution of these various SNPs in disruption of B cell anergy.

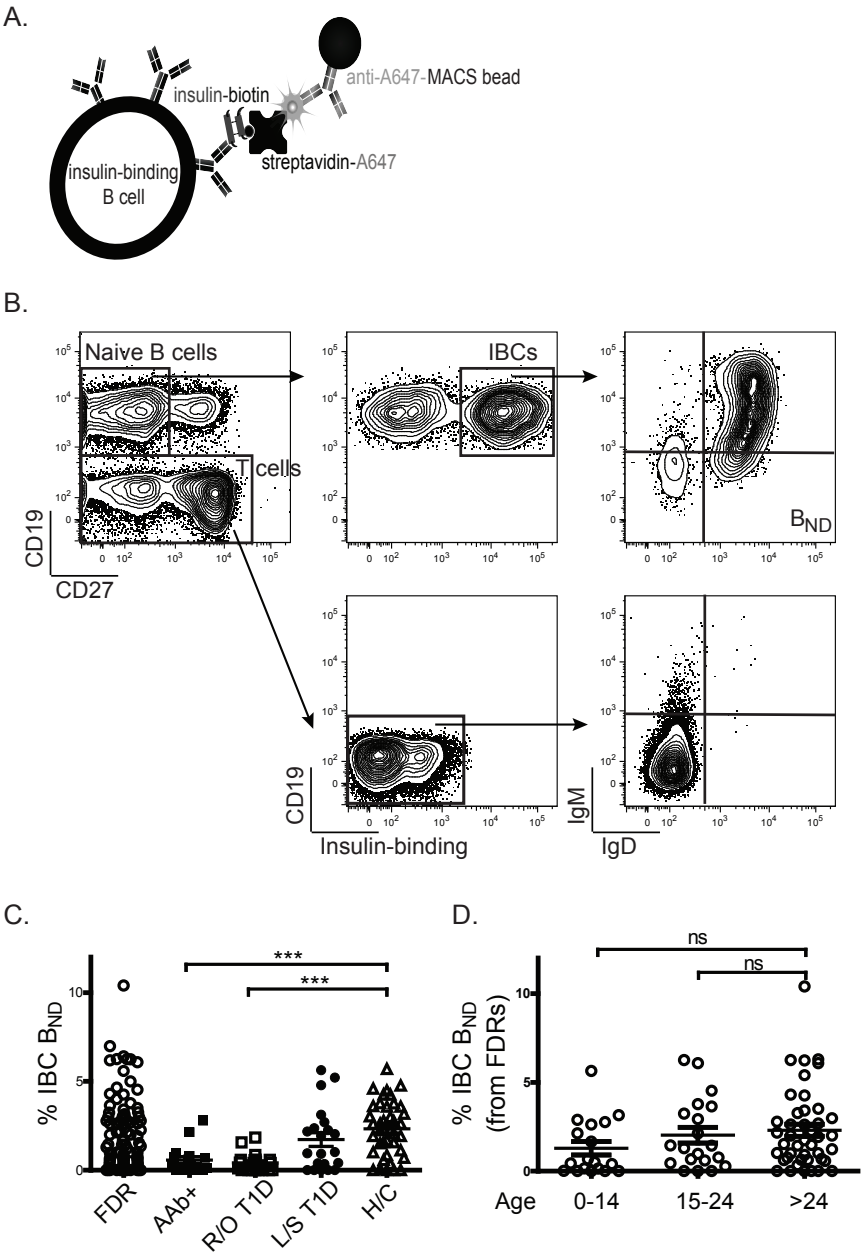



**Figure 4.1 Loss of IBC BND cells in autoantibody positive, recent onset type 1 diabetes, and some first-degree relatives. A.** Diagram of adsorbant used to identify and enrich for insulin-binding B cells (IBCs). **B.** Representative gating strategy to identify anergic (BND) IBCs. Gates for IgM and IgD are drawn based on CD19- T cells. **C.** BND cells (as percentage of CD27- IBC+ B cells) in peripheral blood of first-degree relatives (FDRs), autoantibody positive (AAb+), recent onset type 1 diabetes (R/O T1D), long-standing type 1 diabetes (L/S T1D), and healthy controls (H/C). **D.** BND cells (as percentage of CD27- IBC+ B cells) in blood of FDRs of various age groups.

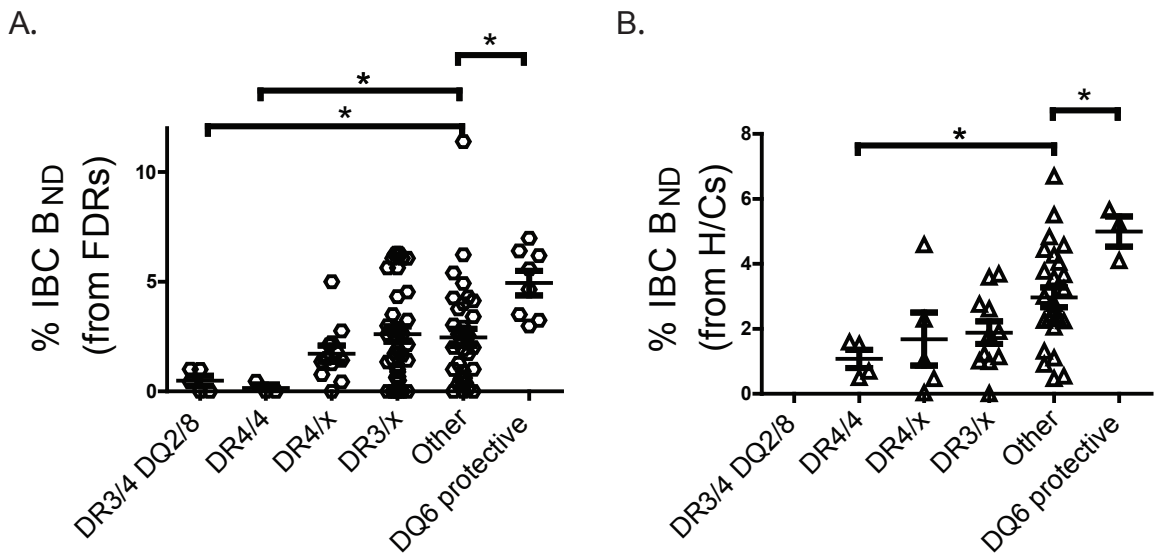



**Figure 4.2 Loss of IBC BND cells is associated with high risk T1D HLA genotypes.** **A.** Percentage of IBC BND cells in FDRs stratified by HLA genotypes. **B.** Percentage of IBC BND cells is healthy controls (H/C) stratified by HLA genotypes. HLA genotypes with highest risk for development of T1D are on the left and decrease toward the right.

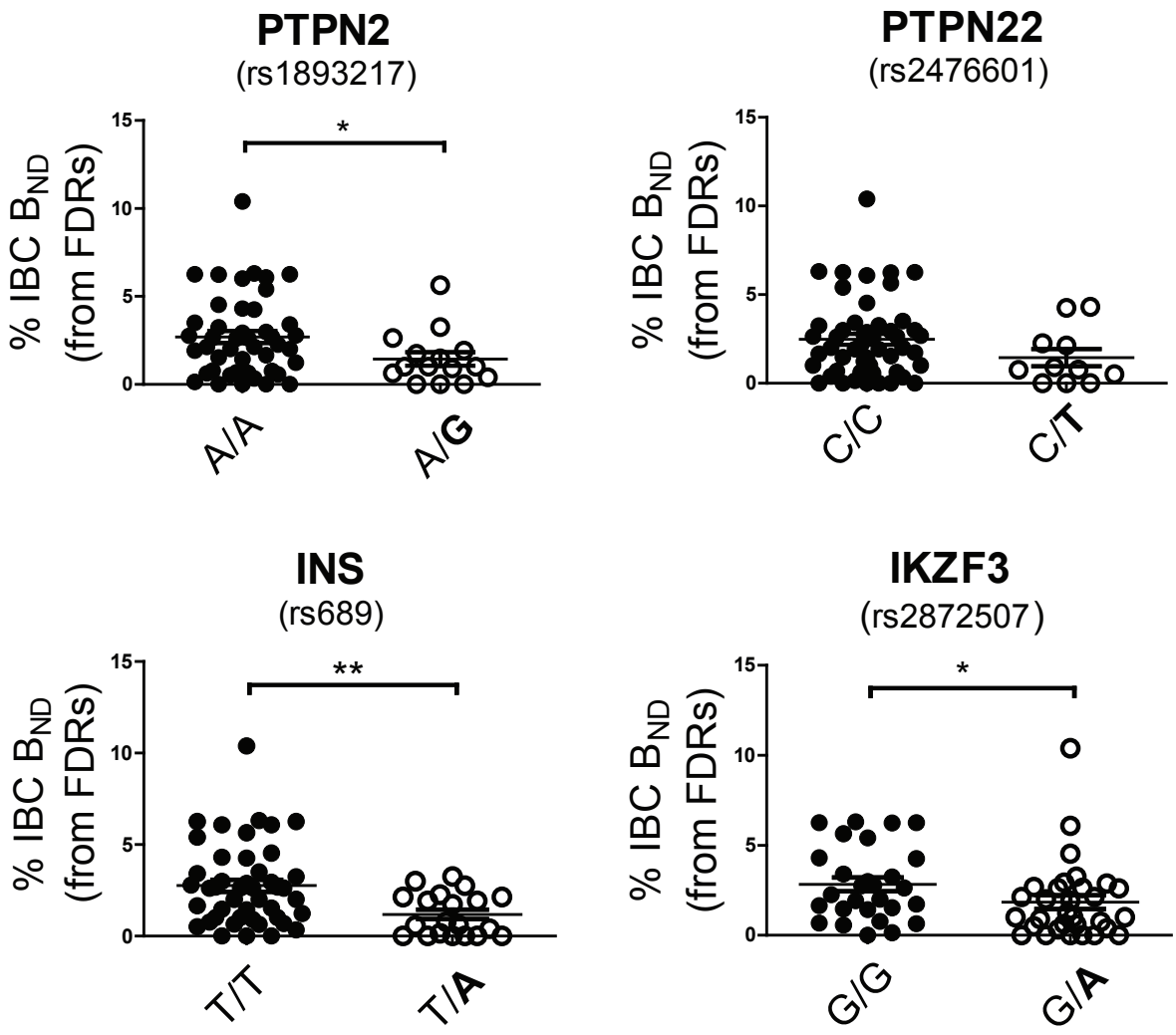



**Figure 4.3 Loss of IBC BND cells is associated with high risk type 1 diabetes non-HLA genotypes.** Percentage of IBC BND cells in FDRs who carry the risk allele for a particular SNP or do not. Risk alleles are in bold. Closed circles are FDRs who carry the non-risk allele and open circles are FDRs who carry the risk allele.

# CHAPTER 5

## Loss of tolerance and activation of thyroid antigen-binding B cells in recent onset autoimmune thyroid disease patients


### SUMMARY

Autoimmune thyroid disease (AITD), which includes Hashimoto's thyroiditis (HD) and Graves' disease (GD), is the most common autoimmune disorder in the United States, affecting over 20 million people. At the time of diagnosis, both HD and GD are characterized by the accumulation of B and T lymphocytes in the thyroid gland and production of autoantibodies targeting the thyroid, indicating that a breach in tolerance of autoreactive lymphocytes has occurred. However, few studies have sought to understand the underlying pathogenesis of AITD that ultimately leads to production of autoantibodies and loss of thyroid function. In this study, we analyzed the phenotype of thyroid antigen-reactive B cells in the peripheral blood of recent onset and long standing AITD patients. We found that in recent onset patients thyroid antigen-reactive B cells in blood no longer appear anergic, rather they express CD86, a marker of activation. This likely reflects activation of these cells leading to their production of autoantibodies. Hence, this study reports the early loss of anergy in thyroid antigen-reactive B cells, an event that contributes to development of AITD.


### INTRODUCTION

While autoimmune thyroid disease (AITD) is rarely life threatening, it is one of the most common autoimmune disorders and carries with it many serious comorbidities. The AITD constellation includes Hashimoto's thyroiditis (HT) and Graves' disease (GD), and common comorbidities include weight gain/loss, heart abnormalities, nervousness/agitation, and increased risk for thyroid cancer [119]. In addition, AITD is one of the most common secondary autoimmune

disorders associated with primary autoimmunities such as type 1 diabetes (T1D), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) [120-122]. Secondary AITD is an immune related Adverse Event (irAE) commonly seen following treatment with immune-checkpoint antibodies, such as anti-PD-1/PD-L1 and anti-CTLA4 [123-125]. In addition, up to 30% of multiple sclerosis patients treated with Alemtuzumab (anti-CD52), which depletes both B and T cells and some myeloid cells, develop secondary AITD. The majority of these patients develop GD [126, 127]. Hence, studies aimed at understanding the underlying pathogenesis of AITD and identification of possible biomarkers to stratify individuals at risk for primary or secondary AITD is needed.

Both HT and GD are characterized by the production of autoantibodies directed against the thyroid that either lead to hypothyroidism in HT or hyperthyroidism in GD. Autoantibodies against thyroglobulin (Tg) and thyroid peroxidase (TPO) are common in the serum of both HT and GD patients, while autoantibodies against the thyroid-stimulating hormone receptor (TSH-R) is more characteristic in GD patients. Mouse models of HT and GD have demonstrated an important role for B cells in the pathogenesis of disease [128-131]. In humans and mice, B cells are thought to not only produce autoantibodies, but also act as antigen presenting cells to T cells. Analysis of the thyroid gland in AITD patients has revealed the presence of B cells in and around the thyroid, forming lymphoid follicles, which are not present in healthy controls [132, 133]. While we know B cells are essential in the pathogenesis of AITD, nothing is known regarding changes in the status of thyroid antigen reactive B cells during disease development.

Development of a B cell-mediated autoimmunity, such as AITD, requires a breach in tolerance that allows self-reactive B cells to become activated and participate in disease. In healthy individuals, it has been shown that up to 70% of B cells produced in the bone marrow bind to self-antigens. However, most autoreactive B cells appear to be eliminated before reaching the periphery as indicated by the fact that autoreactive B cell frequency in the periphery is reportedly ~20% [5]. In the bone marrow, B cells that bind to self with a high avidity undergo a

process of receptor editing, in which they will rearrange their B cell antigen receptor (BCR) immunoglobulin light chains until self-reactivity is eliminated. If they fail to edit away their self-reactivity, these cells can undergo apoptosis [6]. B cells that have moderate avidity for self-antigen can escape these tolerance checkpoints in the bone marrow and enter the periphery where chronic antigen binding induces anergy. Anergic B cells are unresponsive to antigenic stimulation and are characterized by down regulation of surface IgM and an inability to become activated and produce antibodies [8, 10, 11]. Though most of what we know about anergic B cells has come from studies in mice, an anergic B cell population has been identified in the peripheral blood of healthy individuals [9]. These cells bear the markers of mature naïve (CD19+CD27-) while expressing low surface IgM expression but normal levels of IgD. They are termed termed $B_{ND}$ ("B cells that are naïve IgD+").

Previously, we analyzed the frequency of anergic insulin-binding B cells in the peripheral blood of subjects along a continuum of type 1 diabetes development, using a method we devised to identify and enrich insulin binding B cells (IBC) using magnetic particles [68, 104]. We found that autoantibody positive and recent onset T1D subjects have a significant decrease in the frequency of anergic IBC and total anergic B cells. Interestingly, we found in a portion of first-degree relatives the frequency of anergic B cells is also very low, suggesting that loss of B cell anergy precedes development of autoantibodies and could be a biomarker for increased risk for development of T1D [68]. Another group reported that SLE patients have decreased numbers of anergic anti-nuclear B cells in peripheral blood compared to healthy controls [74]. Based on these studies, we hypothesized loss of anergic B cells could be an early event in other autoimmune disorders, such as AITD, and therefore, could be utilized as a biomarker for individuals at risk for developing primary or secondary AITD. Using our method to enrich for antigen-specific B cells, we analyzed the phenotype of Tg and TPO-specific B cells in the peripheral blood of recent onset and long standing AITD subjects compared to healthy controls. We found that recent onset AITD subjects have reduced numbers of anergic Tg/TPO-specific B cells in blood, and this is correlated

with increased autoantibody titers. In addition, Tg-specific B cells show increased expression of CD86, suggesting activation and increased capacity to function in antigen-presentation to thyroid antigen-reactive T cells. Hence, this is the first study to identify changes in the phenotype of thyroid-antigen-reactive B cells in the peripheral blood during AITD development.

## MATERIAL AND METHODS

### *Subjects and Peripheral Blood Processing*

Samples were obtained with informed consent at the University of Colorado Anschutz Medical Center and the Barbara Davis Center for Childhood Diabetes using protocols approved by the University of Colorado Institutional Review Board. Eligible subjects were male or female, who had been diagnosed with either Graves' disease (GD) or Hashimoto's thyroiditis (HT) within 6 months for early onsets and more than one year ago for long standing. Presence of antibodies against Tg, TPO, and TSH-R, as well as TSH, Free T4, and Total T3 tests were used to confirm a diagnosis of GD or HT. PBMCs from 12 early onsets, 10 long standing, and 19 healthy age/sex matched controls were isolated from heparinized blood by Ficoll-Hypaque fractionation.

### *HLA genotyping*

DNA from AITD and healthy controls was extracted from the granulocyte layer using the DNA midi kit (Qiagen). HLA genotyping was conducted at the Autoantibody/HLA Service Center at the Barbara Davis Center for Childhood Diabetes using established protocols

### *Enrichment of antigen-specific B cells and flow cytometry analysis*

In order to maintain consistency of gating, enrichment of antigen-specific B cells, and changes in day to day flow cytometer settings, each day patient samples were analyzed in parallel with age/sex matched healthy controls. A minimum of 30 million PBMCs were used to enrich for Tg and/or TPO-specific B cells. Blood from all AITD subjects was divided in half and enriched for

Tg or TPO-specific B cells, unless an insufficient amount of blood was collected and then only one antigen was used. Native human Tg (AbD Serotec) and recombinant human TPO (Kronus) were biotinylated according to manufacturer's instructions (Thermo Scientific EZ-Link Sulfo-NHS-Biotin).

PBMCs were stained in PBS/1%BSA/0.02%NaAzide with human FcR Blocking Reagent (Miltenyi Biotec), 0.1 µg/$10^6$ cells Tg-biotin or TPO-biotin or TT-biotin, and mouse monoclonal anti-human antibodies against CD19-BV511 (BioLegend), CD27-BUV395 (BioLegend), IgM-PE (Southern Biotech), IgD-FITC (BD Biosciences), CD38-BV421 (BioLegend), and CD86-BV711 (BioLegend) for 20 minutes at 4ºC. Cells were then fixed with 2% formaldehyde at 4ºC, followed by incubation with streptavidin-AlexaFlour647 for 20 minutes at 4ºC. Cells were washed, suspended in MACS buffer (PBS/0.5%BSA/2mMEDTA), and incubated with 1uL anti-Cy5/Anti-Alexa647 microbeads (Miltenyi) /$10^6$ cells for 15 minutes at 4ºC. Samples were then passed over magnetized LS columns (Miltenyi), which was washed 3 times with 2mL of MACS buffer, and bound cells were eluted with 6 mL of MACS buffer. Flow cytometry was performed on LSR Fortessa X20 (BD) and data analyzed with FlowJo software ver. 9.9.4. Gates for $B_{ND}$ cells were drawn based on CD19- T cells, which are negative for IgM and IgD.

*Intracellular PTEN staining*

In early onset AITD subjects and healthy controls that had an excess of PBMCs, staining for intracellular PTEN expression was completed. Approximately 2 million PBMCs in triplicates were fixed and permeabilized (BD fix/perm) for 20 minutes on ice. Cells were washed twice with BD Perm/Wash, and then stained with anti-CD19-BV510, anti-CD27-BUV395, anti-IgM-PE-Cy7, anti-IgD-BV421, and anti-PTEN-Alexa647 (BD) for 1 hour on ice. Cells were washed twice with BD Perm/Wash and then fixed with 2% paraformaldehyde prior to flow cytometry.

*Statistics*

Data were analyzed using Prism software (GraphPad Software, Inc.). Paired Student's *t* tests were used to determine significance of differences among patient groups. Spearman's Correlation tests were used to determine correlation between two data sets.

## RESULTS AND DISCUSSION

*AITD subjects*

For this study we recruited early onset (E/O) AITD patients (10 GD and 2 HT) diagnosed within the previous 6 months. Only subjects that had not begun treatment or had only had minimal treatment with thyroid replacement or antithyroid drugs were enrolled (Table 5.1), since we suspected treatment could alter the phenotype of their peripheral blood lymphocytes. Initially we sought to recruit an equal number of HT and GD patients. However, this was made extremely difficult by the fact the treatment of HT patients is typically begun prior to entering specialist care. Therefore the majority of HT patients coming to our center did not meet eligibility requirements. TSH, T4, T3, and antibody titers for Tg, TPO, and TSH-R were assayed for each patient within 4 weeks of their blood draw (Table 5.1). We also recruited 10 long-standing (L/S) AITD patients (5 GD and 5 HT) that had been diagnosed more than a year earlier, and 19 closely age/sex matched healthy controls. For each AITD subject that was recruited on a given day, we recruited a healthy control so that they could be analyzed simultaneously. Hence, for all analyses a paired Student's *t* test was used. In addition, whenever possible we enriched and analyzed both Tg and TPO-specific B cells from each subject.

*Enrichment for thyroid antigen-specific B cells is specific*

Given that antigen-specific B cells normally occur at a very low frequency (<0.1%) in the peripheral blood, we utilized our previously published method to enrich thyroid antigen-specific B cells in recent onset AITD, long standing AITD, and healthy controls [104]. As depicted in figure

5.1A, biotinylated Tg or TPO was added to the cells along with antibodies against other cell surface markers, followed by streptavidin Alexa647. Anti-Tg/TPO B cells were enriched using anti-Alexa647 magnetic beads and were analyzed by flow cytometry. In a typical PBMC sample, around 0.8% of total B cells bound Tg and 0.4% bound TPO (Figure 5.1B). Enrichment of these cells yielded a ~20-150-fold increase in antigen-binding cell frequency, which enhanced our ability to conduct phenotypic analysis.

In order to verify that our enriched cells were reactive with Tg and TPO antigens, and not some other component of the adsorbent, such as streptavidin Alexa647, we tested the effect of omission of the thyroid antigens from the enrichment scheme (Figure 5.1B). An insignificant number of cells was enriched supporting the thyroid antigen specificity of antigen binding. In addition, no antigen binding cells were found among CD19- cells, which are primarily comprised of T cells (Figure 5.1B). Finally, addition of ~50 fold molar excess of unlabeled Tg/TPO before addition of the biotinylated antigen blocks binding by ~90% (data not shown), also demonstrating the specificity of antigen binding.

*Total Tg and TPO-binding B cell frequency is increased in blood of AITD subjects*

Comparative analysis of Tg and TPO-binding B cells in peripheral blood of early onset AITD, long standing AITD, and healthy controls, revealed no statistically significant differences in frequency, whether we compared AITD patients as a group or by disease classification (HT or GD) or duration of time since diagnosis (E/O or L/S) (Figure 5.1C). However, while not statistically significant, the frequency of Tg and TPO-reactive B cells per $10^7$ PBMCs is increased in the AITD patients as a group, independent of their classification or time since diagnosis [Tg: Mean (per $10^7$ PBMCs) (95% CI) 3,565 (349.7, 6779); TPO: Mean (per $10^7$ PBMCs) (95% CI) 21,817 (9,061, 34,573)] compared to healthy controls [Tg: Mean (per $10^7$ PBMCs) (95% CI) 1,814 (228.5, 3,400); TPO: Mean (per $10^7$ PBMCs) (95% CI) 14,221 (6,625, 21,814)].

*Loss of anergic Tg/TPO-reactive B cells from peripheral blood in early onset AITD subjects*

In order to analyze the distribution of Tg/TPO-specific B cells in various B cell subpopulations, we enriched antigen binding cells as in figure 5.1 and applied the gating strategy shown in figure 5.2A. As depicted, we were able to determine the proportion of antigen-binding cells that reside in the naïve, memory, and plasmablast subpopulations, as well as the previously described anergic ($B_{ND}$) and double negative (DN) B cell subpopulations [9, 91, 134]. As mentioned previously, $B_{ND}$ cells are characterized as mature naïve (CD19+ CD27-) but have greatly reduced surface IgM while retaining normal levels of IgD [9]. Previous studies have shown that these cells have are autoreactive, but refractory to stimulation through their BCR based on decreased intracellular calcium flux and phosphorylation of BCR signaling pathway intermediaries, such as Syk, PLCy2, and Akt (data not shown) [9, 68, 79]. The double negative (DN) B cells are CD27 and IgD negative, but despite lack of expression of the CD27 memory B cell marker are class-switched memory B cells [91]. It is thought that these cells arise outside the germinal center or are a distinct B cell lineage that never acquires the CD27 marker [91, 92]. These DN cells have been found to be increased in SLE and RA patients [91, 135].

Interestingly, analysis of the frequency of Tg and TPO-specific B cells in the naïve, DN, memory, and plasmablast populations in blood of AITD subjects and healthy controls revealed no significant differences between groups (data not shown). This is similar to observations in studies of insulin-binding cells in type 1 diabetics. In that study we found based on analysis of affinity of recombinant BCR cloned from single insulin binding B cells that most antigen-binding cells enriched by this method have such a low affinity that they are ignorant of ambient antigen. We suspect the same is true in the case of enriched thyroid antigen-binding B cells, especially since Tg and insulin are present in the blood at similar concentrations, ~50 pmol/L [136, 137] and TPO is not thought to circulate in the blood at detectable levels. Hence, ignorant Tg and TPO-binding cells should be innocuous and occur in all major B cell subpopulations depending on their antigen experience.

The frequency of Tg and TPO-reactive B cells that reside in the $B_{ND}$ compartment was significantly decreased in early onset AITD subjects compared to long standing AITD and healthy controls (Figure 5.2B). These results suggest a breach in tolerance occurs early in AITD and is transient, since in patients diagnosed for more than one year prior to analysis the anergic B cell population returns to near normal levels. The transient nature of this loss suggests that an environmental trigger may initiate a breach in tolerance of thyroid antigen-reactive B cells.

When we analyzed the frequency of total (Tg/TPO-) anergic $B_{ND}$ cells, we found a significant decrease in the early onset AITD subjects compared to the long-standing and healthy controls (Figure 5.2C). Given that the transient loss of anergic thyroid antigen-reactive B cells is likely due to an environmental factor, one would expect this event to affect the anergy of auoreactive B cells regardless of their autoantigen specificity. Hence, this finding suggests loss of anergic B cells is a general phenomenon that occurs early in disease and may even precede it, as is the case in T1D.

*Loss of anergic Tg-specific B cells is inversely correlated with autoantibody titers*

Early diagnosis of AITD in part depends on the presence of autoantibodies targeting thyroid antigens. Thus it presupposes that a breach in B cell tolerance must occur prior to production of autoantibodies. Hence, we suspected loss of B cell anergy in early onset AITD subjects may correlate with development of autoantibodies. In figure 5.3, the frequency of anergic Tg/TPO binding B cells is plotted against the subject's respective Tg/TPO antibody titer levels. We found loss of anergic B cells inversely correlated with appearance of Tg antibody (Tg correlation coefficient -0.756) and to a much lesser extent with TPO (correlation coefficient -0.018). The inverse correlation was significant for Tg ($p = 0.015$).

*Tg-reactive B cells show evidence of activation in early onset AITD subjects*

The inverse correlation between loss of anergic Tg, and to a lesser extent TPO, reactive B cells and appearance of autoantibodies suggests activation of these thyroid antigen-binding B cells. Hence, we next wanted to investigate whether the Tg/TPO-reactive B cells showed evidence of activation in the AITD subjects compared to healthy controls by analyzing the expression level of CD86, a marker for B cell activation, on these cells. Interestingly, the expression of CD86 on Tg-reactive B cells in early onset AITD subjects was significantly increased compared to healthy controls (Figure 5.3B). This finding was somewhat surprising, given that based on our T1D study we hypothesize the majority of the Tg-reactive B cells we enrich for have such a low affinity for their antigen they are virtually ignorant. When we analyzed the expression of CD86 in the various Tg-binding B cell subpopulations, we found that in early onset AITD subjects CD86 was significantly increased on all major subpopulations, including the the few remaining $B_{ND}$, naïve, and memory B cells compared to healthy controls (Figure 5.3C). However, the fold increase in CD86 expression was highest in the $B_{ND}$ population, which we know have a high antigen affinity. Studies in mice and humans have shown that anergic B cells can upregulate CD86 *in vitro* in response to anti-BCR stimulation [79, 138] or IL-4/CD40L [9, 10, 139, 140]. These findings also suggest Tg-reactive B cells in AITD subjects have an increased capacity to activated and present antigen to Tg-reactive T cells, since CD86 is also a co-stimulatory molecule required for T cell activation.

The above findings were only found in the Tg-reactive B cell population in the early onsets. When we analyzed CD86 expression in the TPO-binding B cells (Figure 5.3B) or the non-Tg/TPO binding B cells (data not show), or analyzed long standing AITD subjects (Figure 5.3B), no statistical differences were found. These results are consistent with our finding that loss of anergic B cells was only found to be inversely correlated with autoantibody titers in the Tg but not TPO-binding cells. In addition, stratifying the AITD subjects based on their subclass (HT or GD) did not make a difference, and therefore, they were grouped together for the above analyses.

CONCLUSIONS

Recently diagnosed AITD subjects were found to have a significant decrease in the frequency of anergic Tg and TPO-reactive B cells, as well as total anergic B cells compared to long standing AITD subjects and healthy controls. This finding suggests loss of anergy is transient and is not limited to relevant antigen-specific B cells during onset of autoimmunity. Such a phenomenon is likely due to an environmental trigger, such as increased dietary iodine, especially since both the environment and genetics are important contributors to development of AITD. In addition, recent GWAS studies have identified several risk alleles associated with development of AITD, some of which could be helping drive loss of B cell tolerance. A polymorphism in the *PTPN22* gene, which encodes the lymphoid tyrosine phosphatase (Lyp) protein, is highly associated with AITD [61, 141], and has previously been shown to be a negative regulator of B cell signaling [52]. In addition, individuals who carry this polymorphism have increased numbers of autoreactive B cells in their periphery [42]. In addition, expression of PTEN, a negative regulator of B cell signaling, has been shown to be increased in anergic B cells in mice [14, 83]. I analyzed the relative level of PTEN in B cells in a portion of the early onset AITD patients, as well as early onset T1D patients, and compared them to their healthy control ran on the same day (Figure 5.4). I found that in autoimmune patients PTEN is significantly decreased in B cells compared to controls, and even more so in their total $B_{ND}$ cells. Hence, we suspect a combination of susceptibility genes and/or changes in negative regulators are involved in driving loss of B cell anergy. Studies in our lab are currently underway to better understand the contribution of genetic risk alleles to loss of B cell anergy.

Loss of anergic B cells was inversely correlated with autoantibody titers, particularly anti-Tg antibodies, demonstrating a break in B cell anergy likely precedes differentiation into autoantibody producing cells, something known to occur in mice but has not been shown in humans [15]. In addition, Tg-reactive B cells in AITD subjects had increased expression of the activation marker CD86 compared to healthy controls, suggesting early activation of these cells

and competence to act as antigen-presenting cells to Tg-reactive T cells. Although all Tg-binding B cell populations showed increased CD86 expression compared to healthy controls, the fold increase in CD86 expression was higher in the few remaining $B_{ND}$ cells in the early onset AITD subjects. Historically anergic B cells in both mice and human have been shown to be negative for CD86 expression, though they can be induced to express it *in vitro* after stimulation with anti-BCR or IL-4/CD40L [9, 10, 79, 138]. Further, Chang et al. found that an equivalent population to $B_{ND}$ cells has increased expression of CD86 *ex vivo* in SLE patients but not healthy controls [142]. Hence, we hypothesize that the few Tg-reactive $B_{ND}$ cells left in the peripheral blood of early onset AITD subjects have recently received strong interaction with multimeric Tg, which could be in the form of immune complexes, thus upregulating CD86. Given that loss of anergic Tg-reactive B cells inversely correlated with Tg antibody titers, this seems plausible. Alternatively, these cells may be recently activated mature naïve Tg-reactive B cells that upon seeing their antigen have internalized their BCR, with reduced mIgM emulating the phenotype of $B_{ND}$ cells.

Dogma suggests autoreactive B cells become anergic in the periphery due to chronic antigen binding in the absence of second signals provided by “helper” T cells (signal 1 without signal 2). Hence, if an anergic B cell receives T cell help, it could drive them out of a state of anergy. The gene conferring the highest susceptibility for AITD is in the *HLA* locus. It is has been shown that the HLA class II genes DR3 and DR4 confer the highest risk [141] and in our study 18/22 AITD subjects carried one or both of these genes (Figure 5.5). It is hypothesized these class II molecules are permissive to binding of Tg and TSH-R peptides that when presented to T cells, gives rise to AITD [143]. Hence, it is possible loss of B cell anergy in our early onset AITD subjects enables these B cells to present antigen and thus receive T cell help. We suspect other factors are in play, such as the environment and other genetic risk alleles, that allow escape of tolerance of thyroid-specific B and T cells.

This is the first study comparing the status of Tg and TPO-reactive B cells in the peripheral blood of early onset AITD, long standing AITD, and healthy controls. Loss of B cell anergy was

found in the early onset subjects, but may precede onset of disease, as suggested by loss of anergic B cells in first-degree relative of T1D patients [68]. Hence, loss of B cell anergy could be an early indicator of increased risk of developing primary or secondary AITD. Studies in our lab are focused at addressing this possibility in high risk first-degree relatives and MS patients treated with Alemtuzumab.

## Table 5.1 Early onset subject test results

| Patient ID | Diagnosis | TPO ab (0-34 IU/mL) | TG ab (0.0-4 IU/mL) | TSHR ab (0.0-1.75 IU/L) | TSH (0.45-4.5 uIU/mL) | Thyroxine/T4 Free (0.82-1.77 ng/dL) | Total T3 (71-180 ng/dL) | % $B_{ND}$ (of TG+) | % $B_{ND}$ (of TPO+) |
|---|---|---|---|---|---|---|---|---|---|
| **2838** | Graves' | 1230 (H) | 94 (H) | 3.7 (H) | 0.02 (L) | 1.17 | 116 | 2.14 | 0 |
| **2899** | Graves' | 1202 (H) | 2 | 26.75 (H) | 81.84 (H) | 1.99 (H) | 132 | 3.37 | 0 |
| **2946** | Graves' | 3.2 | <0.9 | 13.87 (H) | <0.015 (L) | 1.96 (N) | 179 (H) | 0.409 | NA |
| **2983** | Graves' | 202 (H) | 5 (H) | 33.35 (H) | 0.02 (L) | 3.52 (H) | 359 (H) | 0.291 | 0.778 |
| **3000** | Hashimoto | 2874 (H) | 1150 (H) | <0.90 | 9.11 (H) | 0.92 | 92 | 0.425 | 0.659 |
| **3066** | Graves' | 589 (H) | <1 | 27.2 (H) | 0.03 (L) | 5.75 (H) | 778 (H) | 0.32 | 1.14 |
| **3116** | Graves' | 143 (H) | 0 | NA | <0.006 (L) | 6.56 (H) | 627 (H) | 4.68 | 0.97 |
| **3190** | Graves' | 109 (H) | 247 (H) | 9.23 (H) | 0.18 (L) | 3.53 (H) | 219 (H) | 0 | 2.73 |
| **3236** | Hashimoto | >13000 (H) | 2369 (H) | <0.9 | 8.28 (H) | 1.02 | 154 | 0 | NA |
| **3305** | Graves' | 142 (H) | 2 | 24.23 (H) | 0.04 (L) | 3.46 (H) | 330 (H) | 0.224 | 0.513 |
| **3327** | Graves' | 1882 (H) | >2200 (H) | 5.88 (H) | <0.015 (L) | 1.3 | 124 | NA | 2.33 |
| **3533** | Graves' | 88 (H) | 1 | 2.92 (H) | 0.03 (L) | 1.87 (H) | 256 (H) | NA | 0 |

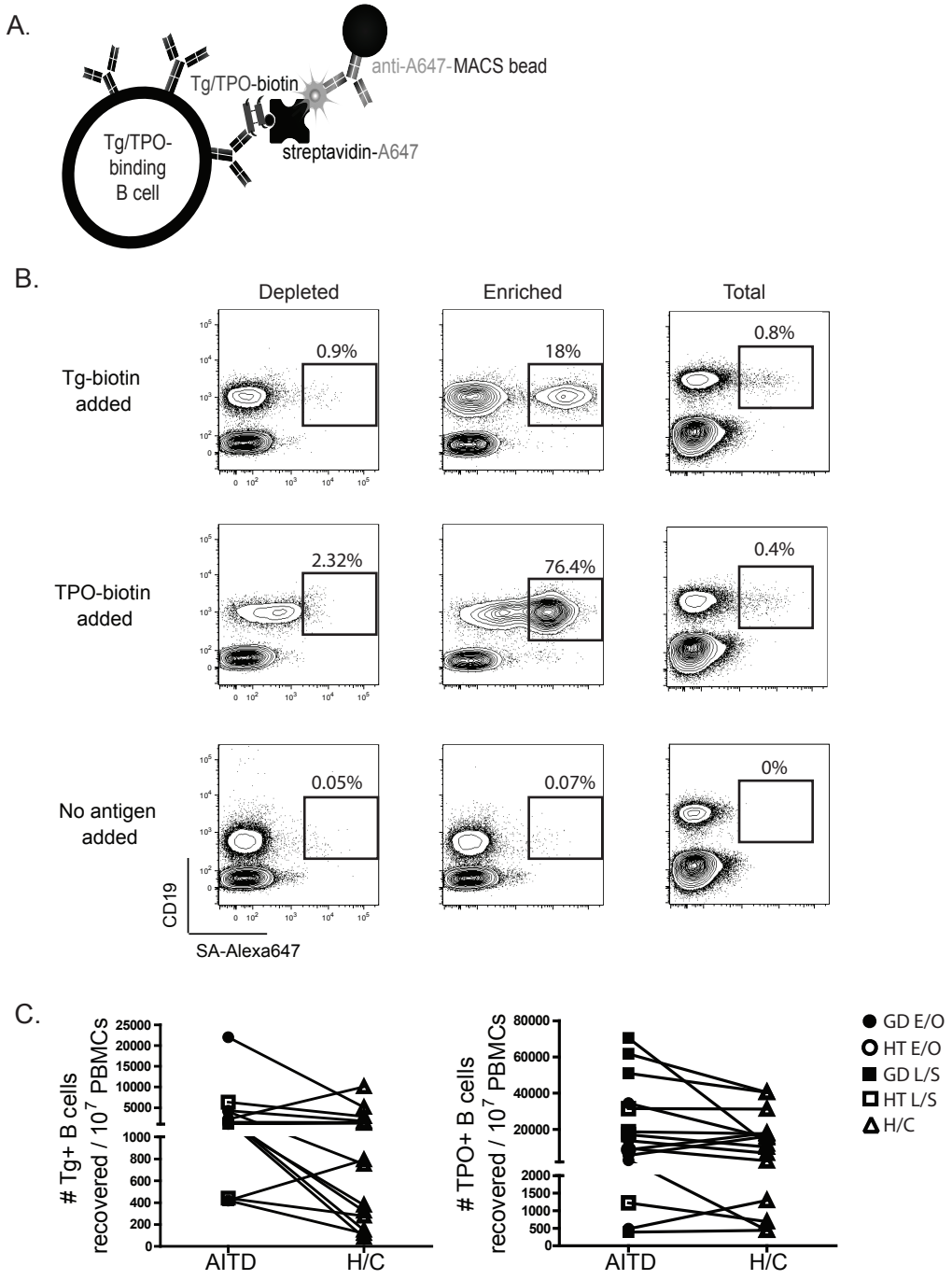



**Figure 5.1 Detection and enrichment of Tg/TPO-binding B cells is specific.** **A.** Diagram of adsorbent used to identify and isolate Tg or TPO-reactive B cells. **B.** Representative cytograms of Tg/TPO-reactive B cells enriched from the blood of a healthy control when antigen is added or when it is omitted. Cells that bound to the adsorbent and eluted from the magnet are termed enriched, non-binders termed depleted, and un-enriched termed total. Percentages shown are the percent of B cells that are Alexa647 positive. **C.** Number of Tg and TPO-binding B cells recovered per 10^7 PBMCS in AITD subjects compared to healthy controls. Each line represents a pair of AITD and healthy control that were ran on the same day.

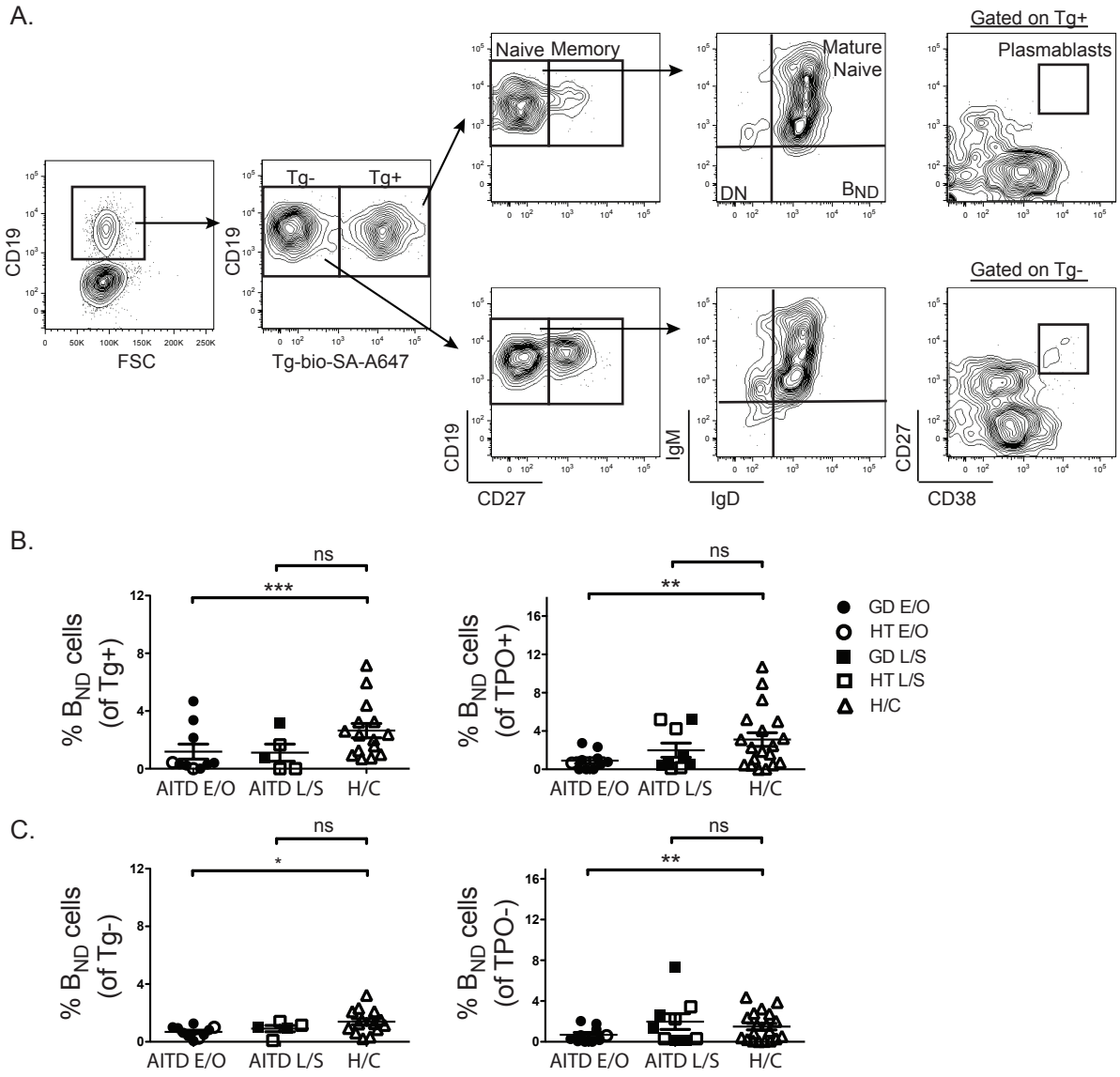



**Figure 5.2 Early onset AITD subjects have a loss of anergic Tg and TPO-bindig B cells in their peripheral blood. A.** Representative gating strategy for identification and analysis of Tg/TPO-reactive B cells using Tg as an example. IgM vs IgD gates are drawn based on staining of T (CD19-) cells, which are IgM-IgD-. **B.** Percentage of anergic Tg and TPO-reactive BND cells in early onset (E/O) AITD, long standing (L/S) AITD, and healthy controls (H/C). **C.** Percentage of total (Tg/TPO-) anergic BND cells in each subject group.

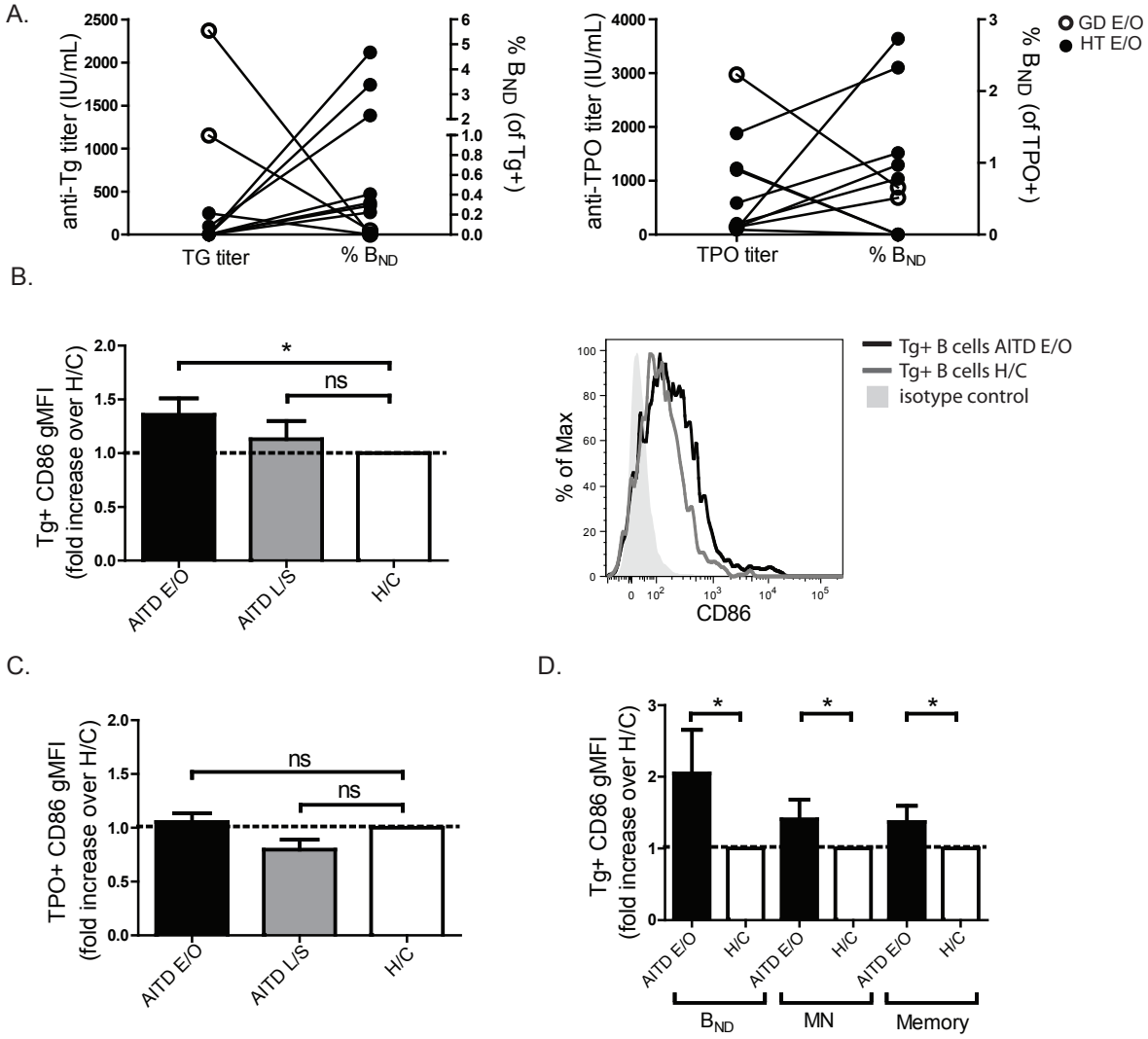



**Figure 5.3 Loss of anergic Tg-reactive B cells is correlated with autoantibody production and activation. A.** Anti-Tg and anti-TPO titers for each early onset AITD subject is plotted with their respective percentage of anergic Tg/TPO-reactive BND cells to demonstrate correlation between the two. Anti-Tg titers had a significant inverse correlation with % Tg-binding BND cells; p=0.015, Spearman's correlation test). **B.** Fold increase of geometric mean florescence intensity (gMFI) of CD86 on Tg-binding B cells from AITD E/O and AITD L/S patients over healthy controls. Representative histogram of CD86 expression from Tg-binding B cells from AITD E/O (black line) and H/C (gray line). **C.** Fold increase of CD86 gMFI on TPO-binding B cells from AITD E/O and AITD L/S patients over healthy controls. **D.** Fold increase in CD86 gMFI on Tg-reactive BND, mature naïve (MN) (CD27-), and memory (CD27+) B cell subpopulations from early onset AITD subjects compared to healthy controls. CD86 gMFI on AITD subjects was normalized to healthy controls in order to account for changes in voltage settings that occurred during the course of the sample collections. Statistical significance determined by paired Student's t tests.

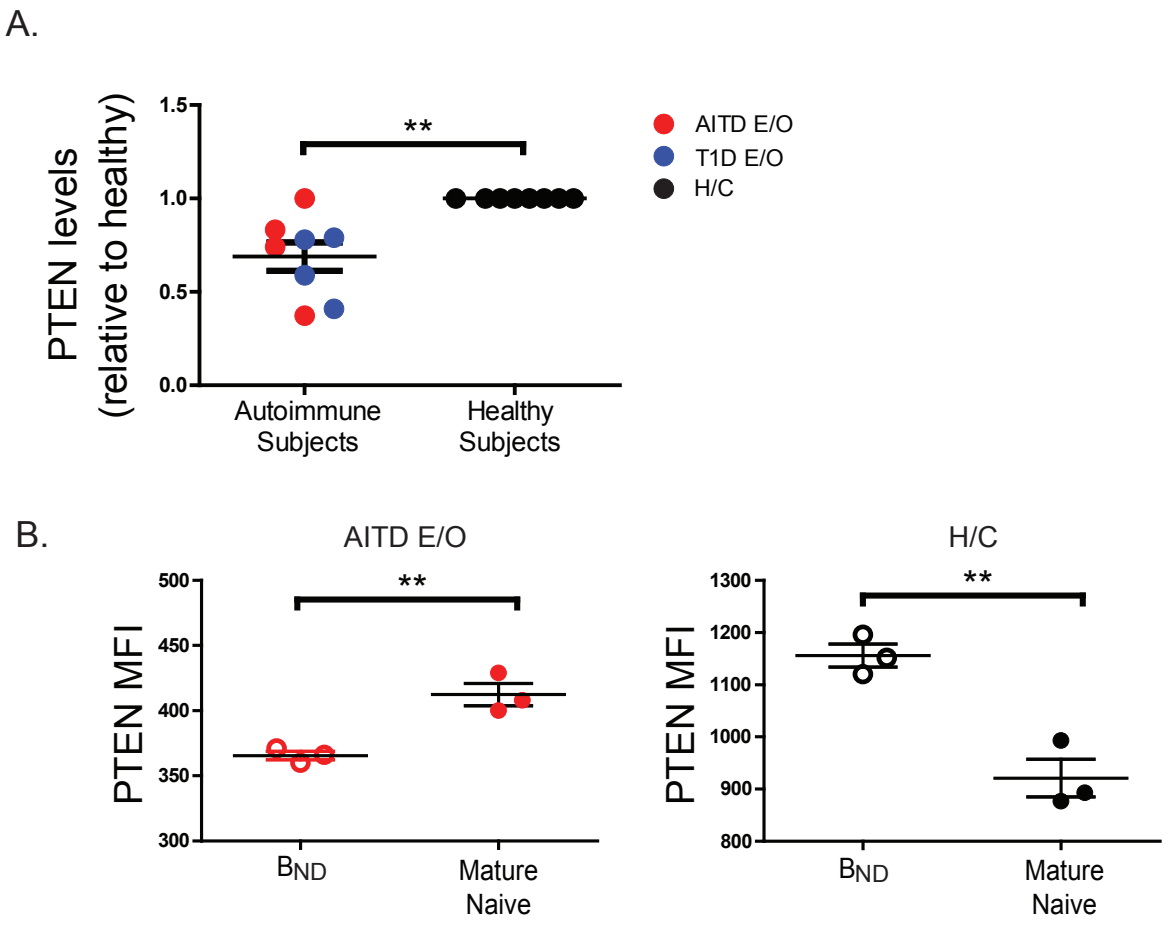



**Figure 5.4 AITD subjects have decreased PTEN expression in B cells compared to controls. A.** Expression of PTEN in total B cells in early onset (E/O) AITD patients normalized to healthy controls (H/C). Early onset type 1 diabetic (T1D) patients are also shown for comparison. **B.** PTEN MFI in BND and mature naive B cells in a representative early onset AITD compared to a healthy control.

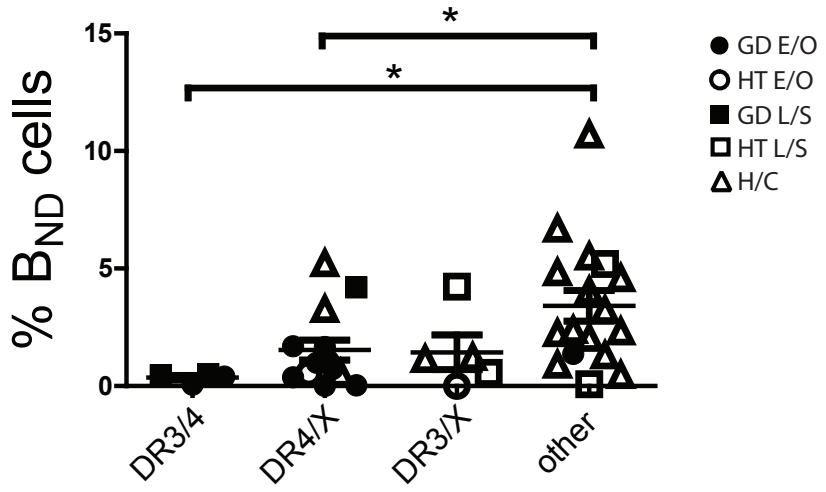



**Figure 5.5 AITD subjects tend to carry the high risk HLA haplotypes associated with development of AITD and also have low frequency of total BND cells.**

# CHAPTER 6

## Insulin-reactive B cells escape tolerance in NOD mice and contribute to the development of T1D by functioning as essential antigen presenters to diabetogenic T cells in the pancreas


## SUMMARY

Although islet antigen-reactive B cells are known to play an important role in the development of type 1 diabetes (T1D) in both man and the non-obese diabetic (NOD) mouse, mechanisms responsible for their apparent break in tolerance and contribution to disease remain unclear. Here we report comparative analysis of the phenotype and function of insulin-reactive B cells in disease sensitive NOD and disease resistant C57BL/6 mice. In disease resistant mice, many insulin-reactive B cells reach the periphery where those with lower affinity are enriched in lambda expression indicating successful receptor editing and those with high affinity for insulin are anergic. Both central and peripheral B cell tolerance appears defective in NOD, as increased numbers of high affinity insulin-reactive cells reach the periphery where they show signs of activation and differentiation. These cells accumulate in the pancreas prior to overt disease development and express insulin B:9-23 peptide in association with cell surface I-$A^{g7}$ indicating successful BCR-mediated antigen uptake and processing. Accumulation of these B cells in the pancreas is uniquely associated with retention and activation of insulin-reactive CD4 T cells. We conclude that during development of T1D islet antigen-reactive B cells lose tolerance and provide critical antigen presenting function for diabetogenic CD4 T cells.


## INTRODUCTION

While it is clear that T cells are the primary pathogenic effectors in type 1 diabetes (T1D), mounting evidence supports the notion that B cells play an important role in disease development. Non-obese diabetic (NOD) mice deficient in B cells from birth fail to develop T1D [144] and

transient B cell depletion renders NOD mice resistant to disease [17, 18, 145]. In new onset human diabetic patients, B cell-depleting Rituximab (anti-CD20) therapy slows the decline of beta cell function, arresting loss of C-peptide until the B cell compartment regenerates [77]. Thus evidence of B cell function is clear, although the mechanism by which B cells contribute to T1D remains elusive.

B cell receptor (BCR) specificity for islet autoantigen is critical for disease. NOD mice in which the BCR repertoire is biased toward insulin reactivity develop disease at an increased rate and higher penetrance [33]. Conversely, skewing the repertoire away from insulin reactivity [33] or fixing the BCR specificity to an irrelevant antigen prevents T1D [32]. Targeted depletion of insulin-binding B cells also impairs development of the disorder [146]. These results indicate that reactivity to islet antigens is critical for development of T1D and that B cell function in this context does not depend on antibody secretion. Islet autoantibodies (Igs) arise during T1D development long before hyperglycemia [29, 147], but are not essential for disease development [41] and do not transfer disease [25]. Transgenic NOD mice in which B cells express membrane-bound, but not secreted Ig, develop T1D at the same rate and frequency as wild-type NOD [41]. Thus we hypothesize that islet antigen reactive B cells participate in disease by non-redundant cytokine production and/or presentation of autoantigen to islet antigen-reactive T cells. Consistent with this possibility B cells have shown to be critical for autoantigen presentation and cross-presentation to CD8 T cells and disease development in NOD [24, 31].

Function of islet antigen-reactive B cells in T1D presumably requires a breach in B cell tolerance or ignorance. Ignorance refers to a situation in which BCR recognize antigen of such low avidity that the B cell does not receive an actionable signal. At least three tolerance mechanisms: receptor editing, clonal deletion, and anergy normally function to inactivate and/or eliminate potentially dangerous autoreactive B cells. Overall, B cell development in NOD bone marrow appears normal, suggesting clonal deletion is generally intact [148]. The number of transitional B cells in spleen, however, is diminished in NOD compared to C57BL/6 [148]. This is

suggestive of problems in maintenance of B cell anergy since anergic B cells tend to reside in this compartment [12].

Fixation of the B cell repertoire by immunoglobulin transgenesis has allowed the study of B cell tolerance to specific autoantigens. B cells in NOD and C57BL/6 mice expressing anti-hen egg lysozyme (HEL) BCR undergo equivalent B cell clonal deletion upon recognition of membrane-bound HEL [148]. However while soluble HEL expression leads to anergy of anti-HEL B cells in C57BL/6, anergy appears compromised in NOD [148]. More pertinent to T1D are studies of the 125 immunoglobulin transgenic mouse in which BCR recognize the islet autoantigen insulin [33]. It has been suggested that in both NOD and C57BL/6, 125 transgenic B cells mature normally, and respond to BCR stimulation by upregulation of CD86, yet do not proliferate or mount antibody responses following stimulation [138]. Nonetheless, NOD 125 B cells exhibit slightly higher surface IgM levels consistent with loss of anergy [33]. Taken together, available evidence suggests that in NOD the silencing of insulin-reactive B cells may be compromised, enabling them to promote T1D. Here we describe studies designed to explore this possibility.

In experiments described here we employed a repertoire-diverse model in which representation of insulin-reactive B cells is nonetheless increased by expression of the BCR heavy chain (VH125) of the 125 insulin reactive hybridoma [149, 150].This heavy chain pairs with endogenous immunoglobulin light chains to achieve repertoire diversity [33]. It is important to note that VH125.C57BL/6 mice and VH125.NOD mice were derived from the same founder VH125 transgenic [151], thus genetic determinants should impact expression equivalently in the two backgrounds [33]. In VH125.NOD and VH125.C57BL/6 mice, insulin-binding B cells are enriched sufficiently to easily detect following antigen adsorbent-based enrichment [104]. From the earliest stages of development, insulin-binding B cells can be tracked as they compete with non-autoreactive B cells for survival factors and physical space. Previous studies using this model indicate that insulin-binding VH125.NOD B cells, despite interacting with autoantigen, exit the bone marrow unimpeded [90]. However, in the autoimmune resistant C57BL/6 background, a

large proportion of insulin-binding B cells are eliminated early in development [90]. Thus confusion exists regarding the tolerance status of autoreactive B cells in NOD, as well as the mechanism(s) by which they contribute to T1D.

Here we interrogate the small, insulin-reactive B cell population that occurs in VH125 mice, and demonstrate that in diabetes-susceptible (NOD) and resistant (C57BL/6) mice carrying the same MHC II allotype ($IA^{g7}$), those with BCR affinity sufficiently high to sense insulin at physiological concentrations are phenotypically and functionally distinct [47]. In VH125.C57BL/6-H2g7, clonal deletion and receptor editing eliminate the majority of high affinity insulin-binding B cells. However, a significant proportion of these cells reach the periphery and are functionally anergic. Insulin-binding B cells in VH125.NOD escape all modes of tolerance, with a large proportion of these cells appearing activated prior to onset of T1D. Importantly, these VH125.NOD insulin-binding B cells accumulate in the pancreas, display the insulin B:9-23 peptide in association with their MHCII, and promote accumulation and activation of insulin-specific CD4 T cells. Thus our results indicate that insulin-reactive B cells provide non-redundant antigen-presenting function for CD4 T cells during T1D development.

## MATERIALS AND METHODS

### *Mice*

Mice expressing the $IgM^a$ heavy chain transgenes VH125 and VH281 on the NOD and C57BL/6 backgrounds were kindly supplied by JW Thomas [33, 138]. VH125.C57BL/6 mice were backcrossed onto the C57BL/6-H2g7 background, obtained from the Jackson Laboratory to create VH125.C57BL/6.H2g7 within our colony. BDC12.4-1.RAG-/-.NOD were supplied by Aaron Michels at the Barbara Davis Center. Female mice were used for all experiments, unless otherwise noted. Mice were monitored for the development of diabetes for a period of 40 weeks. Two consecutive blood glucose readings >250 mg/dl (One Touch), without fasting, identified a mouse as diabetic. All animals were housed in facilities at National Jewish Health and University

of Colorado SOM, Anschutz Medical Campus under specific pathogen free conditions. Each facility's Animal Care and Use Committee approved all experimental procedures.

*Method to identify, enrich, and isolate B cells based on their BCR binding to insulin.*

The approach is described previously [68, 104] and diagrammatically in figure 6.1C. In short, the starting cell population is first depleted of red blood cells. Cells were then stained in PBS/2% BSA/0.02% sodium azide with 0.1 ug/$10^6$ cells of insulin-biotin and a combination of B cell markers (as described below) for 30 minutes at 4°C. After washing, cells were then fixed with 2% formaldehyde at 4°C, followed by incubation with streptavidin– Alexa Fluor 647 for 15 min at 4°C. Cells were washed, suspended in MACS buffer (PBS/0.5% BSA/2 mmol/L EDTA), and incubated with anti-Cy5/anti-Alexa 647 microbeads (Miltenyi Biotec) for 10 min at 4°C. Samples were then passed over magnetized LS columns (Miltenyi Biotec) and washed three times with 2 mL of MACS buffer, and bound cells were eluted with 6 mL of MACS buffer.

*Flow Cytometry Analysis*

Whole pancreas preparation and staining for autoreactive T cells specific for insulin B:9-23 was completed as previously described [152] with tetramers obtained from the Kappler Laboratory. For all other stains commercial antibodies directed against the following molecules were used: AMS-32.1 (BD), B220 (RA3-6B2; BD), CD1d (1B1; BioLegend), CD4 (GK1.5; BioLengend), CD19 (6D5; BioLegend), CD21/35 (eBio8D9; eBioscience), CD23 (B3B4; BD), CD24 (M1/69; eBioscience), CD44 (IM7; BioLegend), CD86 (GL1; BD), CD138 (281-2; BD), Goat Anti-Mouse Lambda-FITC (Southern Biotech), and Steptavidin Brilliant-Violet 421 (BioLegend). Anti-IgM B-7-6 was produced in the Cambier Laboratory and conjugated to DyLight (Pierce) fluorochromes, according to the manufacturer's protocol. mAb287 was produced in the Zhang Laboratory and conjugated to Zenon (Thermo Fisher Scientific) fluorochromes, according to the manufacturer's protocol. Flow cytometry was performed on a CYAN (Beckman Coulter) and

Fortessa (BD Biosciences) and data analyzed with FlowJo software version 8.8.4. VH281.NOD and VH281.C57BL/6 mice were used as gating controls, given they do not generate insulin-binding B cells [33].

*B cell Calcium Flux Analysis*

Splenocytes were harvested from mice and red blood cells were lysed using ammonium chloride. Cells were suspended at $10^7$ cells/mL in warmed 37° C RPMI, 2% BSA, and 1 μM Indo1-AM (Molecular Probes). Anti-CD19-PE, anti-IgM-B.7.6.-FITC Fab, and biotinylated insulin were added during incubation with Indo for 30 minutes and washed two times with warmed RPMI containing 2% BSA. Cells were then stained with anti-biotin-Dylight 650 Fab, made in house, for 15 minutes. Fab antibodies were used in order to not prematurely stimulate the cells. Cells were then placed on a BD Fortessa flow cytometer and after 15 seconds of baseline readings were stimulated with 5 μg/mL of $F(ab')_2$ goat anti-mouse IgM (Jackson Labs) or 1 μL of 1 mg/mL ionomycin as a control. The insulin binding versus non-insulin binding B cell populations were gated based on CD19- T cells, which served as a negative control for insulin-binding. Calcium mobilization analysis was conducted using FlowJo software.

*PhosFlow Syk and PTEN Analysis*

Splenocytes were harvested from mice and red blood cells were lysed using ammonium chloride. Cells were stimulated with 5 ug/mL goat anti-mouse IgM (Jackson Labs) for 5 minutes at 37°C, or left unstimulated. Cells were immediately fixed with 2% PFA at 37°C for 10 minutes, spun down, and then resuspended with ice cold 100% methanol and placed on ice for 30 minutes. Cells were washed and stained with biotinylated insulin and monoclonal anti-mouse antibodies against CD19 and IgM for 15 minutes. Cells were also stained with mouse anti-ZAP70/Syk antibody (BD Phosflow) and anti-PTEN (BD Phosflow), or isotype control simultaneously. Cells

were washed twice, followed by staining with streptavidin-Alexa Fluor 421 to identify insulin binding B cells. Flow cytometry was performed as described above.

*Statistics*

Data were analyzed using Prism software (GraphPad Software, Inc.). Student *t* tests were used to compare differences between groups.

RESULTS

*Insulin-binding B cell frequency is directly correlated with penetrance and rate of onset of T1D in NOD.*

If B cells bearing insulin-binding BCR (IBC) play an essential role in the development of T1D, one would expect that disease development should correlate with the *in vivo* frequency of IBCs. To formally explore this relationship, we analyzed the rate of onset and penetrance of disease in genetically modified NOD mice in which IBCs differ in frequency (Fig 6.1A and 6.1B). IBCs were assayed as shown schematically in figure 6.1C and described below. As shown in figure 6.1, splenic IBC frequency was directly correlated with rate of disease development in these animals. In VH125.NOD mice ten weeks of age, ~1% of splenic B cells (~$8x10^4$/spleen) bound insulin. All of these mice developed disease by 30 weeks of age (Fig 6.1A). In 10 week old non-transgenic female NOD mice, about one tenth the number of splenic IBC were seen and disease onset was delayed, with 70% of mice diabetic at 30 weeks (Fig 6.1A). The IgM heavy chain VH281 differs from VH125 in lacking two amino acids in the CDR2, and this heavy chain is not permissive for insulin binding, regardless of paired light chain [33]. No IBCs were detectable in VH281.NOD mice, and <5% of female VH281.NOD developed T1D during the course of the experiment (Fig 6.1A). The few VH281.NOD mice that developed disease had lost allelic exclusion as indicated by the fact that they expressed endogenous $\mu^b$ heavy chain allotype (data not shown). Thus

disease development may have been supported by rare cells that acquired insulin reactivity by virtue of endogenous Ig gene usage.

As shown in figure 6.1B, female gender bias for development of T1D was maintained in the presence of the VH125 transgene. Though 100% disease incidence was observed in male VH125.NOD, the rate of development was slower than in females. The gender difference was directly correlated with splenic insulin-binding B cell numbers. Thus gender-based factors that affect disease also affect insulin-binding B cell frequency. Taken together, results are consistent with the possibility that the rate and penetrance of T1D in genetically predisposed mice are determined in part by the number of insulin-binding B cells present in peripheral lymphoid organs. Furthermore, gender bias could reflect an effect of sex on autoreactive B cell numbers.

Interestingly, despite containing more peripheral IBCs than WT NOD mice, VH125.C57BL/6-H2g7 mice carrying the $IA^{g7}$ risk allele did not develop disease (Fig 6.1A). The MHC II allotype $IA^{g7}$, which is found in NOD, is uniquely able to present insulin peptide to pathogenic CD4 T cells, supporting disease development [24]. C57BL/6 mice with the NOD MHC haplotype (C57BL/6-H2g7) can develop benign insulitis, but do not progress to overt disease [153]. Thus, on the C57BL/6 genetic background the presence of insulin-binding B cells does not alone suffice to support disease development even when a MHC II allotype competent to present pathogenic insulin peptides is expressed.

*Identification and isolation of insulin-binding B cells*

Experiments described in figure 6.1A and B were made possible by development of methodology to identify and isolate clonally distributed and thus rare B cells that bind insulin via their BCR [68, 104]. In the procedure (Fig 6.1C), splenocytes are depleted of red blood cells and then incubated with biotinylated insulin in combination with antibodies to cell markers of interest. Cells are then washed and fixed with 2% paraformaldehyde, before being incubated in Streptavidin-$Alexa^{647}$ and washed again. Finally, anti-$Alexa^{647}$-conjugated MACS beads are

added to the suspension, and cells that bind the MACS beads are enriched using a magnetic column.

Experiments demonstrated that cell populations isolated using this protocol are highly enriched B cells that bind insulin via their antigen receptors. In support of this conclusion are the following findings. There was no enrichment of “insulin-binding” B cells when biotinylated insulin was omitted during selection (Fig 6.1C). Furthermore, pre-exposure of cells to excess unlabeled insulin at concentrations that block detectable binding of biotinylated insulin blocked cell selection (Fig 6.1C). Thus the method selected B cells with reactivity to insulin without significant contamination by cells recognizing avidin, Alexa$^{647}$ or anti-Alexa$^{647}$-conjugated beads. In further experiments we found that the method does not enrich B cells from MD4 immunoglobulin transgenic mice in which all B cells bear antigen receptors reactive with hen egg lysozyme (data not shown). Thus we conclude that the method does not enrich cells based on insulin binding to CD220 insulin receptors, which would be equivalently expressed on cells from MD4 and WT mice.

We then compared results of analysis of equivalently stained insulin-binding B cells before and after enrichment by magnetic selection. Binding cell frequencies were consistent with those reported in figure 6.1A (Fig 6.1D). Interestingly however, based on staining intensity IBCs from VH125.C57BL/6-H2g7 bind significantly less antigen than IBCs from VH125 NOD, whether one compares magnetic particle enriched or un-enriched populations. This result is consistent with the possibility that IBCs in VH125.C57BL/6-H2g7 are more profoundly anergic than those in VH125 NOD, as previous studies have demonstrated a correlation between anergy and reduced mIgM expression [12, 78]. This conclusion extends previous findings in mice transgenic for anti-hen egg lysozyme (HEL) BCR, which demonstrated that anergy of anti-HEL B cells is compromised in NOD relative to C57BL/6 backgrounds [148].

*VH125.NOD display defects in both central and peripheral tolerance*

The observed reduced frequency of IBCs in VH125.C57BL6-H2g7 spleens relative to VH125.NOD is consistent with defective elimination of IBC by central tolerance mechanisms in NOD. To further explore this possibility, we compared the frequency of insulin-binding B cells in the bone marrow of VH125.NOD and VH125.C57BL/6-H2g7. In VH125.NOD, we found an approximate 3 fold higher frequency of immature insulin-binding B cells than VH125.C57BL/6-H2g7 (Fig 6.2A). This finding and the fact that this differential is not as great as the ~7 fold difference seen in the periphery, suggests that central tolerance is less efficient in NOD (Fig 6.1D). This is consistent with a recent study by Henry-Bonami et al., who showed that in VH125.NOD immature insulin-binding B cells transition from the parenchyma to the sinusoids of the bone marrow and exit to the periphery with no reduction in numbers [90]. As seen in the periphery, surface IgM expression on immature insulin-binding B cells from VH125.NOD was elevated compared to those in VH125.C57BL/6-H2g7 (Fig 6.2A). This may reflect imposition of anergy in the bone marrow compartment where B cells first encounter this systemic autoantigen.

During the course of our studies, we noted the occurrence of two distinct IBC populations in both the VH125.NOD and VH125.C57BL/6-H2g7 that distributed on distinct diagonals when analyzed for IgM expression and insulin binding intensity (Fig 6.2B). In the VH125.C57BL6-H2g7 mice, the low insulin binding B cell population, designated Insulin$^{lo}$, expressed high levels of surface IgM, while the right shifted high insulin binding B cell population (Insulin$^{hi}$) had lower expression of IgM (Fig 6.1B). This is in contrast to the VH125.NOD mouse, in which both the Insulin$^{lo}$ and Insulin$^{hi}$ B cell populations expressed high IgM levels and antigen binding intensity, which is evident when cytograms of the populations from the two strains are overlaid (Fig 6.2B, right panel). Based on these findings, we suspect the Insulin$^{lo}$ B cell population in the VH125.C57BL/6-H2g7 has sufficiently low insulin affinity that they are ignorant of insulin in their environment, despite being captured by the enrichment procedure. The Insulin$^{hi}$ B cell population likely has sufficiently high affinity that it has been rendered anergic, as indicated by IgM

downregulation. On the other hand, it appears that the Insulin$^{hi}$ B cell population in the VH125.NOD mice has not been anergized, since IgM levels remain high.

In order to further analyze the status of these cells, we explored their history of receptor editing by analyzing the ratio of lambda:kappa BCR light chain usage, often used as a surrogate measure of receptor editing (Fig 6.2C) [154]. The frequency of lambda positive cells was higher among Insulin$^{hi}$-binding B cells from VH125.C57BL/6-H2g7 (~7%) than among Insulin$^{hi}$-binding B cells from VH125.NOD (~4%). Thus it appears that fewer of these IBC have attempted to edit in NOD. This could permit more pathogenic high affinity insulin-binding B cells to enter the periphery in NOD because less attempt is made to reduce affinity by alternate light chain usage.

Analysis of the relative number of IBCs in the periphery as a function of animal age provided additional evidence of a difference in autoreactive B cells in the two genetic backgrounds (Fig 6.2D). The total number of splenic insulin-binding B cells in VH125.NOD increased nearly five-fold between weaning (~0.5x10$^5$/spleen) and onset of overt T1D (~2x10$^5$/spleen) (Fig 6.2D), while in VH125.C57BL/6 no such accumulation of insulin-binding B cells occurred. The total number of splenic insulin-binding B cells in VH125.C57BL/6 remained low and constant (~0.25x10$^5$/spleen) regardless of age, consistent with maintenance of peripheral tolerance in these mice.

To better understand their potential contribution to disease development, we assessed the relative numbers of B cells with high versus low insulin affinity as a function of age (Fig 6.2E). In VH125.NOD, both high and low insulin-binding B cells were present in spleen at the earliest time point examined (weaning), and increased in number until the B cell compartment matured, i.e. about 6 weeks of age. While the frequency of low insulin-binding splenic B cells remained relatively constant after six weeks of age, the frequency of high affinity B cells continued to increase until onset of diabetes, consistent with contribution to disease.

Next, we determined which splenic B cell compartments Insulin$^{lo}$ and Insulin$^{hi}$ B cell populations were occupying in the two strains. Analysis of CD21, CD23, and CD1d revealed that

the Insulin$^{hi}$ B cell population in the B6 is low in CD21 and high in CD23 (Fig 6.2F) consistent with the previously defined phenotype of anergic An1 cells found in the T3 population [12]. In contrast, Insulin$^{lo}$ B cells in the B6 are distributed in all major splenic B cell compartments, consistent with the possibility they are ignorant of ambient antigen. Further, Insulin$^{hi}$ cells are homogeneously low in CD1d while Insulin$^{lo}$ cells exhibit a distribution of CD1d expression similar to non-insulin binding cells. Importantly, both the Insulin$^{hi}$ and Insulin$^{lo}$ B cell populations in the NOD have high surface IgM levels, and are distributed in all major splenic B cell populations, consistent with lack of anergy (Fig 6.2B and 6.2F).

*Insulin-binding B cells in VH125.NOD show signs of activation, ability to differentiate into plasma cells, and enter the pancreas prior to overt disease onset.*

Hypothesizing that high affinity VH125.NOD insulin-binding B cells have broken tolerance, we next examined whether these cells exhibit increased markers of activation compared to the non-insulin binding B cells contained within the same spleen. It has been shown high CD19 expression correlates with a lower threshold of B cell activation [155, 156]. Insulin-binding B cells and non insulin-binding B cells in VH125.NOD and VH125.C57BL/6-H2g7 had comparable levels of CD19 expression (Fig 6.3A). This was consistent with other models of autoimmunity [155, 157-159]. In humans, an anergic B cell population has been identified as expressing low levels of CD21 [82]. While the insulin-binding B cells in VH125.NOD and Insulin$^{lo}$ B cells in the VH125.C57BL/6-H2g7 had relatively normal levels of CD21 expression compared to their respective non-insulin binding B cell population, the Insulin$^{hi}$ B cell population in the B6 has a significantly lower expression of CD21, consistent with the human anergic phenotype (Fig 6.3A). Next, we compared the expression of CD86 on these cells in order to determine their activation status and potential to act as antigen-presenting cells. Interestingly, both the Insulin$^{hi}$ and Insulin$^{lo}$ B cells in the VH125.NOD have a relatively normal to increased expression of CD86, but both the Insulin$^{lo}$ and Insulin$^{hi}$ B cells in the B6 have a significantly decreased expression of CD86 (Fig

6.3A) compared to non-insulin binding B cells. These findings support the conclusion that T1D resistant VH125.C57BL6-H2g7 Insulin$^{hi}$ B cells are anergic and the Insulin$^{lo}$ B cells are likely ignorant due to their low affinity for insulin. However, in VH125.NOD both the Insulin$^{hi}$ and Insulin$^{lo}$ B cells have increased expression of BCR and have a high affinity for insulin, and therefore, have potential for activation and participation in disease.

Next, we examined whether insulin-binding B cells in the two strains are capable of differentiating into plasmablasts. While no plasmablasts were found in the VH125.C57BL/6-H2g7 mice (data not shown), almost 1% of the insulin-binding B cells in the VH125.NOD were plasmablasts compared to 0.38% of the non-insulin binding B cells (Fig 6.3B). The VH125 heavy chain limits serum Ig to the IgM isotype. We could detect insulin-specific IgM autoantibodies in VH125.NOD, but not VH281.NOD, VH125.C57BL/6, or VH125.C57BL/6-H2$^{g7}$ (data not shown). Insulin-binding B cells were subsequently found to accumulate in the pancreas of VH125.NOD, but not VH125.C57BL/6-H2g7, though these mice can develop benign insulitis (Fig 6.3C). Taken together, these findings indicate that the insulin-binding B cells in disease prone VH125.NOD become activated and accumulate in the pancreas during disease development.

*Insulin-binding B cells in VH125.NOD are functionally responsive to stimulation and have significantly decreased expression of PTEN, a negative regulator of B cell signaling.*

Data described above suggest that high affinity IBC in VH125.C57BL/6 are anergic, but those from VH125.NOD are not. To test this more formally we assessed the responsiveness of these populations to BCR stimulation. In order to test the responsiveness of IBCs we needed to develop a method to identify the cells without prematurely stimulating them with the addition of streptavidin. Direct labeling of insulin with fluorescein did not adequately distinguish the insulin binding from the non-insulin-binding B cells in either strain (data not shown). However, when we added biotinylated insulin followed by a fluorescently labeled anti-biotin Fab we were able to distinguish the insulin-binding B cells from the non-insulin binding B cells without stimulation (Fig

6.3D). Of note, this method did not enable resolution of Insulin$^{hi}$ and Insulin$^{lo}$ B cell populations based on antigen binding, likely due to decreased signal amplification compared to biotin-streptavidin. However, because the IBCs in the VH125.C57BL/6-H2g7 have decreased IgM expression, we believe this method identifies the Insulin$^{hi}$ B cells when these markers are paired. Similarly, because the IBCs in the VH125.NOD have increased IgM expression, we believe we capture the Insulin$^{hi}$ B cells in this stain, as well (Fig 6.3D).

When we stimulated these cells with F(ab')$_2$ anti-IgM, we found that in VH125.C57BL/6-H2g7 the insulin-binding B cells failed to mobilize intracellular calcium (Fig 6.3D), a classic indication of anergy. VH125.NOD insulin-binding B cells responded well, mobilizing calcium comparably to non-insulin binding B cells in NOD and B6 (Fig 6.3D). A similar relationship was seen in tyrosine phosphorylation of the BCR proximal tyrosine kinase Syk. IBCs from VH125.C57BL/6-H2g7 showed significantly less induced Syk phosphorylation than non-insulin binding B cells (Fig 6.3E), demonstrating a dampened signaling response consistent with anergy. Insulin-binding B cells from VH125.NOD showed enhanced pSyk following stimulation compared to non-insulin binding B cells (Fig 6.3E), confirming that they are not anergic and may be easily primed for participation in disease.

The phosphatase and tension homolog, PTEN, has been shown to be increased in the anergic transgenic mouse model MD4/ML5 and is partially responsible for hyporesponsiveness of anergic B cells in this model [83]. When we compared expression of PTEN in insulin-binding vs non-insulin binding B cells, we found that IBCs from VH125.C57BL/6-H2g7 have significantly increased levels of PTEN compared to non-IBCs (Fig 6.3E), similar to the anergic cells in the MD4/ML5 mouse model. However, IBCs in the VH125.NOD mouse had significantly decreased expression of PTEN compared to non-IBCs (Fig 6.3E), a finding consistent with the recent report of reduced PTEN expression in B cells from autoimmune SLE patients [160]. Taken together, these results demonstrate that IBCs in the B6 mouse are functionally anergic while those in NOD are not.

*Insulin-binding B cells mediate the in vivo activation of diabetogenic insulin reactive T cells.*

We observed that insulin-binding B cells in disease prone VH125.NOD can be activated (Fig 6.3A) and can differentiate into plasma cells (Fig 6.3B). However, the insulin-autoantibodies generated by plasma cells have been previously shown to be largely non-pathogenic [25]. Therefore, we next examined another possible role for these insulin-binding B cells, namely in presentation of autoantigen to diabetogenic T cells. It has been shown previously that development of T1D in NOD mice requires that B cell express MHCII I-Ag7 suggesting that B cell disease promoting function involves antigen presentation [24].

T cells recognizing the insulin B chain amino acid sequence 9 to 23 (B:9-23) in the context of MHCII I-Ag7 can be diabetogenic. The B:9-23 insulin peptide can occupy the binding groove of the NOD MHCII molecule, IAg7, in a number of registers. Pathogenic insulin-specific T cells primarily recognize the MHC II complex when the insulin peptide is bound in register 3 [152, 161]. We have recently produced and described an antibody, designated mAb287, that binds specifically to B:9-23 insulin peptide bound to IAg7 in register 3 [162]. Availability of this antibody allows analysis of the uptake, proteolytic processing and loading of insulin peptide into MHCII of splenic insulin-binding B cells. As shown in figure 6.3A, *ex vivo* insulin-binding B cells from VH125.NOD, but not VH125.C57BL/6-H2g7 mice showed significant staining with this antibody. Comparable levels of total MHC II were observed on IBC from the two mouse strains (Fig 6.4A). These findings are consistent with the possibility that B cells from VH125.NOD mediate their function by antigen presentation. It also indicates that, despite possessing the ability to bind antigen, anergic B cells are unable to successfully process antigen and load it into MHCII. These findings led us to explore the *in vivo* effect of insulin peptide-presenting B cells on the biology of diabetogenic insulin reactive CD4 cells.

Crawford et al. recently developed a set of fluorescent insulin-IAg7 tetramers that bind specifically to B:9-23-reactive T cells [152]. We used these tetramers to mark B:9-23-reactive T cells in the VH125.NOD, NOD, VH281.NOD, and VH125.C57BL/6-H2g7 mice. On the NOD

background, the frequency of pancreatic B:9-23-reactive T cells correlated with the frequency of insulin-binding B cells present (Figs 6.1 and 6.4B,C), with the highest numbers seen in VH125.NOD (Fig 6.4B,C). Since B:9-23 reactive T cells accumulate in the pancreas of VH125.NOD but very few do in VH281.NOD mice, B cell specificity for insulin must be critical. Very few B:9-23-reactive T cells were found in VH125.C57BL/6-H2g7. This may be the consequence of anergy in these IBC, or the result of some other NOD genotype dependent effect. Importantly, the B:9-23 reactive T cells that accumulated in VH125.NOD also expressed the highest levels of the memory marker CD44, consistent with their activation by insulin reactive B cells (Fig 6.4B).

## DISCUSSION

Previous studies have demonstrated a critical role for islet antigen-reactive B cells in the development of T1D in the NOD mouse, and have implicated B cells in human T1D. While B cell function in this context would be expected to require loss of tolerance to islet antigens, the only study that has directly tested this possibility concluded that the offending B cells remain anergic [138]. This conundrum prompted us to examine and compare the status of insulin-reactive B cells in disease permissive NOD compared to disease resistant C57BL/6.H2g7 genetic backgrounds, and to further explore how these cells contribute to disease in NOD. Results demonstrate that in C57BL/6 mice high affinity insulin-reactive B cells are silenced by both central and peripheral mechanisms. In the periphery these cells appear anergic based low mIgM expression, T3 marker phenotype, low expression of CD1d and CD21, high PTEN expression, and unresponsiveness to BCR stimulation as measured by calcium mobilization and Syk tyrosine kinase phosphorylation [8, 12, 78, 82, 83] Conversely, in NOD these cells are less efficiently eliminated by central tolerance mechanisms, occurring in the periphery at 7-8 fold higher frequency. High insulin affinity B cells progressively accumulate from birth in the spleens of these mice. They occupy all major B cell compartments in the spleen and are not anergic as indicated by high mIgM expression and

robust calcium mobilization and Syk phosphorylation in response to BCR stimulation. In NOD but not C57BL/6, insulin-reactive cells show propensity to differentiate into B220 low, CD138 high plasmablasts.

Functional studies revealed that insulin-binding NOD B cells internalize and process antigen *in vivo*, successfully loading insulin B:9-23 peptide into MHCII. Insulin binding cells in C57BL/6.H2g7 do not accomplish MHCII loading. Activated insulin-reactive cells in NOD accumulate in the pancreas, and this is associated with accumulation of diabetogenic, insulin B:9-23-reactive T cells at this site. Accumulation of insulin-reactive T cells in the NOD pancreas requires that B cells be insulin reactive, as it is reduced in NOD and hardly detectable in VH281.NOD. Taken together, data demonstrate that B cell tolerance to insulin fails in NOD. Rather than being rendered anergic as is seen in C57BL6.H2g7 mice, high affinity insulin reactive cells become activated and accumulate in spleen and pancreas. These cells capture, process and present antigen to diabetogenic insulin T cells that show signs of activation as indicated by increased CD44 expression.

The inability of anergic B cells to internalize, process and present antigen represents a significant advancement in our understanding of the biology of anergic B cells. It indicates that in anergic cells signaling machinery downstream from the antigen receptor is unable to sustain this process. Studies have shown antigen presentation by B cells requires cross-linking of the BCR, phosphorylation of ITAMs on the Igα:Igβ co-receptor, recruitment and phosphorylation of kinases such as Syk, and activation of downstream signaling pathways including the PI3K pathway that results in rapid endocytosis of antigen-engaged receptors and entry into late endosomal antigen processing compartments [163]. Hence, inhibitory molecules, such as PTEN, that are upregulated in anergic IBCs in B6 likely inhibit antigen presentation from occurring. Thus it appears that the inability of anergic cells to present antigen is a checkpoint in their participation in immune responses to exogenous antigens.

These studies are also informative regarding the relative importance of various antigen presenting cell populations in the development of T1D in NOD. Lack of significant diabetogenic insulin-reactive CD4 T cell accumulation in the pancreas of NOD mice in which the B cell repertoire lacks insulin reactivity (VH281.NOD) leads to the inescapable conclusion that antigen-specific B cells are critical antigen presenting cells in this process. Efficacy of B cell depleting therapy in human T1D suggests that the same may be true in man.

This study also raises an important question regarding whether and how the immune system perceives soluble monomeric autoantigens. There is general agreement that induction of BCR signaling, like that of other ITAM-containing receptors, requires receptor aggregation. Now classical studies of Chiller and Weigle and others showed that antigen valence was critical for induction of B cell tolerance *in vitro* [164, 165]. It is likely that tolerance measured in these models reflected anergy. In view of this and the demonstrated requirement for chronic antigen exposure to induce and maintain anergy [10], it seems most likely that this response requires BCR aggregation. How could this occur in the VH125 model where the autoantigen is monomeric? An obvious possibility is that B cells see insulin that is cell associated by virtue of association with the ubiquitous insulin receptor CD220. However the parent hybridoma, 125, antibody has been shown not to bind insulin that is associated with its receptor [150]. Another possibility is that these B cells recognize insulin that is associated with BCR on other insulin-reactive B cells. This too is very unlikely because the VH125 expressed on all B cells in this transgenic mouse would direct specificity of all insulin reactive cells to the same epitope regardless of light chain usage. Resolution of this question will clearly require more study.

The lessons learned in this study regarding the role of loss of B cell anergy in T1D almost certainly extends to other autoimmunities seen in NOD, including autoimmune sialitis and autoimmune peripheral polyneuropathy, as well as the autoimmunities that develop after genetic manipulation or crosses to NOD, including the KRN model of arthritis and the NOD.H2h4 model

of autoimmune thyroiditis [166, 167]. Thus, it is likely that loss of B cell anergy is an important contributor to autoimmunity in NOD due to genetic risk factors in all of these cases.

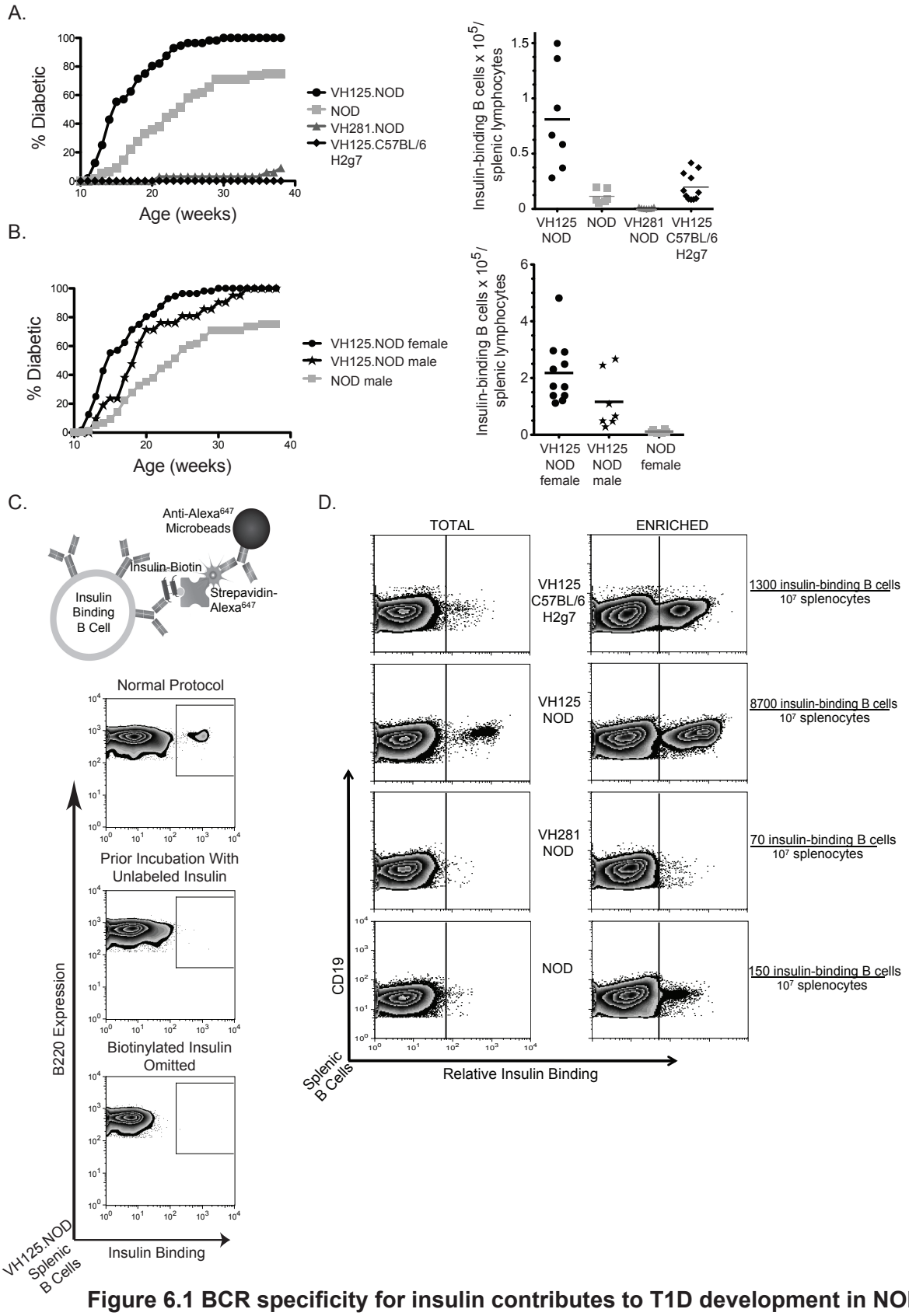



**Figure 6.1 BCR specificity for insulin contributes to T1D development in NOD**

**Figure 6.1 BCR specificity for insulin contributes to T1D development in NOD.**
**A.** Disease curve for female VH125.NOD, NOD, VH281.NOD, and VH125.C57BL/6-H2g7 mice monitored weekly from weaning until disease onset or 40 weeks of age. Total splenic insulin-specific B cell numbers (CD19+) for 8-12 week old female mice are shown. **B.** A diagram of the absorbent used for magnetic particle based staining and enrichment of IBCs. Splenic cells from VH125.NOD were subjected to the enrichment protocol in the presence of 50X unlabeled insulin, or in the absence of biotinylated insulin. **C.** Comparison of the number of splenic IBCs from female 8-10 week old VH125.C57BL/6.H2g7, VH125.NOD, VH281.NOD and NOD mice.

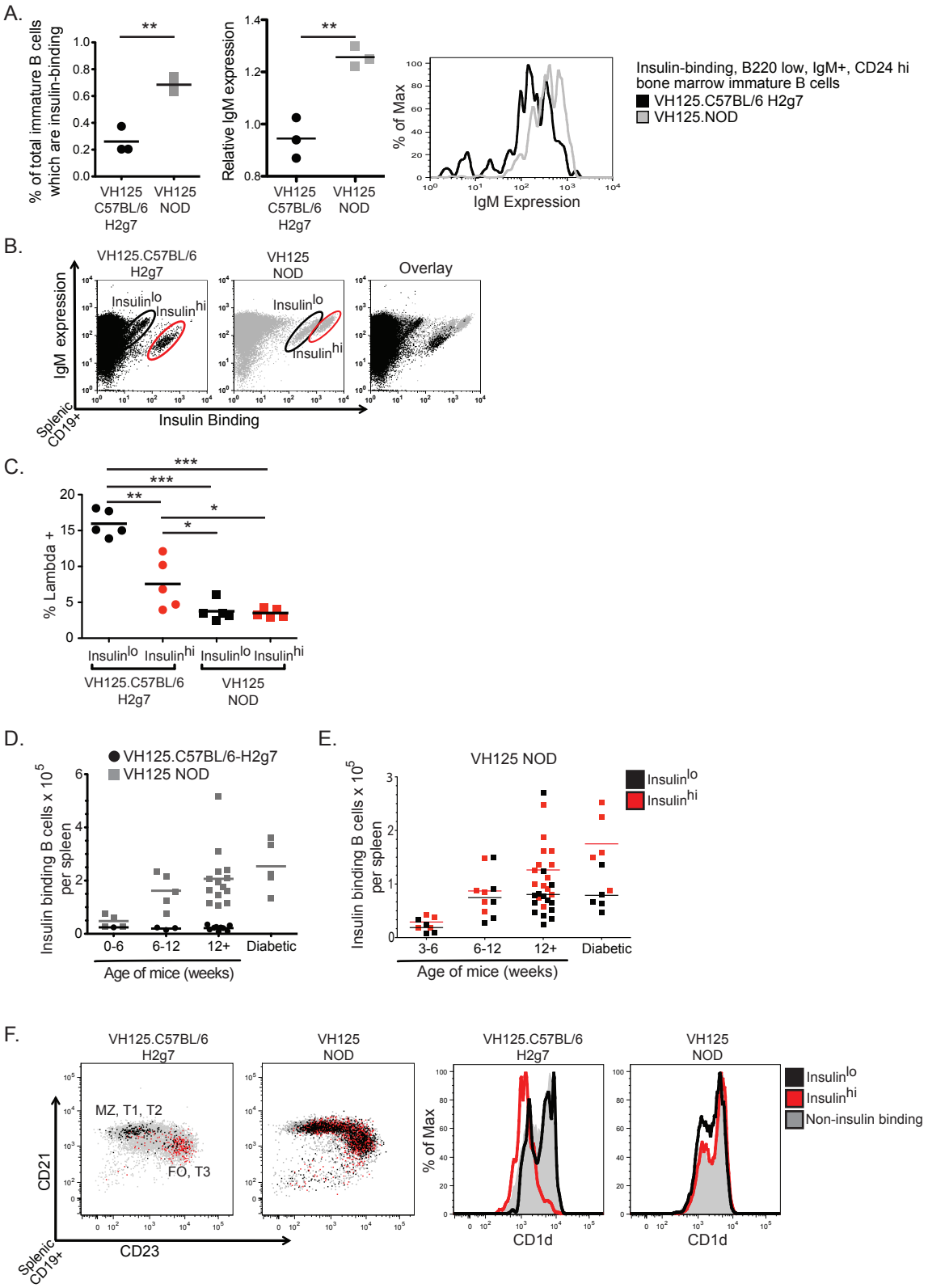



**Figure 6.2 Defective central and peripheral silencinig of insulin-reactive B cells in NOD**

**Figure 6.2 Defective central and peripheral silencing of insulin-reactive B cells in NOD.** **A.** Insulin-reactive B cell frequency and relative IgM expression (IgM MFI of IBCs divided by non-insulin binding B cells) among immature B cells in bone marrow of female VH125.NOD and VH125.C57BL/6-H2g7 mice at 8-10 weeks of age. Representative histogram of IgM expression shown. **B.** Representative cytograms of IgM expression versus insulin reactivity in the VH125.C57BL/6-H2g7 and VH125.NOD mice. Staining reveals a low affinity IBC population, designated $Insulin^{lo}$ (black oval), and a high affinity IBCs population, $Insulin^{hi}$ (red oval), in the VH125.C57BL/6-H2g7 mouse. In the VH125.NOD mouse both the $Insulin^{lo}$ and $Insulin^{hi}$ B cell populations have a high affinity for antigen. The two cytograms are overlaid for comparison of IgM levels and insulin reactivity. **C.** Percentage of splenic lambda positive CD19+ B cells in the $Insulin^{lo}$ and $Insulin^{hi}$ B cell populations from the two strains. **D.** Changes in the number of splenic IBCs with age and progression to T1D in the two strains. **E.** Changes in the number of $Insulin^{lo}$ and $Insulin^{hi}$ splenic B cells in VH125.NOD mice with age and progression to T1D. **F.** Splenic B cell subpopulation distribution of $Insulin^{lo}$ (black), $Insulin^{hi}$ (red), and non-IBCs (grey) B cells in spleens of 10 week old VH125.C57BL/6-H2g7 and VH125.NOD mice. Marginal zone (MZ), transitional type 1 (T1), and transitional type 2 (T2) B cells are $CD21^{hi}$ $CD23^{lo}$, whereas follicular (FO) and transitional type 3 (T3) are $CD21^{lo}$ $CD23^{hi}$. Marginal zone B cells are CD1d+.

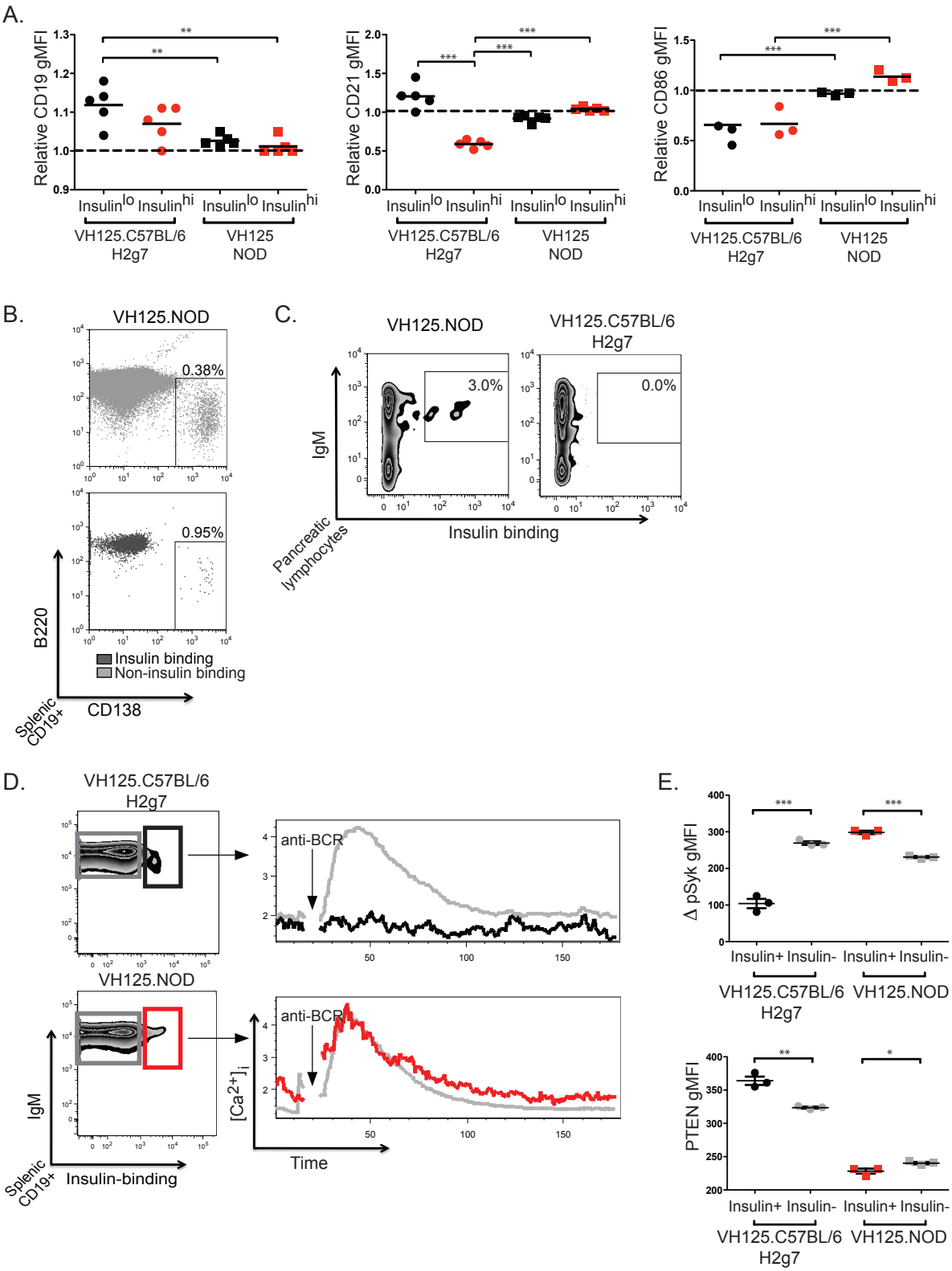



**Figure 6.3 Insulin-binding B cells in VH125.NOD show signs of activation and are responsive to stimuli prior to disease onset.**

**Figure 6.3 IBCs in VH125.NOD show signs of activation and are responsive to stimuli prior to disease onset. A.** Relative geometric mean fluorescence intensity of the surface markers CD19, CD21, and CD86 in splenic IBCs compared to non-IBCs in healthy female VH125.NOD and VH125.C57BL/6-H2g7 mice (8-12 weeks of age) mice. **B.** The frequency of insulin-binding $B220^{lo}$ $CD138^{+}$ plasmablasts is increased in the spleen of VH125.NOD compared to non-IBCs. No insulin-binding plasma cells are detected in the VH125.C57BL/6-H2g7 mouse (data not shown). **C.** IBCs can be detected in the pancreas of VH125.NOD, but not T1D resistant VH125.C57BL/6-H2g7. **D.** IBCs in the VH125.C57BL/6-H2g7 (black line) do not mobilize intracellular calcium following stimulation compared to non-IBCs (grey line), whereas the IBCs in VH125.NOD (red line) do. **E.** Change in geometric mean fluorescence intensity of phosphorylated Syk following stimulation and PTEN levels in IBC versus non-IBC in VH125.C57BL/6-H2g7 and VH125.NOD mice. Cells were stimulated with 5 ug/mL of goat anti-mouse IgM F(ab)'2 in all stimulation experiments. Data are representative of at least three separate experiments.

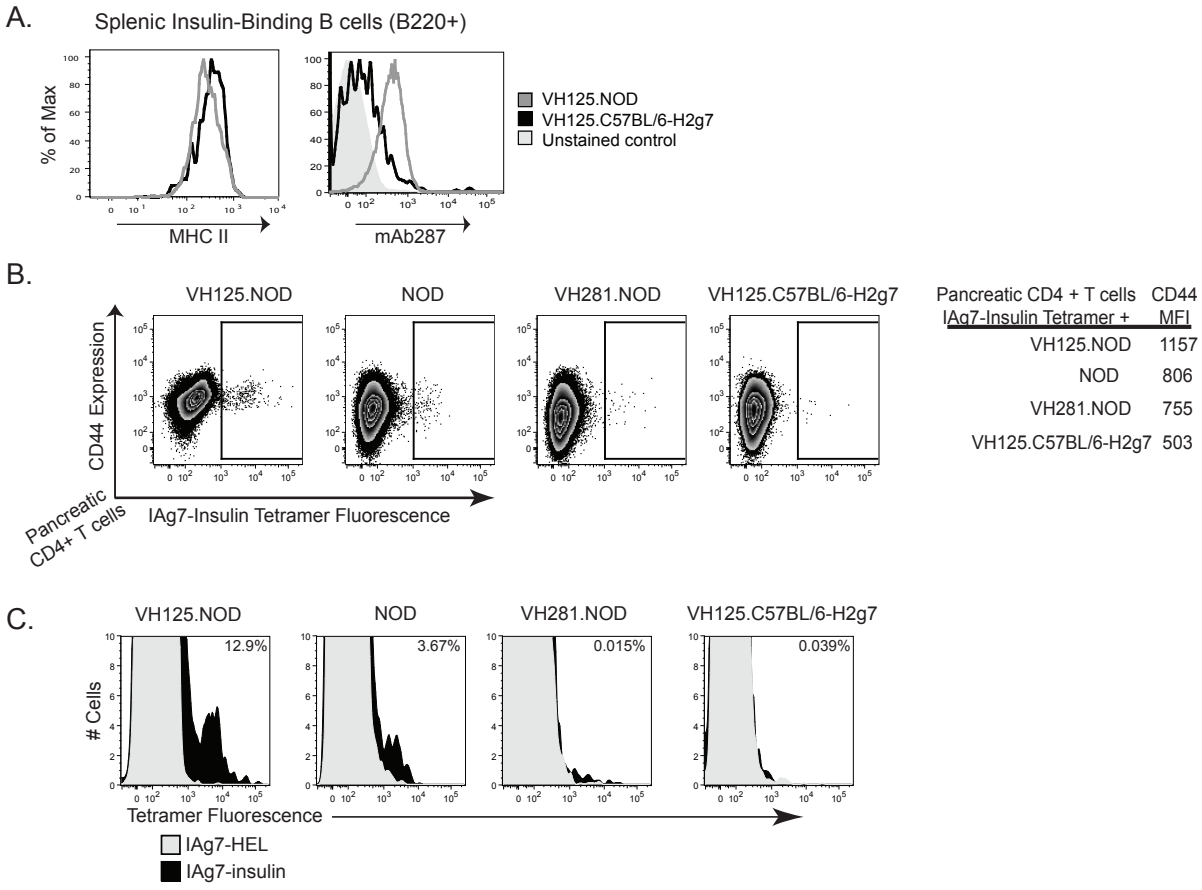



**Figure 6.4 Functional IBCs are required for in vivo activation of pancreatic anti-insulin T cells. A.** VH125.NOD, but not VH125.C57BL/6-H2g7, splenic IBCs present insulin peptide (B:9-23) in their MHC II complex as measured by staining with mAb287. **B.** The number of splenic IBCs (shown in figure 1D) correlates with the frequency of pancreatic insulin-reactive CD4+ T cells as measured by tetramer staining. CD44, as a measure of T cell activation, is highest in the VH125.NOD. **C.** Overlaid histograms for IAg7-insulin tetramer staining (black) compared to control IAg7-HEL tetramer staining (grey). The frequency of IAg7-Insulin tetramer+ T cells (percentage in upper right corner) is highest in the VH125.NOD.

# CHAPTER 7

## Discussion and future directions

These studies are the first to identify B cell anergy is compromised early in autoimmune patients. I found both early onset T1D and AITD patients show defects in maintaining autoreactive B cells in the anergic B cell population. Importantly, I also found this defect occurs before onset of disease, since it is also seen in autoantibody positive, but not yet diabetic patients, and even some first degree relatives of T1D patients. Loss of anergic B cells is associated with high risk T1D HLA and non-HLA alleles, suggesting genetics is likely the major contributor to defects in maintenance of anergy. To support this, I show in mice B cell anergy of IBCs is compromised in NOD mice, an autoimmune prone strain with known genetic loci that contribute to autoimmunity, but not in C57BL/6 mice, and autoimmune resistant strain, harboring the same MHC II haplotype as NOD.

These studies highlight some important findings regarding anergy in humans. First, an inability to sustain autoreactive B cells in the anergic compartment is common in at least three autoimmune disorders, T1D and AITD, as demonstrated here, and SLE as previously published [74]. I suspect in time the same will be shown for other autoimmune disorders, including MS and RA. Studies in our lab are currently addressing this possibility.

Secondly, loss of B cell anergy could be an early biomarker for individuals at risk for developing autoimmune disorders. Future studies to follow $B_{ND}$ sufficient and deficient FDRs longitudinally for development of autoantibodies and disease are underway.

Third, these studies demonstrate particular genetic risk alleles likely prevent autoreactive B cells from becoming anergic. Given that T1D and most other autoimmune diseases are polygenic, it is expected a combination of risk alleles, which may vary with each autoimmune disorder, work in concert to undermine B cell anergy. For T1D it appears high risk HLA haplotypes

and polymorphisms in the *PTPN2, INS, IKZB3*, and likely *PTPN22* genes are involved. Future studies to validate these findings are needed. While the 1858C>T polymorphism in *PTPN22* was not shown to have a statistically significant association with loss of B cell anergy, there was a trend, and the lack of significance was likely due to the small sample size and rarity of the polymorphism in the general population. This SNP is of particular interest, since it is a common risk allele among T1D, AITD, SLE, and RA and has been shown to alter negative regulation of B cell signaling [52, 168]. Hence, more studies to determine how this risk allele affects B cell anergy are needed. Previous studies in our lab have shown acute expression of the mouse ortholog of the human *PTPN22* risk allele, PEP-R619W, induces activation, proliferation, and differentiation of anergic B cells upon transfer into a C57BL/6 recipient (unpublished). One could extend these findings to the VH125.C57BL/6-H2g7 mouse, in order to determine whether conditional expression of the PEP-R619W allele is sufficient to break anergy in the IBCs and possibly lead to diabetes. I have begun to breed these mice already in the lab in hopes of doing these experiments. Further, similar studies analyzing the status of anergic B cells in mice expressing any one of the mouse orthologs of these risk alleles or more simply conditional deletion of the gene, such as *PTPN2*, in B cells only could be accomplished. Studies in our lab are also currently underway to explore what the effect of loss of *PTPN2* expression in B cells has on anergic B cells.

Another important finding from this work demonstrates that anergic B cells in healthy humans have high PTEN expression, a negative regulator of B cell signaling, compared to non-anergic B cells. While this has been shown in mice using transgenic models for B cell anergy [14, 83], this is the first report to demonstrate increased PTEN expression occurs in anergic B cells in humans. Moreover, while decreased PTEN expression in B cells has been shown in SLE patients [160], this is the first report to show similar findings in T1D and AITD patients. Perhaps more important, PTEN expression is decreased even further in the few remaining autoreactive $B_{ND}$ cells in these subjects. These findings were mirrored in the mouse, in which PTEN was increased in all B cells in the disease resistant VH125.C57BL/6.H2g7 mouse, but particularly so in the IBCs

that remain anergic. However, PTEN was significantly decreased in all B cells in the disease susceptible VH125.NOD mouse, but especially in the non-anergic IBCs. Hence, it is possible PTEN levels may govern the responsiveness of autoreactive B cells in humans. I have begun studies in our lab to explore this possibility using various PTEN inhibitors. In addition, since PTEN levels have been shown to be regulated by microRNAs, such as miRNA 7, 21, 22, and 92a [160, 169, 170], I am currently analyzing the relative abundance of these microRNAs in $B_{ND}$ versus mature naïve B cells, with the hypothesis that $B_{ND}$ cells from healthy individuals should express less of these miRNAs, while autoimmune subjects may express more of these regulating miRNAs in all B cells and any remaining $B_{ND}$ cells.

Moreover, these studies highlight the functional consequence of risk alleles on the activation of autoreactive B cells and their subsequent participation in disease. If autoimmune risk alleles, such as in *PTPN22, PTPN2*, or changes in microRNAs that target PTEN, work together to undermine B cell anergy, then this allows autoreactive B cells that normally should reside in the $B_{ND}$ compartment to become activated, express CD86, and act as autoantibody producing cells and/or antigen presenting cells to T cells. This is suggested in the AITD studies in which anergic $B_{ND}$ cells are lost, but the few remaining Tg-reactive $B_{ND}$ cells express high levels of CD86 and Tg-reactive B cells go on to produce autoantibodies. These findings were also shown in the VH125 mice. IBCs in the C57BL/6 mouse remain anergic, have decreased CD86 expression and increased PTEN expression, and are incapable of producing anti-insulin antibodies (data not shown) or placing insulin peptide in their MHC II. On the other hand, in NOD, an autoimmune prone strain, IBCs were functionally able to respond to anti-BCR stimulation, had normal to increased levels of CD86, decreased levels of PTEN, and were capable of producing anti-insulin antibodies and placing insulin-peptide in their MHC II, allowing them to receive T cell help.

Although the mouse studies I have shown here nicely summarize what we hypothesize is occurring in human T1D patients, studies to show this in human have been difficult. Over the past four years we have been members of the Network for Pancreatic Organ Donors with diabetes

(nPOD), which is an organization that procures organs from cadaveric T1D organ donors, including the pancreas, pancreatic lymph nodes, and spleen. From the few samples I have received and analyzed, I have been able to determine that the majority of the B cells present in the pancreatic islets bind to insulin and appear activated (data not shown). However, this needs to be repeated with more T1D organ donors and healthy controls in order to draw clear conclusions. Our lab and the nPOD network are actively seeking more organ donors around the world in order to collect vital information to better understand the pathogenesis of T1D in humans. In addition, we have cryopreserved pancreatic lymph node B cells from over 20 T1D subjects at various times in the disease process and healthy controls that I plan to analyze using over 35 B cell markers with mass cytometry. Results from these studies will hopefully shed light on the phenotype of insulin-binding B cells in the draining lymph node of the pancreas, which will better inform us of their role in T1D. Moreover, studies using mass cytometry will hopefully identify other markers of high affinity anergic B cells, other than low IgM expression, which will better enable us to track these cells in healthy controls and their disappearance in autoimmune subjects.

Lastly, all of the studies presented here utilized the novel method I helped develop to identify and enrich for antigen-binding B cells from blood and tissues of humans and mice. While this method has mainly been used to identify autoantigen reactive B cells in studies of autoimmune disorders, the method could easily be applied to foreign antigens, such as bacteria or viruses, in order to follow the immune response in B cells during the course of infection or immunization. I have conducted preliminary studies tracking tetanus-binding B cells before and after immunization. However, further studies to identify changes in the repertoire of tetanus-binding B cells following immunization could be completed using one of the many single cell dispersing technologies. Such studies would allow a deeper understanding of the somatic mutation and affinity maturation that occurs in antigen-reactive B cells. In addition, tracking antigen-reactive B cells during the course of a foreign antigen infection, such as from Ebola or

Zika virus, could help aid treatment and prevention measures through a better understanding of the role of B cells during disease.

In conclusion, studies presented here demonstrate that anergy as a mode to tolerize autoreactive B cells in autoimmune patients is unstable. It is likely a combination of non-HLA risk alleles that impairs maintenance of B cell anergy through alterations in the threshold for B and T cell signaling and the correct HLA alleles that allow presentation of the relevant peptide which allows these autoreactive B cells to become activated and participate in disease.

# REFERENCES


[1] Chervonsky AV. Influence of microbial environment on autoimmunity. Nat Immunol, 2010;11**:**28-35.

[2] Concannon P, Rich SS, Nepom GT. Genetics of type 1A diabetes. The New England journal of medicine, 2009;360**:**1646-54.

[3] Jeker LT, Bour-Jordan H, Bluestone JA. Breakdown in peripheral tolerance in type 1 diabetes in mice and humans. Cold Spring Harbor perspectives in medicine, 2012;2**:**a007807.

[4] Bluestone JA, Herold K, Eisenbarth G. Genetics, pathogenesis and clinical interventions in type 1 diabetes. Nature, 2010;464**:**1293-300.

[5] Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. Predominant autoantibody production by early human B cell precursors. Science, 2003;301**:**1374-7.

[6] Halverson R, Torres RM, Pelanda R. Receptor editing is the main mechanism of B cell tolerance toward membrane antigens. Nat Immunol, 2004;5**:**645-50.

[7] Meffre E, Wardemann H. B-cell tolerance checkpoints in health and autoimmunity. Curr Opin Immunol, 2008;20**:**632-8.

[8] Cambier JC, Gauld SB, Merrell KT, Vilen BJ. B-cell anergy: from transgenic models to naturally occurring anergic B cells? Nat Rev Immunol, 2007;7**:**633-43.

[9] Duty JA, Szodoray P, Zheng NY, Koelsch KA, Zhang Q, Swiatkowski M *et al.* Functional anergy in a subpopulation of naive B cells from healthy humans that express autoreactive immunoglobulin receptors. J Exp Med, 2009;206**:**139-51.

[10] Gauld SB, Benschop RJ, Merrell KT, Cambier JC. Maintenance of B cell anergy requires constant antigen receptor occupancy and signaling. Nat Immunol, 2005;6**:**1160-7.

[11] Gauld SB, Merrell KT, Cambier JC. Silencing of autoreactive B cells by anergy: a fresh perspective. Curr Opin Immunol, 2006;18**:**292-7.

[12] Merrell KT, Benschop RJ, Gauld SB, Aviszus K, Decote-Ricardo D, Wysocki LJ *et al.* Identification of anergic B cells within a wild-type repertoire. Immunity, 2006;25**:**953-62.

[13] O'Neill SK, Getahun A, Gauld SB, Merrell KT, Tamir I, Smith MJ *et al.* Monophosphorylation of CD79a and CD79b ITAM motifs initiates a SHIP-1 phosphatase-

mediated inhibitory signaling cascade required for B cell anergy. Immunity, 2011;35:746-56.

[14] Getahun A, Beavers NA, Larson SR, Shlomchik MJ, Cambier JC. Continuous inhibitory signaling by both SHP-1 and SHIP-1 pathways is required to maintain unresponsiveness of anergic B cells. The Journal of experimental medicine, 2016;213:751-69.

[15] Getahun A, Wemlinger SM, Rudra P, Santiago ML, van Dyk LF, Cambier JC. Impaired B cell function during viral infections due to PTEN-mediated inhibition of the PI3K pathway. The Journal of experimental medicine, 2017;214:931-41.

[16] Akashi T, Nagafuchi S, Anzai K, Kondo S, Kitamura D, Wakana S *et al.* Direct evidence for the contribution of B cells to the progression of insulitis and the development of diabetes in non-obese diabetic mice. Int Immunol, 1997;9:1159-64.

[17] Xiu Y, Wong CP, Bouaziz JD, Hamaguchi Y, Wang Y, Pop SM *et al.* B lymphocyte depletion by CD20 monoclonal antibody prevents diabetes in nonobese diabetic mice despite isotype-specific differences in Fc gamma R effector functions. J Immunol, 2008;180:2863-75.

[18] Hu CY, Rodriguez-Pinto D, Du W, Ahuja A, Henegariu O, Wong FS *et al.* Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. J Clin Invest, 2007;117:3857-67.

[19] Pescovitz MD, Greenbaum CJ, Bundy B, Becker DJ, Gitelman SE, Goland R *et al.* B-lymphocyte depletion with rituximab and beta-cell function: two-year results. Diabetes care, 2014;37:453-9.

[20] Pescovitz MD, Greenbaum CJ, Krause-Steinrauf H, Becker DJ, Gitelman SE, Goland R *et al.* Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. The New England journal of medicine, 2009;361:2143-52.

[21] Martin S, Wolf-Eichbaum D, Duinkerken G, Scherbaum WA, Kolb H, Noordzij JG *et al.* Development of type 1 diabetes despite severe hereditary B-lymphocyte deficiency. N Engl J Med, 2001;345:1036-40.

[22] Jones JL, Thompson SA, Loh P, Davies JL, Tuohy OC, Curry AJ *et al.* Human autoimmunity after lymphocyte depletion is caused by homeostatic T-cell proliferation. Proceedings of the National Academy of Sciences of the United States of America, 2013;110:20200-5.

[23] Merayo-Chalico J, Rajme-Lopez S, Barrera-Vargas A, Alcocer-Varela J, Diaz-Zamudio M, Gomez-Martin D. Lymphopenia and autoimmunity: A double-edged sword. Hum Immunol, 2016;77:921-9.

[24] Noorchashm H, Lieu YK, Noorchashm N, Rostami SY, Greeley SA, Schlachterman A *et al.* I-Ag7-mediated antigen presentation by B lymphocytes is critical in overcoming a checkpoint in T cell tolerance to islet beta cells of nonobese diabetic mice. J Immunol, 1999;163**:**743-50.

[25] Serreze DV, Fleming SA, Chapman HD, Richard SD, Leiter EH, Tisch RM. B lymphocytes are critical antigen-presenting cells for the initiation of T cell-mediated autoimmune diabetes in nonobese diabetic mice. J Immunol, 1998;161**:**3912-8.

[26] Tian J, Zekzer D, Hanssen L, Lu Y, Olcott A, Kaufman DL. Lipopolysaccharide-activated B cells down-regulate Th1 immunity and prevent autoimmune diabetes in nonobese diabetic mice. J Immunol, 2001;167**:**1081-9.

[27] Harris DP, Haynes L, Sayles PC, Duso DK, Eaton SM, Lepak NM *et al.* Reciprocal regulation of polarized cytokine production by effector B and T cells. Nat Immunol, 2000;1**:**475-82.

[28] Orban T, Sosenko JM, Cuthbertson D, Krischer JP, Skyler JS, Jackson R *et al.* Pancreatic islet autoantibodies as predictors of type 1 diabetes in the Diabetes Prevention Trial-Type 1. Diabetes Care, 2009;32**:**2269-74.

[29] Vehik K, Beam CA, Mahon JL, Schatz DA, Haller MJ, Sosenko JM *et al.* Development of Autoantibodies in the TrialNet Natural History Study. Diabetes Care, 2011.

[30] Verge CF, Stenger D, Bonifacio E, Colman PG, Pilcher C, Bingley PJ *et al.* Combined use of autoantibodies (IA-2 autoantibody, GAD autoantibody, insulin autoantibody, cytoplasmic islet cell antibodies) in type 1 diabetes: Combinatorial Islet Autoantibody Workshop. Diabetes, 1998;47**:**1857-66.

[31] Marino E, Tan B, Binge L, Mackay CR, Grey ST. B-cell cross-presentation of autologous antigen precipitates diabetes. Diabetes, 2012;61**:**2893-905.

[32] Silveira PA, Johnson E, Chapman HD, Bui T, Tisch RM, Serreze DV. The preferential ability of B lymphocytes to act as diabetogenic APC in NOD mice depends on expression of self-antigen-specific immunoglobulin receptors. Eur J Immunol, 2002;32**:**3657-66.

[33] Hulbert C, Riseili B, Rojas M, Thomas JW. B cell specificity contributes to the outcome of diabetes in nonobese diabetic mice. J Immunol, 2001;167**:**5535-8.

[34] Delong T, Wiles TA, Baker RL, Bradley B, Barbour G, Reisdorph R *et al.* Pathogenic CD4 T cells in type 1 diabetes recognize epitopes formed by peptide fusion. Science, 2016;351**:**711-4.

[35] Babon JA, DeNicola ME, Blodgett DM, Crevecoeur I, Buttrick TS, Maehr R *et al.* Analysis of self-antigen specificity of islet-infiltrating T cells from human donors with type 1 diabetes. Nature medicine, 2016;22**:**1482-7.

[36] Wiles TA, Delong T, Baker RL, Bradley B, Barbour G, Powell RL *et al.* An insulin-IAPP hybrid peptide is an endogenous antigen for CD4 T cells in the non-obese diabetic mouse. Journal of autoimmunity, 2017;78**:**11-8.

[37] Bosi E, Boulware DC, Becker DJ, Buckner JH, Geyer S, Gottlieb PA *et al.* Impact of age and antibody type on progression from single to multiple autoantibodies in type 1 diabetes relatives. The Journal of clinical endocrinology and metabolism, 2017.

[38] Barker JM, Barriga KJ, Yu L, Miao D, Erlich HA, Norris JM *et al.* Prediction of autoantibody positivity and progression to type 1 diabetes: Diabetes Autoimmunity Study in the Young (DAISY). The Journal of clinical endocrinology and metabolism, 2004;89**:**3896-902.

[39] Sosenko JM, Yu L, Skyler JS, Krischer JP, Gottlieb PA, Boulware D *et al.* The Use of Electrochemiluminescence Assays to Predict Autoantibody and Glycemic Progression Toward Type 1 Diabetes in Individuals with Single Autoantibodies. Diabetes Technol Ther, 2017;19**:**183-7.

[40] Steck AK, Fouts A, Miao D, Zhao Z, Dong F, Sosenko J *et al.* ECL-IAA and ECL-GADA Can Identify High-Risk Single Autoantibody-Positive Relatives in the TrialNet Pathway to Prevention Study. Diabetes Technol Ther, 2016;18**:**410-4.

[41] Wong FS, Wen L, Tang M, Ramanathan M, Visintin I, Daugherty J *et al.* Investigation of the role of B-cells in type 1 diabetes in the NOD mouse. Diabetes, 2004;53**:**2581-7.

[42] Menard L, Saadoun D, Isnardi I, Ng YS, Meyers G, Massad C *et al.* The PTPN22 allele encoding an R620W variant interferes with the removal of developing autoreactive B cells in humans. J Clin Invest, 2011;121**:**3635-44.

[43] Chamberlain N, Massad C, Oe T, Cantaert T, Herold KC, Meffre E. Rituximab does not reset defective early B cell tolerance checkpoints. The Journal of clinical investigation, 2016;126**:**282-7.

[44] Panigrahi AK, Goodman NG, Eisenberg RA, Rickels MR, Naji A, Luning Prak ET. RS rearrangement frequency as a marker of receptor editing in lupus and type 1 diabetes. J Exp Med, 2008;205**:**2985-94.

[45] Willcox A, Richardson SJ, Bone AJ, Foulis AK, Morgan NG. Analysis of islet inflammation in human type 1 diabetes. Clin Exp Immunol, 2009;155**:**173-81.

[46] Leete P, Willcox A, Krogvold L, Dahl-Jorgensen K, Foulis AK, Richardson SJ *et al.* Differential Insulitic Profiles Determine the Extent of beta-Cell Destruction and the Age at Onset of Type 1 Diabetes. Diabetes, 2016;65**:**1362-9.

[47] Packard TA, Smith MJ, Conrad FJ, Johnson SA, Getahun A, Lindsay RS *et al.* B Cell Receptor Affinity for Insulin Dictates Autoantigen Acquisition and B Cell Functionality in Autoimmune Diabetes. J Clin Med, 2016;5.

[48] Willcox A, Richardson SJ, Walker LSK, Kent SC, Morgan NG, Gillespie KM. Germinal centre frequency is decreased in pancreatic lymph nodes from individuals with recent-onset type 1 diabetes. Diabetologia, 2017;60**:**1294-303.

[49] Erlich H, Valdes AM, Noble J, Carlson JA, Varney M, Concannon P *et al.* HLA DR-DQ haplotypes and genotypes and type 1 diabetes risk: analysis of the type 1 diabetes genetics consortium families. Diabetes, 2008;57**:**1084-92.

[50] Pugliese A, Zeller M, Fernandez A, Jr., Zalcberg LJ, Bartlett RJ, Ricordi C *et al.* The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes. Nature genetics, 1997;15**:**293-7.

[51] Cerosaletti K, Buckner JH. Protein tyrosine phosphatases and type 1 diabetes: genetic and functional implications of PTPN2 and PTPN22. The review of diabetic studies : RDS, 2012;9**:**188-200.

[52] Habib T, Funk A, Rieck M, Brahmandam A, Dai X, Panigrahi AK *et al.* Altered B cell homeostasis is associated with type I diabetes and carriers of the PTPN22 allelic variant. J Immunol, 2012;188**:**487-96.

[53] Dai X, James RG, Habib T, Singh S, Jackson S, Khim S *et al.* A disease-associated PTPN22 variant promotes systemic autoimmunity in murine models. J Clin Invest, 2013;123**:**2024-36.

[54] Simoncic PD, Lee-Loy A, Barber DL, Tremblay ML, McGlade CJ. The T cell protein tyrosine phosphatase is a negative regulator of janus family kinases 1 and 3. Current biology : CB, 2002;12**:**446-53.

[55] Wiede F, Shields BJ, Chew SH, Kyparissoudis K, van Vliet C, Galic S *et al.* T cell protein tyrosine phosphatase attenuates T cell signaling to maintain tolerance in mice. The Journal of clinical investigation, 2011;121**:**4758-74.

[56] Long SA, Cerosaletti K, Wan JY, Ho JC, Tatum M, Wei S *et al.* An autoimmune-associated variant in PTPN2 reveals an impairment of IL-2R signaling in CD4(+) T cells. Genes Immun, 2011;12**:**116-25.

[57] Wiede F, Sacirbegovic F, Leong YA, Yu D, Tiganis T. PTPN2-deficiency exacerbates T follicular helper cell and B cell responses and promotes the development of autoimmunity. Journal of autoimmunity, 2017;76**:**85-100.

[58] Cambier JC. Autoimmunity risk alleles: hotspots in B cell regulatory signaling pathways. J Clin Invest, 2013;123**:**1928-31.

[59] Cho JH, Gregersen PK. Genomics and the multifactorial nature of human autoimmune disease. N Engl J Med, 2011;365**:**1612-23.

[60] Chung SA, Criswell LA. PTPN22: its role in SLE and autoimmunity. Autoimmunity, 2007;40**:**582-90.

[61] Luo L, Cai B, Liu F, Hu X, Wang L. Association of Protein Tyrosine Phosphatase Nonreceptor 22 (PTPN22) C1858T gene polymorphism with susceptibility to autoimmune thyroid diseases: a meta-analysis. Endocr J, 2012;59**:**439-45.

[62] de Wildt RM, Steenbakkers PG, Pennings AH, van den Hoogen FH, van Venrooij WJ, Hoet RM. A new method for the analysis and production of monoclonal antibody fragments originating from single human B cells. J Immunol Methods, 1997;207**:**61-7.

[63] Edelman GM, Rutishauser U, Millette CF. Cell fractionation and arrangement on fibers, beads, and surfaces. Proc Natl Acad Sci U S A, 1971;68**:**2153-7.

[64] Haas W, Schrader JW, Szenberg A. A new, simple method for the preparation of lymphocytes bearing specific receptors. Eur J Immunol, 1974;4**:**565-70.

[65] Kenny JJ, Merrill JE, Ashman RF. A two-step centrifugation procedure for the purification of sheep erythrocyte antigen-binding cells. J Immunol, 1978;120**:**1233-9.

[66] Snow EC, Vitetta ES, Uhr JW. Activation of antigen-enriched b cells. I. Purification and response to thymus-independent antigens. J Immunol, 1983;130**:**607-13.

[67] Egeland T, Hovdenes A, Lea T. Positive selection of antigen-specific B lymphocytes by means of immunomagnetic particles. Scand J Immunol, 1988;27**:**439-44.

[68] Smith MJ, Packard TA, O'Neill SK, Henry Dunand CJ, Huang M, Fitzgerald-Miller L *et al.* Loss of anergic B cells in prediabetic and new-onset type 1 diabetic patients. Diabetes, 2015;64**:**1703-12.

[69] Woda M, Mathew A. Fluorescently labeled dengue viruses as probes to identify antigen-specific memory B cells by multiparametric flow cytometry. J Immunol Methods, 2015;416**:**167-77.

[70] Nair N, Buti L, Faenzi E, Buricchi F, Nuti S, Sammicheli C *et al.* Optimized fluorescent labeling to identify memory B cells specific for Neisseria meningitidis serogroup B vaccine antigens ex vivo. Immun Inflamm Dis, 2013;1**:**3-13.

[71] Amanna IJ, Slifka MK. Quantitation of rare memory B cell populations by two independent and complementary approaches. Journal of immunological methods, 2006;317**:**175-85.

[72] Scheid JF, Mouquet H, Feldhahn N, Walker BD, Pereyra F, Cutrell E *et al.* A method for identification of HIV gp140 binding memory B cells in human blood. Journal of immunological methods, 2009;343**:**65-7.

[73] Doucett VP, Gerhard W, Owler K, Curry D, Brown L, Baumgarth N. Enumeration and characterization of virus-specific B cells by multicolor flow cytometry. Journal of immunological methods, 2005;303**:**40-52.

[74] Malkiel S, Jeganathan V, Wolfson S, Orduno NM, Marasco E, Aranow C *et al.* Checkpoints for Autoreactive B Cells in Peripheral Blood of Lupus Patients Assessed By Flow Cytometry. Arthritis Rheumatol, 2016.

[75] Pape KA, Taylor JJ, Maul RW, Gearhart PJ, Jenkins MK. Different B cell populations mediate early and late memory during an endogenous immune response. Science, 2011;331**:**1203-7.

[76] Abts H, Emmerich M, Miltenyi S, Radbruch A, Tesch H. CD20 positive human B lymphocytes separated with the magnetic cell sorter (MACS) can be induced to proliferation and antibody secretion in vitro. Journal of immunological methods, 1989;125**:**19-28.

[77] Pescovitz MD, Greenbaum CJ, Krause-Steinrauf H, Becker DJ, Gitelman SE, Goland R *et al.* Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. N Engl J Med, 2009;361**:**2143-52.

[78] Goodnow CC, Crosbie J, Adelstein S, Lavoie TB, Smith-Gill SJ, Brink RA *et al.* Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. Nature, 1988;334**:**676-82.

[79] Quach TD, Manjarrez-Orduno N, Adlowitz DG, Silver L, Yang H, Wei C *et al.* Anergic responses characterize a large fraction of human autoreactive naive B cells expressing low levels of surface IgM. J Immunol, 2011;186**:**4640-8.

[80] Smith K, Garman L, Wrammert J, Zheng NY, Capra JD, Ahmed R *et al.* Rapid generation of fully human monoclonal antibodies specific to a vaccinating antigen. Nat Protoc, 2009;4**:**372-84.

[81] Walrand S, Guillet C, Gachon P, Giraudet C, Rousset P, Vasson MP *et al.* Insulin regulates protein synthesis rate in leukocytes from young and elderly healthy humans. Clin Nutr, 2005;24**:**1089-98.

[82] Isnardi I, Ng YS, Menard L, Meyers G, Saadoun D, Srdanovic I *et al.* Complement receptor 2/CD21- human naive B cells contain mostly autoreactive unresponsive clones. Blood, 2010;115**:**5026-36.

[83] Browne CD, Del Nagro CJ, Cato MH, Dengler HS, Rickert RC. Suppression of phosphatidylinositol 3,4,5-trisphosphate production is a key determinant of B cell anergy. Immunity, 2009;31**:**749-60.

[84] Batista FD, Neuberger MS. Affinity dependence of the B cell response to antigen: a threshold, a ceiling, and the importance of off-rate. Immunity, 1998;8**:**751-9.

[85] Henry RA, Acevedo-Suarez CA, Thomas JW. Functional silencing is initiated and maintained in immature anti-insulin B cells. J Immunol, 2009;182**:**3432-9.

[86] Goodnow CC, Crosbie J, Jorgensen H, Brink RA, Basten A. Induction of self-tolerance in mature peripheral B lymphocytes. Nature, 1989;342**:**385-91.

[87] Ichiyoshi Y, Casali P. Analysis of the Structural Correlates for Antibody Polyreactivity by Multiple Reassortments of Chimeric Human-Immunoglobulin Heavy and Light-Chain V-Segments. J Exp Med, 1994;180**:**885-95.

[88] Aguilera I, Melero J, Nunez-Roldan A, Sanchez B. Molecular structure of eight human autoreactive monoclonal antibodies. Immunology, 2001;102**:**273-80.

[89] Meffre E, Davis E, Schiff C, Cunningham-Rundles C, Ivashkiv LB, Staudt LM *et al.* Circulating human B cells that express surrogate light chains and edited receptors. Nat Immunol, 2000;1**:**207-13.

[90] Henry-Bonami RA, Williams JM, Rachakonda AB, Karamali M, Kendall PL, Thomas JW. B lymphocyte "original sin" in the bone marrow enhances islet autoreactivity in type 1 diabetes-prone nonobese diabetic mice. J Immunol, 2013;190**:**5992-6003.

[91] Wei C, Anolik J, Cappione A, Zheng B, Pugh-Bernard A, Brooks J *et al.* A new population of cells lacking expression of CD27 represents a notable component of the B cell memory compartment in systemic lupus erythematosus. J Immunol, 2007;178**:**6624-33.

[92] Fecteau JF, Cote G, Neron S. A new memory CD27-IgG+ B cell population in peripheral blood expressing VH genes with low frequency of somatic mutation. J Immunol, 2006;177**:**3728-36.

[93] Koelsch K, Zheng NY, Zhang Q, Duty A, Helms C, Mathias MD *et al.* Mature B cells class switched to IgD are autoreactive in healthy individuals. J Clin Invest, 2007;117**:**1558-65.

[94] Ercolini AM, Miller SD. The role of infections in autoimmune disease. Clin Exp Immunol, 2009;155**:**1-15.

[95] Munz C, Lunemann JD, Getts MT, Miller SD. Antiviral immune responses: triggers of or triggered by autoimmunity? Nat Rev Immunol, 2009;9**:**246-58.

[96] Wardemann H, Nussenzweig MC. B-cell self-tolerance in humans. Adv Immunol, 2007;95**:**83-110.

[97] Huang SW, Haedt LH, Rich S, Barbosa J. Prevalence of antibodies to nucleic acids in insulin-dependent diabetics and their relatives. Diabetes, 1981;30**:**873-4.

[98] Triolo G, Giardina E, Scarantino G, Seddio G, Cardella F, Meli F *et al.* Cross-reactivity of anti-ssDNA antibodies with heparan sulfate in patients with type I diabetes mellitus. Diabetes, 1989;38**:**718-22.

[99] Triolo TM, Armstrong TK, McFann K, Yu L, Rewers MJ, Klingensmith GJ *et al.* Additional autoimmune disease found in 33% of patients at type 1 diabetes onset. Diabetes Care, 2011;34**:**1211-3.

[100] Barker JM, Yu J, Yu L, Wang J, Miao D, Bao F *et al.* Autoantibody "subspecificity" in type 1 diabetes: risk for organ-specific autoimmunity clusters in distinct groups. Diabetes Care, 2005;28**:**850-5.

[101] Thomas JW, Kendall PL, Mitchell HG. The natural autoantibody repertoire of nonobese diabetic mice is highly active. J Immunol, 2002;169**:**6617-24.

[102] Kinnunen T, Chamberlain N, Morbach H, Cantaert T, Lynch M, Preston-Hurlburt P *et al.* Specific peripheral B cell tolerance defects in patients with multiple sclerosis. J Clin Invest, 2013;123**:**2737-41.

[103] Knip M, Simell O. Environmental triggers of type 1 diabetes. Cold Spring Harb Perspect Med, 2012;2**:**a007690.

[104] Smith MJ, Packard TA, O'Neill SK, Hinman RM, Rihanek M, Gottlieb PA *et al.* Detection and Enrichment of Rare Antigen-specific B Cells for Analysis of Phenotype and Function. J Vis Exp, 2017.

[105] Pugliese A, Gianani R, Moromisato R, Awdeh ZL, Alper CA, Erlich HA *et al.* HLA-DQB1*0602 is associated with dominant protection from diabetes even among islet cell antibody-positive first-degree relatives of patients with IDDM. Diabetes, 1995;44**:**608-13.

[106] Lambert AP, Gillespie KM, Thomson G, Cordell HJ, Todd JA, Gale EA *et al.* Absolute risk of childhood-onset type 1 diabetes defined by human leukocyte antigen class II genotype: a population-based study in the United Kingdom. The Journal of clinical endocrinology and metabolism, 2004;89**:**4037-43.

[107] Schenker M, Hummel M, Ferber K, Walter M, Keller E, Albert ED *et al.* Early expression and high prevalence of islet autoantibodies for DR3/4 heterozygous and DR4/4 homozygous offspring of parents with Type I diabetes: the German BABYDIAB study. Diabetologia, 1999;42**:**671-7.

[108] Kulmala P, Savola K, Reijonen H, Veijola R, Vahasalo P, Karjalainen J *et al.* Genetic markers, humoral autoimmunity, and prediction of type 1 diabetes in siblings of affected children. Childhood Diabetes in Finland Study Group. Diabetes, 2000;49**:**48-58.

[109] Todd JA, Bell JI, McDevitt HO. HLA-DQ beta gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. Nature, 1987;329**:**599-604.

[110] Horn GT, Bugawan TL, Long CM, Erlich HA. Allelic sequence variation of the HLA-DQ loci: relationship to serology and to insulin-dependent diabetes susceptibility. Proc Natl Acad Sci U S A, 1988;85**:**6012-6.

[111] Lee KH, Wucherpfennig KW, Wiley DC. Structure of a human insulin peptide-HLA-DQ8 complex and susceptibility to type 1 diabetes. Nat Immunol, 2001;2**:**501-7.

[112] Vafiadis P, Bennett ST, Todd JA, Nadeau J, Grabs R, Goodyer CG *et al.* Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus. Nature genetics, 1997;15**:**289-92.

[113] Lempainen J, Harkonen T, Laine A, Knip M, Ilonen J. Associations of polymorphisms in non-HLA loci with autoantibodies at the diagnosis of type 1 diabetes: INS and IKZF4 associate with insulin autoantibodies. Pediatric diabetes, 2013;14**:**490-6.

[114] Long SA, Cerosaletti K, Bollyky PL, Tatum M, Shilling H, Zhang S *et al.* Defects in IL-2R signaling contribute to diminished maintenance of FOXP3 expression in CD4(+)CD25(+) regulatory T-cells of type 1 diabetic subjects. Diabetes, 2010;59**:**407-15.

[115] Kinnunen T, Chamberlain N, Morbach H, Choi J, Kim S, Craft J *et al.* Accumulation of peripheral autoreactive B cells in the absence of functional human regulatory T cells. Blood, 2013;121**:**1595-603.

[116] Leonardo SM, De Santis JL, Malherbe LP, Gauld SB. Cutting edge: in the absence of regulatory T cells, a unique Th cell population expands and leads to a loss of B cell anergy. Journal of immunology, 2012;188**:**5223-6.

[117] Wang JH, Avitahl N, Cariappa A, Friedrich C, Ikeda T, Renold A *et al.* Aiolos regulates B cell activation and maturation to effector state. Immunity, 1998;9**:**543-53.

[118] Sun J, Matthias G, Mihatsch MJ, Georgopoulos K, Matthias P. Lack of the transcriptional coactivator OBF-1 prevents the development of systemic lupus erythematosus-like phenotypes in Aiolos mutant mice. Journal of immunology, 2003;170**:**1699-706.

[119] Noureldine SI, Tufano RP. Association of Hashimoto's thyroiditis and thyroid cancer. Curr Opin Oncol, 2015;27**:**21-5.

[120] Pan XF, Gu JQ, Shan ZY. Increased risk of thyroid autoimmunity in rheumatoid arthritis: a systematic review and meta-analysis. Endocrine, 2015;50**:**79-86.

[121] Lu MC, Chang SC, Huang KY, Koo M, Lai NS. Higher Risk of Thyroid Disorders in Young Patients with Type 1 Diabetes: A 12-Year Nationwide, Population-Based, Retrospective Cohort Study. PLoS One, 2016;11**:**e0152168.

[122] Pan XF, Gu JQ, Shan ZY. Patients with systemic lupus erythematosus have higher prevalence of thyroid autoantibodies: a systematic review and meta-analysis. PLoS One, 2015;10**:**e0123291.

[123] Villadolid J, Amin A. Immune checkpoint inhibitors in clinical practice: update on management of immune-related toxicities. Transl Lung Cancer Res, 2015;4**:**560-75.

[124] Abdel-Wahab N, Shah M, Suarez-Almazor ME. Adverse Events Associated with Immune Checkpoint Blockade in Patients with Cancer: A Systematic Review of Case Reports. PLoS One, 2016;11**:**e0160221.

[125] Bertrand A, Kostine M, Barnetche T, Truchetet ME, Schaeverbeke T. Immune related adverse events associated with anti-CTLA-4 antibodies: systematic review and meta-analysis. BMC Med, 2015;13**:**211.

[126] Daniels GH, Vladic A, Brinar V, Zavalishin I, Valente W, Oyuela P *et al.* Alemtuzumab-related thyroid dysfunction in a phase 2 trial of patients with relapsing-remitting multiple sclerosis. The Journal of clinical endocrinology and metabolism, 2014;99**:**80-9.

[127] Mahzari M, Arnaout A, Freedman MS. Alemtuzumab Induced Thyroid Disease in Multiple Sclerosis: A Review and Approach to Management. Can J Neurol Sci, 2015;42**:**284-91.

[128] Yu S, Maiti PK, Dyson M, Jain R, Braley-Mullen H. B cell-deficient NOD.H-2h4 mice have CD4+CD25+ T regulatory cells that inhibit the development of spontaneous autoimmune thyroiditis. The Journal of experimental medicine, 2006;203**:**349-58.

[129] Hong SH, Braley-Mullen H. Follicular B cells in thyroids of mice with spontaneous autoimmune thyroiditis contribute to disease pathogenesis and are targets of anti-CD20 antibody therapy. Journal of immunology, 2014;192**:**897-905.

[130] Yu S, Medling B, Yagita H, Braley-Mullen H. Characteristics of inflammatory cells in spontaneous autoimmune thyroiditis of NOD.H-2h4 mice. Journal of autoimmunity, 2001;16**:**37-46.

[131] Braley-Mullen H, Yu S. Early requirement for B cells for development of spontaneous autoimmune thyroiditis in NOD.H-2h4 mice. Journal of immunology, 2000;165**:**7262-9.

[132] Segundo C, Rodriguez C, Aguilar M, Garcia-Poley A, Gavilan I, Bellas C *et al.* Differences in thyroid-infiltrating B lymphocytes in patients with Graves' disease: relationship to autoantibody detection. Thyroid : official journal of the American Thyroid Association, 2004;14**:**337-44.

[133] Armengol MP, Juan M, Lucas-Martin A, Fernandez-Figueras MT, Jaraquemada D, Gallart T *et al.* Thyroid autoimmune disease: demonstration of thyroid antigen-specific B cells and recombination-activating gene expression in chemokine-containing active intrathyroidal germinal centers. Am J Pathol, 2001;159**:**861-73.

[134] Nicholas MW, Dooley MA, Hogan SL, Anolik J, Looney J, Sanz I *et al.* A novel subset of memory B cells is enriched in autoreactivity and correlates with adverse outcomes in SLE. Clin Immunol, 2008;126**:**189-201.

[135] Adlowitz DG, Barnard J, Biear JN, Cistrone C, Owen T, Wang W *et al.* Expansion of Activated Peripheral Blood Memory B Cells in Rheumatoid Arthritis, Impact of B Cell Depletion Therapy, and Biomarkers of Response. PLoS One, 2015;10**:**e0128269.

[136] Iwase H, Kobayashi M, Nakajima M, Takatori T. The ratio of insulin to C-peptide can be used to make a forensic diagnosis of exogenous insulin overdosage. Forensic Sci Int, 2001;115**:**123-7.

[137] Giovanella L, Imperiali M, Ferrari A, Palumbo A, Furlani L, Graziani MS *et al.* Serum thyroglobulin reference values according to NACB criteria in healthy subjects with normal thyroid ultrasound. Clin Chem Lab Med, 2012;50**:**891-3.

[138] Acevedo-Suarez CA, Hulbert C, Woodward EJ, Thomas JW. Uncoupling of anergy from developmental arrest in anti-insulin B cells supports the development of autoimmune diabetes. J Immunol, 2005;174**:**827-33.

[139] Goodnow CC, Brink R, Adams E. Breakdown of self-tolerance in anergic B lymphocytes. Nature, 1991;352**:**532-6.

[140] Eris JM, Basten A, Brink R, Doherty K, Kehry MR, Hodgkin PD. Anergic self-reactive B cells present self antigen and respond normally to CD40-dependent T-cell signals but are defective in antigen-receptor-mediated functions. Proceedings of the National Academy of Sciences of the United States of America, 1994;91**:**4392-6.

[141] Tomer Y. Mechanisms of autoimmune thyroid diseases: from genetics to epigenetics. Annu Rev Pathol, 2014;9**:**147-56.

[142] Chang NH, McKenzie T, Bonventi G, Landolt-Marticorena C, Fortin PR, Gladman D *et al.* Expanded population of activated antigen-engaged cells within the naive B cell compartment of patients with systemic lupus erythematosus. Journal of immunology, 2008;180**:**1276-84.

[143] Jacobson EM, Huber A, Tomer Y. The HLA gene complex in thyroid autoimmunity: from epidemiology to etiology. Journal of autoimmunity, 2008;30**:**58-62.

[144] Serreze DV, Chapman HD, Varnum DS, Hanson MS, Reifsnyder PC, Richard SD *et al.* B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new "speed congenic" stock of NOD.Ig mu null mice. J Exp Med, 1996;184**:**2049-53.

[145] Noorchashm H, Noorchashm N, Kern J, Rostami SY, Barker CF, Naji A. B-cells are required for the initiation of insulitis and sialitis in nonobese diabetic mice. Diabetes, 1997;46**:**941-6.

[146] Henry RA, Kendall PL, Thomas JW. Autoantigen-specific B-cell depletion overcomes failed immune tolerance in type 1 diabetes. Diabetes, 2012;61**:**2037-44.

[147] Kimpimaki T, Kupila A, Hamalainen AM, Kukko M, Kulmala P, Savola K *et al.* The first signs of beta-cell autoimmunity appear in infancy in genetically susceptible children from the general population: the Finnish Type 1 Diabetes Prediction and Prevention Study. J Clin Endocrinol Metab, 2001;86**:**4782-8.

[148] Silveira PA, Dombrowsky J, Johnson E, Chapman HD, Nemazee D, Serreze DV. B cell selection defects underlie the development of diabetogenic APCs in nonobese diabetic mice. J Immunol, 2004;172**:**5086-94.

[149] Thomas JW, Hulbert C. Somatically mutated B cell pool provides precursors for insulin antibodies. Journal of immunology, 1996;157**:**763-71.

[150] Ewulonu UK, Nell LJ, Thomas JW. VH and VL gene usage by murine IgG antibodies that bind autologous insulin. Journal of immunology, 1990;144**:**3091-8.

[151] Rojas M, Hulbert C, Thomas JW. Anergy and not clonal ignorance determines the fate of B cells that recognize a physiological autoantigen. Journal of immunology, 2001;166**:**3194-200.

[152] Crawford F, Stadinski B, Jin N, Michels A, Nakayama M, Pratt P *et al.* Specificity and detection of insulin-reactive CD4+ T cells in type 1 diabetes in the nonobese diabetic (NOD) mouse. Proc Natl Acad Sci U S A, 2011;108**:**16729-34.

[153] Yui MA, Muralidharan K, Moreno-Altamirano B, Perrin G, Chestnut K, Wakeland EK. Production of congenic mouse strains carrying NOD-derived diabetogenic genetic intervals: an approach for the genetic dissection of complex traits. Mamm Genome, 1996;7**:**331-4.

[154] Mehr R, Shannon M, Litwin S. Models for antigen receptor gene rearrangement. I. Biased receptor editing in B cells: implications for allelic exclusion. J Immunol, 1999;163**:**1793-8.

[155] Inaoki M, Sato S, Weintraub BC, Goodnow CC, Tedder TF. CD19-regulated signaling thresholds control peripheral tolerance and autoantibody production in B lymphocytes. J Exp Med, 1997;186**:**1923-31.

[156] Sato S, Hasegawa M, Fujimoto M, Tedder TF, Takehara K. Quantitative genetic variation in CD19 expression correlates with autoimmunity. J Immunol, 2000;165**:**6635-43.

[157] Saito E, Fujimoto M, Hasegawa M, Komura K, Hamaguchi Y, Kaburagi Y *et al.* CD19-dependent B lymphocyte signaling thresholds influence skin fibrosis and autoimmunity in the tight-skin mouse. J Clin Invest, 2002;109**:**1453-62.

[158] Taylor DK, Ito E, Thorn M, Sundar K, Tedder T, Spatz LA. Loss of tolerance of anti-dsDNA B cells in mice overexpressing CD19. Mol Immunol, 2006;43**:**1776-90.

[159] Tedder TF, Inaoki M, Sato S. The CD19-CD21 complex regulates signal transduction thresholds governing humoral immunity and autoimmunity. Immunity, 1997;6**:**107-18.

[160] Wu XN, Ye YX, Niu JW, Li Y, Li X, You X *et al.* Defective PTEN regulation contributes to B cell hyperresponsiveness in systemic lupus erythematosus. Sci Transl Med, 2014;6**:**246ra99.

[161] Stadinski BD, Zhang L, Crawford F, Marrack P, Eisenbarth GS, Kappler JW. Diabetogenic T cells recognize insulin bound to IAg7 in an unexpected, weakly binding register. Proc Natl Acad Sci U S A, 2010;107**:**10978-83.

[162] Zhang L, Crawford F, Yu L, Michels A, Nakayama M, Davidson HW *et al.* Monoclonal antibody blocking the recognition of an insulin peptide-MHC complex modulates type 1 diabetes. Proc Natl Acad Sci U S A, 2014;111**:**2656-61.

[163] Clark MR, Massenburg D, Siemasko K, Hou P, Zhang M. B-cell antigen receptor signaling requirements for targeting antigen to the MHC class II presentation pathway. Curr Opin Immunol, 2004;16**:**382-7.

[164] Chiller JM, Weigle WO. Cellular events during induction of immunologic unresponsiveness in adult mice. Journal of immunology, 1971;106**:**1647-53.

[165] Cambier JC, Kettman JR, Vitetta ES, Uhr JW. Differential susceptibility of neonatal and adult murine spleen cells to in vitro induction of B-cell tolerance. The Journal of experimental medicine, 1976;144**:**293-7.

[166] Anderson MS, Bluestone JA. The NOD mouse: a model of immune dysregulation. Annual review of immunology, 2005;23**:**447-85.

[167] Aoki CA, Borchers AT, Ridgway WM, Keen CL, Ansari AA, Gershwin ME. NOD mice and autoimmunity. Autoimmun Rev, 2005;4**:**373-9.

[168] Marson A, Housley WJ, Hafler DA. Genetic basis of autoimmunity. The Journal of clinical investigation, 2015;125**:**2234-41.

[169] Ren P, Gong F, Zhang Y, Jiang J, Zhang H. MicroRNA-92a promotes growth, metastasis, and chemoresistance in non-small cell lung cancer cells by targeting PTEN. Tumour Biol, 2016;37**:**3215-25.

[170] Zhang H, Cao H, Xu D, Zhu K. MicroRNA-92a promotes metastasis of nasopharyngeal carcinoma by targeting the PTEN/AKT pathway. Onco Targets Ther, 2016;9**:**3579-88.